Theor Appl Genet (2009) 119:567–575
DOI 10.1007/s00122-009-1066-6

ORIGINAL PAPER

Genetic mapping, cloning, and functional characterization of the *BnaX.VTE4* gene encoding a γ-tocopherol methyltransferase from oilseed rape

Jessica Endrigkeit · Xingxing Wang · Daguang Cai · Chunyu Zhang · Yan Long · Jinling Meng · Christian Jung

Received: 27 November 2008 / Accepted: 11 May 2009 / Published online: 29 May 2009

Abstract Rapeseed (*Brassica napus* L.) is one of the major oilseed crops and an important source for tocopherols known as vitamin E in human nutrition. Increasing the tocopherol content and altering the tocopherol composition is a major goal in rapeseed breeding. The genes encoding enzymes from the tocopherol pathway have been cloned from model species. However, only scant data about tocopherol genes from crop species have been available. We have cloned four sequences of a gene family from *B. napus* with homology to the *Arabidopsis thaliana VTE4* gene. The sequences were amplified by PCR with primers derived from the *A. thaliana* gene. BAC-clones were isolated to analyze the genomic structure of the *BnaX.VTE4*-loci. In contrast to the *A. thaliana* gene all *B. napus* sequences have two additional introns. For functional analysis, the *BnaA.VTE4.a1* sequence was transformed into *A. thaliana*. Seeds from transgenic offspring showed a 50-fold increase of the α-tocopherol fraction which is in accordance with the predicted function of the gene. A marker assay was established and the *BnaA.VTE4.a1* sequence was mapped to the end of chromosome A02 of the Tapidor × Ningyou7 genetic map, where also two QTL for α-tocopherol content had been mapped. Thus, the *BnaA.VTE4.a1* gene is a promising candidate for these QTL and can be used for marker assisted selection for α-tocopherol content in rapeseed.

Communicated by H. Becker.

The nucleotide sequences data reported in this paper have been submitted to GenBank nucleotide sequence database with the accession numbers EU637012 to EU637015 and FJ435091 to FJ435093.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-009-1066-6) contains supplementary material, which is available to authorized users.

J. Endrigkeit · C. Jung (✉)
Plant Breeding Institute, Christian Albrechts University of Kiel, Olshausenstr. 40, 24098 Kiel, Germany
e-mail: c.jung@plantbreeding.uni-kiel.de

X. Wang · C. Zhang · Y. Long · J. Meng
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, 430070 Wuhan, People's Republic of China

D. Cai
Molecular Phytopathology, Christian Albrechts University of Kiel, Hermann-Rodewald-Str. 9, 24118 Kiel, Germany

Introduction

Tocopherols are a class of molecules that are characterized by a fully saturated tail derived from phytyl diphosphate and a chromanol head group. The four naturally occurring tocopherols (α-, β-, γ-, and δ-) differ in their number and position of methyl substituents on this aromatic ring. They are synthesized only by plants, green algae, and some cyanobacteria. Tocopherols, mainly α-tocopherol, are an essential component of human nutrition known as vitamin E (Pongracz et al. 1995). The tocopherol synthesis pathway has been unraveled by Soll et al. (1980) and the corresponding genes from *Arabidopsis thaliana* have been cloned (Bergmüller et al. 2003; Cheng et al. 2003; Collakova and DellaPenna 2001; Motohashi et al. 2003; Norris et al. 1998; Porfirova et al. 2002; Van Eenennaam et al. 2003).

There are two methyltransferases responsible for the different methylation patterns on the aromatic ring (Fig. 1). The 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase (MPBQ MT) catalyzes the methylation of MPBQ

