

การศึกษากลุ่มยีน Aldo-keto reductases ในข้าว (*Oryza sativa*, Indica)

## STUDIES OF OF ALDO-KETO REDUCTASES GENE IN RICE (*ORYZA SATIVA*, INDICA)

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**บทคัดย่อ:** ยีนในกลุ่ม aldo-keto reductase (AKR) เป็นเอนไซม์เกี่ยวข้องกับการเมแทบอลิซึมของโพลีไฮดรอกซีคาร์บอนและแอลดีไฮด์โดยใช้ NADPH หรือ NADH เป็นโคแฟกเตอร์ สามารถใช้สับสเตรตที่หลากหลายเกี่ยวข้องในขบวนการเมตาบอลิซึมที่สำคัญรวมทั้งทำหน้าที่กำจัดแอลดีไฮด์ที่เป็นพิษต่างๆ สำหรับ AKR1B1 ซึ่งเป็นเอนไซม์ที่พบในมนุษย์เกี่ยวข้องกับการกำจัดแอลดีไฮด์ที่เป็นพิษและได้รับการศึกษาอย่างกว้างขวาง เมื่อทำการเปรียบเทียบ AKR1B1 กับยีนกลุ่ม AKR ในข้าว *Oryza sativa* (indica cultivar-group) พบว่ายีน *OsI\_04426* *OsI\_04428* และ *OsI\_04429* มีความใกล้เคียงกันกับยีนดังกล่าว และอาจเกี่ยวข้องกับการกำจัดสารพิษในข้าว จากการศึกษาการแสดงออกในเนื้อเยื่อต่างๆ ของข้าว โดยวิธี RT-PCR พบว่ายีนทั้ง 3 มีการแสดงออกที่แตกต่างกันในแต่ละเนื้อเยื่อ โดย *OsI\_04428* มีการแสดงออกในทุกเนื้อเยื่อ ในขณะที่ *OsI\_04426* และ *OsI\_04429* มีการแสดงออกน้อยในต้นอ่อนและดอกตามลำดับ ผลจากการโคลนและหาลำดับเบสของยีนทั้งสามพบว่า ลำดับเบสมีความแตกต่างกันจากการทำนายของฐานข้อมูล โดยมีความคล้ายคลึงกับ AKR4C9 จากต้น *Arabidopsis* และ AKR4C7 จากข้าวโพด เมื่อผลิตรีคอมบิแนนท์โปรตีนของ *OsI\_04428* โดยใช้เวกเตอร์ pET28-b โดยมีส่วนปลาย N-terminal เชื่อมต่อกับฮิสทีดีนแทกซ์ พบว่าสามารถผลิตรีคอมบิแนนท์โปรตีนได้ แต่โปรตีนดังกล่าวจับตัวเป็น inclusion bodies เมื่อนำไปละลายใน Tris-HCl pH 11 แล้วนำไปทำให้บริสุทธิ์โดย Ni-Colum แล้วทดสอบการจับกับ NADPH พบว่าค่า  $K_d$  เท่ากับ  $13.34 \pm 3.97 \mu\text{M}$  มากกว่าของ AKR4C9 ประมาณ 53 เท่า แสดงว่าโปรตีนถูกผสมอาจสูญเสียกิจกรรมของเอนไซม์

**Abstract:** Aldo-keto Reductase (AKR) is an enzyme superfamily metabolizing aldehydes/ ketones using NADPH or NADH as cofactor. AKRs are capable of metabolizing a broad range of substrates, as well as some toxins. Aldose reductases (AKR1B1) from man is involved in detoxification processes and therefore has been studied as some are exploited as drug target. Searching for AKR1B1 homolog in rice (indica cultivar-group) from protein database found that *OsI\_04426* , *OsI\_04428* and *OsI\_04429* shared highest identities (approximately 40%) and thus, were selected for further studies by investigating their expression patterns and kinetic properties. The results showed that their expression profiles were different to some extent. In flowers, *OsI\_04426* and *OsI\_04428* were strongly expressed, whilst the *OsI\_04429* had very low expression level. In seedlings, *OsI\_04426* was slightly detected, whilst *OsI\_04428* and *OsI\_04429* were found abundantly. The cloning and sequencing results showed that their sequences were different from those predicted from database. *OsI\_04428* recombinant protein was produced by pET28-b and therefore has His-tag in its N-terminal and was detected in the pellet fraction (inclusion bodies) in large amount. The inclusion bodies were resuspended in Tris-HCl pH 11 and subsequently purified on Ni-column. The activity of refolded recombinant *Osi\_004228* was tested by cofactor binding assay. The

result showed that its  $K_d$  is  $13 \pm 4 \mu\text{M}$ , which is higher than that of AKR4C9 by approximately 50 fold. This suggested that the enzyme lost some activity after refolding.

