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cDNA Cloning and Characterization of A Novel Gene Differentially Expressed in Developing Seeds of High-Oleate Safflower (*Carthamus tinctorius* L.)

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Abstract

Transcriptomes of the developing seeds of high-oleate and high-linoleate strains of safflower (*Carthamus tinctorius*) were compared using a fluorescent differential display technique. Two cDNA fragments (O1-1 and O2-3) were identified as the bands originated from the genes highly or specifically expressed in the developing seeds of the high-oleate safflower. A cDNA clone, *CTOS-1*, encoding a novel protein with an isoprenoid binding site at the C-terminus was isolated by screening a cDNA library constructed from the developing seeds of high-oleate safflower using the O2-3 fragment as a probe. Northern blot analysis indicated that *CTOS-1* gene expresses only in the developing seeds of the high-oleate safflower. The transcript cannot be detected either in the developing seeds of the high-linoleate safflower nor in the leaves, stems and flower buds of the two strains. Southern blot analysis using the *CTOS-1* cDNA as a probe indicated the presence of restriction fragment length polymorphism between the high-oleate and high-linoleate strains.

Safflower (*Carthamus tinctorius* L., Compositae) is an important oilseed crop cultivated in Asia and North America (Knowles, 1989). The flowers have also been used as a crude drug in Chinese traditional ("Kanpo") medicine. The seed oil of safflower was originally characterized by its high linoleic acid content and was used as edible polyunsaturated oil. However, high linoleic oil is relatively unstable to auto-oxidation and excessive intake of linoleic acid was pointed out to be a major risk factor for such diseases as arteriosclerosis and allergic hyper-reactivity. Then, high oleic safflower oil has been favorably utilized as a superior cooking oil. High-oleate strain (HO-strain) of safflower was established by selection breeding starting from original safflower containing high linoleic oil (HL-strain) (Knowles, 1972). One major gene (*ol*) and some other genes were reported to be involved in regulation of fatty acid composition of the safflower oil (Fernández-Martinez *et al.*, 1993).

In a previous paper (Huang *et al.*, 1997), we examined the effect of various dietary vegetable oils on the mean survival time of stroke-prone spontaneously hypertensive (SHRSP) rats, and found unexpectedly that SHRSP rats fed a diet supplemented with high-oleate safflower oil had significantly shorter mean survival time than those fed a high-linoleate safflower oil diet. Furthermore, a free fatty acid fraction obtained by lipase-hydrolysis of the high-oleate oil did not shorten the survival time (Miyazaki *et al.*, 1998). These results indicate that the survival time-shortening effect of the high-oleate safflower oil is not due to its high oleic acid content, suggesting the presence of an unknown factor(s) toxic to SHRSP rats in the high-oleate safflower oil.

As one of our efforts towards identification and characterization of the presumed toxic component(s) in the high-oleate safflower oil, we tried to isolate cDNA clones differentially expressed in developing safflower seeds of the HO-strain using a fluorescent differential display (FDD), the results of which are described in the present communication.

Seeds of both HO- and HL-strains of safflower were obtained from Ohta Oil Mill Company, Ltd., Okazaki, Japan, and the plants were cultivated in the Medicinal Plant Garden of Nagoya City University. The date of the first emergence of a petal from a flower bud was recorded as onset of anthesis, and the developing seeds were collected 5, 10, 15, 20, 25 and 30 days after anthesis. The seed oil was extracted with *n*-hexane and its fatty acid composition was determined by GLC after hydrolysis of the extract with sodium methoxide (data not shown). The oil content of the seeds began to