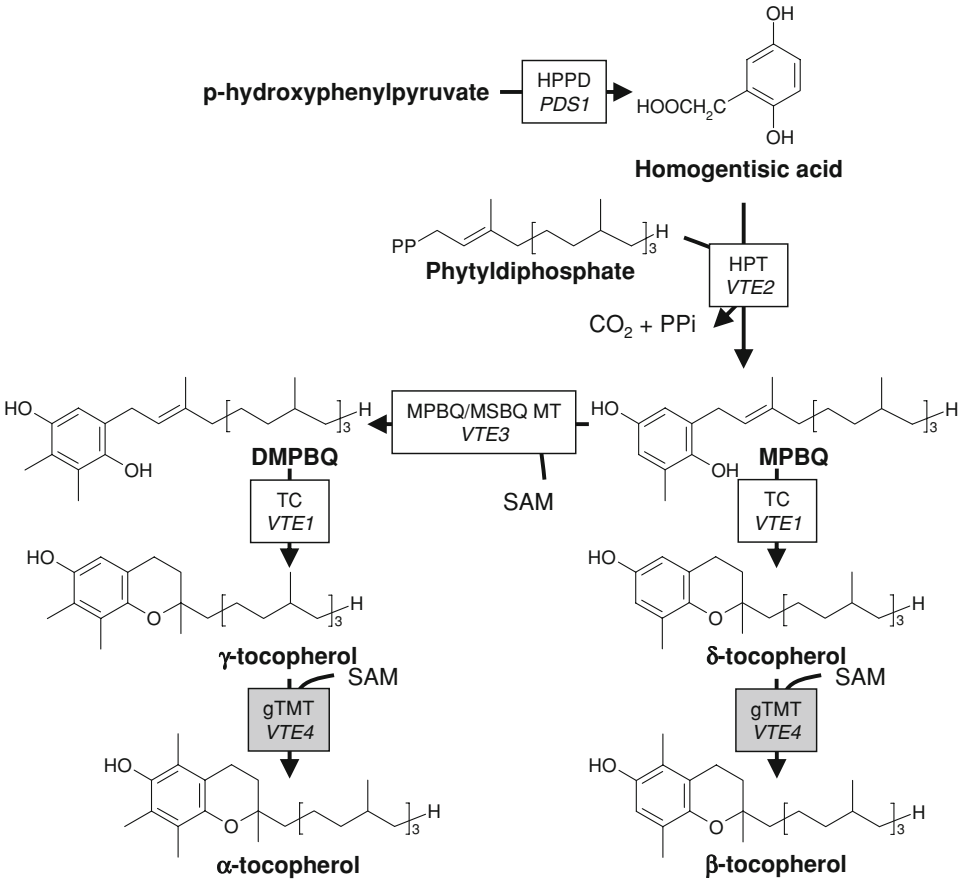

Fig. 1 The tocopherol biosynthetic pathway in plants. DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; SAM, *S*-adenosylmethionine. Enzymes and corresponding genes (*in italic*) from *A. thaliana* are in *boxes*; the enzymatic step investigated here is in gray. HPPD, *p*-hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyltransferase; MPBQ/MSBQ MT, MPBQ/MSBQ methyltransferase; TC, tocopherol cyclase and gTMT, γ-tocopherol methyltransferase

resulting in 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ). This is an important step in the tocopherol synthesis, finally leading to γ- and α-tocopherol. The γ-tocopherol methyltransferase (gTMT) catalyzes the conversion from δ- to β- and from γ- to α-tocopherol, respectively. In *A. thaliana* the gTMT is encoded by the gene *VTE4* (Bergmüller et al. 2003). This gene has been used in transgenic approaches to alter the seed tocopherol composition. The gene *VTE4* was introduced into soybean to improve the seed oil quality by converting the naturally occurring major component γ-tocopherol to α-tocopherol, which has a much higher nutritional value (Van Eenennaam et al. 2003).

Oilseed rape or canola (*Brassica napus* L. ssp. *oleifera* (Metzg.)) is the only oil crop of importance in moderate climates and a major source for vitamin E. So far, none of the endogenous genes for tocopherol synthesis have been functionally described. The seeds of oilseed rape contain α-, γ-, and small amounts of δ-tocopherol (Dolde et al. 1999). The total tocopherol content ranges between 180 and 370 mg kg^{-1} seeds (Goffman and Becker 2002), which is strongly influenced by the environment but differs significantly between cultivars (Goffman and Becker 1999). Two studies on genetics of tocopherol synthesis in oilseed rape have been published so far (Marwede et al. 2004; 2005). Using three doubled-haploid populations grown under three and four environments the heritabilities of α- and γ-tocopherol content in oilseed rapeseeds were calculated to range between 0.23–0.46 and 0.36–0.50, respectively (Marwede et al. 2004). These authors also mapped eight QTL for tocopherol content and composition which were distributed on six linkage groups (Marwede et al. 2005). The tocopherol content of oilseed rape can either be increased by exploiting natural variation during conventional breeding programs or by transgenic approaches. The transformation of *B. napus* with genes for the hydroxyphenylpyruvate dioxygenase, tocopherol cyclase, and homogentisate phytyltransferase from the tocopherol synthesis pathway from different species resulted in doubling of the tocopherol content in some of the transformants (Kumar et al. 2005; Raclaru et al. 2006). However, the use of transgenic plants is avoided in European rapeseed breeding programs. Therefore, natural variation is exploited and molecular markers are needed for selecting high tocopherol genotypes.

Here, we present the first gene from the tocopherol synthesis pathway of *B. napus*. The copy number of *VTE4*-homologous sequences was determined and the function of *BnaA.VTE4.a1* was verified after transformation into *A. thaliana*. The relevance of these findings for cloning other tocopherol synthesis genes and for selecting high tocopherol rapeseed lines is discussed.

Materials and methods

Plant material

For DNA isolation, we used the *B. napus* variety Express (Norddeutsche Pflanzenzucht Hans Georg Lembke KG, Holtsee Germany). For marker development and mapping studies we used the doubled haploid (DH) population, TNDH which had been produced from a cross between the European cultivar Tapidor and the Chinese cultivar Ningyou7 (Yan Long et al. 2007). For transformation experiments, we used the *A. thaliana* ecotype Columbia (Col).