**Introduction:** Aldo-keto Reductase (AKR) is an enzyme superfamily metabolizing aldehydes/ ketones to the corresponding alcohols using NADPH or occasionally NADH as cofactor. All AKRs so far analyzed share almost the same three-dimensional conformation, with approximately 320 amino acids and eight  $\alpha/\beta$  motifs, forming a barrel-like structure. There are also three loops on the top of the enzyme (denoted A, B and C) which are vital for substrate determination. AKRs are able to metabolize a broad range of substrates including steroids, sugars, prostaglandins, chalcones, aliphatic/aromatic aldehydes, as well as some toxins such as aflatoxin. Mammalian AKR(s) are most widely studied as some are exploited as drug targets. One of which is aldose reductase, an enzyme producing sorbitol which serves as osmolyte in kidney cells by metabolizing glucose to its alcoholic forms. In addition, aldose reductase is also involved in detoxification process of several toxic aldehydes generated during lipid peroxidation i.e. HNE. Thus, according to its functions, it may be said that aldose reductase is a stress-protective enzyme in mammals. In attempts to identify plant AKR(s) most related to aldose reductase, it was found that AKR4C whose members are implicated in several stress responses, e.g. cold, water-deficit, osmotic stress, and drought, was the group that shared highest homology to the mammalian enzyme. Over-expression of an AKR in alfalfa (*Medicago sativa*) resulted in drought-tolerance in the plants, suggesting its functions in stress protection. However, so far their actual roles are unclear. The aims of this study are to identify aldose reductase homolog in rice (*Oryza sativa*) and investigate their functions. These were carried out by the following: A) cloning and sequencing the genes B) Analyze their expression patterns C) making recombinant enzymes and study their kinetic properties. In addition, molecular modeling was employed to study the structural differences between the mammalian and plant aldose reductases.

**Methodology:** Rice protein sequences annotated as aldo-keto reductases were obtained by searching GENBANK database. Multiple alignments were done by CLUSTALX and phylogenetic tree was constructed using TreeView. The sequence of AKR1B1, a human aldose reductase (gi|46015262), was selected as a representative of mammalian aldose reductase. The prediction of gene-splicing and coding region was done by GENESCAN. The general properties of the recombinant proteins were predicted by PROTPARAM. The 3D structure of the rice Osi\_04228 was modeled on SWISS-PDB Viewer version 3.7 using AKR4C9 (3H7U) as the template. Rice total RNA was isolated according to the manufacturer's protocols (Plant Total RNA Mini Kit, GENE AID). First Strand cDNA is synthesized using First Strand cDNA Synthesis Kit (New England Biolab). Each reaction contained 800  $\mu\text{g}$  of total RNA. Tissue distribution analysis was carried out on flowers, stems, roots, leaves and seedlings by method of RT-PCR using primer pairs designed for full-length coding sequence. The amplification products were electrophoretically analyzed through 1% agarose gels and visualized under a UV transilluminator after ethidium bromide staining. The coding sequences of AKRs was amplified, gel-eluted, and ligated into pGEM-T easy and subsequently transformed into *E. coli* (DH5 $\alpha$ ) by heat shock. Transformants were selected on LB agar containing ampicillin (50 $\mu\text{g}/\text{ml}$ ), IPTG (100mM) and X-gal (5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) (80 $\mu\text{g}/\text{ml}$ ), by blue-white selection and colony-PCR screening, before

