Validation of an updated Associative Transcriptomics platform for the polyploid crop species *Brassica napus* by dissection of the genetic architecture of erucic acid and tocopherol isoform variation in seeds

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Summary

An updated platform was developed to underpin association genetics studies in the polyploid crop species *Brassica napus*. Based on 1.92 x 10^{12} bases of leaf mRNAseq data, functional genotypes, comprising 355,536 single nucleotide polymorphism markers and transcript abundance were scored across a 383-accession genetic diversity panel using a transcriptome reference comprising 116,098 ordered CDS gene models. The use of the platform for Associative Transcriptomics was first tested by analysing the genetic architecture of variation for seed erucic acid content, as high erucic rapeseed oil is highly valued for a variety of applications in industry. Known loci were identified, along with a previously undetected minor effect locus. The platform was then used to analyse variation for the relative proportions of tocopherol (Vitamin E) forms in seeds and the validity of the most significant markers assessed using a take-one-out approach. Furthermore, the analysis implicated expression variation of the gene Bo2g050970.1, an orthologue of *VTE4* (which encodes a γ-tocopherol methyl transferase converting γ-tocopherol into α-tocopherol), associated with the observed trait variation. The establishment of the first full-scale Associative Transcriptomics platform for *B. napus* enables rapid progress to be made towards an understanding of the genetic architecture of trait variation in this important species and provides an exemplar for other crops.

Significance statement

58 The availability of a full-scale association genetics platform for *Brassica napus*,
59 based on functional genotypes, enables the genetic architecture of essentially
60 any trait to be addressed in this important crop species.
61
62
63 **Introduction**
64 As the demand for ever increasing crop productivity continues against the
65 backdrop of changing climate and diminishing resources, crop improvement has
66 become an important driver for advances in genomic technologies in plants. A
67 broad aim of crop science is the identification of the genetic bases for trait
68 variation, including both the identification of beneficial alleles and the
69 development of molecular markers to accelerate introduction into elite
70 germplasm. Genetic diversity panels, typically comprising past and current
71 cultivars along with wild relatives, are usually available for crop species. Such
72 panels represent ideal resources for genome-wide association studies (GWAS),
73 which exploit historical recombination between molecular markers and loci
74 associated with trait variation. Where recombination between loci is observed
75 proportionately less frequently than expected for unlinked loci (i.e. < 0.5), those
76 loci are said to be in Linkage Disequilibrium (LD). The approach of identifying
77 molecular markers in LD with loci associated with trait variation is an important
78 tool in human genetics studies and has been applied successfully in several plant
79 species (Garrigan and Hammer, 2006; Li et al., 2008; Atwell et al., 2010; Cockram
80 et al., 2010; Tian et al., 2011; Zhao et al., 2011).The recent development of
81 transcriptome-based GWAS, including the technology termed Associative

Transcriptomics (AT), in which both gene sequence variation and transcript abundance variation are used to identify associations with trait variation (Harper et al., 2012) greatly increases the range of crops to which GWAS approaches can be applied.

The Brassicaceae family includes *Arabidopsis thaliana*, the first plant for which a high quality genome sequence was available (AGI, 2000), and the *Brassica* crops. The diploid species *Brassica rapa* and *Brassica oleracea*, which contain the *Brassica* A and C genomes, respectively, are closely related, having shared a common ancestor only *ca.* 3.7 Mya (Inaba and Nishio, 2002). *Brassica napus* is an allopolyploid, arising from the hybridization of these species (U, 1935) and the related (homoeologous) regions of the genomes are clearly discernible (Bancroft et al., 2015). A diverse range of *B. napus* crop types have been developed, including oilseed rape, fodders, leafy vegetables and root vegetables. *Brassica* species have been used extensively in genomics studies, due to their utility in studying the evolution of polyploid genomes (Song et al., 1995; O'Neill and Bancroft, 2000; Pires et al., 2004; Yang et al., 2006; Town et al., 2006; Cheung et al., 2009). A draft genome sequence has been obtained for *B. napus* (Chalhoub et al., 2014). However, at ca. 1.2 Gb, the genome of *B. napus* is relatively large. To address this problem, rapid and cost-effective transcriptome-based technologies, using mRNAseq, have been developed and applied for SNP discovery (Trick et al., 2009), linkage mapping and genome characterization (Bancroft et al., 2011) and transcript quantification (Higgins et al., 2012). Indeed, AT was first developed in *B. napus* with a very small genetic diversity panel,

106 enabling the implication of orthologues of *HAG1* in the control of seed
107 glucosinolate content (Harper et al., 2012).
108
109 Vegetable oils are a major source of dietary vitamin E (Goffman and Becker,
110 2002). Vitamin E occurs in the form of tocopherols, which are lipid-soluble
111 antioxidants that accumulate in the chloroplast. Their function is to protect
112 photosystem II from oxidative damage under the influence of free/released lipid
113 peroxyl radicals and singlet oxygen (Quadrana et al., 2013) and in seeds they
114 play role in preventing oxidation of polyunsaturated fatty acids (PUFAs). The four
115 forms of tocopherol (α-, β-, γ- and δ-), vary in the number and position of methyl
116 substituents attached to the chromanol ring (Munné-Bosh and Alegre, 2002). The
117 most abundant forms of vitamin E in rapeseed oil are γ- and α-tocopherol, with a
118 small proportion of δ-tocopherol (Fritsche et al., 2012, Wang et al., 2012).
119 Besides its nutritional value, α-tocopherol is the most potent vitamin E, whereas
120 the γ- and δ-tocopherol forms are valued for their oil-stabilizing properties
121 (Munné-Bosh and Alegre, 2002), which is particularly relevant for PUFA-rich oils,
122 such as rapeseed. Tocopherol content and composition in rapeseed varies
123 widely; values for total tocopherol content (TTC) have been reported ranging
124 between 166 and 687 $mg.kg^{-1}$, α-tocopherol content between 59 and 286 $mg.kg^{-1}$, and γ-tocopherol content from 107 to 280 $mg.kg^{-1}$. The ratio between α- and γ-
126 tocopherol has also been reported to range between 0.33 and 2.14 (Dolde et al.,
127 1999; Goffman and Becker, 2002; Wang et al., 2012; Fritsche et al., 2012). Genes
128 involved in the tocopherol biosynthetic pathway have been identified in
129 *Arabidopsis thaliana* and other model plants (Valentin et al., 2006; Endrigkeit et

al., 2009; Li et al., 2012), (Figure 1). QTL affecting seed tocopherol content and composition have also been reported (Gilliand et al., 2006), but the control of biosynthesis is poorly understood.

The first AT panel reported for *B. napus* (Harper et al., 2012) comprised only 84 accessions and was smaller than is usually required for association studies (Spencer et al., 2009), meaning that it could be used successfully only for traits with a simple genetic basis. In this study, we report the establishment of a full AT platform for the crop species *B. napus*, based on a widely-shared genetic diversity panel of 383 accessions, which can be used to address the genetic architecture of a broad range of traits. We validated the resource by analysing with the new platform a trait that had been analysed using the original panel (erucic acid content of seed oil), and a new trait (the relative content of γ and α forms of tocopherol in seeds).

Results

The RIPR genetic diversity panel

A diversity panel of 383 *B. napus* doubled haploid (DH) or inbred accessions was assembled, with the aim of covering the breadth of genetic variation available in the species. This panel included the breadth of crop types of *B. napus* and comprised 362 inbred lines previously used by Bus et al. (2011) and Harper et al. (2012) plus 21 further accessions as used by Thomas et al. (2016). The list of

154 accessions is shown in Data S1. The panel is named RIPR after the research
155 project “BBSRC Renewable Industrial Products from Rapeseed (RIPR)
156 Programme” that funded its development and genotyping.
157
158 **Functional genotypes**
159 Functional genotypes were produced for the panel based on leaf RNA, with 100-
160 base read length mRNAseq data produced using the Illumina HiSeq 2000
161 platform. A total of 1.92 x 10^{12} bases of sequence data were produced. The
162 sequence reads were mapped to the CDS gene model-based *Brassica* AC pan
163 transcriptome reference (He et al., 2015), which comprised 116,098 gene
164 models, has an aggregate length of 118,657,829 bases and for which we provide
165 an updated gene order based on a high density SNP linkage map as shown in
166 Data S2. Sequence read mapping statistics are summarised in Data S1. Mean
167 figures of 50,165,125 reads were generated per accession, with 32,275,718
168 being mapped across 61,620,266 bases of the reference sequence, representing
169 52.1-fold coverage of the 51.9% of the predicted transcriptome to which
170 mRNAseq reads were mapped. SNPs were identified and gene expression
171 quantified. Across the panel of 383 lines, 355,536 SNPs were scored, of which
172 the majority (87.0%) were hemi-SNPs, as found in previous *B. napus* studies
173 (Trick et al., 2009). A total of 127,153,561 allele calls were made, with 9,017,727
174 (6.6%) missing values. Significant expression (>0.4 RPKM) was detected for
175 53,889 CDS models (46.4% of all CDS models in the AC pan transcriptome
176 reference), of which 25,834 belong to the A genome and 28,055 to the C genome.

