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COMPARATIVE GENETIC STUDY OF TWO SOURCES OF BETA-TOCOPHEROL IN SUNFLOWER

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SUMMARY

Two different sunflower lines with increased beta-tocopherol concentration in the seeds, LG-15 and T589, have been developed. The objective of the present research was to conduct a comparative genetic study of the increased beta-tocopherol concentration in both lines. Plants of LG-15 were reciprocally crossed with plants of T589 in 2003. The F_1, F_2, BC to LG-15 and BC to T589 seed generations were produced in the same environment in 2004 and analyzed for tocopherol profile. The results indicated a strong environmental effect on the increased levels of beta-tocopherol concentration in LG-15 and T589, which averaged 30.5% and 25.8%, respectively, in 2003 and 43.2% and 54.0%, respectively, in 2004. The comparison of individual plant means and standard errors of individual plant means for the F_2 and BC generations with those in the non-segregating parental and F_1 generations indicated no transgressive segregation for beta-tocopherol concentration in F_2 or BC seeds. These results suggested that increased beta-tocopherol concentration in both lines is the result of genetic alterations at the same locus.

Key words: beta-tocopherol, genetic analysis, *Helianthus annuus* L., tocopherol genes

INTRODUCTION

Tocopherols are a family of four biologically active compounds (vitamin E) named alpha-, beta-, gamma-, and delta-tocopherol. They consist of a chroman head with two rings, one phenolic and one heterocyclic, the latter substituted with a saturated phytyl tail. Although the four tocopherols only differ for the number and position of methyl substituents in the phenolic ring (Kamal-Eldin and Appelqvist, 1996), such chemical changes have important consequences for the biological and technological properties of the tocopherols. Alpha-tocopherol is biologically the most active tocopherol, but it exerts the lowest *in vitro* antioxidant action. The esti-

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mated vitamin E activity of the tocopherols, referred to a relative activity of 100% for alpha-tocopherol, is 50% for beta-tocopherol, 10% for gamma-tocopherol, and 3% for delta-tocopherol (Padley *et al.*, 1994). Conversely, the estimated *in vitro* antioxidant activity of the tocopherols, referred to a relative activity of 100% for alpha-tocopherol at a concentration of 1000 ppm, is 235% for beta-tocopherol, 401% for gamma-tocopherol, and 338% for delta-tocopherol (Pongracz *et al.*, 1995).

Vegetable oils are the most important source of tocopherols (Sheppard *et al.*, 1993). Standard sunflower seeds mainly contain alpha-tocopherol, which accounts for more than 90% of the total tocopherols (Padley *et al.*, 1994). However, sunflower germplasm with alternative tocopherol profiles has been developed, including increased concentrations of beta-, gamma-, or delta-tocopherol (Demurin, 1993; Velasco *et al.*, 2004a,b).

Two different sources of increased beta-tocopherol concentration have been identified so far in sunflower germplasm. Demurin (1993) developed the line LG-15, with about 50% of the total tocopherols in the beta-tocopherol form, from the open-pollinated variety "VNIIMK 8931". Velasco *et al.* (2004a) developed the line T589, with more than 30% of the total tocopherols in the beta-tocopherol form, from an accession of the open pollinated cultivar 'Peredovik'. Demurin *et al.* (1996) concluded that the increased levels of beta-tocopherol in LG-15 were produced by recessive alleles at a single locus, named *Tph*1. Similarly, Velasco and Fernández-Martínez (2003) reported the presence of partially recessive alleles at a single locus underlying the increased beta-tocopherol concentration in seeds of T589. The cross between T589 and the high gamma-tocopherol mutant IAST-1 resulted in additional variability for beta-tocopherol concentration, from which the line IAST-5 was isolated (Velasco *et al.*, 2004b). IAST-5 showed a maximum concentration of 77% of the total tocopherols in the beta-tocopherol form, compared with a maximum of 55% in T589 plants grown in the same environment. These data suggested that genetic factors other than *tph*1 alleles are probably involved in the accumulation of beta-tocopherol in sunflower seeds.

The line T589 has been released and it is available for breeding as well as for basic and applied research on plant tocopherols (Velasco *et al.*, 2004a). However, the genetic relationships between the increased beta-tocopherol concentration in T589 and in the previously developed LG-15 (Demurin, 1993) have not been determined. Accordingly, the objective of the present research was to conduct a comparative genetic study of the increased beta-tocopherol concentration in the sunflower lines LG-15 and T589.

