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Identification and expression of *leu*D Gene in Rice (*Oryza sativa* L.)

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A rice *OsLeuD* gene for small subunit of 3-isopropylmalate isomerase (IPMI) (EC 4.2.1.33) has been isolated. *OsLeuD* gene is located on 109.3 cM of chromosome 2. *OsLeuD* gene was expressed abundantly in metabolically active organs including leaves and developing seeds, indicating that *OsLeuD* gene expression is developmentally regulated. The cDNA of *OsLeuD* gene was coded for 257 amino acids which showed 58% and 48% homology to small subunits of IPMI in *LueD* genes of cyanobacteria and green sulfur bacteria, respectively. The molecular character of *OsLeuD* is closely related to those of photosynthetic bacteria rather than those of eukaryotes including fungi and yeast. This suggests that *OsLeuD* gene in chromosomal genome of plants may possibly be originated from chloroplast genome.

Key words – 3-isopropylmalate isomerase; leucine biosynthesis; leuD; Oryza sativa

Introduction

Leucine biosynthesis genes of *Escherichia coli* [12], *Salmonella typimurium* [9], *Bacillus subtilis* [14], and *Lacococus lactis* [4] are consisted a single operon in the order *leuABCD* [3,15]. The *leuA* and *leuB* genes encode 2-isopropylmalate synthase and 3-isoprophylmalate dehydrogenase, respectively. However, leuC and leuD genes encode the large subunit and small subunits of isoprophylmalate isomerase (IPMI), respectively. Leucine biosynthesis was mediated sequentially by committing enzymes [12]. The 2-isopropylmalate synthase (EC 2.3.3.13) encoded by *leuA* catalyzes the transfer of an acetyl group from acetyl-CoA to 2-ketoisovalerate to produce 3-carboxy-3-hydroxyisocaproate (isopropylmalte). The isoprophylmalate isomerase (IPMI) (EC 4.2.1.33) which is encoded by *leuC* and *leuD*, synonymously called 3-isoprophylmalate dehydratase, causes migration of the hydroxyl group to form 3-carboxy-2-hydroxyisocaprote (3-isoprophylmalate) [5]. The isopropylmalate dehydrogenase (EC 1.1.1.85) encoded by *leuB* decarboxylates 3-isoprophylmalate to 2-ketoisocaproate [8,13]. The branched-chain amino acid aminotransferase (EC 2.6.1.42) reversibly catalyzes the transfer of the amino group of L-leucine amino acid to 2-oxoglutarate to form a 4-methyl-2-oxopentanoate and L-glutamate.

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Bacterial isoprophylmalate isomerase (IPMI) is consisted with a heterodimer with two subunits, a large subunit is encoded by *leuC* and a small subunit is encoded by *leuD* [2], although fungal and yeast IPMIs are monomeric enzymes encoded by *LEU1* [1,11,7]. Up to date, although leucine biosynthesis in bacteria and yeast has well been investigated, leucine biosynthesis in plants is largely unexplored. Here we found a rice cDNA encoding a protein homology to small subunit of IPMI of *leuD* gene of bacteria. This is the first report for expression of *leuD* gene of rice.

Materials and Methods

Plant Material and cDNA Cloning

A cDNA clone encoded for β-subunit of 3-isopropylmalate isomerase was selected from early seeds cDNA library of *Oryza sativa* cv. Japonica, cv. Il-poom. Nucleotide sequencing was performed with the Bigdye^{TM} Terminator Cycle Sequencing kits (PE Biosystems, Foster City, CA, USA) using an automated DNA sequencer (ABI 3100, Applied Biosystems, Rockvill, MD, USA). DNA sequences and deduced amino acids were analyzed using the programs DNAsis (Hitachi, Japan), BLAST, and Clustal W programs.