177 The functional genotypes are available from the York Oilseed Rape
178 Knowledgebase (http://www.yorknowledgebase.info/).
179
180 **Genetic architecture of the Population**
181 The 355,536 SNP markers scored across the RIPR panel were used to analyse
182 the relatedness of members of the panel. First, a distance matrix was generated
183 and visualised by the dendrogram shown in Figure 2a. The assigned crop types
184 (Data S1) show the expected clustering, as shown in Figure 2b. Next, the
185 population structure of the panel was analysed using PSIKO (Popescu et al.,
186 2014). The highest likelihood is a subpopulation K=2, with mixture across the
187 panel as illustrated in Figure 2c. Finally, LD was calculated across the genome,
188 as summarised in Figure S1, producing a mean value of 0.031 for the population.
189
190 **Seed erucic acid analysis**
191 Erucic acid is a 22-carbon monounsaturated fatty acid. Its content in rapeseed oil
192 is one of the key determinants of suitability for use as an edible or industrial oil.
193 Detection of the known loci controlling the biosynthesis of erucic acid in seeds
194 was used as a validation study for the first report of AT (Harper et al., 2012). We
195 re-analysed this trait to compare the performance of the original panel with the
196 new RIPR panel. The fatty acid composition of seeds was determined for 376
197 lines of the RIPR diversity panel (summarised in Data S3). The erucic acid
198 content of seeds varied between 0 and 51%, reflecting the range of crop types
199 represented in the panel, which included modern Canola quality rapeseed
200 varieties as well as crop types for which seed composition was not the subject of

active domesticated selection process (hence representative of “unimproved” seed composition).

Associative Transcriptomics of erucic acid content

The first stage of validation of the new AT platform for *B. napus* involved analysis of seed erucic acid content, a trait for which the two main control loci are known and were confirmed previously by AT (Harper et al., 2012). The estimated narrow-sense heritability (h^2) for the erucic acid trait was estimated from the SNP analysis as 0.794. 318 genome-assigned SNP markers above the Bonferroni-corrected significance threshold of $P = 0.05$ (i.e. $-\log_{10}P$ value of 6.7) were detected across association signals on chromosomes A5, A8, A9, A10 and C3 (Data S4, Figure S3), as illustrated in Figure 3a. The main loci controlling erucic content (on chromosomes A8 and C3) provide association signals with a significance eight orders of magnitude greater: $-\log_{10}P > 16$, compared with <8 in the previous study. The known control genes, orthologues of *FAE1* (AT4G34520), represented by gene models Cab035983.1 and Bo3g168810.1, are near the centres of these SNP association peaks, in the distance of 6 genes (~42 kb) and 9 genes (~56 kb) from the closest significantly associated gene, respectively, according of the reference sequence (Data S4). In addition, SNP associations were found for a region of the genome, on chromosome A5, which were not previously detected. This indicates the position of a novel locus with minor effect on the trait. A candidate for the trait control gene in this region is Cab033920.1. This gene is an orthologue of AT2G34770.1, which is annotated as fatty acid hydroxylase 1 which has a potential role in very long chain fatty acid biosynthesis. An association

signal was also detected for a relatively large region of chromosome A9, which we interpret as corresponding to a seed glucosinolate-controlling locus, which was co-selected in modern low erucic rapeseed cultivars to produce Canola quality seed.

In addition to association analysis using SNP markers, AT also reveals associations between gene expression markers (in the tissue of second true leaves used for the development of functional genotypes) and trait variation. In the case of seed erucic acid content, the main control genes (orthologues of *FAE1*) are transcriptionally inactive in the tissue (leaves) sampled for production of the functional genotypes. However, we are still able to detect both SNP and GEM association peaks through markers in linkage disequilibrium (LD) with *FAE1* on A8 and C3 as illustrated in Figure 3b. The lower resolution observed for the A8 peaks may reflect the influence of two strong bottlenecks during the breeding selection (Hasan et al., 2008) for low glucosinolate content (controlling loci on chromosome A2, A9, C2 and C9) and zero seed erucic acid (controlling loci on chromosome A8 and C3), or perhaps the presence of additional minor effect genes located on A8 that are also contributing to the erucic trait. Indeed there are many potential candidate genes in the region which could have an effect, including an orthologue of FAD6 (AT4G30950) which could act to reduce the pool of oleic acid available for elongation to erucic acid. In addition, there is a signature of slightly inflated LD on the first half of A8, which may further contribute to reducing the resolution of association peaks in this region (Figure S1).

The clear signals in the transcript abundance-based association analysis confirms the stability of differential gene expression across the panel and its utility for the identification of association signals. Regions of the genome previously associated with seed glucosinolate content (selected alongside erucic content in Canola quality rapeseed) show particularly strong transcript abundance associations, which we interpret as consequences of the extensive structural variation in these regions of the genome (He et al, 2016). The new AT platform generates strong signals due to the large, diverse panel and superior number of markers assigned to homoeologues, properties lacking in the platform reported previously (Harper et al, 2012).

Tocopherol phenotype analysis

We selected tocopherols in seeds as test traits of unknown genetic basis, quantifying α, γ and δ forms. Tocopherols were purified from seeds and quantified for 377 accessions of the RIPR panel. The results are summarised in Data S5 and Figure S2. Total tocopherol in seeds varied from 197 to 445 $mg.kg^{-1}$, with the main types being γ-tocopherol (78 to 347 $mg.kg^{-1}$) and α-tocopherol (51 to 229 $mg.kg^{-1}$), the relative proportions of which (measured as the γ/α-tocopherol ratio) varied greatly, ranging from 0.485 to 5.00, δ-tocopherol was a minor component (1.8 to 9.9 $mg.kg^{-1}$). Analysis of tocopherol characteristics by crop type showed that γ-tocopherol content tended to be higher in spring crop types and α-tocopherol content tended to be higher in winter crop types, as illustrated in Figure 2d.

Given that the purpose of tocopherols in seed oil is to protect against oxidation, we assessed the diversity panel for correlation of tocopherol traits with the proportions of the fatty acids found in seed oil that are most susceptible to oxidation, the polyunsaturated fatty acids (PUFAs) linoleic and linolenic. The content of these fatty acids had been determined alongside that of erucic acid (Data S3). A weak positive correlation between total tocopherol and PUFA content was, indeed, identified (R^2 = 0.13; $p<0.001$)..

Associative Transcriptomics of tocopherol composition

To undertake AT for tocopherol traits, we analysed the population for loci controlling the proportion of tocopherol occurring in the γ form rather than the α form by using γ/α ratio as the trait. The SNP-based association analysis, as illustrated in Figure 4a, revealed exceptionally strong associations with markers in a very small regions of chromosome C2, along with weaker associations with a few markers in regions of chromosomes A2 and A10. Unlike seed erucic acid, tocopherol composition has not been selected by *B. napus* breeders. We interpret the very sharp association signal as indicative of this lack of selection and to be consistent with LD across most of the genome. The association peak on chromosome C2 includes 33 genome-assigned markers above the Bonferroni-corrected significance threshold (alpha = 0.05 ; – $\log_{10}P$ value of 6.7) (Data S6, Figure S3). These delineated a genomic region containing 39 genes, including an orthologue of *VTE4*, which encodes a γ-tocopherol methyl transferase (γ-TMT), an enzyme that converts γ-tocopherol into α-tocopherol (Figure 1). A homoeologous region including a duplicate copy of *VTE4* gene within association

297 peak on chromosome A2 was observed, while there was no obvious candidate
298 gene in the region of chromosome A10 showing associations. Four transcript
299 abundance-based markers above the Bonferroni-corrected significance
300 threshold (– $\log_{10}P$ value of 6.03 for GEMs) were identified on chromosome C2,
301 C5 and C7 (Figure 4b).The identification of the gene *VTE4* as the most highly
302 associated GEM on chromosome C2 demonstrated the ability for AT to efficiently
303 provide candidate genes associated with traits of interest.
304 To investigate whether the top selected markers are predictive for γ/α ratio, we
305 performed a set of "take-one-out" permutations for the SNP and GEM markers
306 identified from association analysis of 377 accessions adapted from Harper et al
307 (2016). Markers above the Bonferroni line (Data S6 and S7) were selected for
308 each round of permutations. For SNP data, the allelic effects of each of these
309 markers was used to predict trait values for the missing accessions based on
310 their scored genotypes. For GEM data, RPKM values were fitted to the regression
311 line to predict trait values. The predicted trait values against the observed trait
312 are illustrated, as scatter plots, in Figure 5 and confirmed excellent predictive
313 ability (R^2 = 0.59 for SNPs and R^2 =0.47 for GEMs between predicted and
314 observed values; $p<0.001$), which reflect the estimated narrow-sense heritability
315 (h^2) of 0.452 for γ/α ratio. These SNPs and GEMs can therefore be used as
316 promising markers in marker assisted breeding.
317
318
319

In order to confirm the role of the *VTE4* orthologue in the associated region of C2 (Bo2g050970.1), we used the transcript quantification data that were obtained alongside the transcriptome SNP data as part of the functional genotypes. As illustrated in Figure 6, these show that the expression level of Bo2g050970.1 in the tissue sampled to produce the functional genotypes (leaves) is negatively correlated with the γ/α ratio (R^2 = 0.41, $p<0.001$). This is consistent with the predicted γ-TMT activity of the gene encoded by Bo2g050970.1 (*i.e.* lower expression leading to less conversion of γ-tocopherol to α-tocopherol). There had been no significant associations between SNPs within Bo2g050970.1 and the γ/α ratio, consistent with the basis of the allelic variation being variation in gene expression rather than variation in gene sequence.

