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SHORT COMMUNICATION

Lycopene-ε-cyclase (*e-LCY3A*) is functionally associated with quantitative trait loci for flour b* colour on chromosome 3A in wheat (*Triticum aestivum* L.)

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Abstract Flour b* colour is an important grain quality parameter for specific wheat end-products. The genetic control of b* colour in Australian wheat accessions is controlled by quantitative trait loci (QTL) on chromosomes 3A, 3B, 7A and 7B accumulating lutein, a compound of the carotenoid biosynthetic pathway. The relationship between lutein accumulation and flour b* colour provides an opportunity to identify sequence variants of genes encoding enzymes from the biosynthetic pathway that may control trait variation. This study identified a single nucleotide polymorphism (SNP) in the gene encoding lycopene-ε-cylcase on chromosome 3A (*e-LYC3A*) between two wheat accessions Ajana and WAWHT2074, identifying two alleles, *e-LYC3Aa* and *e-LYC3Ab*, respectively. *e-LCY3Ab* was present in 62.5 % of the wheat accessions analysed. A highly significant ($P < 0.01$) association with QTL on chromosome 3A in two mapping populations indicated that *e-LYC3A* is functionally associated with b* colour variation in some Australian wheat accessions. The SNP induced a serine/glycine substitution at amino acid residue 123 and a subtle change in protein folding at amino acid residue 119. The *e-LYC3A* SNP may be considered along with other alleles and genes on homoeologous group 3 and 7 chromosomes for selecting desirable flour b* colour variation in marker-assisted breeding.

Keywords Lycopene-ε-cyclase · Phytoene synthase · Lycopene-β-cyclase · Flour b* colour · Lutein · Wheat

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The degree of wheat flour whiteness or yellowness, measured as Commission Internationale de l'Eclairage (CIE) b* value, is an important grain quality parameter selected in breeding for intentional use in a specific end-product. Flour b* colour is under complex genetic control and the most common quantitative trait loci (QTL) controlling variation in Australian wheat accessions are located on chromosomes 3A, 3B, 7A and 7B (Crawford et al. 2011; Kuchel et al. 2006; Mares and Campbell 2001; Parker et al. 1998). The homoeologous group 7 chromosomes account for up to 60 % of the phenotypic variation (Kuchel et al. 2006; Parker et al. 1998), whereas chromosomes 3A and 3B account for up to 20 % of variation (Crawford et al. 2011; Mares and Campbell. 2001).

Flour b* colour in wheat is determined by the degree of yellow pigmentation through the accumulation of lutein, a xanthophyll of the carotenoid group of compounds (Fratianni et al. 2005; Howitt and Pogson 2006; Mares and Campbell 2001). Therefore, genes encoding enzymes of the xanthophyll

Table 1 Primer sequence information for amplifying and sequencing *e-LCY3A*

Primer	Sequence	Position	T_m (°C)	Annealing (°C)	Mg^{2+} (mM)
e-LCY3A	AGCCAGCTCGCAATTCCCGC	102–121	60	58	1.5
e-LCY3A	GCGCCTTTGGATGGAAGCGGA	1,258–1,238	60		
e-LCY3A	CCCCAACAAATGTGTCTGTGCCG	982–1,003	59	58	1.5
e-LCY3A	GCATGCTGCTACATTTCACAGTGGTG	2,174–2,149	59		
e-LCY3A	GCAGCAAATAACTAGACCGGGCA	1,782–1,804	57	60	1.5
e-LCY3A	TGAACTGGGGCACAAACCACG	2,805–2,785	58		
e-LCY3A	GGGGACATCGAGTAGTATATTGTGA	2,532–2,556	53	52	1.5
e-LCY3A	CGGGACCTGAAAACAAGCAA	3,858–3,839	53		
e-LCY3A	GGGAGAAATGAGCACAAGTGTCTGC	3,630–3,654	58	64	2.5
e-LCY3A	ACGGCGATGCTCCGATCACT	4,594–4,575	58		

