Tocopherol mutations in sunflower

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ABSTRACT

- The scope of presentation includes review on genetic variability, inheritance analysis, allelic test, expressivity and molecular genetics of tocopherols in sunflower.
- All *tph1*, *tph2* and *tph3* mutations are monogenic recessive and non-lethal. These mutations are mapped to 1, 8 and 4 linkage groups respectively. Recombination of the mutations allows producing nearly any types of tocopherol profiles with four known alpha-, beta-, gamma- and delta-homologues. The influences of genetic background, ontogenesis and environment on tocopherol mutations in sunflower were found. Tocopherol mutations express their phenotypes in different parts of a plant such as roots, hypocotyls, leaves and pollen. The only one exception was absence of *tph1* expressivity in the green tissue of the leaves. The *tph1* mutation was affected by the temperature during seed maturing in the way of positive correlation of increased temperature with percentage of alpha-tocopherol.
- The main current problem is to construct a transparent bridge between classical, molecular and biochemical genetics of tocopherols. Future directions of the research will focus on the reasons of different expressivity of the mutations, particularities of the genetic blocks in biosynthesis of homologues and breeding applications of new types of oil.

Key words: expressivity, seed, genetic background, modifier

INTRODUCTION

Tocopherols including alpha-, beta-, gamma- and delta-homologues are natural fat-soluble powerful antioxidants protecting lipids from autooxidation as free radical scavengers (Nadirov, 1991). There is an inversion in vitamin E *in vivo* and antioxidant *in vitro* activities in this line of tocopherols where alpha-homologue has the highest vitamin and the lowest antioxidant properties (Pongracz et al., 1995; Kamal-Eldin and Appelqvist, 1996).

Sunflower tocopherol complex is known to contain a prevalence content of alpha-homologue about 95% (Coors, 1984; Dolde et al., 1999; Velasco et al., 2002; Velasco et al., 2004d). The main oil crops possess the high percentage of other homologues, especially gamma-tocopherol (Dompert, 1976; Muller-Mulot et al., 1976; Sheppard et al., 1993) making the complex more balanced in vitamin and antioxidant activities.

It appears possible to increase an oxidative stability of sunflower oil via the higher level of antioxidant protection of gamma- and delta-homologues by breeding for enhanced tocopherol composition in the seeds (Demurin et al., 1996; Fernandez-Martinez et al., 2004; Hunter and Cahoon, 2007; Warner et al., 2008).

Genetic variability of tocopherol composition in sunflower seeds

The earliest data about changed tocopherol profiles were obtained with two strains of a Peredovik variety from VNIIMK planted in Hungary (Kurnik, 1966). Both of them did not contain alpha-homologue and possessed only beta- and gamma-isomers undivided with thin layer chromatography (TLC). Unfortunately this observation did not result in further investigation (Kurnik, 1967; Jaky et al., 1980).

The first spontaneous mutation of tocopherol composition with increased content of beta-tocopherol about 50% was revealed after selfing of a plant of the open pollinated variety of VNIIMK 9831 in Krasnodar, Russia in 1983 (Demurin, 1986). It was done with half-seed technique on the base of TLC followed by Emmerie-Engel reaction (Popov and Aspiotis, 1991). An inbred line of LG15 with increased beta-tocopherol phenotype was developed (Table 1). Another inbred line of LG17 with modified tocopherol composition was developed at VNIIMK by selfing of the specimen No.44 of the VIR world germplasm collection in 1985. This specimen was introduced from the USA in 1922. The mutant phenotype had 5% alpha- and 95% gamma-forms in the tocopherol profile with TLC (Popov and Demurin, 1987). A frequency of occurrence of spontaneous mutations was 0.7% for the seeds with increased beta-tocopherol content and 96% for the seeds with high gamma-tocopherol content in corresponding accessions. Moreover the frequency of these mutations in a gene pool of three OP varieties and seven VIR accessions of cultivated sunflower were below 2% (Demurin, 1999).