several positive clones were submitted to DNA sequencing (Pacific Science Co.,LTD.). The correct nucleotide sequence of each gene was concluded from multiple alignments using at least three sequencing results. Correct fragment was sub-cloned via *Nde I/Xho I* sites to pET28-b, an expression vector allocating six histidine codons for fusion of the His-tag at the N-termini of target proteins, using *E. coli* (DH5 $\alpha$ ) as the host strain. In attempts to make recombinant enzyme, the *Osi\_04228*- pET28-b were transformed into *E. coli* strains BL21(DE3) and Rosetta by heat shock method. Then, 400  $\mu$ l of saturated culture of each strain was added to a 400 ml fresh LB broth containing 30  $\mu$ g/ml kanamycin. The cultures were shaken for 3.5 h at 37°C, 220 rpm (O.D.  $\approx$  0.5-0.8) before adding IPTG to a final concentration of 1mM. Then, the culture was shaken further at 37°C for 30 min, before that the recombinant enzyme was allowed to expressed at 15°C for 16 h. The cells were harvested by centrifugation (5min, 3000g, 4°C) and resuspended in cold sonication NaP buffer (50 mM NaP, 300mMNaCl, Trital-X 100 1%, pH8.0). The cells are broken by adding 1 mg of lysozyme and protease inhibitor, and then sonicating on ice at three bursts with 20 sec each and 20 s intervals. The lysate was centrifuged at 4°C 20,000 rpm for 10 min.The pellet was resuspended in Tris-HCl pH 11 and subsequently dialysed in potassium phosphate buffer pH7 until pH is dropped to pH8. The solution is incubate in Ni resin. The solution containing Ni resin was loaded onto an empty plastic column. The mixture was washed with 6 ml of sodium phosphate buffer (50 mM NaP, 300mMNaCl, pH8.0). The refolded protein was eluted by 1 ml of elution buffer (50 mM NaP, 300mM ,NaCl, pH8.0 add imidazole 250 mM) and collect separately. The activity of refolded recombinant *Osi\_004228* was tested by cofactor binding assay using fluorescent titration. Fluorescence titration was carried out using excitation wavelengths at 280 and emission at 340 nm. Small amounts of cofactor were added incrementally to the reaction and the observed fluorescence values were recorded after mixing. The Kd was then calculated by plotting the percentage drop in fluorescence against the cofactor concentration. The curves were fitted to a hyperbola using Enzfitter. The reaction consisted of 700ul of potassium phosphate buffer pH7 , 10 ul of enzyme and 0.5 ul of 0.3 mM the NADPH cofactor.

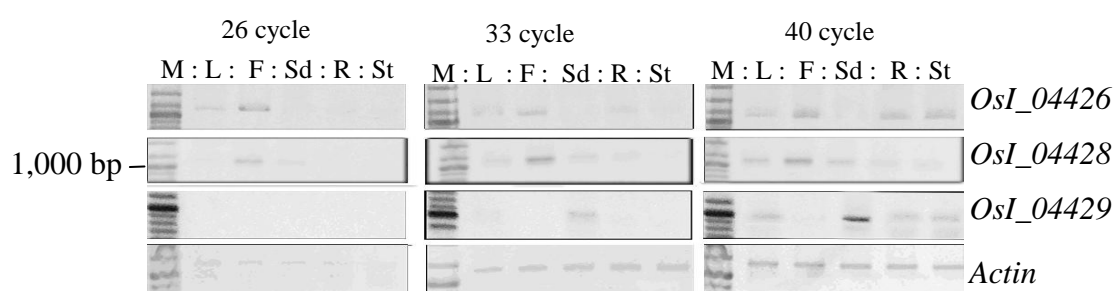
## **Results, Discussion and Conclusion:**

**1. Classification of rice aldose- keto reductases :** Currently, based on amino sequence identity, there are more than 120 proteins annotated as members of aldo-keto reductase superfamily, distributed throughout 14 families (AKR1–AKR14) . Members within a given family share less than 40% amino acid sequence identity with other families, but members within the same subfamily have greater than 60% sequence identity. Searching protein database from NCBI for the presences of AKR(s) in rice (*Oryza sativa* Indica Group) found that there were 47 sequences of rice putative AKR(s) available. When using these sequences aligned with AKR1B1, initially, *Osi\_04333* *Osi\_04335* *Osi\_04336* *Osi\_019521* and *Osi\_019522* genes have % amino acid identity around 40% . However, after the database was updated in November, 2008 .The locus of genes have been changed to *Osi\_04426* , *Osi\_04427* , *Osi\_04428* , *Osi\_04429* *Osi\_20196* and *Osi\_20197*, respectively. However, the sequences derived from the database were incorrect and resulted in failure in the initial attempt. These were accomplished afterward by using GENESCAN to re-predict spicing of each gene from genome sequence basing on homology to AKR4C9 whose C-termini ended with “WDGEI”. Comparison of amino acid sequence identities was shown in table1.

