Breeding and genetic studies on vitamin E content in sunflower seeds

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ABSTRACT

- Tocopherols are natural antioxidants with vitamin E activity. They are synthesized exclusively in photosynthetic microorganisms and plants. Plant tissues vary enormously for total tocopherol content and composition, with the highest concentrations being found in seeds. The predominant tocopherol forms in seeds are gamma- and delta-tocopherol, which have only about one tenth the vitamin E activity of alpha-tocopherol, whereas the latter is the predominant form in photosynthetic tissues. Sunflower seeds are a notable exception as they primarily accumulate alpha-tocopherol, which makes sunflower seeds and oils one of the richest sources of dietary vitamin E. Nutritional studies have pointed to beneficial effects of moderate vitamin E supplementation on human health. Accordingly, sunflower seeds with naturally enhanced vitamin E content might be a promising alternative for producing healthier sunflower oils. The aim of this research was to select sunflower germplasm with increased vitamin E content, to study the inheritance of the trait, and to identify associated quantitative trait loci (QTL).

- A large germplasm collection of cultivated sunflower accessions was evaluated for seed vitamin E content using HPLC analysis with fluorescence detection. A line with increased seed vitamin E content was developed and the inheritance of the trait was studied through evaluation of the F_1, F_2, F_3 and BC_1 plant generations from crosses with a line with conventional seed vitamin E content. A genetic linkage map was also constructed using a set of 156 SSR and INDEL polymorphic and evenly spaced markers. QTL analysis was conducted using phenotypic data for vitamin E content in the F_2 and F_3 plant generations.

- Selection on the germplasm accessions with the highest vitamin E content allowed the isolation of an $S_{4:5}$ line with an increased total tocopherol content, mainly in the alpha-tocopherol form (>95% of total tocopherols) of 467 mg kg^{-1} seed compared to 250.9 mg kg^{-1} seed in the line with conventional tocopherol content used as a control. Increased vitamin E content was also expressed in the pollen. Classical genetic studies revealed broad-sense heritabilities between 0.67 and 0.78 and narrow-sense heritabilities between 0.60 and 0.70. Additional estimates of heritability through parent-offspring correlation and realized heritability gave consistent values of 0.67 and 0.64, respectively. A major QTL on LG 10 was responsible for more than half of the explained phenotypic variation for increased tocopherol content, whereas six additional minor QTL were identified.

- Our results revealed the feasibility of selecting for enhanced vitamin E content in sunflower seeds. Increased vitamin E levels are under oligogenic control and show moderate to high heritabilities, which anticipates good prospects for introgressing the trait into sunflower elite lines. The identification of a major QTL is a promising starting point for identifying the main gene underlying increased vitamin E content and for developing diagnostic markers to assist in backcrossing programmes. The role and consistency of minor QTL should be further investigated.

- Sunflower oils with naturally high levels of vitamin E possess potential market niches. This research led to the isolation of a line with increased seed vitamin E content and contributed to the identification of the genetic basis underlying the trait, which is a good starting point for future breeding advances in this field.

Key words: Alpha-tocopherol – QTL – sunflower – trait breeding – vitamin E

INTRODUCTION

Oil seeds contain compounds with antioxidant activity that protect unsaturated fatty acids from oxidation, both *in vivo* as well as *in vitro*. The most important of them are the tocopherols, which are oil-soluble compounds with vitamin E activity that become part of the oil during extraction (Padley et al., 1994). Tocopherols have a molecular structure comprising a chromanol ring with a given number of methyl substituents and a saturated phytyl side chain. They occur in nature in four forms known as alpha-, beta-, gamma-, and delta-tocopherol, which differ in the number and position of methyl substituents and also in their antioxidant properties (Hunter and Cahoon, 2007). Alpha-tocopherol shows a maximum *in vivo* vitamin E activity but poor *in vitro* protection of the extracted oil, whereas beta-, gamma- and delta-tocopherol are more powerful *in vitro* antioxidants with a lower vitamin E value than alpha-tocopherol (Pongracz et al., 1995; Warner and Moser, 2009).

