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Prediction and Annotation of ABC Transporter Genes from *Magnaporthe oryzae* Genome Sequence

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Magnaporthe oryzae is destructive plant-pathogenic fungus and causes rice blast. The pathogen uses several mechanisms to circumvent the inhibitory actions of fungicides. ATP-binding cassette (ABC) transporters are known to provide protection against toxic compounds in the environment. PC facilitated bioinformatic analysis, particularly with respect to accessing and extracting database information and domain identification. We predicted ABC transporter genes from the *M. oryzae* genome sequence with computation and bioinformatics tools. A total of thirty three genes were predicted to encode ABC transporters. Three of thirty three putative genes corresponded to three known ABC transporter genes (ABC1, ABC2 and ABC3). Copy numbers of the ABC transporter genes were proven by Southern blot analysis, which revealed that twenty genes tested exist as a single copy. We amplified the DNA complementary to RNA corresponding to eleven of these by reverse transcriptase polymerase chain reaction.

Key words : ABC transporter gene, *Magnaporthe oryzae*, gene expression

Introduction

Rice blast disease is caused by the hemibiotrophic fungus *M. oryzae* [10]. *M. oryzae* causes economically significant crop losses annually, each year it is estimated to destroy enough rice to feed more than 60 million people. It can also infect a number of other agriculturally important cereals including wheat, rye, barley, and pearl millet causing diseases called blast disease or blight disease. The fungus is known to occur in 85 countries worldwide. In 2005 the complete genome of *M. oryzae* was sequenced [3]. The organism is predicted to have over 11,000 genes. It is sometimes thought of as a model organism in the study of phytopathogenic fungi. Individual plant species are susceptible to only a limited number of pathogens because of the presence of effective general defense mechanisms. The defense responses mediated by gene-for-gene interactions include rapid localized cell death, known as the hypersensitive response [7], the production of phytoalexins and other antimicrobial secondary metabolites [15], and the expression of pathogenesis-related (PR) proteins [13]. Some PR proteins exhibit antimicrobial properties. The signaling molecules implicated in these inducible defense systems include salicylic acid (SA), jasmonic acid (JA) [7], ethylene (ET) [2], and reactive oxygen species (ROS) [12]. In particular, microorganisms often encounter an array of pre-formed and inducible chemical barriers during their attempts to infect plants. To defend themselves against fungal pathogens [4], plants produce an array of antifungal proteins as well as specialized antibiotics called phytoalexins [12]. Only pathogens which can evade these plant defense responses during early infection stages are able to survive and cause disease. ATP-binding cassette (ABC) proteins constitute one of the largest protein superfamilies and are present in all organisms from bacteria to human [18,21]. ABC transporters are evolutionary ancient and involved in the biochemical defense against toxicants [11,17]. ABC transporters actively transport chemically [8,15] or functionally diverse compounds [11,12,17] from the inside of the cell to the outside using the energy from ATP hydrolysis. The common feature of all ABC transporters is that they consist of two distinct domains, the transmembrane domain (TMD) and the nucleotide-binding domain (NBD). The N-terminal transmembrane domain and the C-terminal ABC domains are fused as a single polypeptide chain, arranged as TMD-NBD-TMD-NBD. The TMD, also known as consists of α-helices, embedded in the membrane bilayer. It recognizes

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a variety of substrates and undergoes. On the other hand, the NBD is located in the cytoplasm and has a highly conserved sequence [Walker A motif, Walker B motif (or P-loop, XXXXD, X is a hydrophobic residue), C motif (LSGGQ) and so on] [6,16]. We report here a genome-wide survey of ABC transporter genes, providing for the first time information on gene family in *M. oryzae*.

Materials and Methods

Computation analysis

To identify ABC transporters in *M. oryzae*, we used the InterPro profile consisting of six terms: IPR000412 (ABC-2), IPR001140 (ABC transporter, transmembrane region), IPR003439 (ABC transporter related), IPR010929 (CDR ABC transporter), IPR011527 (ABC transporter, transmembrane region, type 1), and IPR013283 (ABC transporter, ABCE). These six terms were retrieved from 22 ABC transporters in *Saccharomyces cerevisiae* (REF). Among six terms, IPR001140 and IPR011527 should exist on ABC transporters because these terms indicate transmembrane helixes. Protein databases used were Comparative Fungal Genomics Platform (CFGP) (http://cfgp.riceblast.snu.ac.kr/main.php) and The Broad Institute of MIT and Harvard (http://www.broadinstitute.org). Transmembrane helices were analyzed with TOPCONS (http://topcons.cbr.su.se/). Gene models were refined on the basis of sequence homology and the subfamily assignment confirmed by protein BLAST analysis on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov) [18].

