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Short communication

Genetic mapping of the Tph1 gene controlling beta-tocopherol accumulation in sunflower seeds

Elsa M. Vera-Ruiz[1], Leonardo Velasco[1], Alberto J. Leon[2], Jose M. Fernández-Martínez[1] and Begoña Pérez-Vich[1,*]
*[1]Instituto de Agricultura Sostenible (CSIC), Apartado 4084, E-14080 Córdoba, Spain; [2]Advanta Semillas, Ruta 226, Km 60.0. 7620 Balcarce, Buenos Aires, Argentina; *Author for correspondence (e-mail: bperez@cica.es; phone: +34-957-499281; fax: +34-957-499252)*

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Abstract

Tocopherols are a family of fat soluble antioxidants of great value for both nutritional and technological properties of seed oils. The four naturally occurring tocopherols (alpha-, beta-, gamma- and delta-tocopherol) widely differ for their relative *in vivo* (vitamin E) and *in vitro* antioxidant properties. Sunflower (*Helianthus annuus* L.) seeds mainly contain alpha-tocopherol (95% of the total tocopherols), which has a great vitamin E value but a low *in vitro* activity. Conversely, beta-tocopherol shows more balanced *in vitro* and *in vivo* antioxidant properties, which is desired for specific uses of the oil. The sunflower line T589 is characterised by an increased beta-tocopherol content in the seeds (>30%), which is determined by the single gene Tph1. The objectives of this study were to map the Tph1 gene by molecular markers (SSRs) and to develop a linkage map of the Tph1-encompassing region. High performance liquid chromatography (HPLC) was used to phenotype 103 F_2 and 67 F_3 progeny from the mapping population CAS-12 × T589, which segregates for Tph1. Bulk segregant analysis identified two SSR markers on linkage group (LG) 1 linked to Tph1. A large linkage group was constructed by genotyping additional SSRs and INDEL markers. Tph1 mapped to the upper end of LG 1 and cosegregated with the SSR markers ORS1093, ORS222, and ORS598. The availability of tightly linked PCR-based markers and the location of the Tph1 gene on the sunflower genetic map will be useful for marker-assisted selection in sunflower and provides a basis for the physical mapping and positional cloning of this gene.

Results

Mapping population and phenotypic segregation for beta-tocopherol

Crosses between the lines CAS-12 (Fernández-Martínez et al. 1997), with a standard low beta-tocopherol content (<3% of the total tocopherols), and T589 (Velasco et al. 2004a), with high beta-tocopherol levels (>30% of the total tocopherols) were made in 2000. The F_1 generation from this and subsequent crosses were analysed using the half-seed technique (Conte et al. 1989), which has been adapted for the nondestructive

analysis of tocopherol composition in sunflower (Demurin et al. 1996). F_1 half-seeds, which showed an average beta-tocopherol content of 4.2% (from 2.5% to 7%), were sown and F_2 seeds were obtained by self-pollination of individual F_1 plants. One-hundred and three F_2 half-seeds were analysed for tocopherol profile as described in Velasco et al. (2004b). The beta-tocopherol content of individual F_2 half-seeds ranged from 0.7% to 53.6% and showed a bimodal distribution (Figure 1). The first class ranged from 0.7% to 7.5%, which was similar to the range observed in CAS-12 and the F_1, and the second class ranged from 32.8% to 53.6%, which was coincident with the range observed in T589. The observed distribution of F_2 seeds (80 F_2 seeds with beta-tocopherol $<7.5\%$: 23 F_2 seeds with beta-tocopherol $>30\%$) satisfactorily fit a phenotypic ratio of 3:1 ($\chi^2 = 0.39$, $p = 0.53$) indicating segregation of a single, partially recessive gene. According to recent allelic studies (Vera-Ruiz et al. 2005), the altered locus in T589 is the Tph1 locus described by Demurin et al. (1996). The 103 F_2 half-seeds were sown and F_3 seeds were obtained from a total of 67 F_2 plants through self-pollination. From each of the 67 F_2 plants, 12 to 24 F_3 seeds were analysed for tocopherol profile. F_2 plants were classified as *Tph1Tph1* if their F_3 seeds had a uniform low beta-tocopherol content ($<3\%$), *Tph1tph1* if their F_3 seeds segregated for beta-tocopherol, and *tph1tph1* if their F_3 seeds showed a uniform high beta-tocopherol content ($>30\%$). The distribution of the F_2 plants in the three classes was 15:37:15 (*Tph1Tph1*:*Tph1tph1*:*tph1tph1*), which fit the expected 1:2:1 segregation ratio ($\chi^2 = 0.73$, $p = 0.69$) that confirms inheritance of the single gene Tph1.