Discussion

Association studies are becoming increasingly widely-used in crops for identifying molecular markers linked to trait-controlling loci (Rafalski, 2010). However, polyploid crops present additional difficulties that must be overcome, including the intrinsic genome complexity and increased genome structural instability, such as the copy-number variations (CNV) which affect gene families (Zhang et al., 2013; Renny-Byfield and Wendel, 2014). Such difficulties occur in *B. napus*, as was recently shown by Chalhoub et al. (2014) and He et al. (2016). Association studies have to meet many demands to maximize the probability of identifying marker-trait associations. In addition to good planning of experimental design,

along with access to all the necessary equipment and available funds, there is also the need to choose a permanent and sufficiently large set of diverse and preferably homozygous individuals, the larger size and higher genetic diversity of which providing sufficient power for association analysis (Spencer et al., 2009; Huang and Han, 2014). Once assembled, association panels need to be genotyped with molecular markers in a sufficiently high density to identify polymorphisms in linkage disequilibrium with trait-controlling loci. The development of suitable association panels is challenging for individual research groups, providing a driver for the development of community resources.

In this study, we introduce a new genetically diverse AT panel of 383 rapeseed accessions, together with a mapping platform that comprises complete genotype information for this panel, which may be used for a broad range of association studies suitable for re-phenotyping any trait, without the need of additional genotyping. This panel, being made available with all transcriptomic data, offers a large range of potential applications: identifying causative genes, uncovering unknown pathways, identifying regulatory genes or transcription factors, and screening of available germplasm for allelic variants and to support the development of molecular markers for marker-assisted breeding. Our resource provides 355,536 SNP markers, equivalent to one SNPevery 0.33 kb across our *Brassica napus* AC pan-transcriptome reference. The SNP density is much higher than the density of the commercially available 60K Brassica Infinium® SNP array, which only provided 26,841 or 21,117 SNPs for recent *B. napus* GWAS studies (Li et al., 2014, Xu et al., 2016). Although the number of SNPs

368 can even be greater when using whole genome re-sequencing, as shown by
369 Huang et al. (2013), the advantage of transcriptome re-sequencing using
370 mRNAseq is the availability of transcript abundance data; in our case for 46% of
371 the genes present in the AC pan-transcriptome reference sequence. In this study,
372 we demonstrate a significant step-change in resolution from our original AT
373 platform based on a panel of 84 accessions, as reported in Harper et al. (2012).
374 The unigene-based transcriptome reference sequence used by that platform had
375 relatively poor capability to resolve homoeologous loci, due to its construction
376 based on a Brassica-wide transcriptome assembly and subsequent “curing” to
377 more closely match the progenitor genomes. In the absence of the ability to map
378 sequence reads unambiguously to the correct homoeologue, most SNPs appear,
379 due to cross-mapping, as “hemi-SNPs”, i.e. where one allele comprises a mixture
380 of two bases (Trick et al., 2009). In the original platform only a small proportion
381 of markers could be assigned with high confidence to a genome, the majority
382 being assigned to both homoeologous positions. The new platform is based
383 mainly on gene models originating from the genome sequences of the progenitor
384 species permits more discriminating read mapping, resulting in a greater
385 proportion of “simple SNPs” (i.e. where the polymorphism is between resolved
386 single bases only), which can be assigned with confidence to a genome. Where
387 there are association peaks comprising pale points in homoeologous positions to
388 the associations identified, such as those observed in regions of A2 depicted in
389 Figure 4a, these can be disregarded as homoeologous “shadows” of the regions
390 genuinely containing causative variation. SNP discovery for particular genes from
391 juvenile leaves can be limited by their transcription in different phenological stage

392 or tissue, but candidate loci/genes associated with trait manifesting in different
393 time or place can be still identified, as demonstrated here in case of *FAE1* and in
394 previous AT studies (Lu et al., 2014; Wood et al., 2017). This is possible due to
395 the presence of variation in genes in LD with the causative gene, resulting in an
396 associated region including the control gene. In addition, the new platform
397 provides much greater resolution of the contributions to the transcriptome of pairs
398 of homoeologous genes. This permitted efficient detection of association peaks
399 based solely on transcript abundance variation, as illustrated in Figure 3.
400 Moreover, the current platform also allows deeper insight of the structural
401 changes and functional interactions between *B. napus* AC genomes. Information
402 about respective homologous genes including their copy number, sequence
403 variation and transcript prevalence provides important information in polyploid
404 research.
405
406 In addition to extending previous association studies of the control of seed erucic
407 acid content, a trait selected recently by rapeseed breeders, we applied the
408 platform to a trait not previously selected by breeders, or studied extensively: the
409 control of tocopherol (Vitamin E) forms accumulated in seeds. We analysed seed
410 tocopherols in 377 rapeseed accessions for their type and content. The profiles
411 presented here showed a high degree of variability for the γ-/α-tocopherol ratio
412 (CV=53%), displaying distinct patterns for different crop types, which allowed us
413 to identify gene Bo2g050970.1 (an orthologue of the Arabidopsis gene *VTE4*) on
414 chromosome C2 as a candidate gene, based on inference of gene function based
415 on studies of its orthologue in *A. thaliana*. Although there was no evidence of the

presence any specific allelic form of the *VTE4* orthologue associated with γ-/α-tocopherol ratio, this gene has been easily identifiable by the presence of SNPs in surrounding genes. This set of tightly linked markers exhibited excellent predictive ability (Figure 5), which we attribute to the broad (species-wide) range of genetic variation represented by the RIPR diversity panel, overcoming the lack of predictive capability that can be encountered when applying markers to test material (Bush and Moore, 2012). The association we observed between transcript abundance of Bo2g050970.1 in leaves and the tocopherol γ/α ratio in seed is consistent with our understanding that tocopherols are synthesized and localized in plastids and accumulate in all tissues with generally highest content in seeds (Sattler et al., 2004). In Arabidopsis, γ-TMT (*VTE4,* AT1G64970) is known to use δ- and γ-tocopherols as substrates to produce β- and α-tocopherols respectively (Shitani and DellaPenna, 1998) and the effect of *VTE4* gene from *B. napus* on α-tocopherol content has been also proved by overexpression in soybean and Arabidopsis (Chen et al., 2012, Endrigkeit et al., 2009).

By assembling and developing functional genotypes (i.e. comprising both gene sequence variation and gene expression variation) for a diversity panel representing species-wide genetic diversity, we have established a resource for the whole rapeseed research community to use. Furthermore, the success of the approach of Associative Transcriptomics for the identification not only of linked markers, but of candidates for causative genes, serves as an exemplar for plant and crop science more broadly.

Experimental procedures

Growth of the genetic diversity panel

The panel of 383 *B. napus* accessions is available from the John Innes Centre, Norwich, UK. It was planted in a randomized block design of five biological replicates under controlled conditions of two polytunnels at University of Nottingham as described by Thomas et al. (2016). The accessions comprise inbred derivatives of both recent and historic varieties and some research lines. Plants were bagged before flowering to prevent cross-pollination. Seeds were collected from individual plants at maturity. Seeds from 377 and 376 accessions were used for the tocopherol and erucic acid measurement, respectively. Based on descriptors originally received with the material and analysis of relatedness, they were attributed to one of seven different groups, namely spring oilseed rape (123), semi-winter oilseed rape (11), swede (27), kale (3), fodder (6), winter oilseed rape (169) or crop type not assigned (44), as listed in Data S1.

Measurement of fatty acid content and composition

For fatty acid methyl esters (FAME) analysis, 30 mg of seeds were homogenized in a glass vial with 5 ml of heptane. To the homogenate, 500 µl of 2 M potassium hydroxide was added, left for one hour and neutralised with sodium hydrogen sulphate monohydrate. The upper phase was transferred into a crimp cap Chromacol 0.8 ml vials for analysis using a DANI Master GC fitted with an SGE-BPX70 double column.

464

465 **Measurement of tocopherol content and composition**

466 The α-, γ- and δ-tocopherol (the sum of which formed total tocopherol, TTC) were
467 extracted from a homogenous mixture of 80 mg rapeseed seeds and analyzed
468 by normal-phase HPLC as described previously (Fritsche et al., 2012). Modified
469 mobile phase A was heptane (Rathburn, Walkerburn, UK), phase B
470 heptane:dioxane (Sigma-Aldrich, Gillingham, UK) (90:10, v/v). Internal standard,
471 α-tocopherol acetate (Sigma-Aldrich), was added to each sample at a
472 concentration of 25.4 μM (12 $\mu g \cdot mL^{-1}$).