Primer sequences and PCR conditions

For amplification of *BnaX.VTE4* the primers vte4-3fw (5′TTT CTC CAA CCA ACC TCT CAT TAT AA) and vte4-3rv (5′ACG ACA ATT TTA TTG) were used. The sequences were derived from a RACE-experiment. To integrate *Xho*I restriction sites for recombinant vector preparation, we used the modified primers vte4-3Xho-fw (5′TTT **CTC GAG** CCA ACC TCT CAT TAT AA, *Xho*I site in bold) and vte4-3Xho-rv (5′**CTC GAG** TTC AAT CTC C, *Xho*I site in bold). The primers vte4-1fw (5′AGA GAG CCA ATG ATC TCG) and vte4-2rv (5′GCG AGA TCA TTG GCT CTC T) were utilized for 3′ and 5′ RACE, respectively. The primer combination vte4-1fw (5′AGA GAG CCA ATG ATC TCG)/vte4-1rv (5′CCC TTC AAT CAT CAA TGG), was used for probe preparation for genomic Southern hybridization and for BAC-library screening. To check the transgenic nature of regenerated plants from the transformation experiments, we used the 35S-promotor specific primer (5′CAA TCC CAC TAT CCT TCG) in combination with one gene-specific reverse primer. For mapping the primer combinations vte4-4fw (5′TCACGT TTTTCTATTTAATT) and vte4-4rv (5′TCTGTCCAAGA GGTTCTGCT) were used.

Nucleic acid isolation and BAC-library screening

Total RNA was isolated from leaves of 4-week-old plants, using the TRIZOL®-reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. For first-strand amplification with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Karlsruhe, Germany) oligo(dT) primers were used. DNA was extracted from leaves using the CTAB-protocol (Saghai-Maroof et al. 1984).

For Southern hybridization with the *BnaX.VTE4* probe, total DNA was digested with *Eco*RI, *Hin*dIII, and *Xba*I. The *BnaX.VTE4* probe carries one *Hin*dIII and one *Xba*I restriction site. The DNA was separated in a 0.75% agarose gel and transferred to Hybond®-nylon membranes (GE Healthcare, Munich, Germany). The probe was $\alpha[^{32}P]$-labeled (Feinberg and Vogelstein 1983) and hybridized at 60°C for 16 h. The band detection was carried out by autoradiography (Hyperfilm MP, GE Healthcare, Munich, Germany) after 36 h.

Genomic *BnaX.VTE4* sequences were isolated from the JBnB BAC library with Tapidor as a DNA donor (Rana et al. 2004). The BAC library was pre-screened with a probe mixture containing three tocopherol genes. To identify the *BnaX.VTE4* carrying BACs we performed a dot-blot hybridization with the same probe as that used for Southern analysis. Positive BACs were analyzed by PCR with the same primers used for *BnaX.VTE4* amplification and the resulting PCR products were sequenced.

Plasmid cloning and *Agrobacterium tumefaciens* mediated transformation of *A. thaliana*

The PCR products were cloned into the pGEM®T-Vector (Promega, Madison, USA) and one-shot competent *E. coli* cells (Invitrogen, Karlsruhe, Germany) were transformed. Candidate genes were cloned into the binary vector pAM194 carrying a GUS gene and the *nptII* gene for selection (Kifle et al. 1999). For *A. thaliana* transformation the *A. tumefaciens* nopaline strain GV3101 (Koncz and Schell 1986) harboring the helper plasmid pMP90RK was used. The sequence *BnA.VTE4.a1* was modified using the primer combination vte4-3Xho-fw/rv and was cloned into the *Xho*I site of the binary vector pAM194 between the 35S promoter and the terminator sequence. Root transformation was performed essentially as described by Valvekens et al. (1988). The stable integration of the transformed genes was tested by a GUS-assay with regenerating shoots. For this, young leaves were incubated over night with a sodium phosphate buffered solution (pH 7), containing 300 mg l^{-1} X-Gluc at 37°C.

5′- and 3′-RACE experiments and cloning of full length cDNAs

We used 900 ng total RNA to perform 5′ and 3′-RACE experiment (SMART™ RACE cDNA amplification kit, BD Biosciences, Heidelberg, Germany) following the manufacturer's instructions. A PCR was carried out with 25 cycles at 68°C and 10 cycles at 67°C. The resulting products were ligated into the pGEM®-T Vector (Promega, Madison, USA) and transformed into *E. coli*. Five independent recombinant clones were sequenced. The primers vte4-3fw and vte4-3rv were synthesized and used for RT-PCR. *BnaX.VTE4* full-length cDNAs were cloned and the inserts of 39 recombinant plasmids from independent positive clones were sequenced.

Genetic mapping of *BnaA.VTE4a*

For mapping of *BnaA.VTE4a*, we genotyped 190 DH-lines of the DH–TN mapping population using the primer combination vte4-4fw/vte4-4rv. The PCR fragments 280 bp in size were separated in 3% metaphor agarose gels. The sequence was integrated into the existing map consisting of 411 markers using the Joinmap 3.0 software (Van Ooijen and Voorrips 2001). Groups of significantly associated loci at a LOD score of 6.0 were chosen to calculate the linkage map using Haldane's mapping function.