increase rapidly 20 days after anthesis and reached a maximum 30 days after anthesis in both the HO- and HL-strains. The fatty acid composition characteristic to each fatty acid strain was also observed 20 days after anthesis and thereafter. Based on these results, we prepared total RNA from the seeds collected 20 days after anthesis using TRIZOL Reagent (Gibco BRL). Single strand cDNA was synthesized from the total RNA (2.5 μg) using a Superscript II reverse transcriptase (Gibco BRL) and three different fluorescein labeled 3'-anchored oligo(dT) primers (5'-fluorescein-$GT_{15}N$; N is either G, C or A). Fluorescent differential display screening was carried out essentially as described by Kuno *et al.* (2000). For northern hybridization analysis, total RNAs (20 μg) were electrophoretically separated on a 1.2% agarose gel containing formaldehyde and transferred to a Hybond N+ membrane (Pharmacia Amersham). Labeling of the probes with digoxigenin was carried out using PCR DIG Probe Synthesis kit (Roche Molecular Biochemicals), and hybridization and chemiluminescent detection of the digoxigenin-labeled probes were performed according to the manufacture's instructions.

Using the three different 3'-anchored oligo(dT) primers, each in combination of 10 arbitrary 12-mer primers, we screened about 2,100 cDNA bands and identified 11 fragments as candidate cDNAs differentially expressed in the developing seeds of the HO-strain. These bands were eluted from the gel, re-amplified, and cloned into pGEM-T vectors (Promega). By northern hybridization using the cloned cDNA fragments as probes, two fragments (O1-1 and O2-3) confirmed that the corresponding transcript level was higher in the HO-strain seeds than in the HL-strain seeds. Then, we tried to identify the full length cDNAs of the respective clones by library screening. The cDNA, *CTOS* (*Carthamus tinctorius* high-oleate strain specific gene)-*1*, was isolated by screening of λZAP II cDNA library (Stratagene) constructed from the developing seeds of the HO-strain of 20 days post-anthesis using the O2-3 fragment as a probe. Isolation of the full length cDNA corresponding to the O1-1 fragment has been unsuccessful so far by either library screening or RACE method.

The nucleotide sequence of *CTOS-1* was 2,301 bp long, which is roughly consistent with the transcript size estimated by the northern analysis, and contained an open reading frame of 2,097 bp encoding 699 amino acids forming a 81 kDa polypeptide (**Fig. 1**). The nucleotide and deduced amino acid sequences were used to search for similar sequences in the GenBank and SWISS-PROT databases, respectively, using Blast as a search engine, but exhibited no significant similarity to any known genes or proteins in the databases, suggesting that *CTOS-1* represents a novel gene. The CTOS-1 protein contains the reported specific binding site of an isoprenoid (a farnesyl or geranylgeranyl moiety), the Cys-A-A-X box where A is an aliphatic and most often hydrophobic amino acid and X is any amino acid placed at the carboxy terminus. Isoprenoid binding to this site has been considered to mediate the protein movement to cellular membranes as reported in yeast **a**-factors and mammalian Ras proteins (Rodríguez-Concepción *et al.*, 1999), and/or to modulate the protein function as recently reported for an *Arabidopsis* transcriptional factor APETALA1 (Yalovsky *et al.*, 2000).

To analyze *CTOS-1* mRNA accumulation during the seed development, total RNAs were prepared from the seeds collected at 5, 10, 15, 20, 25 and 30 days post-anthesis. As shown in **Fig. 2A**, the transcript level of *CTOS-1* started to increase rapidly between 15 days and 20 days after anthesis, reached a maximum at 20 days after anthesis and then gradually decreased in the HO-strain, whereas *CTOS-1* transcript was not detected throughout the seed development in the HL-strain. We also examined the organ distribution of *CTOS-1* mRNA (**Fig. 2B**). The *CTOS-1* transcript was present solely in the developing seeds of the HO-strain. It was present neither in any other organs of the HO-strain nor in any organs examined in the HL-strain. Thus, it is clear that the expression of *CTOS-1* is strictly limited to the developing seeds of the HO-strain.