**2. Tissue-specific expression pattern of *OsI\_04426*, *OsI\_04428* and *OsI\_04429*:** Due to the five genes were located in different chromosomes, in which *OsI\_04426*, *OsI\_04428* and *OsI\_04429* were clustered on rice chromosome I, whilst the other two, *OsI\_20196* and *OsI\_20197* were located on chromosome V. This study would only focused on those on chromosome I for their distribution in rice organs using semi-quantitative RT-PCR analysis. The expression of these genes were detected in every tissues used in this study including flowers, stems, roots, leaves and seedlings. However, their expression profiles were different to some extent. In flowers, *OsI\_04426* and *OsI\_04428* gene were strongly expressed, whilst the *OsI\_04429* gene had very low expression level. In seedlings, *OsI\_04426* was slightly detected, whilst *OsI\_04428* and *OsI\_04429* were found abundantly (Figure 1).

**Table 1.** Showing % amino acid sequence identities between *OsI\_04426*, *OsI\_04428*, *OsI\_04429* with *AKR4C9* and *AKR1B1*.

Amino acid	<i>OsI_04426</i>	<i>OsI_04429</i>	<i>AKR4C9</i>	<i>AKR1B1</i>
<i>OsI_04428</i>	89%	67%	70%	45%
<i>OsI_04426</i>		68%	68%	45%
<i>OsI_04429</i>			64%	39%
<i>AKR4C9</i>				43%



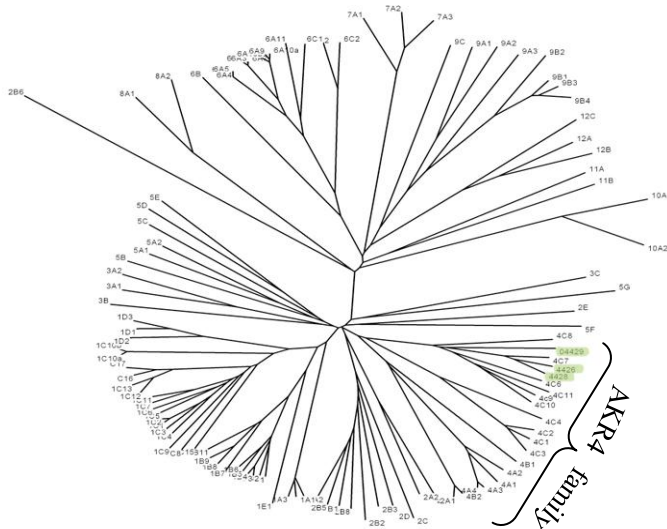
**Figure 1.** Expression pattern of *OsI\_04426*, *OsI\_04428* and *OsI\_04429* in leaves(L), Seedlings (Sd),flowers (F), stems(St) and roots(R). Actin was used as the control.

**3. Cloning, expression rice aldose-keto reductases enzyme :** The PCR products of the *OsI\_04426*, *OsI\_04428* and *OsI\_04429* were ligated to pGEM-T easy. At least three clones with presence of the gene fragment were submitted to DNA sequencing. The correct coding sequences were submitted to GENBANK database. Their accession numbers, together with some general properties of the proteins from prediction were shown in table 1.