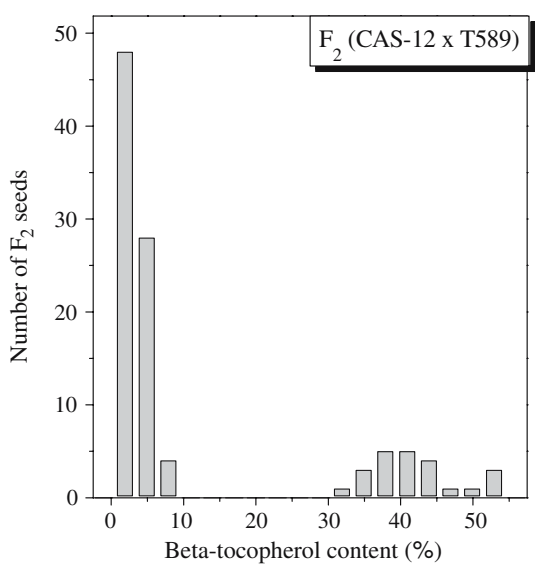

Figure 1. Distribution of beta-tocopherol content (% of the total tocopherols) in F_2 seeds from the cross CAS-12 × T589.

Bulk segregant analysis

Two fully expanded leaves were cut from each of the 103 F_2 plants from the mapping population CAS-12 × T589 and frozen at −80 °C. The leaf tissue was lyophilised and ground to a fine powder in a laboratory mill. DNA was isolated from ground leaf tissue from each F_2 plant as described in Pérez-Vich et al. (2004). DNA was also isolated from two plants of each parental line, CAS-12 and T589. For bulk segregant analysis (BSA; Michelmore et al. 1991), a low beta-tocopherol bulk was made up from eight F_2 individuals classified as *Tph1Tph1*, and a high beta-tocopherol bulk was constructed from 10 individuals classified as *tph1tph1*. Homozygosity of F_2 individuals included in the bulks was verified through the analysis of their respective F_3 seeds. Two replicate samples of each bulk and the parental lines were screened with a genome-wide framework of 95 sunflower simple sequence repeats (SSRs) (Tang et al. 2003). For SSRs analyses, PCRs were performed as described by Pérez-Vich et al. (2004), and the amplification products were resolved by electrophoresis on denaturing polyacrylamide gels following Pérez-Vich et al. (2004) or on 3% Metaphor® (BMA, Rockland, ME, USA) agarose gels in 1× TBE buffer with ethidium bromide incorporated in the gel. Forty-five out of 89 SSR markers that produced amplification products were polymorphic between the parental lines CAS-12 and T589. Two markers from linkage group (LG) 1 (ORS371 and ORS716) were also polymorphic between the low-tocopherol and the high-tocopherol bulks (Figure 2). The CAS-12 allele only amplified in the low beta-tocopherol bulk, and the T589 allele only amplified in the high beta-tocopherol bulk (Figure 2). These results indicated that Tph1 might reside on LG 1. Other

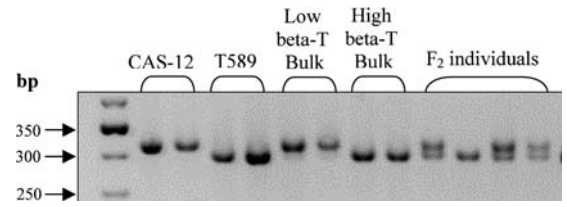

Figure 2. Amplification products of the SSR marker ORS716. Replicate samples of the low beta-tocopherol parental line CAS-12, the high beta-tocopherol parental line T589, the low beta-tocopherol (beta-T) bulk (including eight homozygous low beta-T F_2 individuals), the high beta-T bulk (including 10 homozygous high beta-T F_2 individuals) and four F_2 individuals from CAS-12 × T589 are shown.

three SSR marker loci on LG 1 from the genome-wide framework (ORS610, ORS543, and ORS837) were monomorphic between CAS-12 and T589.