The genomic DNAs from the HO- and HL-strains were subjected to the southern hybridization analysis to compare the genomic organization of *CTOS-1* gene. The total DNA (10 μg each) was digested with *Eco* RI, *Eco* RV, *Hae* III, *Nci* I and *Sty* I, all of which have no restriction site in *CTOS-1* cDNA, and subsequently applied to hybridization analysis using the digoxigenin-labeled cDNA as a probe. As shown in **Fig. 3**, multiple signals were detected in both of the two strains under stringent conditions. *Hae* III and *Sty* I digestions produced the third band in the HO-strain in addition to the two major bands present in the HL-strain. A restriction fragment length polymorphism between the two strains was also observed in the *Nci* I digestion. These results indicate the difference in the genomic organization of *CTOS-1* gene between the HL- and HO-strains, which may result in the strain- and organ-specific expression of the *CTOS-1* gene in the developing seeds of the high-oleate safflower.

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GAATCCTCAACCGGCATGTTCATCGGAATCATCATCAGGAGTTGGTTGATTTGTCTCATCGGGTTGAAGGGGCGGTTTTATGTGCCGATG  90
                                                                                          M
TTAATGTTGATCAAGCAGAAGATGGGAACGTTTTTCTGTCCGAAGATGAAGATAACATTTCTCAAACTATGGAGGAGGATGATGAGGGAT 180
L  M  L  I  K  Q  K  M  G  T  F  F  C  P  K  M  K  I  T  F  L  K  L  W  R  R  M  M  R  D
GTAAGTTCTACAAGCCTAATGTTGATGAATCAATACGACCGAAGTGGTGACGTTTTCCCAACCGTTGAAGCTGCAGAGAAGATGTATCGT 270
V  S  S  T  S  L  M  L  M  N  Q  Y  D  R  S  G  D  V  F  P  T  V  E  A  A  E  K  M  Y  R
AAGTACGCTAGTGCTGCTGGGTTTGATGTGCGCTTGTCAAACAAAAAAACAAACAAGTTTGGGATTACTATAGCTAGGTTCTTTGTTTGC 360
K  Y  A  S  A  A  G  F  D  V  R  L  S  N  K  K  T  N  K  F  G  I  T  I  A  R  F  F  V  C
AATAAGGAGGGTCATCCTACTCCGAAATTGTACGATTCACTAAATAAGAAATCTGGGGAGCGACGTCGACGCAACTCTAACCTTAAGAGG 450
N  K  E  G  H  P  T  P  K  L  Y  D  S  L  N  K  K  S  G  E  R  R  R  R  N  S  N  L  K  R
GCTGGGTGCATGGCATGCATGAAGGTTCATTATGTGAAGAGTATAGGTCGATATGAGGTTTATAAGTTTAATGAAAAACATAGTCATATG 540
A  G  C  M  A  C  M  K  V  H  Y  V  K  S  I  G  R  Y  E  V  Y  K  F  N  E  K  H  S  H  M
TTGTTTAGTGGTGATGAGATGTCATTATCTCGGTCAAATCGCGAACTTACTTTTGGTGATCAATGTAATGTTTTCAATGCGTGTGTAACG 630
L  F  S  G  D  E  M  S  L  S  R  S  N  R  E  L  T  F  G  D  Q  C  N  V  F  N  A  C  V  T
AAGGTTGGTGTGTCTAAGTCACATAGGTTGCGTAATATTAGTAAAGGGAATGTTGGTTTGTCGGGAGGAACAGTTAGAGATTACCAGAAC 720
K  V  G  V  S  K  S  H  R  L  R  N  I  S  K  G  N  V  G  L  S  G  G  T  V  R  D  Y  Q  N