MATERIALS AND METHODS

The line T589 was selected from a strain of the cultivar 'Peredovik' in which seeds with increased beta-tocopherol concentration were identified (Velasco *et al.*,

2004a). Seeds of the line LG-15 (Demurin, 1993) were kindly provided by Dr. Y. Demurin, All-Russia Research Institute of Oil Crops, Krasnodar, Russia.

Twenty-four half seeds of both lines were nondestructively analyzed for tocopherol profile as described below, germinated and planted in pots in a field screenhouse in spring 2003. Plants of LG-15 were reciprocally crossed with plants of T589. Crossing was done by emasculating florets of the female parent followed by pollination of their stigmas with pollen from the male parent. Half seeds of the parents as well as F_1 seeds were analyzed for tocopherol profile. Half seeds were sown in January 2003. The corresponding plants were grown in the greenhouse and they started flowering in spring 2004. F_1 plants were self-pollinated to obtain the F_2 generation and backcrossed to both parents. Reciprocal crosses between LG-15 and T589 were repeated in order to obtain F_1 seeds under the same environment as the F_2 and BC_1 seeds. Plants of the line HA89, with standard tocopherol profile, were used as check.

Seeds of the parents, F_1, F_2 and BC generations were analyzed for tocopherol profile. The number of single seeds analyzed were: 288 seeds from six plants of LG-15, 312 seeds from six plants of T589, 300 F_1 seeds from five plants of reciprocal crosses, 191 F_2 seeds from two F_1 plants, 251 BC to LG-15 seeds from three plants, 108 BC to T589 seeds from two plants, and 240 seeds from five plants of HA89. Means and standard errors for beta-tocopherol concentration were calculated from the seeds analyzed for each individual plant. Presence or absence of segregation for beta-tocopherol concentration in F_2 and BC phenotypes was evaluated by comparing the range of variation and the standard error of the mean with those of the parents grown in the same environment.

The analysis of tocopherol profile followed the method of Goffman *et al*. (1999). Half seeds were placed into 10 ml tubes with 2 ml iso-octane. The half seeds were then crushed with a stainless steel rod as fine as possible. The samples were stirred and extracted overnight at room temperature in the dark (extraction time about 16 h). After extraction, the samples were stirred again, centrifuged, and filtered. 5 μl of the extract were analyzed by HPLC using a fluorescence detector at 295 nm excitation and 330 nm emission and iso-octane/tert-butylmethylether (94:6) as eluent at an isocratic flow rate of 0.8 ml min^{-1}. Chromatographic separation of the tocopherols was performed on a LiChrospher 100 diol column (250 mm $\times$ 3 mm I.D.) with 5 μm spherical particles, connected to a silica guard column (LiChrospher Si 60, 5 mm $\times$ 4 mm I.D.). The peak areas of the individual tocopherols were corrected according to their previously calculated response factors: alpha-tocopherol=1.0; beta-tocopherol=1.80. Only alpha- and beta-tocopherol were detected in all the material analyzed in the present study.

RESULTS AND DISCUSSION

Seeds from T589 plants grown in spring 2003 had an average beta-tocopherol concentration of 25.8% of the total tocopherols, with individual plant means ranging from 23.0% to 28.2% (Table 1). Seeds from LG-15 plants grown in the same environment averaged 30.5% beta-tocopherol, with a range for individual plant means from 24.9% to 37.2%. Differences for beta-tocopherol concentration between both lines were significant at $P<0.01$, suggesting a greater beta-tocopherol concentration in LG-15. However, opposite results were obtained in spring 2004, where seeds from T589 averaged 54.0% beta tocopherol, with individual plant means ranging from 46.0% to 59.8%, whereas seeds from LG-15 averaged 43.2% beta-tocopherol, with average plant means from 37.3% to 49.3% (Table 1). These results suggested a strong environmental influence on beta-tocopherol concentration in both lines, which determined large differences in plant means in the two environments included in the study, as well as a possible genotype by environment interaction, as suggested by the relative performance of both lines in different environments. The influence of the environment on the expression of increased beta-tocopherol levels has not been studied so far. Demurin *et al*. (1996) reported that the increased beta-tocopherol trait was stable in different genetic backgrounds, but its stability over environments was not studied by the authors. Velasco and Fernández-Martínez (2003) reported a wide range of variation for beta-tocopherol concentration in T589 plants grown under the same environment, from about 30% to 50% of the total tocopherols.