**Table 1.** Accession numbers and some properties of the new versions *OsI\_04426*, *OsI\_04428*, *OsI\_04429*.

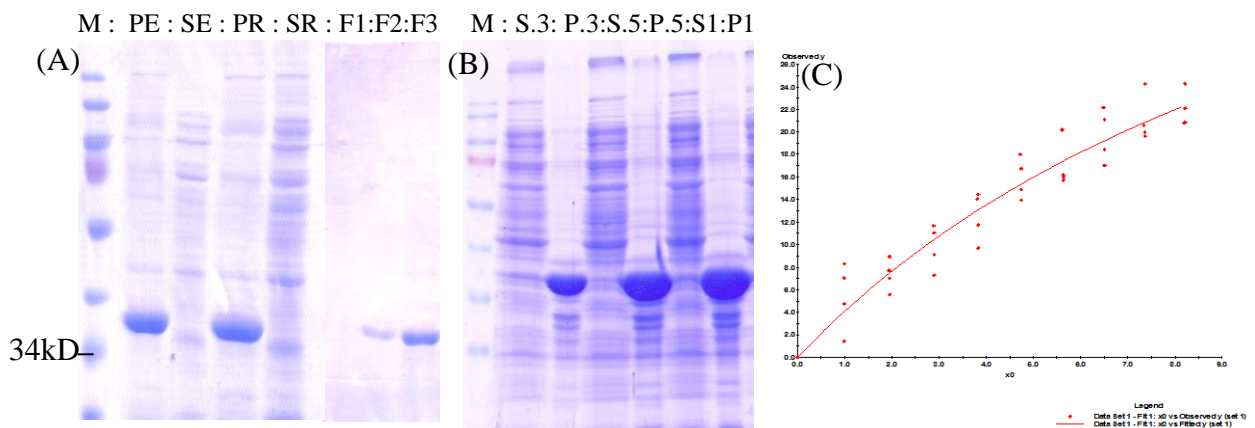
Gene	Name	Ac. No.	nucleotides	Amino acid	pI	Molecular weight
<i>OsI_04426</i>	AKR1	GQ227708	936 bp	311 aa	5.89	34640.6 Da
<i>OsI_04428</i>	AKR2	GQ227709	936 bp	311 aa	6.37	34501.5 Da
<i>OsI_04429</i>	AKR3	GQ219963	915 bp	304 aa	5.85	33435.3 Da

Based on amino acid identities and phylogenetic analysis (Figure 2.), the three rice AKRs were members of AKR4C(s), a subfamily found restrictively in plant kingdom. Two of their closest relatives have been characterized so far, were AKR4C7 from maize (*Zea mays*) with 67-87% identity, following by AKR4C9 from *Arabidopsis thaliana* (64-70% identity).



**Figure 2.** Phylogenetic tree showing evolutionary relationship of the *OsI\_04426*, *OsI\_04428* and *OsI\_04429* to current members of the AKR superfamily.

In the attempt to produce recombinant enzyme, *OsI\_04428*-pET28-b-BL21(DE3) and *OsI\_04428*-pET28-b-Rosetta were cultured as described. From SDS-PAGE analysis, the expression of the recombinant protein, with expected molecular mass, was detected in the pellet fraction (inclusion bodies) of both strains (Figure 3A). Optimization done by dropping the concentration of IPTG did not improve the solubility of the enzyme (Figure 3B). In attempts to refold the protein, the pellet of inclusion bodies were resuspended in Tris-HCl pH 11 and subsequently purified by Ni-resin. The activity of the protein was monitored by cofactor binding test done by fluorescent titration to analyze for its  $K_d$ . The  $K_d$  was  $13 \pm 4 \mu\text{M}$ , higher than that of AKR4C9 by approximately 50 fold. This suggested that the enzyme lost some activity after refolding.



**Figure 3** A) Expression of rice *OsI\_04428* protein analyzed by SDS/10% polyacrylamide gel stained by coomassie blue. Lanes 2 and 3, pellet and supernatant fraction of *E. coli* BL21 (DE3); lane 4 and 5, pellet and supernatant fraction of *E. coli* Rosetta. F1-3 is elution fraction. Sizes of molecular mass standard (Fermentas) in kDa are shown in the first lane. (B) Expression of the recombinant *OsI\_04428* in BL21 (DE3) using a variation of IPTG concentration (0.2, 0.4, 0.5 mM). Lanes 1, Marker lane 2 to lane 6, the supernatant and pellet with IPTG 0.2 mM, 0.3 mM, 0.5 mM. (C) The graph shows % drop fluorescent compared to NADPH concentration. Technology of Thailand

**4. Modeling of OsI\_04428 protein on AKR4C9:** The model of OsI\_04428 protein was constructed by using AKR1B1 and AKR4C9 structures as the templates. AKR1B1 was chosen as it is most well-known aldose reductase; and for AKR4C9 as it shared highest identity to the rice one and its 3D structure is available in protein databank. From the comparison of the three enzymes (Figure 4.), the overall structure of OsI\_04428 was similar to that of AKR4C9, except for its N-termini was shorter and was more similar to AKR1B1. However, all loops (A, B and C) of OsI\_04428 were shorter than AKR1B1 as also did AKR4C9. This can be seen clearly by the superimposition of the two proteins (Figure 5.). ↓