The position of primers are provided relative to template sequence, EU649785. The melting temperature (T_m), optimised annealing temperature and $MgCl_2$ concentration for amplification of overlapping DNA fragments are shown

biosynthetic pathway are targets for determining the molecular basis and genetic control of flour b* colour and identifying functionally associated markers for breeding. Phytoene synthase is the first committed step of the carotenoid biosynthetic pathway and its corresponding gene, *Psy1*, was shown to have a functional association with QTL for flour b* colour on chromosome 7A (Crawford et al. 2011; He et al. 2008; Howitt et al. 2009; Singh et al. 2009) and 7B (Pozniak et al. 2007). Lycopene-ε-cyclase catalyzes the conversion of lycopene to δ-carotene and is an important step for downstream conversion to lutein that gives rise to yellow flour colour in wheat (Howitt and Pogson 2006; Howitt et al. 2009). The genes encoding lycopene-ε-cyclase on chromosomes 3A, 3B and 3D (*e-LCY3A, e-LCY3B* and *e-LCY3D,* respectively) have been sequenced and characterised in wheat (Howitt et al. 2009). Although *e-LCY3B* has been functionally associated with QTL on chromosome 3B controlling flour b* colour, it is unknown whether *e-LCY3A* controls phenotypic variation for flour b* colour on chromosome 3A. Therefore, the aim of this study was to sequence *e-LCY3A* from parents of mapping populations, identify sequence variants and determine functional association of *e-LCY3A* alleles with flour b* colour variation based on QTL mapping. Development of a functionally associated marker for flour b* colour QTL on chromosome 3A is a key outcome of the study.

The genomic sequence of *e-LCY3A* from Chinese Spring (EU649785) was used as a template for sequencing. PCR primers were designed to amplify five overlapping DNA fragments, including the full coding region represented by ten exons and nine introns for a total gene length of 4,473 bp (Table 1). PCR fragments were amplified from accessions Ajana and WAWHT2074 and purified using QIAquick gel cleanup kit (Qiagen, CA, USA) following agarose gel electrophoresis, sequenced using Big Dye® Terminator v3.1 cycle sequencing (Applied Biosystems, CA, USA) and chromatograms analysed using Geneious (Drummond et al. 2007). Sequence assembly and comparison showed 100 % sequence identity between WAWHT2074 and the e-LCY3A gene (EU649785) described by Howitt et al. (2009). However, one single nucleotide polymorphism (SNP) located within the second exon at position 2,028 bp (A substituted for G) was identified between Ajana (Genbank accession number JX288762) and WAWHT2074, creating an additional *Hpa*II restriction site in WAWHT2074 (data not shown). The SNP and subsequent *Hpa*II restriction site difference was used to develop a diagnostic allele marker for *e-LCY3A*.

A 1024-bp DNA fragment containing the SNP was amplified using primers e-LCY3A-3F and e-LCY3A-3R (Table 1). Analysis of aneuploid lines showed the presence of this DNA fragment in lines nullisomic for 3B and 3D but absent from a line nullisomic for 3A (Fig. 1a), confirming that the SNP was derived from *e-LCY3A* and not *e-LCY3B* or *e-LCY3D*. PCR amplification of the 1024-bp fragment and subsequent digestion with *Hpa*II identified a diagnostic band at 537-bp for Ajana (allele *e-LCY3Aa*), whereas Chinese Spring and WAWHT2074 had two smaller diagnostic