Table 1. Putative ABC transporter genes from the *M. oryzae* genome sequence were predicted with bioinformatics tools

	Locus name	Number of exon	Gene length	Predicted Structure	Amino acid length
1	MGG00141	3	4143	(TMD6-NBD)*2	1333
2	MGG00239	6	4100	(TMD6-NBD)*2	1240
3	MGG00447	3	4615	(NBD-TMD6)*2	1484
4	MGG01563	4	3350	(TMD1-NBD-TMD6)	1041
5	MGG01674	13	6660	(TMD11-NBD)*(TMD6-NBD)	1607
6	MGG02348	4	4013	(TMD6-NBD)*2	1269
7	MGG03406	2	2200	(TMD4-NBD)	700
8	MGG03736	2	4776	(TMD11-NBD)*(TMD6-NBD)	1571
9	MGG04855	6	5614	(TMD11-NBD)*(TMD6-NBD)	1683
10	MGG04899	3	5205	(TMD6-NBD)*2	1655
11	MGG05009	1	3959	(TMD6-NBD)*2	1320
12	MGG05044	11	5111	(TMD11-NBD)*(TMD7-NBD)	1446
13	MGG05190	3	3258	(TMD12-NBD)	1028
14	MGG05595	14	5634	(TMD4-NBD)*(TMD6-NBD)	1303
15	MGG05746	6	5176	(TMD9-NBD)*(TMD4-NBD)	1490
16	MGG06024	6	4967	(TMD4-NBD)*(TMD6-NBD)	1432
17	MGG06707	4	2439	(TMD4-NBD)out	732
18	MGG06878	1	2471	(TMD6-NBD)	824
19	MGG07375	6	4850	(NBD-TMD6)*2	1469
20	MGG07567	2	5019	(TMD10-NBD)*(TMD6-NBD)	1571
21	MGG07848	2	4445	(NBD-TMD6)*2	1463
22	MGG08309	2	4578	(TMD6-NBD)*2	1500
23	MGG08970	3	4252	(TMD6-NBD)*2	1369
24	MGG09931	10	4972	(TMD4-NBD)*2	1194
25	MGG10277	2	4758	(NBD-TMD6)*2	1559
26	MGG10410	4	4219	(NBD-TMD6)*2	1262
27	MGG11025	6	4700	(TMD5-NBD)*2	1401
28	MGG11382	7	2369	X	599
29	MGG11862	3	2116	X	636
30	MGG13624	7	5386	(NBD-TMD6)*2	1547
31	MGG13762	13	4561	(TMD5-NBD)*(TMD5-NBD)	1170
32	MGG14069	6	2864	(TMD6-NBD)	815
33	MGG14400	4	4657	(TMD6-NBD)*2	1350

Fungal strains and culture conditions

M. oryzae strain KJ-201 was maintained on V8 Juice agar media (4% V8 juice and 1.5% agar, pH 7.0) with constant fluorescent lighting at 25±1°C for conidiation. Mycelia for DNA isolation were prepared by culturing in CM liquid media (7.5 g of yeast, 7.5 g of casein acid hydrolysate, and 10 g of sucrose per liter) with shaking at 25±1°C in the dark for 3 or 4 days.

Polymerase chain reaction (PCR)

Twenty eight degenerate primers were designed from highly conserved regions of ABC transporter genes from *M. oryzae* 70-15 and PCR was performed with *M. oryzae* KJ-201 genomic DNA as template. The primers used in this study are described in (Table 3). Hairpins that form below 50°C generally are not such a problem so the temperature of primer annealing step was raised over 55°C. PCR was performed with Prime Taq DNA polymerase (GenetBio) using the following cycling parameters: 1 min at 95°C, followed by 30 cycles (30 sec at 95°C, 30 sec at 60°C, 1 min at 72°C), with a final extension of 72°C for 10 min. The PCR products were isolated from the gel using a Gel Extraction kit (GENE All, General biosystem).