Later in 2001, two sunflower germplasms were jointly developed and released by the Institute for Sustainable Agriculture (CSIC) and the Center of Agricultural research and Development at Cordoba, Spain (Velasco and Fernandez-Martinez, 2003; Velasco et al., 2004a). A half-seed technique and HPLC of tocopherols were used. The T589 with increased to more than 30% of beta-tocopherol content is derived from PI 307937, a selection of Peredovik variety. Another T2100 with high gamma-tocopherol content more than 95% was a selection from CO-77-256, an old accession of Peredovik. A frequency of spontaneous mutation occurrence was 15% for the seeds with increased beta-tocopherol content and 8% for the seeds with high gamma-tocopherol content in corresponding accessions.

The seeds of Peredovik variety were used for chemical mutagenesis with ethyl methane sulfonate (EMS) to produce more than 90% of gamma-tocopherol content in the line of IAST-1 (Velasco et al., 2004b).

Another approach to widen the genetic variability of tocopherol composition was genes recombination.

The cross of LG15 and LG17 has led to the recombinant F_2 seeds with increased delta-tocopherol content from 8 to 20%. These seeds were used to develop an inbred line LG24 at VNIIMK, Russia (Demurin et al., 1991; Demurin, 1993). The same result was obtained with the cross of IAST-1 and T589 at CSIC, Spain. Moreover transgressive levels of F_2 segregants of 77% for beta-tocopherol and 68% for delta-tocopherol were found (Velasco et al., 2004b, 2004c).

Table 1. Independent origin of tocopherol mutations in sunflower

Mutation	Type	Line	Origin	Reference
tph1	spontaneous	LG15	VNIIMK 8931	Demurin (1986)
	spontaneous	T589	Peredovik	Velasco et al. (2003)
tph2	spontaneous	LG17	accession No.44, VIR	Popov and Demurin (1987)
	spontaneous	T2100	Peredovik	Velasco et al. (2003)
	EMS induced	IAST-1	Peredovik	Velasco et al. (2004b)

To compare the all above mentioned phenotypes correctly it should be taken into account that the TLC profiles (indirect tocopherol determination) may differ from HPLC ones (direct determination) in the way of lowering the percentage of beta-, gamma- and especially delta-tocopherols due to relatively low level staining ability of the homologues in this line with Emmerie-Engel reaction (Berezovsky, 1983; Carpenter, 1979).

Inheritance and allelic test of tocopherol mutations

Sunflower (*Helianthus annuus* L.) is the first plant species with the identified genes controlling tocopherol composition in the seeds. These genes were described with classic genetic analysis.

Two non-allelic unlinked genes designated *Tph1* (Demurin, 1986; Popov et al., 1988) and *Tph2* (Popov and Demurin, 1987) were identified at VNIIMK, Krasnodar, Russia. The *Tph1* gene controls the ratio of alpha- and beta-tocopherols in LG15, whereas *Tph2* gene affects that of alpha- and gamma-gomologs in LG17. The *tph2* mutation has an epistatic action over *tph1* with appearance of delta-tocopherol in a recombinant double recessive homozygote of LG24 (Demurin, 1993). Interactions between wild and recessive mutant alleles for both genes correspond to incomplete dominance of a wild type with the same degree of 0.87 (Demurin, 1999).

Crosses of T589 with the standard line HA89 showed that increased beta-tocopherol content is controlled by a recessive allele of a single gene. Crosses of T2100 with HA89 revealed that a high level of gamma-tocopherol was also inherited as a monogenic recessive trait (Velasco et al., 2004a).

Seed exchange of mutant lines between VNIIMK and CSIC was made to develop genetic collections. Genetic identification with allelic test showed a new medium beta-tocopherol mutation of T589 to be allelic to *tph1* of LG15 (Demurin et al., 2004; Vera-Ruiz et al., 2005). A new high gamma-tocopherol mutation of T2100 was allelic to *tph2* of LG17 (Demurin et al., 2004). Moreover the high gamma-tocopherol lines IAST-1 and IAST-540 which were isolated after chemical mutagenesis possess *tph2* mutation (Garcia-Moreno et al., 2006).