Tocopherols are synthesized exclusively in photosynthetic organisms. The plant tocopherol biosynthetic pathway utilizes cytosolic aromatic amino acid metabolism for head group synthesis and the plastidic deoxyxylulose-5-phosphate pathway for the synthesis of the hydrophobic isoprenoid-derived side chain (Dellapenna and Mène-Saffrané, 2011). The first step in tocopherol biosynthesis involves the production of the aromatic head group, homogentisic acid (HGA), from p-hydroxyphenylpyruvic acid (HPP) by the enzyme HPP dioxygenase (HPPD). HGA is a substrate for prenylation with phytyl diphosphate (phytyl-PP), catalyzed by homogentisic acid phytyl transferase (HPT), to yield the first committed intermediate in tocopherol synthesis, 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ). MPBQ can be methylated at ring position C-3 by 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone methyltransferase (MPBQ/MSBQ-MT) yielding 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ). The prenylquinols MPBQ and DMPBQ are then cyclized into delta- and gamma-tocopherol, respectively. This enzymatic reaction is catalyzed by tocopherol cyclase (TC). Finally, delta- and gamma-tocopherol are methylated at ring position C-5 by the gamma-tocopherol methyltransferase (gamma-TMT) to yield beta- and alpha-tocopherol, respectively (DellaPenna and Mène-Saffrané, 2011).

Plant tissues vary enormously in their tocopherol content and composition. In general, photosynthetic tissues contain relatively low tocopherol levels mainly in the alpha-tocopherol form, while seeds accumulate higher tocopherol levels, predominantly in the form of gamma-, and delta- tocopherol (DellaPenna and Pogson, 2006). Sunflower is a notable exception, since, unlike other major oilseeds, its seeds mainly contain alpha-tocopherol, which accounts for more than 90% of total tocopherols in seeds and subsequently in sunflower oil (Padley et al., 1994). Such a great proportion of alpha-tocopherol confers great nutritional value to sunflower oil as a source of natural dietary vitamin E.

The importance of vitamin E in the human diet to maintain optimal health was established in early experiments on Vitamin E deficiencies. Accordingly, appropriate minimum vitamin E dietary levels were established (DellaPenna and Mène-Saffrané, 2011). Additionally, there is a large body of medical evidence pointing to the benefits for human health associated with daily, moderate vitamin E supplementation (Bramley et al., 2000), which has encouraged breeding research to enhance alpha-tocopherol levels in oilseed crops. The present research was aimed at developing sunflower lines with increased total tocopherol content in the alpha-tocopherol form, and to characterize them at the phenotypic, genetic and molecular level.

MATERIALS AND METHODS

A germplasm collection of 952 sunflower entries was analysed for total seed tocopherol content by high performance liquid chromatography (HPLC) as described by Goffman et al. (1999). Analyses were conducted on six bulked achenes from each accession. Twenty-eight accessions with low and high total tocopherol levels were selected and planted in the field in 2000. S_1 seeds from each plant were further analyzed for tocopherol content, which resulted in a head to row selection scheme of the best entries until the $S_{4:5}$ generation. At this stage, $S_{4:5}$ families were selected for low and high seed tocopherol content and their seeds pooled to constitute the selected lines. Total tocopherol content in these lines was also measured in plant tissues other than seeds. To that end, tissue of roots, leaves and pollen of self-fertilized plants selected for total high or low seed tocopherol levels was collected, freeze-dried, lyophilized, and analysed for total tocopherol content using around 200 mg lyophilized tissue.

Genetic studies of selected lines with high and low total tocopherol content were conducted through reciprocal crosses of these lines with a line with standard total tocopherol content, HA-89. F_1, F_{1r}, F_2, F_{2r}, BCP_1, and BCP_2 plant generations were obtained and evaluated, together with the parental lines,

in two replicate trials in 2006 and 2007, respectively. The 2007 field plot also included the evaluation of the F_3 plant generation. Broad-sense heritability and narrow-sense heritability were estimated on a single-plant basis as described in Allard (1960). Estimate of heritability through parent-offspring correlation was computed as the correlation coefficient between tocopherol content in the F_2 and the F_3 plant generations. The minimum number of genes controlling the total tocopherol content was also estimated (Wright, 1968).