RNA blot and Genomic DNA blot Analysis

For genomic Southern blot analysis, a radioactive labeled probe was generated by random primer labeling system (Promega Co. Medison, WI., USA). For Southern blot analy-

sis, α-[^{32}P] dCTP labeled DNA probe was generated by a random primer labeling system (Promega, Madison, WI., USA). Genomic DNA from rice seedlings was isolated using the CTAB method (10). Twenty micrograms of genomic DNA were digested to completion with *Bam*HI, *Eco*RI and *Hin*dIII. The enzyme treated genomic DNAs were electrophoretically separated for 8 h (25V) in 1X TAE buffer on a 0.8% agarose gel, denatured and then transferred onto nylon membrane (Amersham, Piscataway, NJ) in 25 mM Na_2HPO_4/NaH_2PO_4 buffer(pH 7.0). The membranes were UV-cross linked with 1,200 J under a UV crosslinker. Prehybridization with DNA blotted nylon membranes was performed at 42℃ for 3 h in 50% (v/v) formamide, 6X SSC (1X SSC is 0.15 M NaCl, 0.25 M NaH_2PO_4 and 25 mM Na_2EDTA), 5X Denhardt's solution [1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin], 0.5% (w/v) SDS, and 0.1 mg/mL Herring sperm DNA. DNA blotted nylon membranes were hybridized in the prehybridization buffer with radioactive labeled probes at 42°C for 12 h. After completion of hybridization, the DNA blotted membranes were washed twice in 2X SSPE, 0.1% SDS at room temperature for 5 min, once in 1X SSPE, 0.1% SDS at room temperature for 10 min, once in 0.1X SSPE, 0.1% SDS at room temperature for 20 min and once in 0.1X SSPE, 0.1% SDS at 42°C for 5 min.

For Northern blot analysis, total RNA was extracted from different organs including, roots, young and mature leaves and flowers in meiosis stage, 5 days after pollination (5-DAP). Twenty micrograms of total RNA was electrophoretically separated on a 1.4% agarose gel using 1X MOPS [3-(N-morpholino)-propanesulfonic acid] buffer and transferred to nylon membranes in 25mM phosphate buffer, pH 7.0 for 12 hrs. The membranes were UV-cross linked with 1200 J. Prehybridization was performed at 42℃ for 3 h in 50% (v/v) formamide, 6X SSC (1X SSC is 0.15 M NaCl, 0.25 M NaH_2PO_4 and 25 mM Na_2EDTA), 5X Denhardt's solution [1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin], 0.5% (w/v) SDS, and 0.1 mg/mL Herring sperm DNA. Hybridization was then performed in a prehybridization solution with α-[^{32}P] CTP labeled probe at 65°C for 14 h. The α-[^{32}P] CTP labeled probe labeled RNA probe were synthesized using T7 RNA polymerase (Promega Co.) according to manufactures' protocol. After performing hybridization, the RNA blotted nylon membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 5 min, once in 1X SSC, 0.1% SDS at room temperature for 10 min, once in 0.1X SSC, 0.1% SDS at room temperature for 20 min, and once in 0.1X SSC, 0.1% SDS at 65°C for 5 min. Membranes were exposed for 24 h to X-ray film and developed for their autoradiographic images.

Results and Discussion

Luecine D gene of rice (*OsLueD*)

The cDNA encoded a protein of 257 amino acids which showed 33.3% identity to 3-isopropylmalate dehydratase small subunit (EC 4.2.1.33) (isopropylmalate isomerase 2, IPMI). IPMI was encoded by the *LueD* gene of cyanobacteria. The deduced amino acids of the *LueD* gene of rice contained three protein kinase C phosphorylation sites, one glycosylation site and one cAMP-dependent protein kinase phosphorylation site were contained (Fig. 1).