Confirmation of gene copy number

Genomic DNA of *M. oryzae* KJ201 was prepared by using a modification of the rapid isolation procedure developed by M. G. Murray and W. F. Thomson (1980). Genomic DNA (20 μg) was digested with restriction enzymes (*Bam*H Ⅰ, *Eco*R Ⅰ, *Hind* Ⅲ, *Pst* Ⅰ) and separated via electrophoresis on 1% agarose gel and transferred to Hybond N+ membrane (Amersham Bioscience) according to the standard methods including 0.2N HCl, Denaturation buffer (1.5 M NaCl, 0.5 M NaOH), Neutralization buffer (1 M Tris-HCl, 1.5 M NaCl),

Table 2. Homology of ABC transporter using BLAST

	Locus name	Homology of ABC transporter
1	MGG00141	*Aspergillus clavatus*, ABC multidrug transporter Mdr1
2	MGG00239	*Aspergillus clavatus*, ABC multidrug transporter Mdr1
3	MGG00447	*M.oryzae* ABC2
4	MGG01563	ABC-2 type transporter family protein
5	MGG01674	*Aspergillus fumigatus*, ABC metal ion transporter
6	MGG02348	*Aspergillus clavatus*, ABC multidrug transporter Mdr1
7	MGG03406	*Schizosaccharomyces pombe*, ABC family iron transporter Atm1
8	MGG03736	Multidrug resistance-associated protein, ABC bile acid transporter
9	MGG04855	ABC multidrug transporter, ABC bile acid transporter
10	MGG04899	*Penicillium chrysogenum*, ABC multidrug transporter Pc18g03100
11	MGG05009	*Pyrenophora tritici* canalicular, multispecific organic anion transporter 1
12	MGG05044	*Aspergillus fumigatus*, ABC multidrug transporter
13	MGG05190	*Aspergillus fumigatus*, vacuolar ABC heavy metal transporter Hmt1
14	MGG05595	*Aspergillus terreus*, lipid A export ATP-binding/permease protein msbA
15	MGG05746	Multidrug resistance-associated protein, ABC bile acid transporter
16	MGG06024	ABC multidrug transporter, ABC bile acid transporter
17	MGG06707	Adrenoleukodystrophy protein
18	MGG06878	ABC multidrug transporter
19	MGG07375	ABC multidrug transporter
20	MGG07567	ABC multidrug transporter, ABC bile acid transporter
21	MGG07848	*Aspergillus fumigatus*, ABC multidrug transporter
22	MGG08309	ABC multidrug transporter
23	MGG08970	*Talaromyces stipitatus*, ABC multidrug transporter SitT
24	MGG09931	*Aspergillus terreus*, lipid A export ATP-binding/permease protein msbA
25	MGG10277	*Pyrenophora tritici*, ABC drug exporter AtrF
26	MGG10410	ABC multidrug transporter
27	MGG11025	*Arabidopsis thaliana*, multidrug resistance-associated ATMRP1
28	MGG13624	*M.oryzae* ABC1
29	MGG13762	*M.oryzae* ABC3
30	MGG14069	Vacuolar ABC heavy metal transporter
31	MGG14400	*Aspergillus clavatus*, ABC multidrug transporter Mdr1

and 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, adjust pH 7.0 with NaOH). Hybridization was done at 65°C in Amersham Rapid-hyb buffer (GE Healthcare, UK). The probes were made from PCR product gel extracts radio-labeled with [α-^{32}P]dCTP using a Random Prime Labelling System (Amersham, Rediprime Ⅱ, GE Healthcare, UK). Washings were performed twice at 65°C in wash buffer (2× SSC, 0.5% SDS) and wash buffer (0.5× SSC, 0.1% SDS) in series. The signal on the membrane was detected by exposing on X-ray film.

Expression of ABC transporter genes

For mRNA induction experiments, precultures were grown in 200 ml of CM medium at 25°C and 100 rpm for 3 days. Fungal biomass was harvested and frozen in liquid nitrogen. Total RNA was isolated using solution D (99% Guanidine thiocyanate, 0.1 M β-mercaptoethanol) and clean up using DNase I (TAKARA). One microgram of total RNA was to synthesize the first-strand cDNA using PrimeScript™ Reverse Transcriptase (TAKARA) and oligo (dT) 12 in a 10 μl reaction system.