For the molecular analyses, two genetic linkage maps were constructed from two populations segregating for total seed tocopherol content: IAST-413 x HA-89, segregating for increased seed tocopherol content and IAST-522 x HA-89, segregating for reduced seed tocopherol content. Both populations were phenotyped for total seed tocopherol content at the F_2 and the F_3 plant generations. The genetic maps were constructed with a set of about 155 SSR and INDEL polymorphic markers using the software MAPMAKER/EXP 3.0b (Lander et al., 1987). Composite interval mapping (CIM) was used for mapping QTL and estimation of their effects. Computations were performed using the software PLABQTL Version 1.1 (Utz and Melchinger, 1996).

RESULTS

Isolation of lines with modified total seed tocopherol content and phenotypic characterization
A range of variation for total seed tocopherol content from 119 to 491 mg kg^{-1} was detected in the analysis of 952 germplasm accessions, with alpha-tocopherol being the predominant tocopherol derivative in most of them (Velasco et al., 2010). Selection cut-offs for low and high total tocopherol content were established at 145 and 400 mg/kg, respectively, based on the distribution of the trait in the germplasm collection. This resulted in a selection of a total of 28 accessions, all with alpha-tocopherol as the predominant form. Analyses of tocopherol content at the S_0 level (S_1 seeds averaged) and in further selections conducted from S_1 to S_4 plant generations led to the isolation of lines IAST-413 and IAST-522. Line IAST-413 showed the highest total tocopherol content, averaging in the four S_1 to S_4 generations 467 mg/kg compared to 251 mg/kg in the standard line HA-89, and corresponded to a selection from the Russian variety Peredovik. Line IAST-522 showed the lowest seed tocopherol content, averaging from S_2 to S_4 generations 73 mg/kg, and was derived from a selection for broomrape (*Orobanche cumana* Wallr.) resistance within a collection of old Spanish cultivars. Increased total tocopherol content was also detected in the pollen of line IAST-413, but not in leaves and roots. Reduced tocopherol content was detected in roots and pollen of line IAST-522, but not in leaves (Del Moral et al., 2012).

Genetic characterization of lines with modified total seed tocopherol content
Evaluation of the inheritance of increased seed tocopherol content in line IAST-413 under two environments resulted in broad-sense heritability estimates of 0.78 and 0.67, narrow-sense heritability estimates of 0.70 and 0.60, and values of 1.04 and 1.95 for the minimum number of genes (k) controlling the increased tocopherol content trait (Del Moral et al., 2011a). Inheritance studies for reduced tocopherol content in sunflower line IAST-522 under two environments revealed broad-sense heritability estimates of 0.81 and 0.67, a narrow-sense heritability of 0.49 in the only environment in which it could be estimated, and values of 1.55 and 1.62 for the minimum number of genes controlling the reduced tocopherol content trait (Del Moral et al., 2011b.)

Molecular characterization of lines with modified total seed tocopherol content
Seven and six QTL determining increased and reduced tocopherol content, respectively, were detected. Of these, a major QTL (peak LOD of 8.7 in the F_2 and 11.4 in the F_3) on linkage group (LG) 10 was responsible for more than half of the explained phenotypic variation for increased tocopherol content in the IAST-413 x HA-89 population. Similarly, a major QTL on LG 11 (peak LOD of 5.5 in the F_2 and 3.5 in the F_3) was responsible for more than half of the explained phenotypic variation for reduced seed tocopherol content in the IAST-522 x HA-89 population. Three QTL on LG 2, 4, and 17 were detected at overlapping support intervals in both mapping populations. A candidate gene locus (*MT-2*) corresponding to a MPBQ/MSBQ-MT gene was found co-locating with the QTL on LG 4 (L. Del Moral et al., unpublished results).