Sequence analysis

The sequences of the different plasmid inserts and PCR products were analyzed using the SeqMan tool from the DNASTAR software (Lasergene, GATC Biotech, Konstanz, Germany). Contig alignment was performed with a similarity threshold of 99%. The four *BnaX.VTE4.a–d* sequences were checked for ORFs and translated with the EditSeq tool. We analyzed the predicted amino acid sequences for transit peptide structures and cleavage sites using the TargetP 1.1 tool (Emanuelsson et al. 2000; Nielsen et al. 1997). For detecting functional domains we compared the sequences with the pfam database (www.sanger.ac.uk/Software/Pfam/, Sanger-Institute). The sequence alignments were performed with the AlignX program of the VectorNTI software (Invitrogen, Karlsruhe, Germany).

Tocopherol analysis

Mature seeds between 3 and 15 mg were ground in 2 ml reaction tubes with the Geno/Grinder 2000 (SPEX SamplePrep, Metuchen, USA) using *n*-heptane and 0.2–2.0 mm metal beads. The samples were incubated for 12 h at −20°C. Further steps and HPLC analyses were performed as described (Dähnhardt et al. 2002; Falk et al. 2003; Schledz et al. 2001).

Results

Cloning of *BnaX.VTE4*-sequences

The primers were designed with the *B. oleracea* sequence AF381248 (representing a *VTE4* homolog sequence) and the *VTE4* sequence from *A. thaliana*. Sequence alignment revealed several highly conserved regions between the two

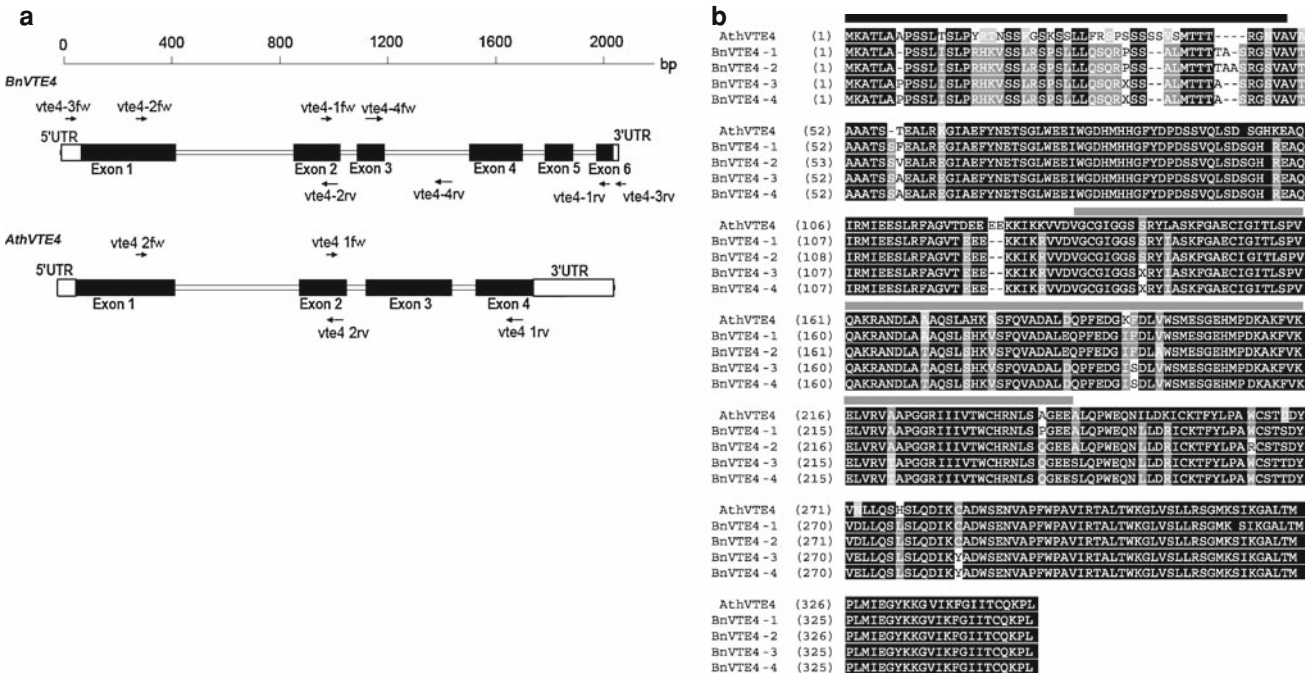

Fig. 2 **a** Structure of the *BnaX.VTE4* genes in comparison to the *A. thaliana* gene *VTE4*. The *A. thaliana* exon 3 is homologous to the exons 3 and 4 from *B. napus*; the exon 4 of *Arabidopsis* is homologous to the exons 5 and 6 from rapeseed. *Black boxes*: translated regions; *white boxes*: untranslated regions. Primers used for amplification are indicated by *arrows*. **b** Comparison of the predicted polypeptides *BnaX.VTE4.a–d* and *A. thaliana* gTMT (AthVTE4). For aligning the sequences the AlignX program was used. Identical amino acids are highlighted in *black*, similar amino acids are highlighted in gray. The *black bars* on top of the sequences indicate the plastid import sequences; the gray bars indicate the SAM-dependent methyltransferase domains