Results

Computation analysis

Thirty three ABC transporters were identified, of which thirty two genes have not been previously identified in *M. oryzae*. Finally we identified thirty ABC transporters on *M. oryzae* genome (Table 1). ABC transporters have a variety of transmembrane domain from 4 to 12 α-helix. A total of thirty sequences possibly involved with putative ABC transporter in *M. oryzae* genome sequence.

The results of BLAST showed nineteen sequences related to drug resistance transporters, two sequences to metal ion transporter, two sequences to heavy metal ion transporter, two sequences to lipid A exporter, one sequence to adreno-leukodystrophy protein, two sequence to lipid A exporter, one sequence to organic anion transporter and one sequence

Table 3. Primers for probe amplification and RT-PCR

	Locus name	5′-Forward primer-3′	5′-Reverse primer-3′
1	MGG00141	CAACGAGAGGGTCATAGAAG	GACCAAGGTCATTGTCAAGT
2	MGG00239	AGTCGCGACACCTTCCTG	GTCGCCAATGTCCACCTC
3	MGG01563	CCGGCCCATTCCATCAGT	AGCATCGGGCACCAGTCC
4	MGG01674	TATCGGTACGACTCGAACTT	CGTCTGCACAAGAGTGAGTA
5	MGG02348	AGCGGACCTACAGAGAACGA	GAACTCGACCATCCCTTCAA
6	MGG03406	ATTAGCTTTTTGCTCACCAG	GGTAAGGGTCAGATCACGTA
7	MGG03735	GGGACTCTGGACAGTGATAA	GTCTGATGGTCGAAATGTCT
8	MGG04855	GGGCGATTTCGTTTCCTT	AAGCTGACGTTGGCCTTG
9	MGG04899	ATTCGAACCAAGATGGTATG	CAGGTTGGTGATGGTACTTT
10	MGG05009	AGGGCAGTTGCAGACGAC	GGCATCGGGTTTGTTGAG
11	MGG05044	TCGGGCTCCATTGACATT	GTGCCGTCGACGTCTTCT
12	MGG05190	GGCACGACCACTGCTTTT	TCCGCCTGTGCTCTTGTTTC
13	MGG05595	GGCCGCTGACGAGAGTGC	CCCGAGTGCCCCAAAGTC
14	MGG05746	GGCCAATCGATGTCGAAC	CCTGCTCCGTAGCTGCTC
15	MGG06024	GCGTGCGTTTCAAGTCCT	AGGCATCTCCACCATCCA
16	MGG06707	GTTCCTCAAGCGCATCGT	CGAACACCGGTACCGAAC
17	MGG06878	TAAGAAGGATGGAGATGTGG	CAACCCGTAGATCCATAGAA
18	MGG07375	CCCTTCCCTGTTCGACCT	GCTGTCCGCCAGTCACTT
19	MGG07567	GGTGCTTTCGCCCTGTTA	TGATTGCTGCCGTCAAAA
20	MGG07848	GCCATCCTTTGCACCATC	CGGCTGTCAACTGTGTCG
21	MGG08309	CTGCCCATTTTTGCATCC	TGATCAAGGGGGTCTGGA
22	MGG08970	CCGACAAGACACCGATCC	CCAGACGTTGGTGTGCAG
23	MGG09931	CACGTGCTGGACTCGATG	GGGCGCATCGATAATGAC
24	MGG10277	AAGCAAGAAAAGCTCGACTA	GTCATGAAGACGGAGAAGAG
25	MGG10410	CAAGATCCCCCTCCGAGT	GCCGTTGACGTTGAGCTT
26	MGG11025	TCATCGCTACGACGGACA	CGACTTGAGGCGTGACCT
27	MGG14069	GGCGACTGACGACGAAGT	GGTTTCTCGCTCCTGCAA
28	MGG14400	GACGCTGATCCGCTTCAT	AACACGATGGCCGAAAAA