473

474 **SNP identification and Transcript quantification for RNA-seq data**

475 The growth conditions, sampling of plant material, RNA extraction and
476 transcriptome sequencing was carried out as described by He et al. (2016). The
477 RNA-seq data from each accession line were mapped on to recently-developed
478 ordered Brassica A and C pan-transcriptomes (He et al., 2015) as reference
479 sequences Maq v0.7.1 (Li et al., 2008). SNPs were called by the meta-analysis
480 of alignments as described in Bancroft et al. (2011) of mRNAseq reads obtained
481 from each of the *B. napus* accessions. SNP positions were excluded if they did
482 not have a read depth in excess of 10, a base call quality above Q20, missing
483 data below 0.25, and 3 alleles or fewer. An additional noise threshold was
484 employed to reduce the effect of sequencing errors, whereby ambiguous bases
485 were only allowed to be called if both bases were present at a frequency of 0.2
486 or above. This resulted in a set of 355,536 SNPs, of which 256,397 had the
487 second most frequent allele in the population, so called here as a minor allele

frequency (MAF) > 0.01. The markers were also classified as those that can be assigned with confidence to the genomic position of the CDS model in which they are scored (simple SNPs and hemi-SNPs genetically mapped into the appropriate genome using the TNDH mapping population), and those that cannot as the polymorphism may be in either homoeologue of the CDS model in which they are scored (hemi-SNPs not genetically mapped into the appropriate genome using the TNDH mapping population). Transcript abundance was quantified and normalized as reads per kb per million aligned reads (RPKM) for each sample for 116,098 CDS models of the pan-transcriptome reference. Significant expression (>0.4 RPKM) was detected for 53,889 CDS models.

Clustering based on SNP genotypes

Clustering and dendrogram visualisation on SNP data was performed by in-house R script. R package “phangorn” was used for generating distance matrix with JC69 model (Schliep, 2011).

Assessment of LD

Pairwise linkage disequilibrium was calculated and heatmaps produced for each individual chromosome, and these values used to calculate the mean LD across the genome. SNPs were removed from the analysis if they were not confirmed by TNDH population (Qiu et al., 2006) that assigned to the A or C genome and if their minor allele frequency was below 0.01. A single SNP was selected at random from each CDS model to reduce the effect of many linked SNPs in the

same gene. Pairwise r2 LD matrices and heatmaps were calculated for each chromosome using the R package LDheatmap (version 0.99-2; Shin et al., 2006).

Associative Transcriptomic analysis

SNPs and gene expression markers (GEMs) association analysis was performed using R as previously described by (Harper et al., 2012,Sollars et al., 2017), with modifications: to deal with the greatly increased sizes of the datasets, PSIKO (Popescu et al., 2014) was used for Q-matrix generation and GAPIT R package with a mixed linear model (Lipka et al., 2012) was used for GWAS analysis. For SNP association Manhattan plots, SNP markers were filtered to include only those with minor allele frequency > 0.01, markers that could be assigned with confidence to the genomic position of the CDS model are rendered as dark points and markers that could not be assigned with confidence were rendered as pale points. For GEM association, CDS models were filtered prior to regression to include only those with mean expression across the panel > 0.4 RPKM. The association between gene expression and traits was calculated by fixed effect linear model in R with RPKM values and the Q matrix inferred by PSIKO as the explanatory variables and trait score the response variable. R^2 regression coefficients, constants and significance values were outputted for each regression. Genomic control (Devlin and Roeder, 1999) was applied to the GEM analysis to correct for spurious associations, with p-value adjustment applied when the genomic inflation factor (λ) was observed to be greater than 1.

Validation of marker association by trait prediction

The predictive power of the best GEMs and SNPs were assessed using a “take one out” approach (Harper et al. 2016) whereby each accession is removed from the SNP or GEM analysis in turn. An in-house R script was performed with adaptation from Harper 2016, with a modification of incorporating all SNPs and GEMs above bonferroni lines. When permutations finishes, an r square value is calculated from predicted trait values regressed against the observed trait values which indicates the predictive power of the top selected GEMs and SNPs.

Accession numbers

Sequence data from this article can be found in the SRA data library under accession number PRJNA309367.

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Supporting Information

559 Supporting data are provided. The largest datasets, representing the functional
560 genotypes of the RIPR panel, are accessible via a data distribution website:
561 http://www.yorknowledgebase.info/.The smaller datasets accompany the
562 manuscript, as MS Excel files:
563
564 Supporting figures:
565 Figure S1. Genome-wide Linkage Disequilibrium analysis for the RIPR diversity
566 panel: Figure S1_LD_SAF_1perc_26-9-17.pdf
567
568 Figure S2. Histograms of seed tocopherol composition of the RIPR diversity
569 panel in different crop types: Figure S2_histograms of seed tocopherol
570 composition.pdf
571
572 Figure S3. QQ plots from GEM and SNP association analysis for erucic acid
573 and γ/α tocopherol ratio: Figure S3_QQ_plots.pdf
574
575 Supporting data:
576 Data S1. List of cultivars, crop type classifications and Illumina read mapping
577 statistics: Data S1_cultivars and read mapping_20-12-16.xlsx.
578
579 Data S2. Ordered list of CDS gene model-based *Brassica* AC pan
580 transcriptome: Data S2_v11 pan-tanscriptome_20-12-16.xlsx.
581

582 Data S3. Seed fatty acid composition of the RIPR diversity panel: Data S3_fatty
583 acids_10-04-17.xlsx.
584
585 Data S4. Markers and genomic regions showing association with variation for
586 erucic acid content: Data S4_erucic-associated regions_30-3-17.xlsx.
587
588 Data S5. Seed tocopherol composition of the RIPR diversity panel: Data
589 S5_tocopherols_14-10-16.xlsx.
590
591 Data S6. Markers and genomic regions showing association with variation for
592 γ/α tocopherol ratio: Data S6_tocopherol-associated regions_SNPs.xlsx.
593
594 Data S7. Gene expression markers showing association with variation for γ/α
595 tocopherol ratio: Data S7_tocopherol-associated regions_GEMs.xlsx
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597
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Figure legends

Figure 1. Simplified tocopherol biosynthesis pathway in plants. HPP, p-hydroxyphenylpyruvate; HGA, homogentisic acid; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; PDP, phytyl-diphosphate; *HPPD*, HPP dioxygenase; *VTE1*, tocopherol cyclase; *VTE2*, homogentisate phytyltransferase; *VTE3*, MPBQ methyltransferase; *VTE4*, γ-tocopherol methyltransferase; *VTE5*, phytol kinase.

Figure 2. Population structure and trait variation across the RIPR panel. A. Relatedness of accessions in the panel based on 355,536 scored SNPs. B. Main crop types in the panel, colour-coded: orange for spring oilseed rape, green for semi-winter oilseed rape, light blue for swede, dark blue for kale, black for fodder and red for winter oilseed rape, grey for crop type not assigned. C. Population structure for highest likelihood K = 2. D. Variation for seed content of α- tocopherol (light blue), γ-tocopherol (dark blue) and δ-tocopherol (magenta).

Figure 3. Association analysis. A. Transcriptome SNP markers with seed erucic acid content. The SNP markers are positioned on the x-axis based in the genomic order of the gene models in which the polymorphism was scored, with the significance of the trait association, as –log10*P*, on the y-axis. A1 to A10 and C1 to C9 are the chromosomes of *B. napus*, shown in alternating black and red colours to permit boundaries to be distinguished. Hemi-SNP markers (i.e. polymorphisms involving multiple bases called at the SNP position in one allele of the polymorphism) for which the genome of the polymorphism cannot be assigned are shown as light points whereas simple SNP markers (i.e. polymorphisms between resolved bases) and hemi-SNPs that have been directly linkage mapped, both of which can be assigned to a genome, are shown as dark points. The broken light blue horizontal line marks the Bonferroni-corrected significance threshold of 0.05.

B. Transcript abundance with seed erucic acid content. The gene models are positioned on the x-axis based in their genomic order, with the significance of the trait association, as –log10*P*, on the y-axis. The broken dark blue horizontal line marks the 5% false discovery rate.

Figure 4. Association analysis. A. Transcriptome SNP association analysis for seed γ/α tocopherol ratio. The SNP markers are positioned on the x-axis based in the genomic order of the gene models in which the polymorphism was scored, with the significance of the trait association, as –log10*P*, on the y-axis. A1 to A10 and C1 to C9 are the chromosomes of *B. napus*, shown in alternating black and

892 red colours to permit boundaries to be distinguished. Hemi-SNP markers (i.e.
893 polymorphisms involving multiple bases called at the SNP position in one allele
894 of the polymorphism) for which the genome of the polymorphism cannot be
895 assigned are shown as light points whereas simple SNP markers (i.e.
896 polymorphisms between resolved bases) and hemi-SNPs that have been directly
897 linkage mapped, both of which can be assigned to a genome, are shown as dark
898 points. The broken light blue horizontal line marks the Bonferroni-corrected
899 significance threshold of 0.05.
900 B. Association analysis of transcript abundance with seed γ/α tocopherol ratio.
901 The gene models are positioned on the x-axis based in their genomic order, with
902 the significance of the trait association, as –log10*P*, on the y-axis. The broken
903 dark blue horizontal line marks the 5% false discovery rate.
904
905 Figure 5. Test of the predictive ability of SNP and GEM markers associated with
906 γ/α tocopherol ratio by using ”take-one-out” permutation. The allelic effects of
907 each of 36 SNP markers associated with γ/α tocopherol ratio was used to predict
908 the γ/α tocopherol ratio for the missing accessions. For GEM data, RPKM values
909 for each of 4 GEMs were fitted to the regression line to predict γ/α tocopherol
910 ratio. The strong correlation between predicted and observed γ/α tocopherol ratio
911 values ($R2 = 0.59$; $p<0.001$ for SNPs and $R2 = 0.47$; $p<0.001$ for GEMs)
912 demonstrates excellent predictive ability.
913
914 Figure 6. Relationship between expression in leaves of Bo2g050970.1 and the
915 tocopherol γ/α ratio in seed. The ratio of γ-tocopherol / α-tocopherol measured in