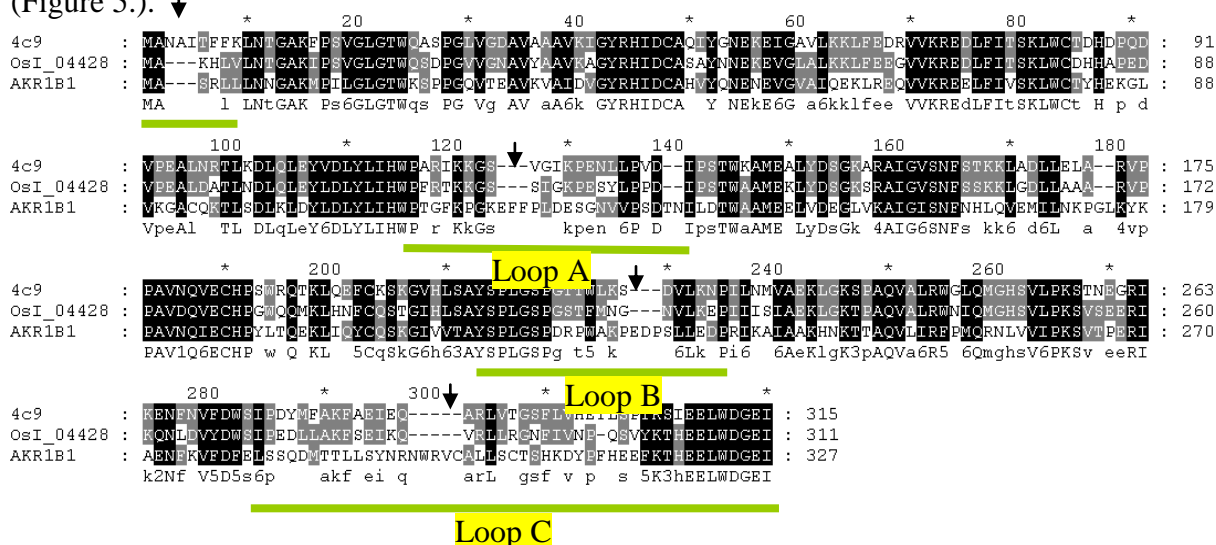
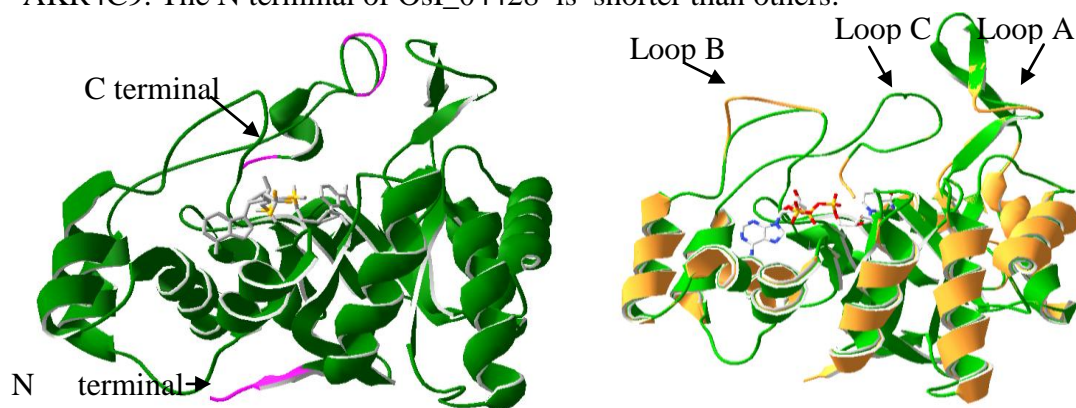


Figure 4. A sequence alignment of AKR1B1, AKR4C9 and OsI\_04428 showing deletions (arrows) in region of all loop versus AKR1B1 and deletion in region of loop C versus AKR4C9. The N terminal of OsI\_04428 is shorter than others.



**Figure 5.** Superimposition of ribbon models of OsI\_04428 (green), AKR1B1(yellow), AKR4C9 (pink). The fold of OsI\_04428 are almost the same to AKR4C9

**References:**

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**Keywords:** rice , aldo-keto reductase , expression pattern.