Linkage of ORS371 and ORS716 with Tph1 was verified by genotyping these SSR markers on 103 F_2 individuals from the mapping population used to construct the bulks. The significance of each marker's association with the beta-tocopherol content was determined by one-way analysis of variance (ANOVA) using the statistical package SPSS v 12.0 (SPSS for Windows; SPSS Inc., Chicago, IL, USA). Marker genotypes were classes. Clear significant differences ($p < 0.001$) between the marker class means for the beta-tocopherol content were detected, which confirmed linkage of ORS371 and ORS716 with Tph1. Additionally, a preliminary linkage analysis was run (MAPMAKER/EXP v 3.0b; Whitehead Institute, Cambridge, MA, USA; Lander et al. 1987) using segregation data from ORS371, ORS716, and Tph1. The genotypes for the Tph1 gene were inferred from beta-tocopherol phenotypes in F_2 and F_3 seeds, as described above. Those F_2 individuals for which no F_3 seeds were available (36 out of a total of 103) were scored as *tph1tph1* if they had an increased beta-tocopherol content (> 30%), and *Tph1_* if they had a low beta tocopherol content (< 7.5%). Linkage was considered significant if the LOD score was > 5.0. For consideration of the positions of the SSR marker loci relative to the target locus Tph1, linkage distances were calculated as two point data. Two-point analysis showed ORS371 and ORS716 to be 15.8 and 9.3 cM, respectively, from Tph1.

F_2 SSR genotyping, map construction, and Tph1 mapping

In order to construct a complete genetic map of LG 1 including the Tph1 gene, all public SSR markers known to map to LG 1 (Tang et al. 2002, 2003), excluding those already used for BSA, were screened for polymorphisms between the parental lines CAS-12 and T589. Additionally, four INDEL markers mapped to LG 1 (ZVG1, ZVG2, ZVG3, and ZVG4) (Yu et al. 2003) were also screened for polymorphisms. Four dominant and four codominant SSR polymorphisms, and one dominant and one codominant INDEL polymorphisms were detected. The 10 SSR and INDEL polymorphic markers were genotyped in the 103 F_2 individuals. A linkage map for LG 1, including Tph1, was constructed with MAPMAKER. The genotypes for the Tph1 gene were deduced as described above, and mapped accordingly. Two-point analysis was used to group all the 12 SSR and INDEL marker loci (the 10 new markers plus ORS371 and ORS716) and Tph1 at a LOD score of 3 and a maximum recombination frequency of 0.35. Three-point and multi-point analyses were used to determine the order and interval distances between the markers in LG 1. The Kosambi mapping function was used to compute the map distances in centiMorgans. LG 1 spanned a 55.2 cM distance, with an average marker interval of 4.6 cM (Figure 3). The locus orders for SSR markers and INDEL markers were identical between this map and reference LG 1 maps (Tang et al. 2002, 2003; Yu et al. 2003), except for ORS509. Tph1 mapped on the upper end of LG 1 and cosegregated with ORS1093, ORS222, and ORS598 (Figure 3).

