Regulatory of polyunsaturated fatty acids accumulation and characterization of linolenic acid after germination of sunflower seed

Gian Paolo Vannozzi , Zohreh Rabiei, Sattar Tahmasebi Enferadi*

Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Udine, via delle Scienze 208, 33100 Udine, Italy
*To whom correspondence should be addressed (email thamasebi@uniud.it)

Abstract

The metabolism of triacylglycerols (TAG) and the conversion of them into new tri-glycerids and polar lipids like linolenic acid takes place during the first phase of development of *Hellianthus annuus* L. seed after seeding. Oleate desaturase and linoleate desaturase are two major enzymes responsible for the synthesis of these compounds. To determine whether enzymatic mechanisms function the same in catabolizing seed store lipids of normal and mutant sunflower lines, the modification of fatty acids composition of seeds and the first stages of growth (seeding - VE) at the controlled conditions of temperature were assessed. The higher increase of linoleic acid during all stages of developing in mutant line revealed more activity of oleic acid desaturase (Δ12-desaturase) in transforming oleic acid to linoleic acid probably due to the higher availability of the substrate of this enzyme in this line, however in the normal line its amount increased moderately. The activity of FAD2-3 in developing sunflower seeds is recognised even two days after seeding (DAS) in both lines, however linolenic acid accumulation starts seven DAS .

Introduction

Most TAG are hydrolyzed in the first 10 DAS depending on the action of lipase (Ichihara et al. 1980), But desaturation of fatty acids with increasing of unsaturated ratio involved with different types of desaturase; oleic acid desaturase (membrane-bound enzyme FAD2; EC 1.3.1.35) (Shanklin and Cahoon, 1998) and linoleic acid desaturase which are present at the endoplasmic reticulum in the cytosol (ER fad2 and ER fad3 respectively) and Plastid fad6 and Plastid fad7in the chloroplasts respectively, which are responsible of the modification of oleic acid to linoleic acid and linoleic acid to linolenic acid (Mollers, 2002). This enzyme catalyzes the first extra-plastidial desaturation in plants, converting oleic acid, preferentially esterified in the *sn*-2 position of phosphatidylcholine, to linoleic acid. The reaction involves the concomitant reduction of molecular oxygen to water and requires the presence of NADH, NADH-cyt *b*5reductase and cyt *b*5 as electron donor system (Smith et al., 1990).
FAD2 (FAD2; EC 1.3.1.35) (Shanklin et al., 1994) uses 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine as a substrate (Stymne and Appelquist 1978) and requires oxygen, NADH as a reductant (Vijay and Stumpf 1972), NADH:Cytb5 oxidoreductase and Cytb5 (Smith et al., 1990) to produce the polyunsaturated fatty acids linoleate (cis 9,12-18:2) and alpha-linolenate (cis 9,12,15-18:3). Those are major acyl groups found esterified to compounds present in plant membrane lipids.
It is very well known that environmental temperature during oilseed development modifies the proportion of linoleic acid Graces et al., (1992) and Martinez-Rivas et al., (2000) depending on the geographical area and year, resulting in an unwanted variation of the oleic to linoleic ratio in the final composition of the oil (Canvin et al., 1965). High temperatures decrease the linoleic acid content of oilseed oils. However, the extension of this temperature effect varies depending on the plant species. While in sunflower seeds the percentage of linoleic acid is highly influenced by growth temperature (Lajara et al., 1990)/ Indeed, a cold-inducible plastid ω-3 desaturasesgene (FAD7) has been isolated from Arabidopsis (Gibson et al., 1994). In Arabidopsis , microsomal ω-6 desaturase is encoded by a single FAD2 gene, and expression of this gene is not regulated by low growth temperature (Okuley et al., 1994).
Thus, we suggest the decrease in 18:1 content in seed lipids at low temperature may not be due to the transcriptionally induced or enhanced expression of ω-6 desaturase gene in soybean plants.
The FAD2-1 gene is strongly expressed in developing seeds, whereas the FAD2-2 gene is constitutively expressed in both vegetative tissues and developing seeds. Thus, the FAD2-2 gene-encoded omega-6 desaturase appears to be responsible for production of polyunsaturated fatty acids within membrane lipids in both vegetative tissues and developing seeds. The seed-specifically expressed FAD2-1 gene is likely to play a major role in controlling conversion of oleic acid to linoleic acid within storage lipids during seed development. In both soybean seed and leaf tissues, linoleic acid and linolenic acid levels gradually increase as temperature decreases. However, the levels of transcripts for FAD2-1, FAD2-2, and the plastidial omega-6 desaturase gene (FAD 6) do not increase at low temperature. These results suggest that the elevated polyunsaturated fatty acid levels in developing soybean seeds grown at low temperature are not due to the enhanced expression of omega-6 desaturase genes Heppard et al., (1996).
There are traceable quantities of linoleic acid (0.1% or trace) in sunflower seed whereas the abundant amount of linolenic acid was found in green tissues of plant, which associated with cellular and sub-cellular membranes (Salisbury and Ross, 1988).