916 seeds was regressed against the transcript abundance in leaves of the VTE4
917 orthologue Bo2g050970.1 (R2=0.26; p<0.001), measured as reads per kilobase
918 per million aligned reads (RPKM).
919

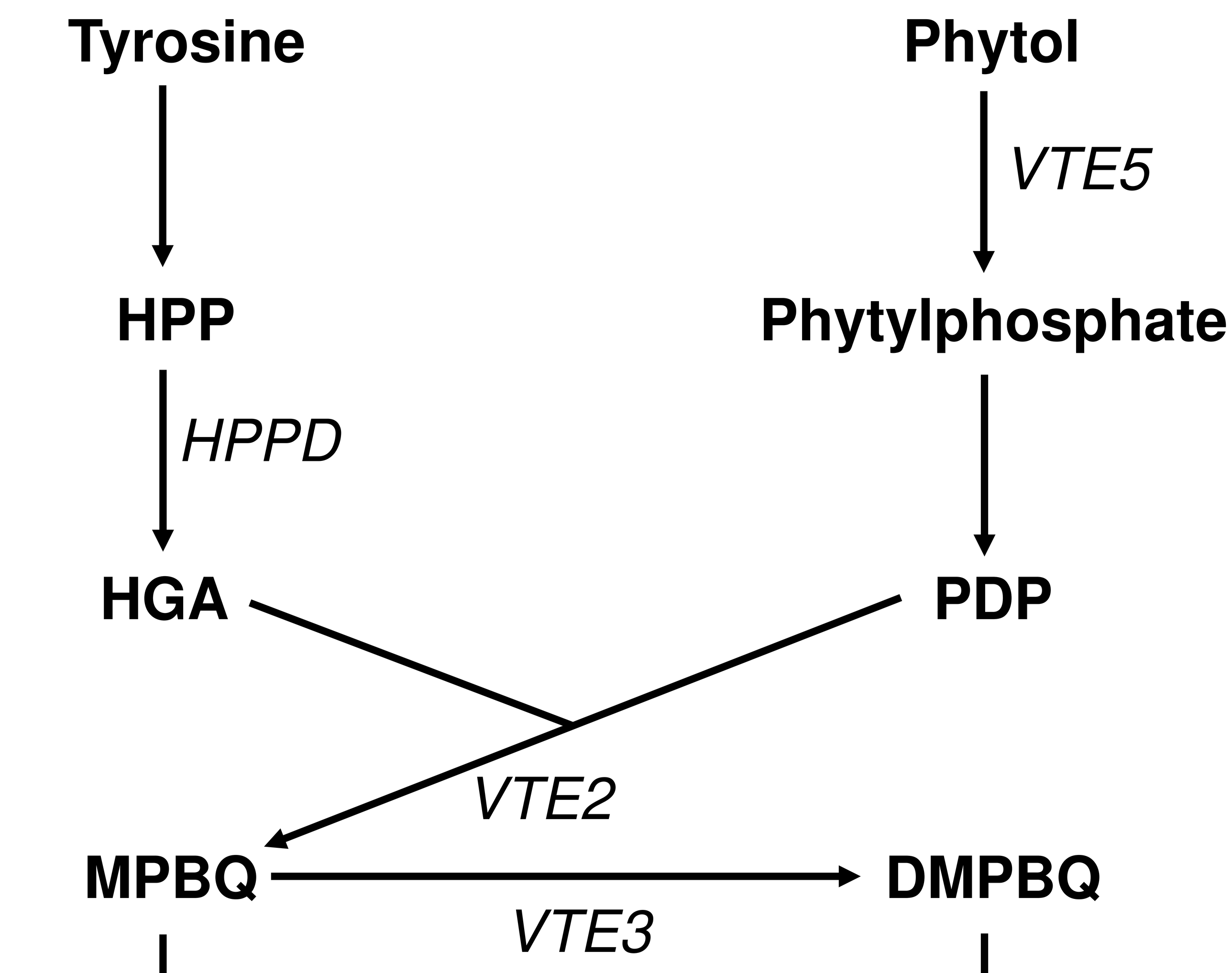


Figure 1. Simplified tocopherol biosynthesis pathway in plants. HPP, p-hydroxyphenylpyruvate; HGA, homogentisic acid; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; PDP, phytyl-diphosphate; *HPPD*, HPP dioxygenase; *VTE1*, tocopherol cyclase; *VTE2*, homogentisate phytyltransferase; *VTE3*, MPBQ methyltransferase; *VTE4*, γ-tocopherol methyltransferase; *VTE5*, phytol kinase.

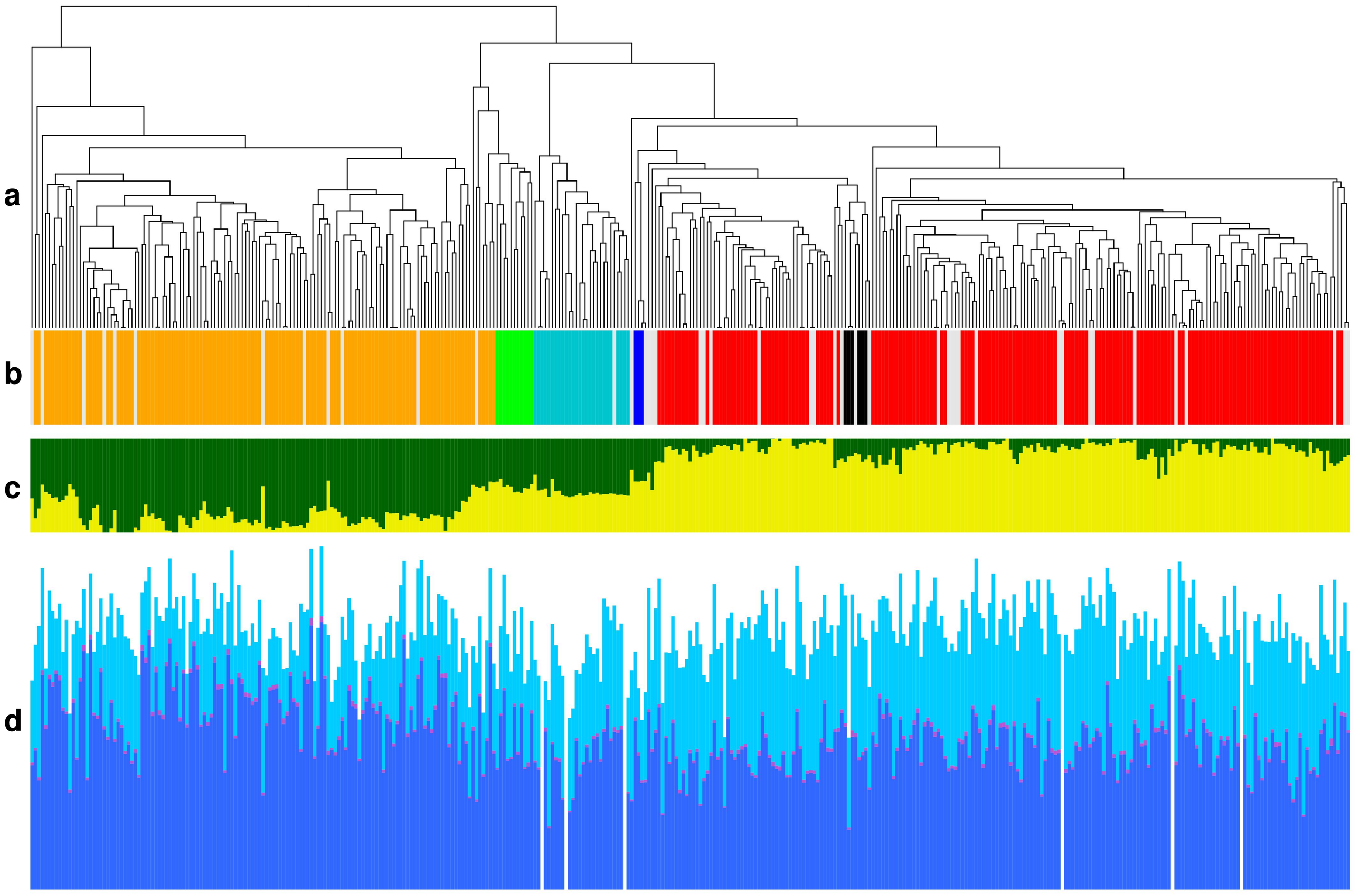
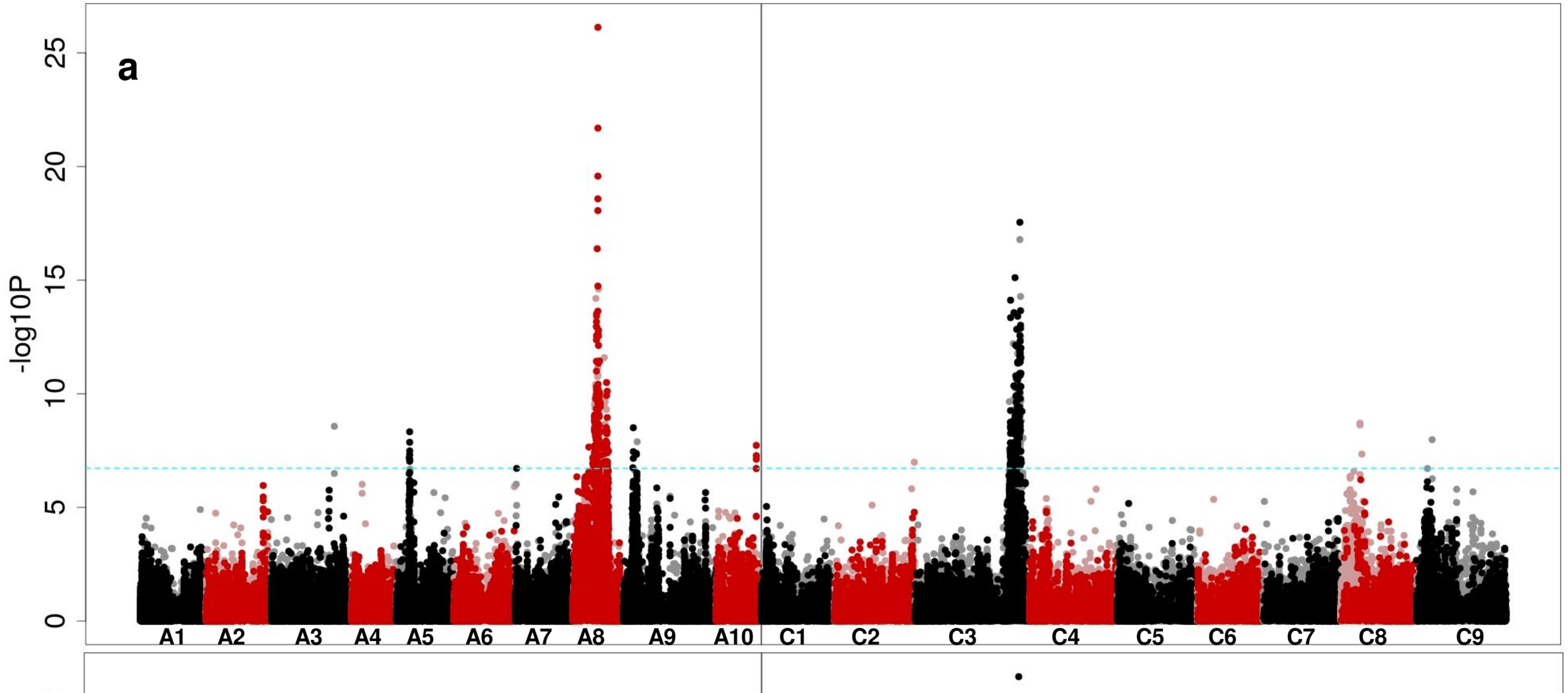