sequences. These were used for designing the 3′- and 5′-RACE primers, vte4-1fw, and vte4-2 rv (Fig. 2a). The full length *BnaX.VTE4* cDNA sequences were amplified from the *B. napus* cultivar Express with the primers vte4-3fw and vte4-3rv, derived from the *B. napus* RACE experiment. As a result the RT-PCR reaction with *B. napus* leaf-RNA gave a single fragment of about 1,160 bp which is within the range of the expected size. Sequence analysis of 39 recombinant clones revealed four different classes of sequences which were named *BnaA.VTE4.a1* and *BnaX.VTE4.b1–d1* (Acc. No. EU637012 to EU637015). Among the four sequences, *BnaA.VTE4.a1* was the most abundant, representing 62% of the analyzed clones (Table 1). The sizes of the predicted translated regions ranged between 1,044 bp (*BnaA.VTE4.a1*, *BnaX.VTE4.c1* and *BnaX.VTE4.d1*) and 1,047 bp (*BnaX.VTE4.b1*) (Table 1). The similarities between the *BnaX.VTE4* sequences and the *VTE4* gene ranged between 86% and 87% (Table 1) and all *BnaX.VTE4* sequences covered the translated region of the *A. thaliana* sequence. In a next step, the predicted BnaVTE4 polypeptide sequences were investigated in silico. A comparison between the *A. thaliana* and the *B. napus* polypeptide sequences is shown in Fig. 2b. The TargetP1.1 tool (Emanuelsson et al. 2000) was used for predicting the subcellular localization of the polypeptide and the pfam-database (http://pfam.sanger.ac.uk/) was used for domain determination. All *B. napus* sequences showed both, the expected transit peptide consisting of about 50 amino acids for plastidal import and the SAM-dependent methyltransferase domains (aa 130 to 228) (see supplementary data S1).

A BAC-library from the *B. napus* variety Tapidor was screened with a *BnaX.VTE4* specific probe. Eight positive BACs could be identified and verified by PCR. The *BnaX.VTE4* sequences were amplified by PCR and sequenced. Three different sequence variants were found which were representing the sequences *BnaA.VTE4.a2*, *BnaX.VTE4.b2*, and *BnaX.VTE4*, which had been identified by RT-PCR. By comparison of the genomic sequences (Acc. No. FJ435091 to FJ435093) with the cDNA sequences two additional introns with sizes of 312, 322 bp (intron 3), and 86 bp (intron 5), respectively, were detected in relation to the *A. thaliana* gene *VTE4* (Fig. 2a).

Table 1 Comparison of the four *BnaX.VTE4* sequences identified through multiple sequence alignment of the insert sequences of 39 full-length EST clones

Sequence	Accession number	ORF (bp)	Similarity to *VTE4* (%)	Number of sequenced clones
BnaA.VTE4.a1	EU637012	1,044	87	24
BnaX.VTE4.b1	EU637013	1,047	86	8
BnaX.VTE4.c1	EU637014	1,044	87	4
BnaX.VTE4.d1	EU637015	1,044	86	3

Genomic copy number estimation

The copy number of the genes was estimated in two different ways: first by sequencing the PCR products, second by genomic Southern hybridization. The sequencing of PCR products yielded four different cDNA (Express) and three genomic sequences (Tapidor), respectively. For Southern analysis, DNA from the rapeseed cultivar Express was restricted with *Eco*RI, *Hin*dIII, and *Xba*I. The genomic fragment obtained after PCR with the primer combination vte4-1fw/vte4-1rv was used as a probe for Southern-blot hybridization. Depending on the restriction enzyme, three, four, and five fragments were detected, respectively (Fig. 3).

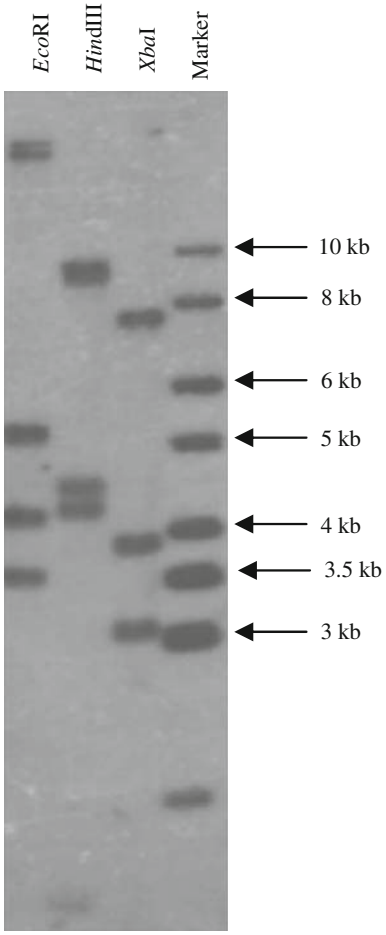

Fig. 3 Southern analysis of genomic *B. napus* DNA (variety Express) to determine the copy numbers of the *BnaX.VTE4* gene family. Nuclear DNA was digested with different enzymes, separated on agarose gels and blotted on nylon membranes. The membranes were hybridized with a PCR fragment from a partial *BnaX.VTE4* genomic sequence