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   1 CCTCACACACTGAACACCATGGCGGCGGCGGCGGCGGCTCCGGCTCTATCCTTGGCCGAG
                       M A A A A A A P A L S L A E 14
  61 GCGGCGCCGGTGACAGCAGTTCTGGCACCGTGTCCCACGCCCTCGAGGACGTTCCGCCGC
     A A P V T A V L A P C P T P S R T F R R 34
 121 CGCAGCTGGGTCGCGGCTATCTGCCGGCCCGCTCTGAAATGCCACCACAGTCGTCCCCTG
     R S W V A A I C R P A L K C H H S R P L 54
 181 ACCGCCGTGGCCGCCGCGGCTGCGGCTGCCGCTGCGGCGGGGACTCGACGTCGGCCGGC
     T A V A A A A A A A A A A G D S T S A G 74
 241 GTATTCCACGGCGAGTGCTTCGTCGTGGGGATAACATCGACACCGACCAGATCATCCCG
     V F H G E C F V V G D N I D T D Q I I P 94
 301 GCCGAGCACCTGACCCTGGTCCCGTCCAAGCCCGACGAGTACCGCAAGCTCGGCTCGTTC
     A E H L T L V P S K P D E Y R K L G S F 114
 361 GCCTTCGTTGGCCTCCCCACCGCGGCCTACCCGACGCCGTTCGTCGCCCCCGGCGAGGAG
     A F V G L P T A A Y P T P F V A P G E E 134
 421 ACCACCCGCTACGCCGTCATCATCGGCGGCGCCAACTTCGGCTGCGGCTCCTCCCGCGAG
     T T R Y A V I I G G A N F G C G S S R E 154
 481 CACGCGCCCGTCGCCCTGGGCGCCGCCGGCGCCCGCGCCGTCGTGGCCGAGGGCTACGCG
     H A P V A L G A A G A R A V V A E G Y A 174
 541 CGCATCTTCTTCCGCAACTCCGTGGCCACCGGTGAGGTCTACCCGTTGGAGCTAGCGGAC
     R I F F R N S V A T G E V Y P L E L A D 194
 601 ACTGGAGCCTGGAAGGAGTGCAAGACCGGGGATGTGGTCACGGTGGAACTTGATAATTGC
     T G A W K E C K T G D V V T V E L D N C 214
 661 GTCATGATCAACCACACATCCGGCAAGCAGTACAAGCTGAAGCCTATCGGCGATGCCGGG
     V M I N H T S G K Q Y K L K P I G D A G 234
 721 CCGGTTATTGAGGCAGGCGGGATCTTTGCCTATGCCCGGAAGACCGGAATGATCGCATCC
     P V I E A G G I F A Y A R K T G M I A S 254
 781 AAGTCTGCGTGAGGGAAAGGCGAGTTTGGTCTGCTGTCAAGATAGTCGAGGCCTCTGCAG
     K S A * 257
 841 ATAGCAAGACTGGGTTGTGGATTTGAACCTATTGCACCTCTATGCGATTGTCCATCAGTT
 901 GTACTGCTGTTTTTACCTAGGTTGTGTGTCATCAGTGGTGTTTTTGGAATAAGTTAAAAG
 961 TTACAGAGTACTGAACTATGATGTATTAGTCCATGTGATCTTATGTAACCCCCTTATGTA
1021 ATAACACTCGTTTATACCTGCCAAAAAAAAAAAAAAAAAAAAA 1063
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Fig. 1. Nucleotide sequence of *OsLeuD* cDNA and its deduced amino acid sequence. Amino acids sequences for either chloroplast signaling for thylakoid space or mitochondrial localization signaling sequences for mitochondria intermembrane space were *shaded*. The putative poly (A) signal is *bolded*. Three protein kinase C phosphorylation sites were *italic bolded* and *underlined*. Glycosaminoglycan attachment site is *bold* and *underlined* (218-NHTS). cAMP-dependent protein kinase phosphorylation site (33-RRRS) was *bolded* and *double underlined*. GenBank Accession of *OsPIG-F* is AY363174.

Further analysis for amino acid composition showed that both chloroplast signaling for thylakoid space or mitochondrial localization signaling sequences for mitochondria intermembrane space were contained from amino terminus sequences to amino acid residue from 54 to 68 in the 3-isopropylmalate dehydratase small subunit of rice (Fig. 1). This suggested that the predicted locations of this protein in cellular organelles could be in chloroplast thylakoid space. Alignment of amino acid sequences of rice IPMI small subunit revealed that the protein shared high homology with those of algae and cyanobacteria (Fig. 2) [3,5,8,14]. Moreover, phylogenic analysis showed that rice IPMI was grouped with that of green algae, cyanobacteria, eudicots and green sulfur bacteria (Fig. 3). This phylogenic