Figure 2. Population structure and trait variation across the RIPR panel. A. Relatedness of accessions in the panel based on 355,536 scored SNPs. B. Main crop types in the panel, colour-coded: orange for spring oilseed rape, green for semi-winter oilseed rape, light blue for swede, dark blue for kale, black for fodder and red for winter oilseed rape, grey for crop type not assigned. C. Population structure for highest likelihood K = 2. D. Variation for seed content of α- tocopherol (light blue), γ-tocopherol (dark blue) and δ-tocopherol (magenta).



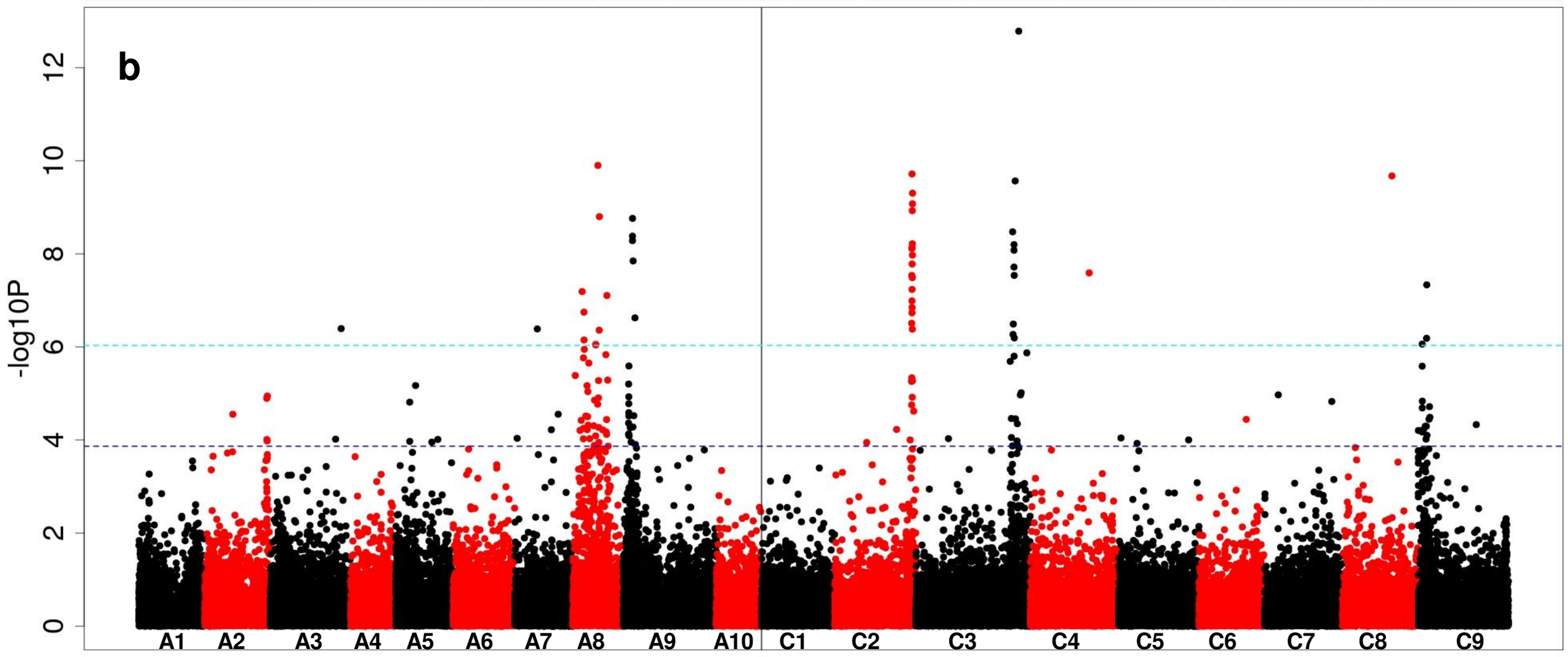
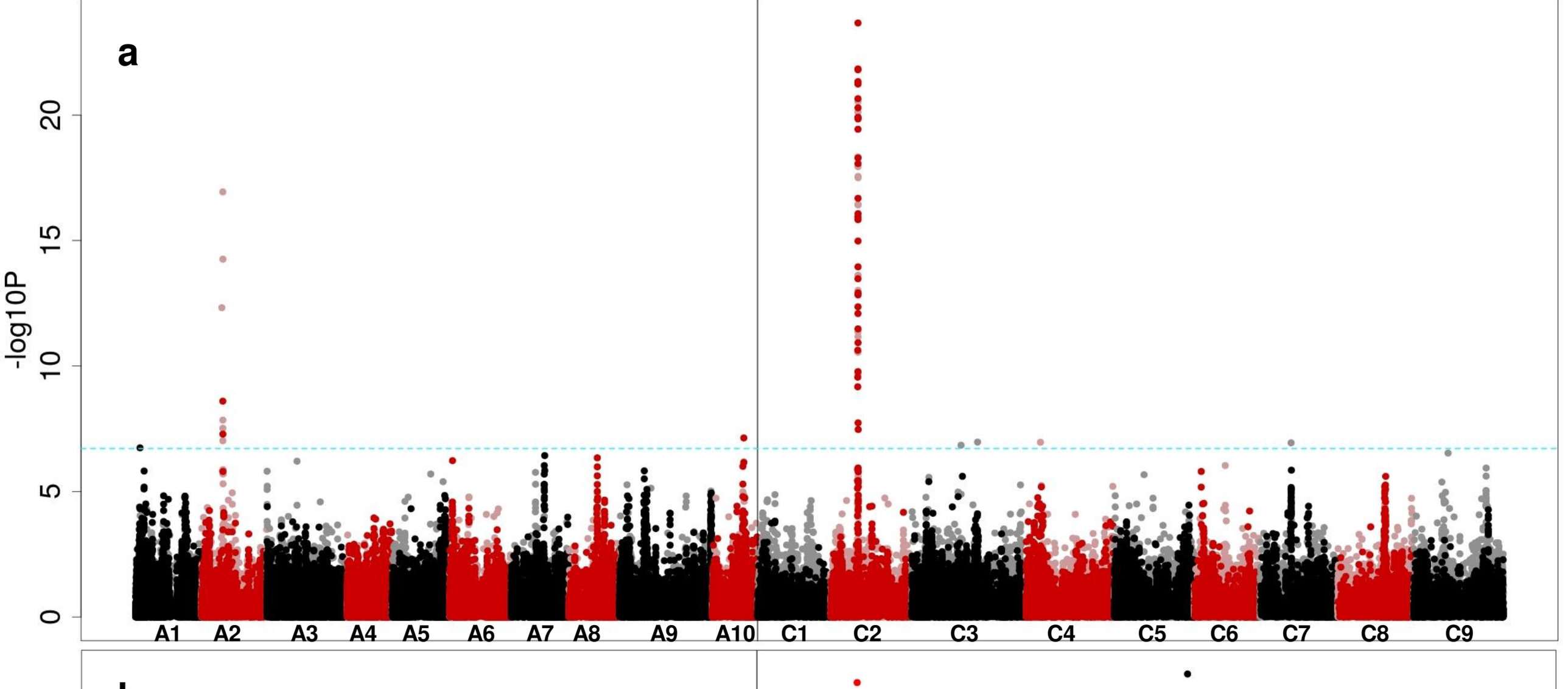