Genetic mapping of *BnaA.VTE4.a*

To map the *BnaX.VTE4a–d* sequences as SNPs, locus specific primer combinations were established. The primer combination vte4-4fw/vte4-4rv (Fig. 2a) distinguishes in Tapidor between the *BnaA.VTE4.a2* sequence and the other sequence variants by a 20 bp deletion within the 3rd intron (see supplementary data S1). As expected, a 300 bp fragment representing *BnaX.VTE4.b2* and *BnaX.VTE4.d2* and a 280 bp fragment representing *BnaA.VTE4.a2* were found in Tapidor, whereas only the larger fragment was present in Ningyou7 (Fig. 4a). Out of 190 DH-lines 75 lines displayed the Tapidor genotype and 115 the Ningyou7 genotype which indicates a distorted segregation ratio and does not fit to the expected 1:1 segregation ($\chi^2 = 8.4 > \alpha_{0.05} = 3.84$). The *BnaA.VTE4.a* locus maps on chromosome A02 in distances of 2.4 and 5.1 cM to the markers sN3761a and HAU69, respectively, which had been previously located on the TN-map (Long et al. 2007; Fig. 4b). Interestingly this gene maps to the same position as two QTL for α-tocopherol content with LOD = 2.85 and 3.23, respectively. These QTL explain 7.0% and 5.3% of the phenotypic variation, respectively (X. Wang, in preparation).

Functional analysis of the *BnaA.VTE4.a1*-sequence by overexpression in *A. thaliana*

To prove its function the *BnaA.VTE4.a1* gene was expressed in *A. thaliana* under the transcriptional control of the 35S promoter (Fig. 5a). The amount and composition of tocopherols were determined by HPLC using T1 seed bulks from three individual transgenic plants tracing back to three different transformation events. Seeds from non-transformed *Arabidopsis* shoots served as a control (Table 2). The α-tocopherol contents of T1 seeds varied from 175 to 348 mg kg^{-1} representing an increase up to 50-fold in the α-tocopherol fraction over the non-transformed control. Correspondingly, the γ-tocopherol fraction was much lower in the transgenic plants (Fig. 5b). β-tocopherol was only found in transgenic plants, whereas the δ-tocopherol fraction of the T1-seeds was slightly lower (Fig. 5b). However, the total tocopherol content in seeds was not significantly changed. T2 and T3 seeds were produced from plant pAM194_*BnVTE4*-90. The altered tocopherol contents and compositions found in the T1 seeds were confirmed by T2 and T3 seed analysis thus demonstrating stable inheritance of this character (Table 2).

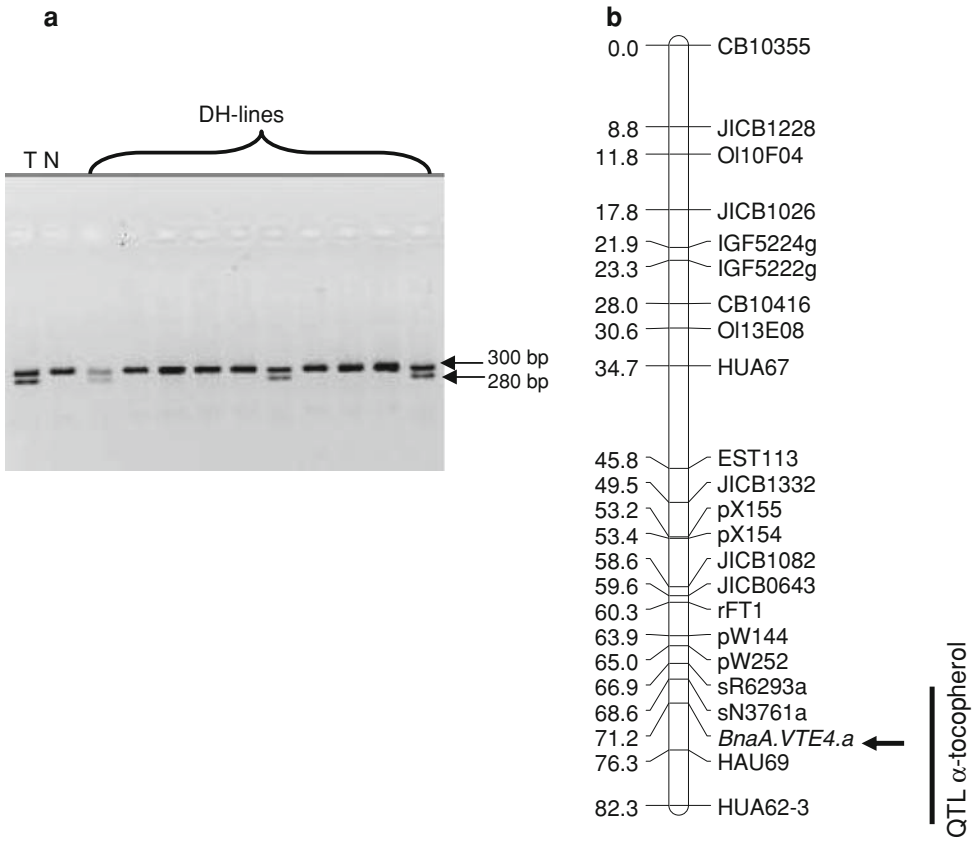

Fig. 4 Mapping of the *BnaA.VTE4.a* sequence as a PCR marker. **a** Gelelectrophoretic separation of *BnaA.VTE4.a*-PCR products from both parents, Tapidor (T), Ningyou7 (N) and 10 DH-lines. The polymorphic banding pattern was used to genotype 190 DH-lines. **b** Map position of the *BnaA.VTE4.a* gene and the α-tocopherol QTL on chromosome A02 (Long et al. 2007; X. Wang et al. in preparation). The confidence interval is indicated by the *vertical bar*