TTTAAAAAGGATATGGTTACGTTTGTTGGCAACAAAGATGCAAAAATGCTCATAAATACGATGGTAAATCGGCAGAAAATTTCTCCACAG 810
F  K  K  D  M  V  T  F  V  G  N  K  D  A  K  M  L  I  N  T  M  V  N  R  Q  K  I  S  P  Q
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F  F  F  E  F  K  C  N  E  K  E  L  L  A  I  F  W  A  D  E  V  A  R  M  N  Y  R  E  F  G
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D  A  I  S  F  D  A  T  Y  R  T  Q  K  H  A  M  I  F  V  P  F  V  A  V  D  N  H  K  K  S
GTGGTGGTTGGTGCTGCATTGATTCGGAAGGAAAATGCTGATTACTTTACTTGGGTCTTGAATGCGTTTGTTAAAGCGCATGGTAGCCTA 1080
V  V  V  G  A  A  L  I  R  K  E  N  A  D  Y  F  T  W  V  L  N  A  F  V  K  A  H  G  S  L
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P  K  L  V  I  T  D  Q  C  P  A  M  K  Q  A  I  S  I  A  F  P  N  T  I  H  R  L  C  L  W
CACATTACCAAGAATGTAAAAAAGCAGGTTAGCGTCCATCTTGTGAAAAAAACGTCGTTTGTAGCCGACTGGAATAAGATGATTTGGAAT 1260
H  I  T  K  N  V  K  K  Q  V  S  V  H  L  V  K  K  T  S  F  V  A  D  W  N  K  M  I  W  N
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L  H  L  G  P  A  E  F  D  N  K  W  E  E  F  L  D  L  Y  G  L  T  G  V  S  W  F  N  H  M
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Y  E  I  R  E  S  W  I  P  A  F  F  K  D  T  P  M  S  G  L  M  K  T  T  S  R  S  E  S  I
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N  A  S  S  V  Y  T  R  K  V  F  F  E  V  Q  K  E  L  I  K  A  V  W  C  C  G  W  D  E  I
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V  D  R  I  P  D  K  Y  I  L  R  R  W  R  R  D  L  I  P  P  Q  W  L  P  A  R  V  R  Y  G
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E  V  D  V  E  K  E  R  L  M  A  R  A  F  A  A  V  D  R  M  I  G  R  V  R  N  E  K  D  V
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L  E  R  V  V  E  K  L  E  N  M  D  E  E  L  D  E  V  V  P  L  K  S  S  R  E  R  K  K  E
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K  R  L  K  G  V  R  E  R  V  D  E  E  A  K  K  P  K  R  L  C  R  T  C  N  D  F  V  R  H
GACTCTCGTAACTGCCCTATGCGTTGAAGGTCATTGTATATCTGGGTTTTACTTTTGTTTTGGTAGCTGGAAGTTTTCC TATAAATGGTT 2250
D  S  R  N  C  P  M  R  *
TCATACTGGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2301
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Fig. 1 The nucleotide and deduced amino acid sequences of *CTOS-1* cDNA obtained from the developing seeds of the high-oleate safflower. The C-A-A-X motif is boxed and the putative polyadenylation site is shown in italics. The sequence data will appear in the DDBJ/EMBL/GenBank databases under the accession number AB047859.