Table 1: Ranges of individual plant means and their standard errors (SE) for beta-tocopherol concentration (% of the total tocopherols) in seeds of T589 and LG-15 plants in two environments, as well as F_1, F_2, and BC seed generations (plants of the line HA89, with standard tocopherol profile, were used as check)

Generation	Spring 2003		Spring 2004	
	Means[a]	SE[b]	Means	SE
T589	23.0-28.2	0.6-1.1	46.0-59.8	0.5-1.2
LG-15	24.9-37.2	0.5-1.2	37.3-49.3	0.7-1.5
T589 x LG-15	33.9	0.4	45.3-52.6	0.5-1.0
LG-15 x T589	43.3	0.4	39.1-48.9	0.6-1.2
F_2	-----	-----	36.3-45.3	0.5-0.6
BC to T589	-----	-----	42.3-50.8	0.4-1.2
BC to LG-15	-----	-----	43.5-46.7	0.5-0.9
HA89 (check)	-----	-----	0.8-1.1	0.1-0.2

[a]Range of individual plant means
[b]Range of standard errors of individual plant means

F_1 seeds from the cross T589 × LG-15 averaged 33.9% beta-tocopherol in spring 2003, whereas those from the reciprocal cross averaged 43.3% (Table 1). Conversely, F_1 seeds from the cross T589 × LG-15 averaged 51.6% beta-tocopherol in spring 2004, whereas those from the reciprocal cross averaged 45.0% in the

same environment. Such differences for beta-tocopherol concentration between reciprocal F_1s paralleled the observed differences between the parental lines T589 (25.8% in 2003 and 54.0% in 2004) and LG-15 (30.5% in 2003 and 43.2% in 2004), which is probably just the expression of the environmental effect on the female plants, rather than a true maternal effect.

The analysis of F_2 and BC seeds revealed similar ranges of variation for individual plant means and standard errors of individual plant means in comparison with the non-segregating parental and F_1 generations (Table 1). These results indicated no transgressive segregation in F_2 or BC seeds, which would have produced a significant effect on the standard errors of the individual plant means. The lack of transgressive segregation was also observed at the single-seed level. Individual seeds from different T589 and LG-15 plants showed ranges of variation for beta-tocopherol concentration from 26.0% to 65.1% and from 18.9% to 61.7%, respectively, which were not surpassed by the ranges of variation of single F_2 (18.7% to 58.0%), BC to T589 (19.2% to 56.9%), or BC to LG-15 (20.7% to 64.6%) seeds. In all cases, the minimum values for beta-tocopherol were significantly higher than the maximum beta-tocopherol concentration found in the wild type line HA89 (Table 1). A wide range of variation for increased beta-tocopherol concentration in genetically stable germplasm was previously reported by Velasco and Fernández-Martínez (2003) in the line T589. The results of the present research indicate that such a wide range of variation is also a characteristic of the line LG-15.

Increased beta-tocopherol concentration has been reported to be produced by recessive alleles at a single gene in LG-15 (Demurin *et al*., 1996) or by partially recessive alleles at a single gene in T589 (Velasco and Fernández-Martínez, 2003). The gene in LG-15 was named *Tph*1 by Demurin *et al*. (1996), with the LG-15 line carrying the allelic configuration *tph*1*tph*1. Our results revealed that T589 also shows a genetic alteration at the same locus *Tph*1 as LG-15. The abovementioned small differences in the mode of inheritance (recessive in LG-15 and partially recessive in T589) were probably produced by environmental effects or methodological differences in the analysis of the tocopherol profile. From a breeding point of view, the possibility of further increasing beta-tocopherol concentration in sunflower seed through recombination between T589 and LG-15 lines can be discarded after the results of the present research.

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REFERENCES

Demurin, Y., 1993. Genetic variability of tocopherol composition in sunflower seeds. Helia 16: 59-62.

Demurin, Y., D. Škorić, and D. Karlović, 1996. Genetic variability of tocopherol composition in sunflower seeds as a basis of breeding for improved oil quality. Plant Breeding 115: 33-36.

Goffman, F.D., L. Velasco, and W. Thies, 1999. Quantitative determination of tocopherols in single seeds of rapeseed (*Brassica napus* L.). Fett/Lipid 101: 142-145.