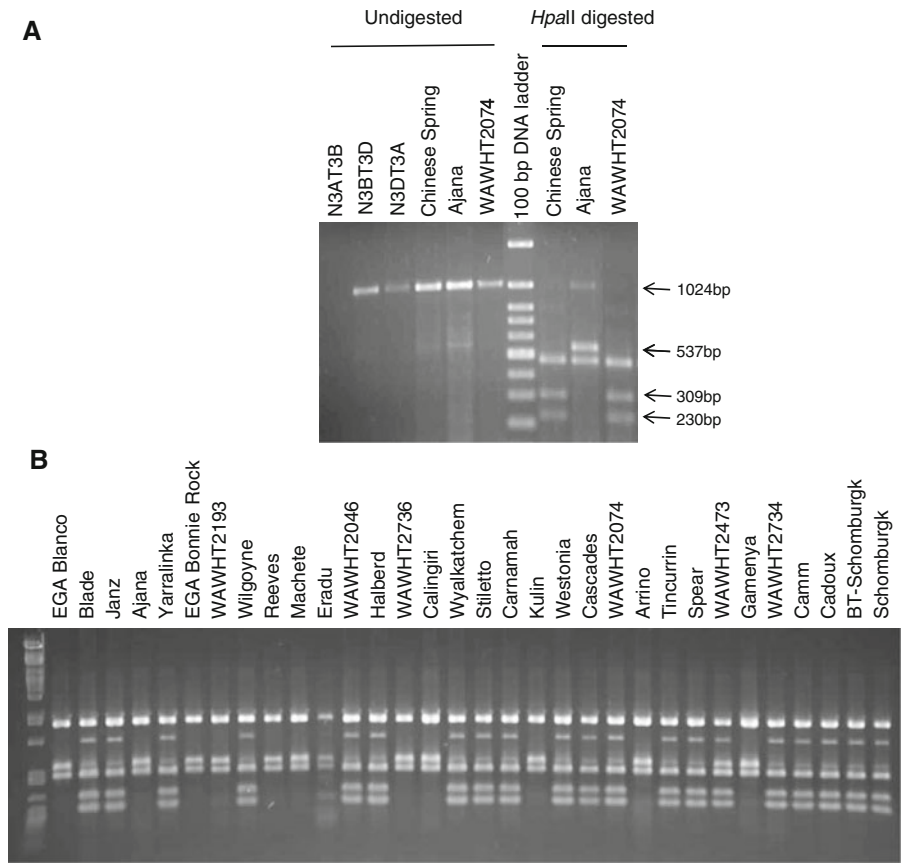

Fig. 1 **a** Agarose gel electrophoresis of PCR amplicon harbouring SNP undigested and digested with *Hpa*II. **b** Selected Australian accessions screened using *e-LCY3A* CAPS marker

bands at 309 and 230 bp (allele *e-LCY3Ab*) (Fig. 1a). This cleaved amplified polymorphic sequence (CAPS) marker was therefore able to discriminate the SNP at 2,028 bp, and was deemed suitable for determining the presence of the 2028-bp sequence variant amongst selected Australian wheat accessions varying in flour b* colour.

Wheat accessions grown in multi-environment trials in Western Australia in 2005 were milled and CIE values of flour samples were measured using a Minolta CR-400 Chromameter (Crawford et al. 2011). A panel of 32 wheat accessions were chosen for analysis of *e-LCY3A* alleles based on wide variation for flour b* colour, ranging in mean CIE values (±SD) from 7.27 (±0.48) for EGA Blanco (white flour) to 13.67 (±0.50) for Schomburgk (extreme yellow flour) as described in Crawford et al. (2011). The majority of wheat accessions (62.5 %) contained the 2028-bp SNP identified by the *e-LCY3Ab* allele (Fig. 1b). Schomburgk and Yarralinka were of particular interest because of highly significant differences ($P < 0.01$) in b* values with mean values of 13.67 (±0.50) and 8.47 (±0.53), respectively (Crawford et al. 2011), combined with a previous report of QTL controlling flour b* colour on chromosome 3A from a recombinant inbred population derived from these cultivars (Parker et al. 1998). In this study however, Schomburgk and Yarralinka both had the *e-LCY3Ab* allele (Fig. 1b). This indicates that the *e-LCY3A* SNP identified here may not be functionally associated with flour b* variation in Australian cultivars, with other unidentified SNPs within the gene possibly playing this role. Alternatively, *e-LCY3A* may not be involved in flour b* variation, with other genes on chromosome 3A contributing to flour b* colour variation.