The gene *Tph1* had no association in F_2 with eleven genes of morphological characters *T, T_1, M, P, F, Er, Sn, Dl, O, L* and *Fl* (Demurin, 1999). Linkage test showed the *tph1* and *tph2* mutations to be independently inherited from: seven isozyme loci *Est1, Gdh1, Gpi1, Mdh2, Mdh5, Pgd1* and *Pgm4* (Loskutov et al., 1994), a high oleic mutation *Ol* (Demurin, 1999) and an *Imr* gene for imidazolinone resistance (Demurin et al., 2006a).

Genetic collection of sunflower at VNIIMK for tocopherol composition in the seeds includes about 30 inbreds, eight of which are breeding lines. The *tph1* mutation is used in the first commercial hybrid Krasnodarsky 917 with increased to 50% of beta-homologue in tocopherol profile of the seed oil (registered in 1992). A new hybrid Oxy contains the double mutation *tph1, tph2* with gamma- and delta-tocopherols (60 and 40% respectively) on a high oleic background.

Expressivity of tocopherol mutations

Expressivity of tocopherol mutations can be estimated with a range of its phenotypic variability under influence of the modifier genes of genetic backgrounds, plant ontogenesis and environment factors.

Genetic background of different lines was found to influence essentially the expressivity of *tph1, tph2* mutations and a double recessive homozygote. The beta-tocopherol percentage varied from 60 to 30% with corresponding changes of alpha-tocopherol content from 40 to 70%. The gamma-tocopherol percentage for *tph2* varied in case of maximum expressivity from of 100% to the minimum level of 20% (Table 2). The double recessive homozygote showed maximum level of expressivity with 60% of gamma- and 40% of delta-homologues. The minimum level of expressivity of the double homozygote corresponds

to the tocopherol profile with 40% of alpha-, 25% of beta-, 25% of gamma- and 10% of delta-forms. The last phenotype is unique with the equivalent content of four tocopherols.

Table 2. Expressivity of tocopherol mutations in different genetic background of inbred lines in VNIIMK collection (Demurin, 1999; Demurin et al., 2006b)

Mutation	Expressivity	Tocopherol composition (TLC), %			
		alpha	beta	gamma	delta
tph1	max	40	60	0	0
	min	70	30	0	0
tph2	max	0	0	100	0
	min	80	0	20	0
tph1, tph2	max	0	0	60	40
	min	40	25	25	10

The constant sublines of sunflower with maximum and minimum expressivity of *tph2* mutation for each of VK175 (*tph2*) and VK876 (*tph1, tph2*) lines were developed and crossed. Different level of expressivity of the *tph2* mutation was shown to be inherited intermediately in the F_1. Continual variation without discrete phenotypic classes was observed in the F_2. The mean value of F_1, F_2 and the parent's mean did not differ. That indicates the additive action of the modifier genes controlling the differences in gamma-tocopherol content between corresponding sublines of VK175 and VK876 (Demurin and Peretyagina, 2007). The same results were obtained in the crosses of the sublines possessing different levels of expressivity with a normal line (Demurin and Peretyagina, 2009).

Seed maturation from 10 to 38 days after flowering (DAF) influenced tocopherol composition for both normal and mutant genotypes with increasing of alpha-tocopherol content. The trait varied for normal genotype from 81 to 97%, for *tph1* from 33 to 50% and for *tph2* from 0 to 6% (Demurin, 1999).