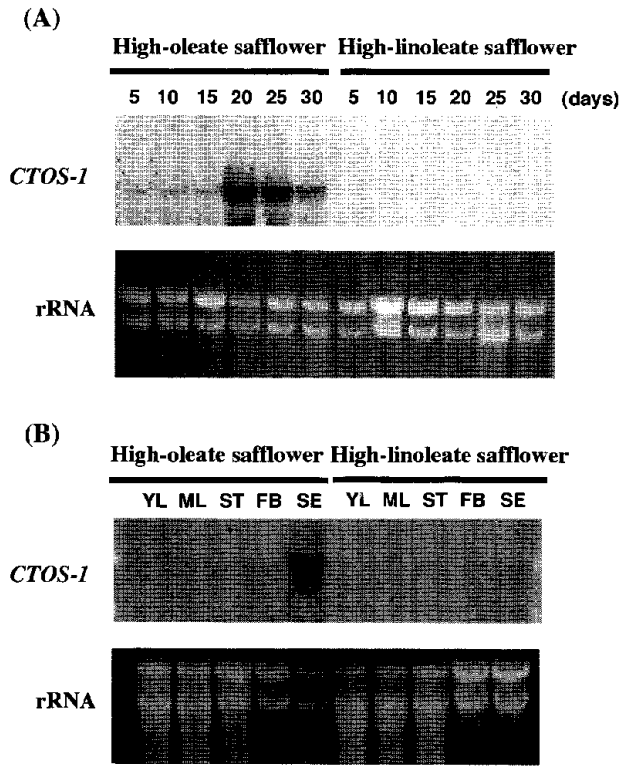

Fig. 2 (**A**) Time-course of the changes in abundance of mRNAs for CTOS-1 in the developing seeds of the high-oleate (left) and high-linoleate (right) safflower after anthesis. (**B**) Expression of the *CTOS-1* gene in various organs of the high-oleate (left) and high-linoleate (right) safflower. YL, young leaves; ML, mature leaves; ST, stems; FB, flower buds; SE, developing seeds (20 days after anthesis). Twenty μg of total RNAs were fractionated on a 1.2% agarose gel containing formaldehyde and then transferred to a nylon membrane. The membrane was allowed to hybridize with a digoxigenin-labeled *CTOS-1* cDNA.

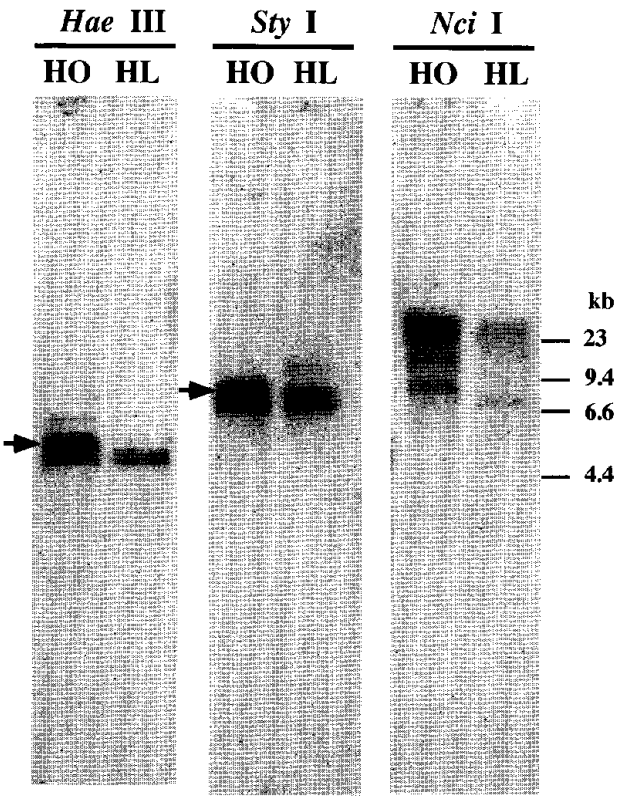

Fig. 3 Southern blot analysis of *CTOS-1* gene in the high-oleate (HO) and high-linoleate (HL) safflower. Ten μg of total DNAs prepared from the leaves were digested with *Hae* III, *Sty* I or *Nci* I, fractionated on a 1% agarose gel, and transferred to a nylon membrane. The membrane was allowed to hybridize with a digoxigenin-labeled *CTOS-1* cDNA. Arrows indicate the polymorphic bands present in the high-oleate safflower strain.

In conclusion, we have isolated a cDNA clone presumably encoding a novel prenylated protein which may locate in cellular membranes from the developing seeds of HO-strain of *Carthamus tinctorius*. It is unclear whether or not the gene product is involved in the toxic effect of the high oleic-safflower oil on SHRSP rats. However, it will be interesting to investigate the function of the gene and/or the gene product, because it is expressed in a highly organ- and fatty acid strain-specific manner.

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