DISCUSSION

The results of this research revealed the feasibility of selecting for enhanced vitamin E content in sunflower seeds. Line IAST-413 with increased seed tocopherol levels, mainly in the alpha-tocopherol form and consistently expressed across environments, constitute a unique source of naturally derived vitamin E and provide the basis for development of vitamin E-enriched specialty sunflower oil. Interestingly, the phenotypic genetic analysis suggested that tocopherol content in this line is controlled by a reduced number of genes, which has recently been confirmed in a molecular study in which a QTL on LG 10 with a major effect and detected across generations was responsible for more than half of the explained phenotypic variation for total seed tocopherol content (L. Del Moral et al., unpublished results). It is important to note that the present research was conducted on the tocopherol content of sunflower seeds. Most studies on the evaluation of genetic and environmental factors influencing tocopherols in sunflower have been based on the study of oil tocopherol content rather than seed tocopherol content, though no clear global conclusion can be extracted from the different studies (Marquard, 1990; Alpaslan and Gündüz, 2000; Velasco et al., 2002). Selection for increased tocopherol content at the seed level is preferable to selection at the oil level, as analysis of tocopherols can be made on small seed samples such as half seeds (Velasco et al., 2004). Conversely, extraction of the oil consumes extra time and requires larger amounts of seeds. Previous studies have revealed no correlation between oil and tocopherol content, neither in sunflower (Velasco et al., 2002) nor in rapeseed (Marwede et al., 2004). Additionally, Nolasco et al. (2004) concluded that seed oil tocopherol content in sunflower is mainly accounted for by the oil content of the seeds, which suggests that studies on seed oil tocopherol content may provide primarily information on variation for seed oil content rather than on seed tocopherol content. Since the biosynthesis of tocopherols and fatty acids, the major component of seed oils, are not related, a breeding strategy based on identifying germplasm with increased alpha-tocopherol content in the seeds and then to introgress the alleles that promote maximum alpha-tocopherol accumulation in the seeds into elite germplasm with high oil content is suggested to develop cultivars producing oils with naturally enhanced vitamin E content.

Breeding for enhanced tocopherol content has been carried out in other oilseed crops such as soybean (Raclaru et al., 2006; Hunter and Cahoon, 2007), rapeseed (Goffman and Becker, 2001; Marwede et al., 2004) and safflower (Velasco and Fernández-Martínez, 2004). However, whereas sunflower and safflower seeds primarily accumulate alpha-tocopherol, gamma-tocopherol is the predominant form in soybean seeds, while canola seeds show varying balanced levels of gamma- and alpha- tocopherol. As the tocopherol profile in the seeds of soybean and canola is different to sunflower, comparison of breeding results for enhanced tocopherol content between these crops is not feasible. In fact, the most successful breeding efforts for vitamin E enhancement in oilseed crops with low proportion of alpha-tocopherol such as soybean and canola have been directed to modifying the tocopherol profile towards a higher alpha-tocopherol accumulation, both using traditional and transgenic approaches (Van Eenennaam et al., 2003; Ujiie et al., 2005; Karunanandaa et al., 2005; Tavva et al., 2006).

Although the enzymatic steps for tocopherol biosynthesis are well defined and the genes for the tocopherol pathway are all known (DellaPenna and Mène-Saffrané, 2011), there are still several factors determining tocopherol accumulation that remain unidentified. Gilliland et al. (2006) and DellaPenna and Mène-Saffrané (2011) reported that less than half of the natural variation for tocopherol content and composition found in *Arabidopsis* corresponded to QTL underlying known tocopherol biosynthetic genes, the rest of the QTL lacking known candidate genes in their intervals. Additionally, transgenic approaches have been unable to engineer large increases in tocopherol content in oilseeds, probably because of gaps in our understanding of factors enhancing the flux towards the tocopherol pathway (Hunter and Cahoon, 2007). In the present research, we have identified a major QTL for increased tocopherol content on LG 10 as well as a major QTL for reduced tocopherol content on a different linkage group (LG 11). Identification of the enzymes underlying these QTL and their role in the tocopherol biosynthetic pathway will be crucial for understanding the key biosynthetic steps that must be modified to produce further enhancement of tocopherol accumulation in sunflower seeds. Other minor QTLs were also found for both increased and reduced tocopherol content. One of them on LG 4, identified in the two populations segregating for reduced and increased seed tocopherol content, co-located with a candidate gene locus (*MT-2*) corresponding to a MPBQ/MSBQ-MT gene. Haddadi et al. (2011) found four candidate gene loci exhibiting co-localization with QTL for total oil tocopherol content in sunflower, two of them corresponding to tocopherol biosynthetic loci (HPPD and gamma-TMT). The genetic resources developed, including mapping populations, phenotyping across generations and populations, candidate gene analyses and the recent advances in sunflower genome sequencing (Kane et al., 2011) and high-throughput genotyping should greatly accelerate the identification and utilization of key genes for developing sunflower germplasm with further increased total seed tocopherol content.

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