Mutations for tocopherol composition can be detected in different parts of a plant such as roots, hypocotyl, leaves, pollen and callus from the seeds, hypocotyl and leaves. The only one exception was absence of *tph1* expressivity in the green tissue of the leaves both for single and double mutations (Demurin et al., 2006b). Interesting to note that heterozygous plants can be clearly identified on the basis of analysis for tocopherol composition of the pollen from one head. That is possible due to production of the mixture of heterogeneous pollen grains of two types in 1 normal: 1 mutant ratio for any single mutation and four types of pollen grains for a double heterozygote in the 1 normal: 1 *tph1*: 1 *tph2*: 1 *tph1, tph2* ratio. That is very suitable for discrimination of the genotypes with backcross selection to develop the near-isogenic lines (Demurin, 2003).

Experiment with day/night temperature during seed development at 20/18 and 30/26 °C showed that *tph1* mutation increased alpha-tocopherol content from 39 to 48% at high temperature. Both normal genotype (about 97% of alpha-tocopherol) and the *tph2* mutation (about 98% of gamma-tocopherol) were relatively constant in these two regimes (Demurin et al., 2006).

Molecular and biochemical genetics of tocopherol mutations

Molecular genetics approach revealed a modifier cryptic recessive mutation, designated *d*, which has no effect in a normal genotype but increases both beta-tocopherol content in *tph1tph1 dd* homozygotes up to 70% and delta-tocopherol content in *tph1tph1 tph2tph2 dd* homozygotes to 40% (Hass et al., 2006; Tang et al., 2006). Three methyltransferase mutations *m (tph1), g (tph2)* and *d* were mapped to linkage groups 1 (Vera-Ruiz et al., 2006), 8 (Garcia-Moreno et al., 2006) and 4 respectively (Hass et al., 2006; Tang et al., 2006). The non-lethal knockout mutation *tph1* was shown to be caused by the insertion of a retrotransposon in exon 1 of a MT-1 allele (Tang et al., 2006). The *tph1* mutation has SSR markers (Tang et al., 2006; Vera-Ruiz et al., 2006). The *d* mutation can be designated *tph3* (Fernandez-Martinez et al., 2009). Probably the *tph3* mutation is a part of the genetic background influencing expressivity of *tph1* and *tph2* mutations.

There are two routes of alpha-tocopherol biosynthesis in plants. The route from gamma- to alpha- may be the main route and that from delta- via beta- to alpha-tocopherol may be a minor one. Obviously different "5- carbon" and "7- carbon" methyltransferases are involved. The *tph1* mutation was recently shown to knockout the "5- carbon" type of enzyme: 2-demethylphytylplastoquinol methyltransferase (or

MPBQ/MSBQ-MT) and *tph2* mutation to knockout the "7- carbon" type of enzyme: gamma-tocopherol methyltransferase (γ-TMT) (Hass et al., 2006; Tang et al., 2006).

Different phenotypes of the mutations due to the different expressivity can be explained with the general scheme of proposed genetic blocks in tocopherol biosynthesis where alpha-form is a terminal compound for the wild type (Demurin et al., 2006b). For example, the higher level of *tph2* expressivity in a double mutation leads to the higher content of gamma- and delta-tocopherols. To the contrary, the low level of *tph2* expressivity may result in the additional alpha- and beta-tocopherol accumulation. On the other hand immature seeds are expected to contain increased percentages of precursors, i.e. beta-, gamma- and delta-forms. Finally the high temperature during seed development can not change tocopherol profiles in the case of extremely high percentage of the terminal compounds, i.e. alpha-tocopherol for a normal genotype or gamma-tocopherol for the *tph2* mutation with maximum level of expressivity.

CONCLUSIONS

Tocopherol mutations in sunflower seeds were mainly originated from spontaneous mutagenesis within a gene pool of cultivated plants of *Helianthus annuus* L. All *tph1*, *tph2* and *tph3* mutations are monogenic recessive and non-lethal. The recombination of these mutations allows producing nearly any types of tocopherol profiles with four known alpha-, beta-, gamma- and delta-homologues. That makes both theoretical research and breeding application in the field of tocopherol genetics of sunflower to be promising.

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