We further investigated the functional association of *e-LCY3A* with flour b* colour variation detected on chromosome 3A using genetic mapping and QTL

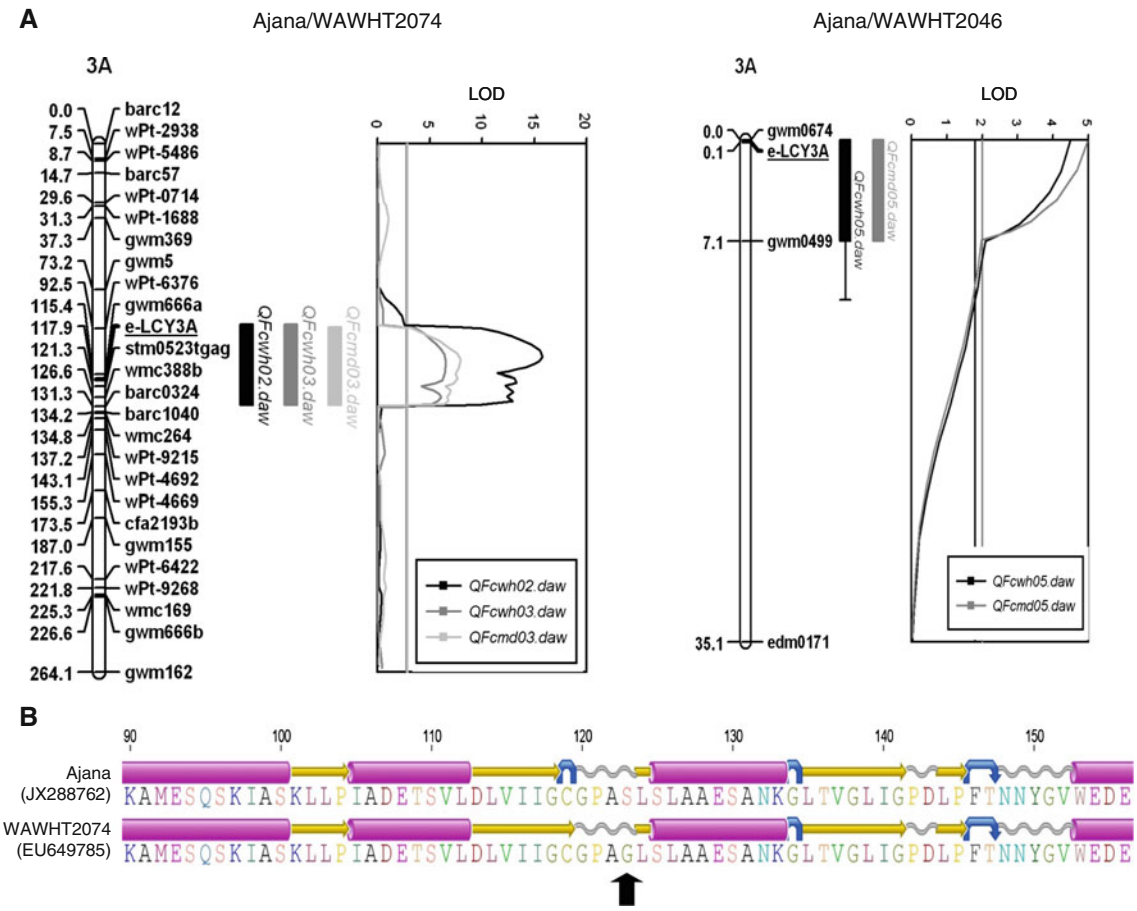

Fig. 2 **a** Genetic linkage map for chromosome 3A showing the position of *e-LYC3A* gene (*underlined*) and corresponding QTL traces for flour b* colour variation in two DH mapping populations, Ajana/WAWHT2074 and Ajana/WAWHT2046. QTL detected from phenotypic analysis of multi-environment trials as described by Crawford et al. (2011). **b** Aligned portion of translated protein sequence encoded by *e-LYC3A* alleles from Ajana and WAWHT2074 with Genbank accession numbers shown in parentheses. Amino acid change at residue 119 is caused by the SNP at 2,028 bp and highlighted by *black arrow*. Predicted protein folding is represented by *pink* (alpha-helix), *yellow* (beta strand), *blue* (turn) and *grey* (coil)

analysis in two doubled haploid (DH) mapping populations, Ajana/WAWHT2074 and Ajana/WAWHT2046 (Crawford et al. 2011), where parents differ in *e-LCY3A* alleles (Fig. 1c). Progeny of individuals from each mapping population were genotyped for *e-LCY3A* using the CAPS marker and integrated into the genetic maps consisting of 575 markers for Ajana/WAWHT207 (Francki et al. 2009) and 163 markers for Ajana/WAWHT2046 (Crawford et al. 2011) populations. Populations were grown in multi-environment trials and phenotyped (Crawford et al. 2011), where flour b* values were used in composite interval mapping using *e-LCY3A* as a co-factor. *e-LCY3A* mapped to chromosome 3A in both populations and association with the QTL for flour b* colour was highly significant (Fig. 2a). It is therefore reasonable to conclude that *e-LCY3A* is functionally associated with flour b* colour variation in some Australian accessions, although we cannot assume that *e-LCY3A* alleles are functionally associated with b* colour variation in all accessions. For instance, QTL have been detected on chromosome 3B but not on 3A in a bi-parental population (Mares and Campbell 2001), therefore either non-functional alleles of *e-LCY3A* or no sequence variation may be present amongst wheat accessions. Since natural genetic variation in lycopene-ε-cyclase gave rise to distinct differences in kernel colour of inbred maize lines (Harjes et al. 2008), the presence of multiple alleles in wheat and their contribution to differences in