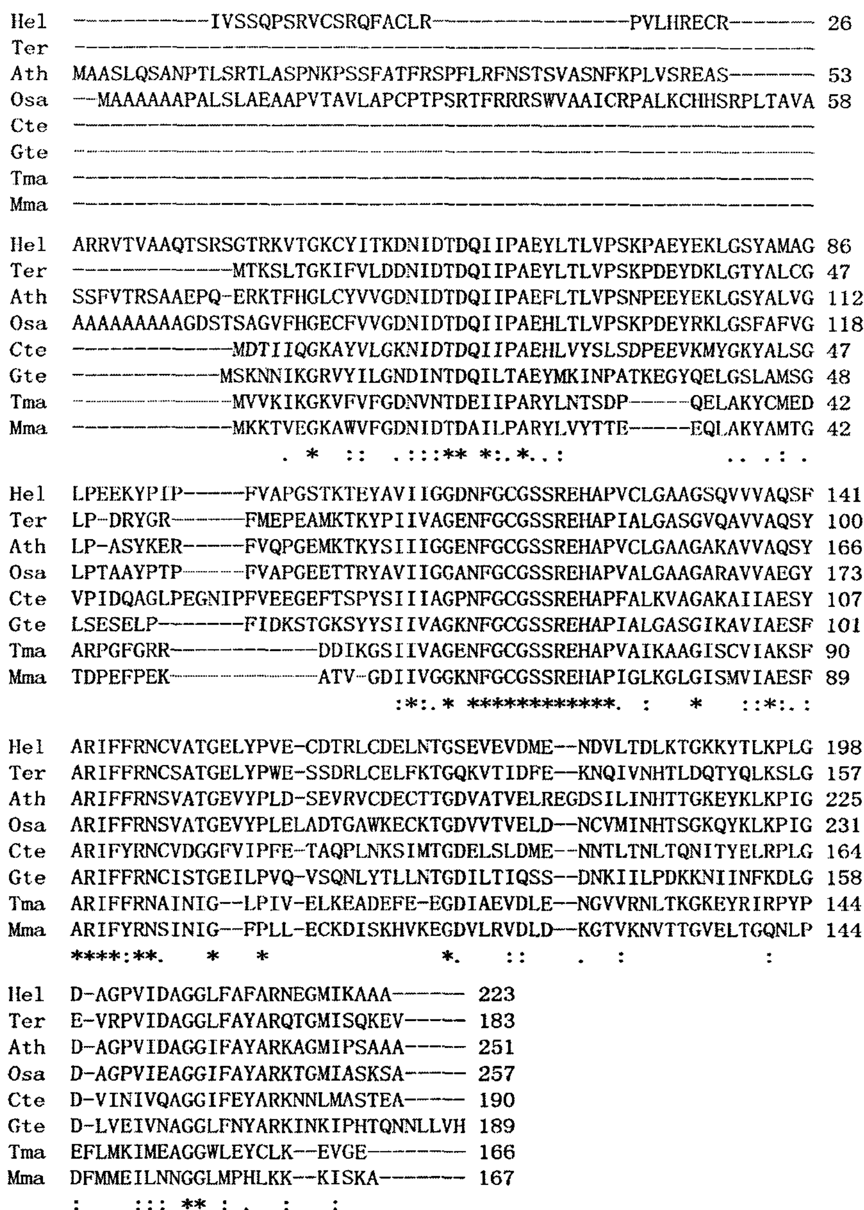

Fig. 2. Multiple sequence alignment of amino acid sequences of IPMI small subunits. Homologus proteins were aligned. Osa is AAQ67235.1 [*Oryza sativa* (japonica cultivar-group)] (monocots); Ath is AAL34203.1 (*Arabidopsis thaliana*: eudicots); Hel is AAU93934.1 (*Helicosporidium sp. ex Simulium jonesi*: green algae); Ter is ZP_00324779.1 (*Trichodesmium erythraeum*: cyanobacteria); Cte is NP_661513.1 (*Chlorobium tepidum* TLS: green sulfur bacteria); Gte is YP_063541.1 (*Gracilaria tenuistipitata* var. liui: red algae); Tma is MSB8, NP_228365.1 (*Thermotoga maritime*: thermotogals); and Mma is NP_987256.1 (*Methanococcus maripaludis*: euryarchaeots). Numbers indicate amino acid positions in the presented proteins. Consensus sequences and homologous sequences are marked (*) and (.), respectively.