High oleic sunflower (HOA) made a widespread industrial utilisation in food and non-food sectors. Technical application of HOA depends on its storage conditions. Humidity causes germination that make active enzymes responsible of transforming of oleic acid to linoleic acid. The aim of the study was to determine effect of early germination because of humidity in store on the expression of fatty acid desaturases (FAD) and consequently the production of linilenic acid as a poly unsaturated fatty acid that plays an important role in decreasing the stability of oil extracted from those germinated seeds. The expression of FAD was identified by cDNA synthesized from mRNA isolated of samples, alternatively, linolenic accumulation was measured with Gas chromatography.

Materials and Methods

Normal-type sunflower (*Helianthus Annuus* L. cv. HA-89) a restorer inbred line released by Dr. J. F. Miller at Fargo, ND, USA and high oleic sunflowers inbred line was R 978, a restorer inbred line selected at the University of Udine from material coming from Pervenets and Kindly supplied by prof. G.P.Vannozzi.
Seeds were cut horizontally on the basis of half-seed analysis Conte et al. (1986) then the portions containing the embryos planted for germination and its rest used for assessing of fatty acid composition by GC .
The total fatty acid composition was determined with a HRGC Mega 2, Fisons gaschromatography equipped with a split injection system and flame ionization detector (FID) a fused-silica capillary column 30m x 0.32mm i.d. and the percentage of fatty acids were obtained by integrating the peak with Chrom-Card, Fisons Ins. Software.
Samples of different stages of sunflower development (2 – 25 DAS) were harvested and used for RNA isolation. Total RNA and mRNA were made by using the SV Total RNA Isolation System Kits (Promega) and Qiagen OligoTex mRNA mini kit (Promega), respectively, according to the manufacturer′s instructions. cDNA prepared according Reverse Transcription System (Promega).
In this study a two way ANOVA Completely Randomized Design, with 10 replicates was used. The first factor (A) days after seeding (in seed, 2, 7, 12, 18, 25 DAS) and the second factor (B) genotype, was constructed by two inbred lines, LOAC and HOAC.

Results and Discussion

The result of GC analysis demonstrated these two inbred lines have completely different metabolism of fatty acids during all phases of growth, even in linolenic acid transforming that its amount was about zero at seed in both lines.
The evaluation of palmitic and Stearic acid during the seed development in both lines were the same and there was not any significant difference in all phases of developing of sunflower seed that implies, line (low oleic or high oleic acid) has not any effect on the metabolism of this fatty acid during this experimental period (seeding to VE).
It is worthy to note that stearic acid can transform to oleic acid by the action of Δ9 desaturase as described by (Arao and Yamada, 1994).
In LOAC line, oleic acid content as a monounsaturated acid and also as a substrate for oleate desaturase was 42% and remained constant during first 3 days after seeding, but it gradually decreased to 35% at 7th day as a result of oleate desaturase action.