Figure 3. Association analysis. A. Transcriptome SNP markers with seed erucic acid content. The SNP markers are positioned on the x-axis based in the genomic order of the gene models in which the polymorphism was scored, with the significance of the trait association, as –log10*P*, on the y-axis. A1 to A10 and C1 to C9 are the chromosomes of *B. napus*, shown in alternating black and red colours to permit boundaries to be distinguished. Hemi-SNP markers (i.e. polymorphisms involving multiple bases called at the SNP position in one allele of the polymorphism) for which the genome of the polymorphism cannot be assigned are shown as light points whereas simple SNP markers (i.e. polymorphisms between resolved bases) and hemi-SNPs that have been directly linkage mapped, both of which can be assigned to a genome, are shown as dark points. The broken light blue horizontal line marks the Bonferroni-corrected significance threshold of 0.05.

B. Transcript abundance with seed erucic acid content. The gene models are positioned on the x-axis based in their genomic order, with the significance of the trait association, as –log10*P*, on the y-axis. The broken dark blue horizontal line marks the 5% false discovery rate.



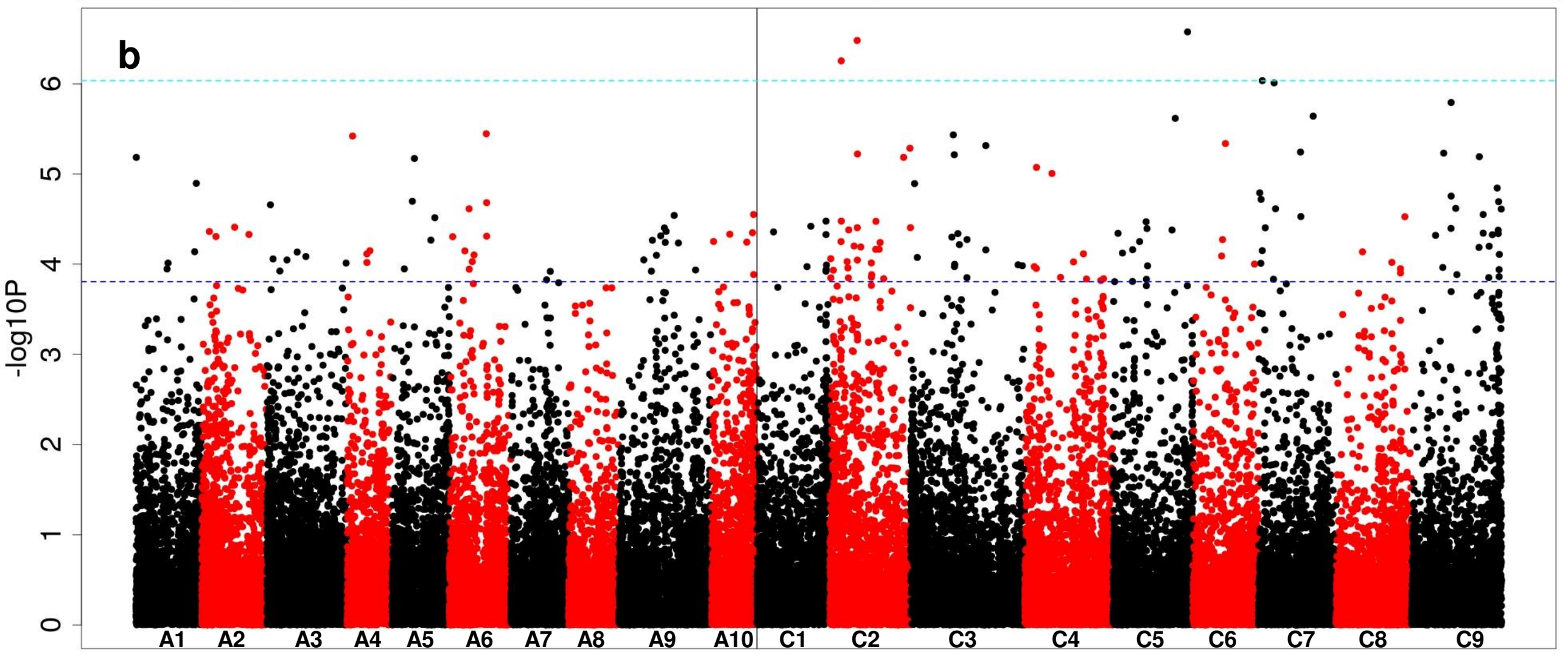
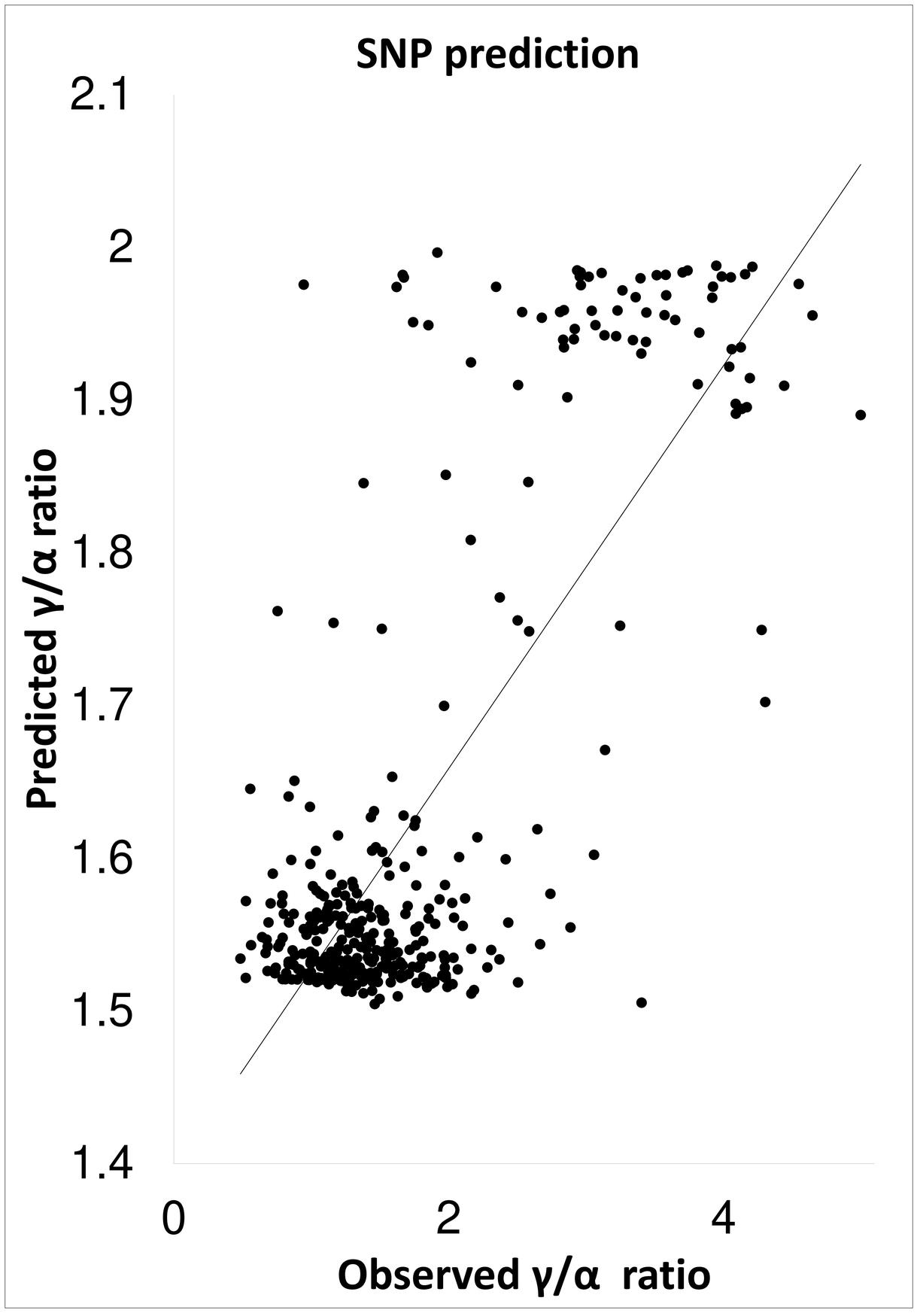


Figure 4. Association analysis. A. Transcriptome SNP association analysis for seed γ/α tocopherol ratio. The SNP markers are positioned on the x-axis based in the genomic order of the gene models in which the polymorphism was scored, with the significance of the trait association, as –log10*P*, on the y-axis. A1 to A10 and C1 to C9 are the chromosomes of *B. napus*, shown in alternating black and red colours to permit boundaries to be distinguished. Hemi-SNP markers (i.e. polymorphisms involving multiple bases called at the SNP position in one allele of the polymorphism) for which the genome of the polymorphism cannot be assigned are shown as light points whereas simple SNP markers (i.e. polymorphisms between resolved bases) and hemi-SNPs that have been directly linkage mapped, both of which can be assigned to a genome, are shown as dark points. The broken light blue horizontal line marks the Bonferroni-corrected significance threshold of 0.05.