Fig. 1. PCR for making probes from chromosomal DNA. M: 100 bp DNA ladder marker, lane 1: MGG00141, lane 2: MGG00239, lane 3: MGG 01563, lane 4: MGG01674, lane 5: MGG02348, lane 6: MGG03406, lane 7: MGG03736, lane 8: MGG04855, lane 9: MGG04899, lane 10: MGG05009, lane 11: MGG05044, lane 12: MGG05190, lane 13: MGG05595, lane 14: MGG05746, lane 15: MGG06024, lane 16: MGG06707, lane 17: MGG06878, lane 18: MGG07375, lane 19: MGG07567, lane 20: MGG07848, lane 21: MGG08309, lane 22: MGG08970, lane 23: MGG09931, lane 24: MGG10277, lane 25: MGG10410, lane 26: MGG11025, lane 27: MGG14069, lane 28: MGG14400.

to unknown function (Table 2).

Amplification of probe template

Twenty eight hybridization probe template probes were amplified from *M. oryzae*. A single band corresponding to the amplified probes were observed; these fragments ranged in size from 700 to 1,200 bp (Fig. 1).

Confirmation of gene copy number

Hybridization of the probe to ABC transporter genes on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe. Southern blot analysis performed with restriction enzyme-digested genomic DNA was used to determine the number of sequences in a genome [18]. A probe that hybridizes only to a single DNA segment that had not been cut by the restriction enzyme will produce a single band on a Southern blot analysis. Copy numbers of the ABC transporter genes were proved by Southern blot analysis, which revealed that twenty genes tested exist as a single copy (Fig. 2).

Expression of ABC transporter genes

RT-PCR has a problem with genomic DNA contamination during isolation of total RNA. RT-PCR result of β-tubulin showed that it was amplified without genomic DNA contamination. RT-PCR was carried to monitor mRNA expression of ABC transporter genes. We have amplified the DNA complementary to RNA corresponding to eleven of these by RT-PCR (Fig. 3). RT-PCR was used in the determination of the abundance as a measure of eleven ABC transporter gene expression.

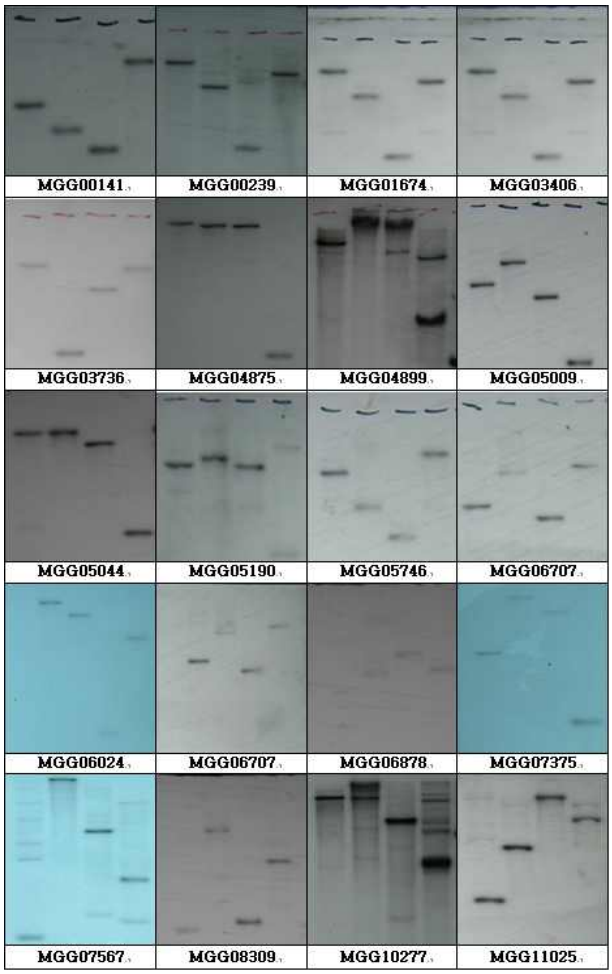

Fig. 2. The copy number of putative ABC transporter genes was confirmed by Southern blot analysis, which revealed that 20 genes exist as a single copy, respectively. Genomic DNA digested with restriction enzymes, 1st lane: *Bam*H I, 2nd lane: *Eco*R I, 3rd lane: *Hind* III, 4th lane: *Pst* I.