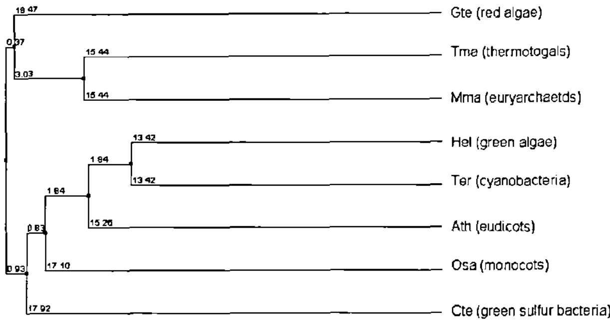

Fig. 3. Phylogeny of IPMI small subunits. Osa is AAQ67235.1 *Oryza sativa* (monocots); Ath is AAL34203.1 (*Arabidopsis thaliana*: eudicots); Hel is AAU93934.1 (*Helicosporidium sp. ex Simulium jonesi*: green algae); Ter is ZP_00324779.1 (*Trichodesmium erythraeum*: cyanobacteria); Cte is NP_661513.1 (*Chlorobium tepidum* TLS: green sulfur bacteria); Gte is YP_063541.1 (*Gracilaria tenuistipitata* var. liui: red algae); Tma is MSB8, NP_228365.1 (*Thermotoga maritime*: thermotogals); and Mma is NP_987256.1 (*Methanococcus maripaludis*: euryarchaeots). Each distance is indicated on the line.

analysis suggests that *OsLeuD* (AY363174) in genomic DNA is chloroplast origin.

OsLeuD allele on chromosome 2 in *Oryza sativa* Japonica group

Further genomic BLAST search in the Rice Genomic Program (RGP; http://rgp.dna.affrc.go.jp) [6] revealed that *OsLeuD* cDNA was a transcript of genomic DNA sequence of accession number AP005006 (P0519E06), which was located on the 109.3 cM of chromosome 2 of the high density linkage maps of *O. sativa* cv. Nipponbare of japonica genome [6]. BLAST analysis of view gene in GRAMENE database (http://www.gramene.org) revealed that *LueD* gene is located in 26.45 Mb of genomic DNA (accession AP004676_3715_131955) on chromosome 2 of *Oryza sativa* (japonica cultivar-group) (Fig. 4A). *OsLeuD* mRNA is the same as a CDS of a predicted coding sequence of The Institute for Genomic Research (TIGR) model 3642.m00172. *LeuD* gene of rice is closely linked to between RM7245 and RM5706 of the Rice simple sequence repeat (SSR) marks (Fig. 4A). Since a cDNA coded for 3-isopropylmalate dehydratase small subunit had been experimentally cloned it needed for approve whether the transcripts of the CDS on the rice genomic DNA on chromosome 2 was the same transcript as the cloned cDNA by performing genomic Southern blot analysis. On the genomic DNA sequence AP004676, a 3.1 kb of DNA fragment of *Eco*RI restriction

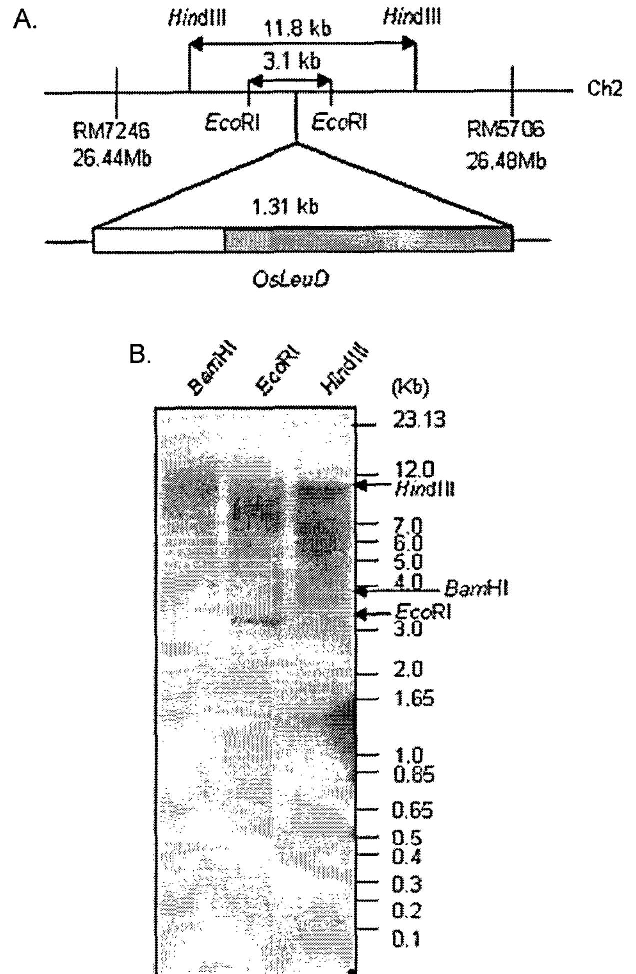

Fig. 4. Map and genomic southern blot analysis of *OsLeuD*. A, Constitution of *OsLeuD* gene in genomic DNA of chromosome 2 in rice *Oryza sativa* Japonica. RM7245 and RM5706 are rice simple sequence repeat (SSR) marks. Restriction enzymes are indicated. B, Genomic DNA in each lane was digested by specific restriction enzymes including *Bam*HI, *Eco*RI and *Hin*dIII.