B. Association analysis of transcript abundance with seed γ/α tocopherol ratio. The gene models are positioned on the x-axis based in their genomic order, with the significance of the trait association, as –log10*P*, on the y-axis. The broken dark blue horizontal line marks the 5% false discovery rate.



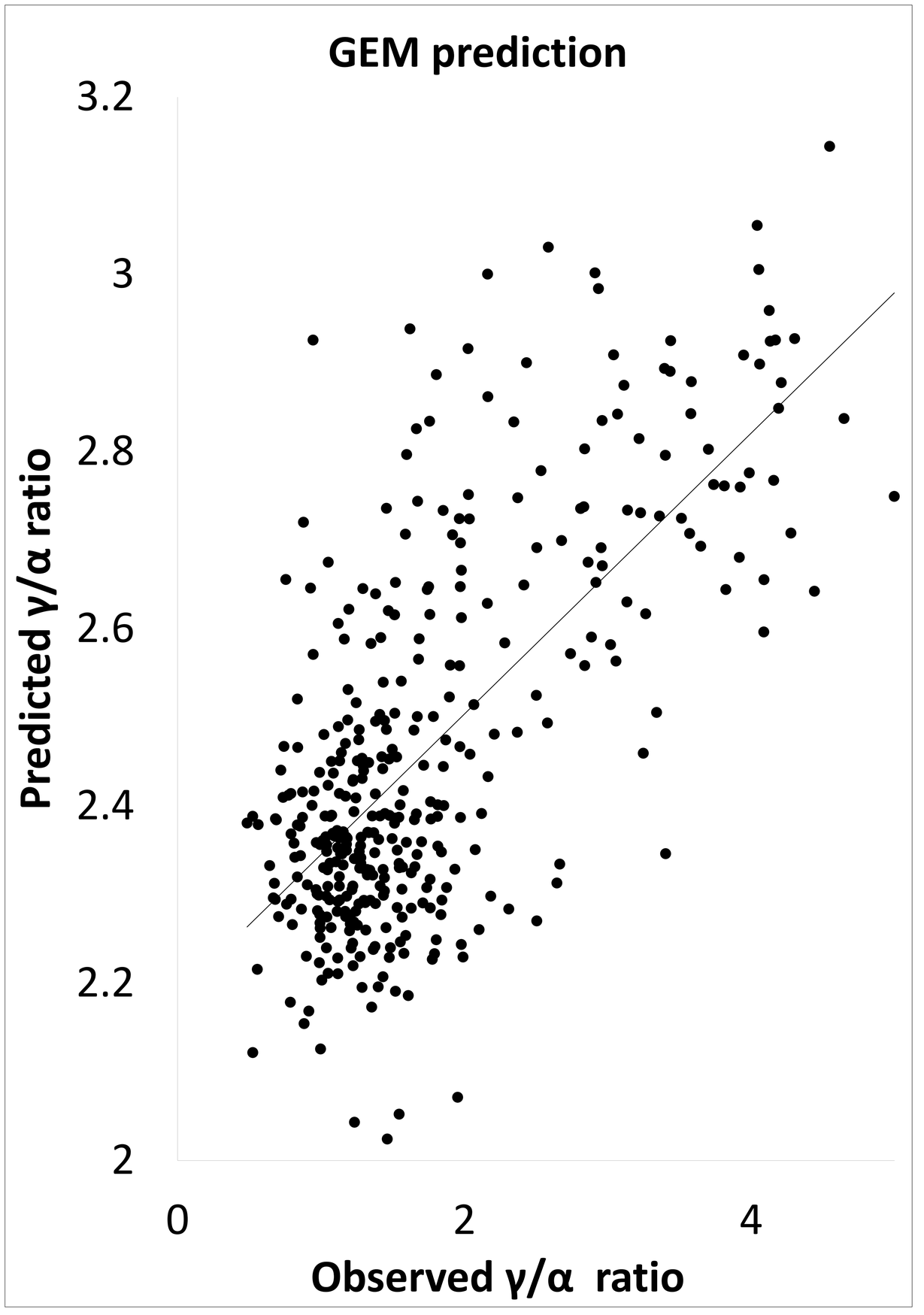


Figure 5. Test of the predictive ability of SNP and GEM markers associated with γ/α tocopherol ratio by using ”take-one-out” permutation. The allelic effects of each of 36 SNP markers associated with γ/α tocopherol ratio was used to predict the γ/α tocopherol ratio for the missing accessions. For GEM data, RPKM values for each of 4 GEMs were fitted to the regression line to predict γ/α tocopherol ratio. The strong correlation between predicted and observed γ/α tocopherol ratio values (R^2 = 0.59; $p<0.001$ for SNPs and R^2 = 0.47; $p<0.001$ for GEMs) demonstrates excellent predictive ability.

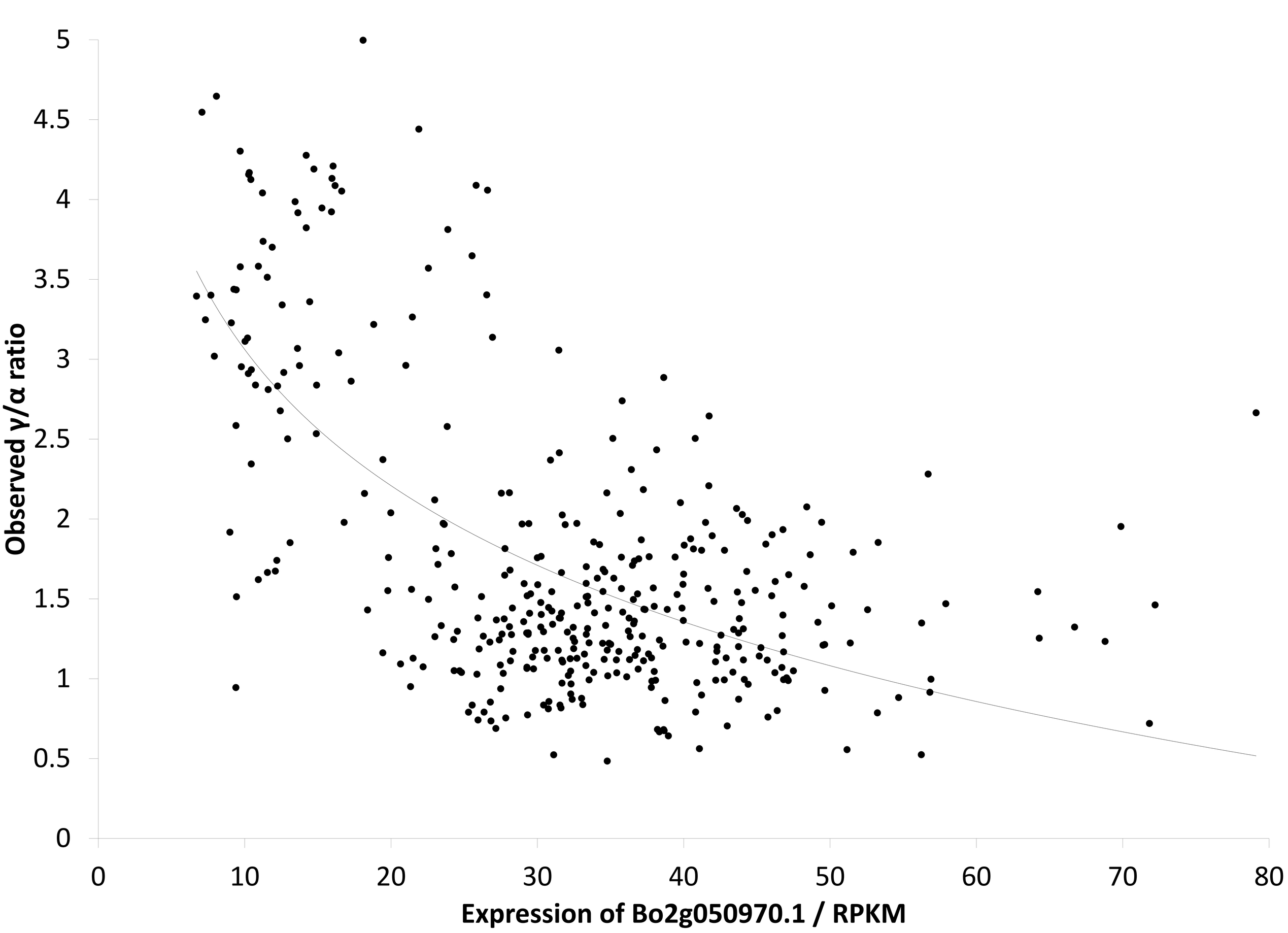


Figure 6. Relationship between expression in leaves of Bo2g050970.1 and the tocopherol γ/α ratio in seed. The ratio of γ-tocopherol / α-tocopherol measured in seeds was regressed against the transcript abundance in leaves of the *VTE4* orthologue Bo2g050970.1 (R^2=0.41; p<0.001), measured as reads per kilobase per million aligned reads (RPKM).