Since Tph1 cosegregated with the three SSR markers ORS1093, ORS222, and ORS598, a one-way analysis of variance (ANOVA) was run using the ORS222 marker, which showed the best score, directly as independent variable in order to asses its effect. The number of individuals per genotype varied (the data were unbalanced), so the least square means and Type III test statistics were estimated. The additive (a) and dominance (d) effects were estimated using linear and quadratic contrasts among the genotype means: $\mu_{AA} - \mu_{aa}$ for the additive effect, and $(\mu_{AA} + \mu_{aa}) - 2\mu_{Aa}$ for the dominance effect, where μ_{aa}, μ_{Aa}, and μ_{AA} are least square means for the *aa*, *Aa*, and *AA*

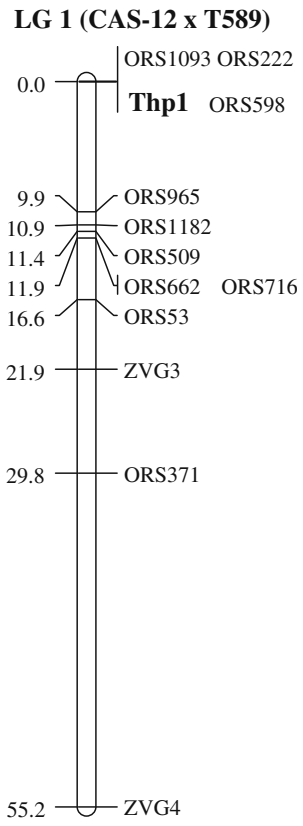

Figure 3. Molecular map of sunflower linkage group (LG) 1 containing the Tph1 gene determining increased beta-tocopherol content. The ORS prefix denotes public SSR marker loci, and the ZVG prefix denotes INDEL marker loci. The cumulative distances in centiMorgans are shown at the left of the map. The map was drawn using the MapChart software (Voorrips 2002).

genotypes, respectively, for locus *A* (ORS222). Analyses of variance were carried out using the SAS GLM procedure (Version 8.01; SAS Institute 1999-2000). Table 1 shows the ANOVA results. ORS222 effect was highly significant, explaining a 97% of the phenotypic variation for the F_2 beta-tocopherol content, and a 87% for the same trait in the F_3, and large ($a = 40.1$ and 26.8 in the F_2 and the F_3 generations, respectively). These results confirm that beta-tocopherol accumulation in the T589 line is determined by the single gene Tph1 located on LG 1 and tightly linked to the ORS222 marker.

Discussion

Tocopherols are fat-soluble compounds with vitamin E activity that exert an effective inhibition of lipid oxidation in oils, fats, and foods containing them (*in vitro* antioxidant activity), as well as in biological systems (*in vivo* antioxidant activity) (Kamal-Eldin and Appelqvist 1996). They occur as a family of four derivatives namely alpha-, beta-, gamma-, and delta-tocopherol, which differ in their relative *in vivo* and *in vitro* antioxidant activities. The seed oil of cultivated sunflower (*Helianthus annuus* L.) is a naturally rich source of alpha-tocopherol, the most bioactive form of vitamin E, whereas most seed oils are naturally rich sources of gamma-tocopherol, which is the most powerful antioxidant *in vitro* (Padley et al. 1994). In contrast, there is no commercial vegetable oil rich in beta-tocopherol, which shows balanced intermediate *in vitro* and *in vivo* antioxidant properties, demanded for specific end uses of the oil (Pongracz et al. 1995).

Sunflower germplasm with increased concentrations of beta-tocopherol has been developed (Demurin 1993; Velasco et al. 2004a, b). The increased beta-tocopherol content in the LG-15 line (with about 50% of the total tocopherols in the beta-tocopherol form; Demurin 1993), and T589 (with more than 30% of beta-tocopherol; Velasco et al. 2004a) is controlled by a single gene, named Tph1 (Demurin et al. 1996; Vera-Ruiz et al. 2005).

Table 1. Association between the marker locus ORS222 and beta-tocopherol content in the CAS-12 × T589 cross determined by variance analysis.

Parameter	F_2 Beta-tocopherol content					F_3 Beta-tocopherol content				
	Effect [A]					Effect [A]				
	a	*d*	*F*	*P*	R^2 [B]	*a*	*d*	*F*	*P*	R^2 [B]
ORS222	40.1	−35.7	1411.7	< 0.0001	0.97	26.8	−12.2	206.6	< 0.0001	0.87

[A] a = Additive effect, calculated as explained in the text, d = Dominance effect, calculated as explained in the text. [B] R^2 = Coefficient of determination.