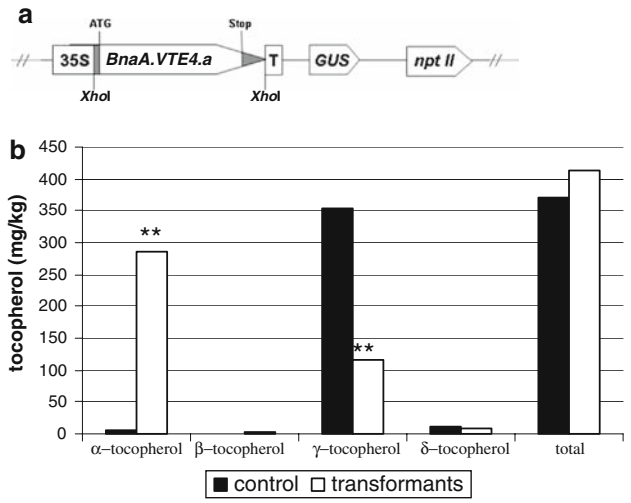

Fig. 5 Functional analysis by overexpression of *BnaA.VTE4.a1* in *A. thaliana*. **a** pAM194 vector construct used for transformation. The sequence was cloned in between the 35S-promotor and a terminator sequence. The vector carries the *nptII* gene for kanamycin resistance and the GUS reporter gene. *BnaA.VTE4.a1* was integrated cloned into the *Xho*I restriction site. Untranslated regions are indicated in gray. **b** Results from tocopherol measurements of T1 seeds of transgenic *A. thaliana* plants and seeds of control plants. Columns are means of separate HPLC analyses (see Table 2). ** Significance level 0.01

Discussion

We have cloned the first gene from *B. napus* involved in tocopherol biosynthesis. The function was verified after transformation into *A. thaliana*. Our study relied on high sequence similarity between species of the *Brassicaceae* which has been demonstrated before (Lagercrantz and Lydiate 1996; Parkin et al. 2002, 2005; Paterson et al. 2001). Based on the fact that all genes for tocopherol biosynthesis have been identified and analyzed from *A. thaliana* the respective sequence was used to clone orthologs from *B. napus*. As demonstrated for other gene families before, consensus primers derived from conserved regions of an *A. thaliana* gene are highly efficient for amplifying homologs from *B. napus* (Brunel et al. 1999). This strategy led to the isolation of partial sequences of 25 *B. napus* genes, which play a role in fatty-acid synthesis, disease defence, and flowering time (Fourmann et al. 2002). We could not find consensus primers for amplifying the 5′ ends of the *BnaX.VTE4* gene, probably due to low sequence conservation within the transit peptide region. Therefore, we used RACE to clone the 5′ end of the *BnaX.VTE4* gene. Low sequence conservation has also been described for the *FAD7* gene involved in fatty-acid

Table 2 Seed tocopherol analysis of transgenic *A. thaliana* plants

	Seed tocopherol content (mg kg^{-1})				
	α	β	γ	δ	Total
A. thaliana control plants	7 ± 1	n.d.	353 ± 52	12 ± 3	372 ± 54
Bulked T1 seeds from selected single T0 plants					
pAM194_*BnVTE4*-103	175 ± 2	2 ± 0	237 ± 37	9 ± 1	423 ± 36
pAM194_*BnVTE4*-176	331 ± 39	6 ± 1	100 ± 1	5 ± 1	442 ± 42
pAM194_*BnVTE4*-90	348	n.d.	9	13	370
Bulked T2 seeds from selected single T1 plants					
pAM194_*BnVTE4*-90-1	499	Traces	2	Traces	501
pAM194_*BnVTE4*-90-2	489	Traces	3	Traces	492
pAM194_*BnVTE4*-90-3	280	Traces	17	Traces	297
Bulked T3 seeds from selected single T2 plants					
pAM194_*BnVTE4*-90-1-1	387	Traces	4	Traces	391
pAM194_*BnVTE4*-90-1-2	318	Traces	5	Traces	313
pAM194_*BnVTE4*-90-1-5	403	Traces	1	Traces	404
pAM194_*BnVTE4*-90-2-1	398	Traces	3	Traces	401
pAM194_*BnVTE4*-90-2-2	466	Traces	1	Traces	467
pAM194_*BnVTE4*-90-2-3	424	Traces	1	Traces	425
pAM194_*BnVTE4*-90-3-1	214	Traces	9	Traces	223
pAM194_*BnVTE4*-90-3-3	346	Traces	8	Traces	354
pAM194_*BnVTE4*-90-3-7	399	Traces	1	Traces	400

T1 seeds harvested on single T0 plants and selected T2 and T3 seed bulks were analyzed by HPLC. Control plants were grown and treated like the transformants

n.d. not detectable; *traces* detectable but not quantifiable

synthesis by (Brunel et al. (1999) who found a low degree of conservation within the first 225 bp between *Arabidopsis* and different *Brassica* species.