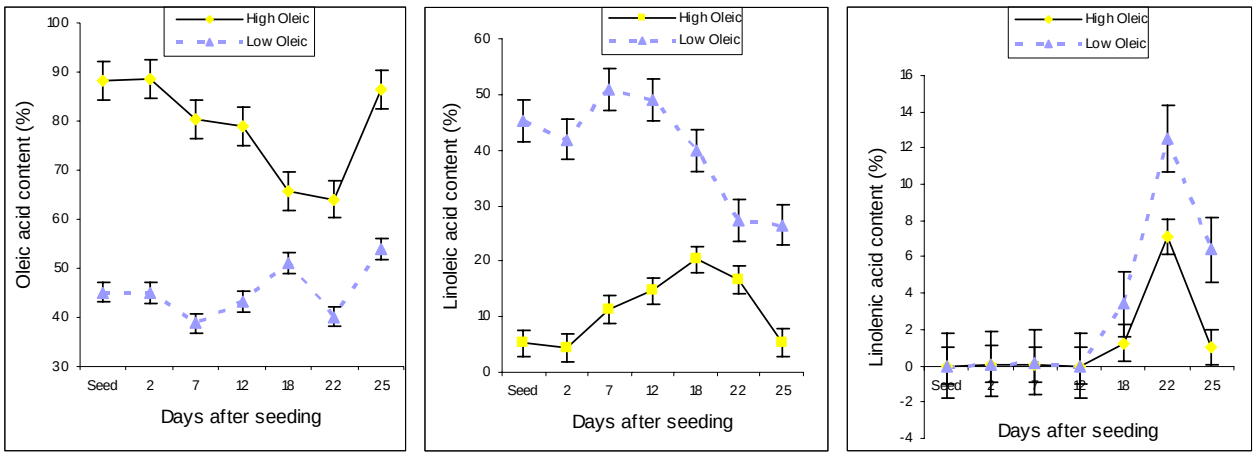

Figure 1 *The modification of fatty acids in HA 89 and R 973 sunflower genotypes during 25 DAS oleic acid (a), linoleic acid (b), linolenic acid (c)*

Considering linoleic acid content revealed a primary reduction until 3rd DAS, then a 9% of increase at 7th DAS. During the rest of the experiment the infinite reduction of linoleic acid was observed. The decrease of linoleic acid kept up with the increase of linolenic acid took place during 7 - 18 DAS that is predictable and normal (Fig. 1a, b, c). After that phase, its content decreased. This phenomena could explain as disactivation of linoleate desaturase and/or the initiation of anabolism of 18: 4 (Δ6- desaturase) and 20:3 (Δ6 – elongase).

In HOAC line, oleic acid catabolized (35% reduction) until 22nd DAS that was coming with the 17% increase of linoleic acid and 7% increase of linolenic acid (Fig. 1a, b, c).
At the last phase (VE) an increase of oleic acid, kept up with the decrease of linoleic and linolenic acid was observed. This could explain the specific expression of FAD2 class in sunflower that its behaviour is not similar to other oil plants like soybean.
In soybean the FAD2-1 gene is specifically induced during seed development when the rate of storage lipid synthesis is at a maximum. In contrast, the FAD2-2 gene was constitutively expressed in both vegetative tissues and throughout seed development, although the highest expression level was found in leaf tissues. The composition of fatty acids in developing seeds is regulated by developmental stage and growth temperature; however, expression of FAD2-1 and FAD2-2 genes controlling the fatty acid desaturation was not induced or inhanced by cold temperature.
It is worthy to follow FAD2 class expression in seed development to justify the fluctuation of fatty acid content and its dissimilarity between low and high oleic acid lines.
Designing an effective primer pair of FAD2 class capable to amplify a fragment of 674 bp from *Helianthus annuus* L. Species. The region selected for specific amplification of FAD2-3 is shown in Fig 2.

```
          87    P  V  Y  W  I  F  Q  G  C  V  L  T  G  V  W  V  I  A  H  E
FAD2-3    278   CCGTTTACTGGATCTTTCAAGGATGTGTTCTAACCGGGGTTTGGGTCATAGCCCATGAAT  337 FAD2-3_FOR
                ||||||| ||||| | |||||| |||||| ||||||| ||||||||||||||||||||
FAD2-2    281   CCGTTTATTGGATTTGTCAAGGTTGTGTTTTAACCGGTGTTTGGGTCATAGCCCATGAAT  340
          88    P  V  Y  W  I  F  Q  G  C  V  L  T  G  V  W  V  I  A  H  E

                                        . . . . . .

          307   V  F  H  N  I  T  D  T  H  V  T  H  H  L  F  S  T  M  P  H
FAD2-3    938   TGTTCCATAACATAACCGATACGCACGTTACACACCATTTGTTCTCCACCATGCCACATT  997 FAD2-3_REV
                ||||||||||||| |||||||| |||||  ||||||||||||||| || ||||| ||||
FAD2-2    941   TGTTCCATAACATTACCGATACTCACGTGGCACACCATTTGTTCTCGACAATGCCTCATT  1000
          308   V  F  H  N  I  T  D  T  H  V  A  H  H  L  F  S  T  M  P  H
```