Discussion

M. oryzae has been able to develop resistance to both chemical treatments and genetic esistance developed by plant breeders in some types of rice. Evolutionary of ABC transporter gene family is developed which both reduces

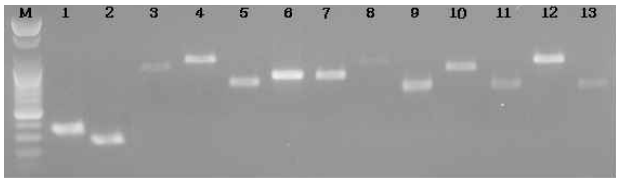

Fig. 3. RT-PCR for confirmation on the gene expression of novel ABC transporter genes. M: 100 bp DNA ladder marker, lane 1: amplification of β-tubulin from genomic DNA, lane 2: amplification of β-tubulin using RT-PCR, lane 3: MGG 00141, lane 4: MGG01674, lane 5: MGG03406, lane 6: MGG03736, lane 7: MGG04855, lane 8: MGG05009, lane 9: MGG05190, lane 10: MGG05746, lane 11: MGG06707, lane 12: MGG06878, lane 13: MGG09931.

selection against gene loss, and leads to the facile sorting of functions among paralogs following gene duplication [17]. ABC transporter knock-out mutant elicits to reduce pathogenic [5,8,12,19,20], but many ABC transporter gene annotation was not completion from *M. oryzae*. The completion of the *M. oryzae* genome project [3] make it possible to analyze entire gene families. The advantage of performing a combined analysis of gene family is that it allows prediction of novel ABC transporter genes from the *M. oryzae* genome sequence. The basic level of annotation is using BLAST for finding similarities, but the BLAST being a particular problem (*M. oryzae* is predicted to have over 11,000 genes) when we predict gene family from genome database. PC facilitated the bioinformatic analysis (InterPro profile consisting), particularly with respect to accessing and extracting database information and domain identification. We predicted twenty eight novel ABC transporters. The total number of ABC transporter genes in *M. oryzae* now stands at thirty four. Nineteen sequences related to drug resistance transporters.

An expressed sequence tag or EST is a short sub-sequence of a transcribed cDNA sequence. They may be used to identify gene transcripts, and are instrumental in gene discovery and gene sequence determination [1]. ESTs can be mapped to specific chromosome locations using physical mapping techniques, such as radiation hybrid mapping or FISH. Alternatively, if the genome of the organism that originated from the EST has been sequenced one can align the EST sequence to that genome. Some authors use the term "EST" to describe genes for which little or no further information exists besides the tag [17]. However, we could not find EST matches for the predicted genes from BLAST search using EST database. And also from the preliminary test for detecting the gene expression of the predicted ABC transporter genes, we found the basic level of mRNA expression is quite weak. These indicate that the level of expression of the ABC transporter genes is low enough to be detected with EST sequencing method and normal Northern blot analysis. We applied RT-PCR for detecting the mRNA expression of the predicted sequences and confirmed at least eleven genes were reasonably expressed in mRNA transcription (Fig. 3).

ABC transporter genes were predicted and annotated from genomic DNA sequence but phenotype analysis was not completed. The first step of understanding gene functions generally involves disruption of individual genes [9,19,20]. Therefore based on these results, we propose that ABC transporter knock-out analysis from *M. oryzae* could be utilized to analyze the function of the important genes.

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초록 : 벼도열병균 게놈서열로부터 ABC transporter 유전자군의 예측 및 특성 분석

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벼의 생산에 있어 가장 큰 문제 요인 중 하나인 벼도열병의 발생 원인균인 벼도열병균은 다양한 기작에 의해 방제 약제에 대한 내성을 가지는 것으로 알려져 있다. 막 운반단백질인 ABC transporter의 경우 환경으로부터의 다양한 독성 물질들을 배출하는 것으로 알려져 있다. 이미 알려진 벼도열병균의 게놈 서열로부터 생물정보학적 분석을 통하여 ABC transporter 단백질의 도메인 특성을 보이는 33개의 유전자군 서열을 예측하였다. 이중 3개의 경우는 이미 알려진 유전자로 판명되었다. Southern Hybridization 분석에 적용한 20개의 유전자들이 모두 게놈 상에 단일 copy로 존재함을 확인하였다. 새로 예측된 30개의 유전자중 11개는 RT-PCR을 통하여 전사단계에서의 유전자 발현이 확인되었다.