flour colour variation is a distinct possibility. Extensive allele mining across a broad collection of Australian wheat accessions and their association with QTL using other bi-parental populations or association mapping would provide further insights into functional and non-functional sequence variants of *e-LCY3A*.

The sequence variant at 2028 bp between Ajana and WAWHT2074 provided an opportunity to predict protein differences contributing to enzyme functionality. Protein sequence was deduced from Ajana and WAWHT2074 based on translation of exons and alignment with protein sequence for e-LCY3A (EU649785). The SNP discriminating Ajana and WAWHT2074 resulted in a single amino acid change at residue 123, a serine/glycine substitution (Fig. 2b). Although the significance of this substitution on enzyme functionality is unknown, analysis of predicted folding structure using Geneious (Drummond et al. 2007) showed a difference at residue 119 resulting in a subtle coil and beta strand disparity (Fig. 2b) that may contribute to changes in enzyme properties. Further studies are warranted to identify the functional significance of the subtle amino acid and predicted protein folding variation in *e-LCY3A*.

This study provided evidence that *e-LCY3A* is functionally associated with flour b* colour on chromosome 3A. However, xanthophyll accumulation is under complex genetic and biochemical control with evidence that alleles for *e-LCY3B* on chromosome 3B and *Psy1* on chromosome 7A and 7B (Crawford et al. 2011; He et al. 2008; Howitt et al. 2009; Pozniak et al. 2007; Singh et al. 2009) also contribute to flour b* variation. Although we have identified a sequence variant for *e-LCY3A*, its application to marker-assisted breeding must take into consideration alleles of genes at other loci on group 3 and group 7 chromosomes to predict and select specific flour b* values.

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