Figure 2 *BLAST analysis of 720 bases of FAD2-2 and FAD2-3 sequences. Above and below the two sequences the CDS translation is shown. The designed primer is bold dark blue. Different aminoacids are evidenced in reverse color. The middle part of the blast was cancelled*

PCR amplification of cDNA for detection of FAD2-3 gene expression was carried out (fig 3). To establish definitively that the designed primer represents the FAD2-3,a constitutive gene like ubquitin (GUbB1) was used.
FAD2-3 expresses immediately after soaking the seed in water for germination and accumulation of Linolenic acid occurs till 20 DAS, in this moment this gene will not expressed more, Fig 3. In fact the transcript of the FAD2-3 gene was moderately expressed in developing seeds, but at four true leaf it would be blocked.

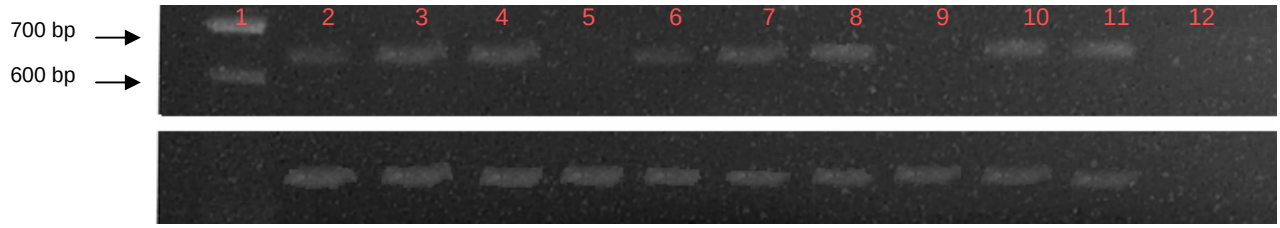

Figure 3 *Agarose gel electrophoresis of the two sunflower genotypes (high and low oleic acid) of different growth stage (seeding to VE) on FAD2-3 sequence. Lane 1 ladder; lanes 2-5 high oleic genotype in seed 2,5,9,14 DAS, respectively ; lanes 6-9 low oleic genotype in seed 2,5,9,14 DAS, respectively; lanes 10, 11 DNA of HOAC and LOAC respectively; lane 12 blank control (PCR mixture without DNA template). Lane 5 and 9 indicate inactivation of gene FAD2-3 in this stage. Lower row shows the amplification in the presence* of a constitutive gene, ubquitin (GUbB1)

Conclusions

The changes of oleic, linoleic and linolenic acids in the vegetative stage in both lines were significant, that could be related to FAD2 and FAD3 activity.
Linoleic acid as a substrate for linoleate desaturase increased during all stages of developing only in mutant line that revealed the more activity of oleic acid desaturase (Δ12-desaturase) in transforming oleic acid to linoleic acid in this line, and the less activity of this enzyme in low oleic acid line, probably due to the low availability of substrate of this enzyme in low oleic acid line, and quantitative analysis can follow by a RT-PCR.
FAD2-3 expresses in seeds two days after seeding for germination and accumulation of Linolenic acid occurs till about 20 DAS, in this moment this gene will not expressed more.