There are more than 810,000 EST entries in GenBbank from *B. napus* and the related species of the U-triangle listed in the sequence databases (www.brassica.info). For *VTE4* in addition to the *B. oleracea* sequence AF381248, which was used for primer design, full-length sequences of *B. juncea* (DQ864978) and *B. napus* (DQ508019) were also available. They show 96% homology to the *BnaA.VTE4.a1* sequence identified in this study; however, no proof of function has been performed yet.

There are three lines of evidence which demonstrate that we have cloned functional copies of *VTE4* from the *B. napus* genome. First, the sequence comparison between the *B. napus* sequences and the *A. thaliana* sequences revealed high similarity between *B. napus* and *A. thaliana* sequences. The *BnaX.VTE4* genes show similar cDNA lengths compared to *A. thaliana.* Second, the overall structure of the genes and the predicted protein is very similar although the *BnaX.VTE4* genes haves two additional introns compared to *A. thaliana*. The similarity between intron sequences of both species was lower as between exons. Third, the tocopherol profiles of transgenic *A. thaliana* plants transformed with *BnaA.VTE4.a1* were different from wild type plants. For transformation we chose the sequence *BnaA.VTE4.a1* because this sequence was the most abundant sequence in among all analyzed cDNA clones and the putative amino-acid sequence showed the highest similarity to *A. thaliana*. HPLC analysis of T1 seeds showed the expected changes in tocopherol composition. The *BnaA.VTE4.a1* transformants displayed a shift from γ-tocopherol to α-tocopherol which is in accordance with the function of the encoded methyltransferase. Likewise, Van Eenennaam et al. (2003) and Shintani and DellaPenna (1998) found a shift from γ-tocopherol to α-tocopherol after transformation of soybean and *A. thaliana* with the *VTE4* gene from *A. thaliana*.

The polyploid nature of the rapeseed genome poses a problem for gene identification. On an average, each *A. thaliana* sequence is represented by four to six copies in rapeseed (Brunel et al. 1999; Fourmann et al. 2002; Parkin et al. 2003). In our study two approaches were chosen to determine the copy number of sequences with homology to the *VTE4* gene. First, amplified fragments were sequenced either directly or after cloning. Second, PCR fragments were hybridized to genomic DNA and the number of bands was determined. Conclusively, the number of copies was estimated to be in a range between three and five.

Tocopherol content and composition can be determined safely only by HPLC which is not applicable for routine selection during breeding. This created a need to establish diagnostic marker assisted selection. Hence, a major focus of our work was to use the tocopherol pathway sequence as molecular markers to map them within the *B. napus* genome and to determine putative coincidence of marker positions and tocopherol QTLs. The polymorphism affecting the *BnaA.VTE4.a* locus in the Tapidor genotype has been successfully used to integrate the gene as a PCR-marker into an existing *B. napus* map. This mapping population has been used before to map QTL for tocopherol content (X. Wang, in preparation). The *BnaA.VTE4.a* gene and two QTL controlling α-tocopherol content both mapped to the same position on chromosome A02. The additive effects of the Tapidor alleles were quite low (4.81 and 3.56 ppm) which could be expected because both parents differ much with regard to their α-tocopherol content and because of strong genotype $\times$ environment interaction (Marwede et al. 2004). Our data suggest that *BnaA.VTE4.a* is a candidate gene for these tocopherol QTLs. Likewise, in *A. thaliana* many tocopherol QTLs could be explained by known tocopherol genes but only a minor QTL was found to be linked with *VTE4* (Gilliland et al. 2006).

Presently the other genes involved in tocopherol biosynthesis from *B. napus*, namely *VTE1*, *VTE2*, *VTE3*, and *PDS1* as well as related genes like *VTE5* are cloned in our lab by their homology to the respective *A. thaliana* sequences. After cloning they will be mapped to the existing linkage maps of *B. napus*. Our final aim is to compare their map positions with the positions of QTL for tocopherol biosynthesis. In this way the sequences will be used as functional markers, a concept presently followed for many characters in crop plants (Andersen and Lübberstedt 2003). Coincidence of QTL and tocopherol gene position would be indicative for the function of the respective gene in tocopherol synthesis and composition. For further identification of ubiquitous markers, tocopherol profiles and tocopherol sequence haplotypes will be determined in a panel of 90 *B. napus* varieties to uncover linkage disequilibrium. Since tocopherol content and composition is difficult to determine and HPLC analysis cannot be used as a routine selection procedure selectable markers would greatly enhance the breeding process to select oilseed rape varieties with improved tocopherol content and composition.

Acknowledgments We wish to thank Martina Bach and Jens Hermann for their excellent technical assistance. This work was funded by the Stiftung Schleswig-Holsteinische Landschaft and the German Research Foundation (DFG) as part of the Research Training Group GRK820.

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