sites and a 11.8 kb DNA fragment of *Hin*dIII sites were identified (Fig. 4B). Southern blot analysis revealed that these predicted fragments of restriction enzymes were exactly identified as 3.1 kb fragment in the *Eco*RI-digested DNA and as 11.8 kb fragment in the *Hin*dIII-digested DNA (Fig. 4B, *Hin*dIII). This indicated that the *OsLeuD* cDNA was the transcripts of a predicted ORF of genomic DNA BAC clone, AJ037662. Further BLAST search revealed that the 3-isopropylmalate isomerase large subunit has been identified (Accession number XM_463952), indicating that 3-isopropylmalate isomerase of plant consists with large and small subunits like that of photosynthetic bacteria, dislike monomeric 3-isopropylmalate isomerases of yeast or

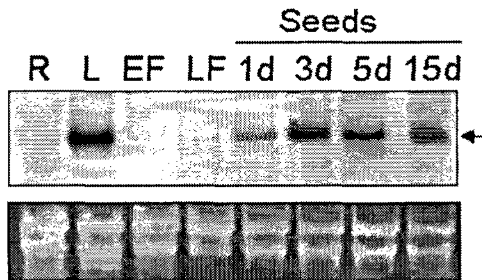

Fig. 5. Expression patterns of *OsLeuD* gene in selected rice organs indicated as R, root; L, leaf; EF, early flower; LF, late flower; and 1d, 3d, 5d and 15d, days after pollination in seed developing seeds.

animal. This suggests that some components of leucine biosyntesis of plants were originated from genome of chloroplast.

OsLeuD gene expression

Based on RNA blot analysis, *OsLeuD* gene expression was developmentally regulated. As shown in Figure 5, the transcripts of *OsLeuD* were abundant in leaves and panicles after pollination, but low in flower before pollination. As we are generally known that leaves are most metabolic active organs for producing organic carbons by photosynthesis in chloroplasts. For photosynthesis, the components of photosynthetic proteins such as RuBisCo, chlorophyll A/B binding proteins and other thylakoid membrane binding proteins are continually synthesized. Therefore, amino acids such as leucine are required for accomplish theses protein synthesis in chloroplasts. As we observed in RNA blot analysis in Figure 5, high level of *OsLeuD* gene expression in leaves is for increasing leucine biosynthesis for supplying leucine to protein synthesis in chloroplasts.

It is interesting that the level of the *OsLeuD* gene expression was markedly induced in developing seeds (Fig. 5). In the initial stage of seed development such as milky and dough stages in rice, the levels of protein synthesis and the rate of metabolism are usually accelerated. For these seed developing stages, recruitment for amino acids and components of protein synthesis are mostly required. Therefore, we may understand that inducible pattern of the *OsLeuD* gene expression is for supplying amino acids valine, leucine, and isoleucine for protein synthesis during seed development.

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초록 : 벼(*Oryza sativa* L.)의 *leuD* 유전자

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식물의 루이신 생합성에 관여하는 3-isopropylmalate isomerase (IPMI) (EC 4.2.1.33) 효소의 소단편을 암호화는 Leucine D유전자를 벼로부터 분리하고 *OsLeuD*유전자로 명명하였다. *OsLeuD*유전자는 257개의 아미노산을 암호화하고 있으며 cyanobacteria의 IPMI 단백질과는 약58% 그리고 green sulfur bacteria들의 IPMI 단백질과는 약48%의 상동성을 갖고 있었다. 벼의 *OsLeuD*유전자는 japonica벼 (*Oryza sativa* L.)의 2번 염색체의26.45 Mb의 위치로서 109.3 cM 거리에 좌위하고 있었다. *OsLeuD*유전자는 잎과 성숙하는 종자에 많이 발현이 되었으므로 대사가 급증하는 발생단계에 발현이 조절되는 것으로 여겨진다. *OsLeuD*유전자와 단백질은 균류와 yeast 보다 광합성 박테리아의 유전자와 높은 동질성을 보이는 것으로 보아 *OsLeuD*유전자는 식물의 엽록체 유전자 genome 에서 기원하여 핵 genome으로 이동 진화된 유전자로 추측된다.