Kamal-Eldin, A., and L.Å. Appelqvist, 1996. The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids 31: 671-701.

Padley, F.B., F.D. Gunstone, and J.L. Harwood, 1994. Occurrence and characteristics of oils and fats, In: F.D. Gunstone, J.L. Harwood and F.B. Padley (Eds.), The Lipid Handbook, Chapman & Hall, London, pp. 47-223.

Pongracz, G., H. Weiser, and D. Matzinger, 1995. Tocopherole. Antioxidantien der Natur. Fat Sci. Technol. 97: 90-104.

Sheppard, A.J., J.L. Weihrauch, and J.A.T. Pennington, 1993. Analysis and distribution of vitamin E in vegetable oils and foods. In: L. Packer and J. Fuchs (Eds.), Vitamin E in Health and Disease, Marcel Dekker, New York, pp. 9-31.

Velasco, L., and J.M. Fernández-Martínez, 2003. Identification and genetic characterization of new sources of beta- and gamma-tocopherol in sunflower germplasm. Helia 26: 17-24.

Velasco, L., J. Domínguez, and J.M. Fernández-Martínez, 2004a. Registration of T589 and T2100 sunflower germplasms with modified tocopherol profiles. Crop Sci. 44: 361-362.

Velasco, L., B. Pérez-Vich, and J.M. Fernández-Martínez, 2004b. Novel variation for tocopherol profile in sunflower created by mutagenesis and recombination. Plant Breeding 123: 490-492.

ESTUDIO GENÉTICO COMPARATIVO DE DOS FUENTES DE BETA-TOCOFEROL EN GIRASOL

RESUMEN

Se han desarrollado hasta la fecha dos líneas diferentes de girasol con niveles elevados de beta-tocoferol en las semillas, LG-15 y T589. El objetivo de este trabajo fue la realización de un estudio genético comparativo sobre los niveles elevados de beta-tocoferol en ambas líneas. Se realizaron cruzamientos recíprocos entre plantas de LG-15 y T589 en 2003. Las generaciones de semillas F_1, F_2, BC a LG-15 y BC a T589 se obtuvieron en el mismo ambiente en 2004 y se analizaron para perfil de tocoferoles. Los resultados indicaron una fuerte influencia ambiental sobre los niveles elevados de beta-tocoferol en LG-15 y T589, con valores medios de 30.5% y 25.8%, respectivamente en 2003, y 43.2% y 54.0%, respectivamente en 2004. La comparación de las medias de plantas individuales y de sus errores estándar de las generaciones F_2 y BC con aquellas de las generaciones no segregantes (parentales y F_1) indicó ausencia de segregación transgresiva para beta-tocoferol en las generaciones F_2 y BC. Estos resultados sugieren que los niveles elevados de beta-tocoferol en ambas líneas es el resultado de alteraciones genéticas en el mismo locus.

ÉTUDE GÉNÉTIQUE COMPARATIVE DE DEUX SOURCES DE TOCOPHÉROL BÊTA DANS LE TOURNESOL

RÉSUMÉ

Deux lignées différentes de tournesol ayant un contenu majoré de tocophérol bêta dans la graine, LG-15 et T589 ont été développées. L'objectif de cette recherche était de faire une étude génétique comparative de la concentration majorée de tocophérol bêta dans les deux lignées. Les plantes de LG-15 ont été croisées avec les plantes de T589 en 2003. Les générations de semences F_1, F_2 et BC à LG-15 et BC à T589 ont été produites dans le même milieu en 2004 et leur profil de tocophérol a été analysé. Les résultats ont démontré une forte influence de l'environnement sur le contenu majoré de tocophérol bêta en LG-15 et en T589 dont les moyennes étaient de 30,5% et 25,8% en 2003 et de 43,2% et 54,0% en 2004. La comparaison des moyennes individuelles des plantes et les erreurs standard des moyennes de certaines plantes des générations F_2 et BC avec les valeurs correspondantes des générations parentales de non ségrégation et de la génération F_1 n'a pas démontré l'existence de ségrégation transgressive sur la concentration de tocophérol bêta dans les graines des générations F_2 ou BC. Ces résultats suggèrent que l'augmentation de la concentration de tocophérol bêta dans les deux lignées est le résultat de changements génétiques au même locus.