In this study we have tagged and mapped the Tph1 gene, which will facilitate the development of sunflower commercial lines with an increased beta-tocopherol content in their seed oils.

The Tph1 gene was mapped to the upper end of LG 1, and the map-based markers linked to this gene can be used to assist selection for increased beta-tocopherol content. High efficiencies for marker assisted selection (MAS) require small distances between markers and genes. No recombinant individuals were detected between ORS598, ORS222, ORS1093, and Tph1. In the maps developed by Tang et al. (2002, 2003), ORS598 and ORS1093 span a distance of about 3 cM. None of the phenotypic or DNA markers were mapped to the resolution needed to ascertain the order and orientation of loci in the ORS598-ORS1093 region. Since Tph1 is tightly linked to the markers in this 3 cM region, these markers can be used for MAS with a high selection efficiency. At least five additional SSR markers are located in the ORS598-ORS1093 segment. These markers were monomorphic in the population used in this study, but could be polymorphic in other populations, increasing the chance of finding suitable polymorphic combinations for each cross.

In addition to LG-15 and T589, Velasco et al. (2004b) developed the IAST-5 line with beta-tocopherol levels of up to 77% through recombination between T589 and the high gamma-tocopherol line IAST-1. These levels are the highest reported so far in oilseed crops. Velasco et al. (2004b) suggested that genetic control of increased beta-tocopherol in this line is determined by other genes in addition to Tph1. The number of allelism tests required to differentiate the genes determining accumulation of specific tocopherols grows rapidly as additional lines with a modified tocopherol profile are described. Using the results of this study as a starting point, mapping other genes of the tocopherol biosynthetic pathway on the sunflower linkage map is probably the quickest method of differentiating them.

Tocopherols are ''minor'' constituents of seed oils. Vegetable oils are mainly made up of triacylglycerols (TAGs), which usually constitute more than 95% of the oil weight. Seed oil quality is determined primarily by the fatty acid composition of TAGs. Therefore, great breeding efforts have been devoted to its modification for special purposes (Velasco et al. 2004c). Nowadays, combination of both a modified fatty acid profile with a modified tocopherol profile in sunflower is possible and is becoming a promising approach to develop tailored oils for specific applications with high potential economic value. Moreover, synergism between fatty acids and tocopherols has been reported (Demurin et al. 1996). The pyramiding of genes for fatty acid and tocopherol biosynthesis into a single cultivar is therefore a major breeding objective in sunflower. This process could be facilitated by the identification of molecular markers tightly linked to these genes that would allow the selection of interesting genotypes in MAS breeding programs.

Other seed oil quality traits have also been mapped to LG 1 of the sunflower genetic map. Pérez-Vich et al. (2002) demonstrated the existence of a major QTL on this LG corresponding to the Es1 gene that determines increased stearic acid content in the seed oil. This QTL was located 24.6 cM proximal to the marker locus ZVG4. Therefore, despite the genetic linkage between Es1 and Tph1, they are distant enough to assure recombination between the traits they determine.

The enzymatic steps for tocopherol biosynthesis were elucidated by biochemical means nearly 20 years ago, but the membrane association of the enzymes has made purification and subsequent gene identification difficult. In recent years, genomics-based approaches have been applied to *Arabidopsis thaliana* and the cyanobacterium *Synechocystis* to identify genes for the tocopherol pathway (Shinatani and DellaPenna 1998; Savidge et al. 2002; Cheng et al. 2003). However, very little research has been carried out in crop species. Mapping the Tph1 gene on the sunflower genome opens up two strategies to identify the genes underlying the high beta-tocopherol phenotype in this crop. The first one is a candidate gene approach, which is facilitated by the understanding of the biochemistry of this trait and by the identification of genes potentially involved in its expression. The second will be a map-based cloning strategy, which requires that the target gene has a clear phenotype and that its position on the genetic map is known, pre-requisites which are fulfilled in the case of increased beta-tocopherol content in sunflower.

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