Acknowledgment

This work was supported by grants from the Regione Autonome Friuli Venezia Giulia on developing the inter regional project “sviluppo di nuove per la produzioni no-food (olio industriali, fibra, cellulose ed amidi) con studi e ricerche sulle technolodie e sulla razionalizzazione dei processi e dei sistemi produttivi” and the support of Prof. Giovanardi is gratefully recognized.

References

Arao T, Yamada M (1994) Biosynthesis of polyunsaturated fatty acids in the marine diatom, *Phaeodactylum triconutum*. *Phytochemistry* 35: 1177-1181

Canvin DT (1965) The effect of temperature on the oil content and fatty acid composition of the oils from several oil seed crops. *Can J Bot* 43: 63-69

Conte L S, Leoni O, Palmieri S, Lercker P (1989) Half-seed Analysis: Rapid Chromatographic Determination Of The Main Fatty Acids Of Sunflower Seed 1989. *Plant Breeding* 102, 158-165

Gibson S, Arondel V, Iba K, Somerville CR (1994) Clonning of a temperature-regulated gene encoding a chloroplast ω-3 desaturase from Arabidopsis thaliana. *Plant Physiol* 106: 1615-1621

Graces R, Sarmiento C, Mancha M (1992) Temperature Regulation Of Oleate Desaturase In Sunflower (*Hellianthus Annuus L.*) Seeds, *Planta*, 186: 461-465

Heppard EP, Kinney AJ, Stecca KL, Miao GH (1996) Development and growth temperature regulation of two different microsomal ω-6 desaturase gene in soybeans. *Plant Physiol* 110: 311-319

Ichihara KI, Noda M (1980) Fatty Acid Composition And Lipid Synthesis In Developing Safflower Seed. *Phytochemistry* 19: 49-54

Lajara JR, Diaz U, Quidiello DR (1990) Definite influence of location and climatic conditions on the fatty acid composition of sunflower seed oil. *J Am Oil Chem Soc* 67: 618-623

Martinez-Rivas LM, Gracia-Diaz MT, Mancha M (2000) Temperature and oxygen regulation of microsomal oleat desaturase (FAD2) from sunflower. *Biochemichal Society*, 28: 890-892.

Mollers C (2002) Development of high oleic acid oilseed rape. Presentation at the 8 th international conference for renewable resources and plant biotechnology. Narossa R 2002. Magdeburg, 10-11. Juni; published on CD only (proceeding).

Okuley J, Lightner J, Feldmann K, Narendra Y, Lark E, Browse J (1994) Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *The Plant Cell* 6: 147-158

Salisbury FB and Ross CW (1988) Fisologia Vegetale, Belmont, California, Pp.219-279

Shanklin J, Cahoon EB (1998) Annu. Rev. *Plant Physiol. Plant Mol. Biol* 49: 611-641

Shanklin J, Whittle E, Fox BG **(**1994) Eight Histidine Residues Are Catalytically Essential In A Membrane-associated Iron Enzyme, Stearoyl-coa Desaturase, And Are Conserved In Alkane Hydroxylase And Xylene Monooxygenase. *Biochemistry* 33:12787-12794

Smith MA, Cross AR, Jones OTG, Griffiths WT, Stymne S, Stobart K (1990) Electron-transport components of the 1-acyl-2-oleoyl-sn-glycerol-3-phosphocoline Δ^{12}-desaturase (Δ^{12}-desaturase) in microsomal preparations from developing sufflower (*Carthamus tinctorius* L.) cotyledons. *Biochem J* 272: 23-29

Stymne S, Appelqvist LA (1978) The Biosynthesis Of Linoleate From Oleoyl-coa Via Oleoyl-phosphatidylcholine In Microsomes Of Developing Safflower Seeds. *Eur. J. Biochem*. 90: 223-229

Vijay I K, Stumpf P K (1972) Fat Metabolism In Higher Plants. 48. Properties Of Oleyl Coenzyme A Desaturase Of Carthamus Tinctorius. *J. Biol. Chem*. 247: 360-366