Genetic dissection of the fuzzless seed trait in *Gossypium barbadense*

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Abstract

Cotton fibres are single-celled trichomes arising from the epidermal cells of the seed coat and may be either long (lint) or very short (fuzz). The dominant fuzzless *N1* of *Gossypium hirsutum* is a defective allele of the At-subgenome homoeolog of *MYB25-like*, but the genetic components underlying the recessive fuzzless trait from *G. barbadense* (Gb) are unknown. We have identified five genetic loci, including a major contributing locus containing *MYB25-like_Dt*, associated with Gb fuzzless seeds based on genotyping of fuzzy and fuzzless near isogenic lines (NILs) from an interspecies cross (*G. barbadense* × *G. hirsutum*). At 3 d post-anthesis when fuzz fibres are initiating, expression of *MYB25-like_Dt* was significantly lower in fuzzless NILs than in fuzzy seeded NILs, while higher *MYB25-like_Dt* expression was associated with more seed fuzz across different cotton genotypes. Phenotypic and genotypic analysis of *MYB25-like* homoeoalleles in cottons showing different fibre phenotypes and their crossing progeny indicated that both *MYB25-like_At* and *MYB25-like_Dt* are associated with lint development, and that fuzz development is mainly determined by the expression level of *MYB25-like_Dt* at ~3 d post-anthesis. Expression of Gb fuzzless seeds depends on genetic background and interactions amongst the multiple loci identified. *MYB25-like_Dt* is one of the best candidates for *N2*.

Keywords: Bulk segregant analysis, fuzzless, fuzz percentage, *G. barbadense*, lintless, mapping-by-sequencing.

Introduction

Mature cotton seeds are covered with two types of fibres: lint (up to ~3.5 cm) and fuzz (<0.5 cm). Both are single-celled, tubular outgrowths that arise from the epidermal cells of the seed coat and are indistinguishable in appearance during the early stages of their growth (*Lang*, 1938; *Stewart*, 1975), suggesting that their growth may involve the same physiological and biochemical processes. Lint fibre initials in *Gossypium hirsutum* (*Gh*) and *G. barbadense* (*Gb*) start developing on the day of anthesis, i.e. 0 d post-anthesis (dpa), with approximately a quarter to a third of the epidermal cells becoming fibre initials and finally lint fibres (*Lang*, 1938; *Stewart*, 1975). Fuzz fibres start developing after the lint at ~4 dpa, do not elongate to the same extent as the lint, and are variable in length and abundance among different genotypes (*Lang*, 1938; *Joshi et al.*, 1967; *Lee et al.*, 2006), with some cottons having no fuzz fibres, but still producing normal lint fibres.
Fuzzless cotton seeds are referred to as ‘naked seeds’ and have advantages during ginning because they generally require much less force to remove the lint from the seed than fuzzy seeded cottons, and hence less power consumption at the gin and less breakage of the lint fibres (Boykin, 2007; Bechere et al., 2009, 2012). A number of fuzzless loci have been reported in cotton, including the dominant N1 and the recessive n2 (Percy & Kohel, 1999). Homozygous N1/n1 and n2/n2 mutants are completely fuzzless, and lack any tuft (seen in the recessive mutants) at the micropylar tip of the seed and also have a significantly reduced lint percentage (Ware, 1940; Turley & Kloth, 2002; Turley et al., 2007) probably because of delayed lint initiation and their shorter lint (Lee et al., 2006; Turley et al., 2007; Zhang et al., 2007; Romano et al., 2011).

In earlier genetic studies, the recessive n2 fuzzless mutant was believed to have a genotype of n2n2. However, Turley & Kloth (2002) demonstrated that a third unlinked recessive locus, n3, is required for expression of the fuzzless trait in accessions carrying n2. This second locus may have confounded some earlier genetic studies of the n2 mutant, which shows variable fuzz development that is influenced by genetic background and environmental conditions (Turley & Kloth, 2002; Rong et al., 2005). Compared with the wild-type TM-1, in which fuzz initiation was observed at 4 dpa, few or no epidermal protrusions were observed in the N1 and n2n2 mutants at the same time point (Zhang et al., 2007). Cotton plants homozygous for all three fuzzless loci (N1/N1n2n2n3n3) are fibreless (i.e. lack both lint and fuzz: Turley & Kloth, 2002), so clearly the genes at these loci have central roles in the development of both types of fibres. A new ethyl methanesulfonate-induced mutant, n4′, was recently reported that appears to be different from the other naked seed loci (Bechere et al., 2009, 2012). The n4′ mutation has been reported to have a less negative effect on lint percentage and hence lint development (Bechere et al., 2012). In addition, no fuzzy seeded but lintless phenotype has ever been observed in cotton, suggesting that fuzz genes are epistatic to lint genes (Du et al., 2001) and/or that development of fuzz and lint are temporally and spatially regulated by the same regulatory genes. The genetic control of fuzz development is clearly very complex and there are many different genes other than these major naked seed genes that modify the amount of fuzz on the seed (Turley & Kloth, 2002) even on the same plant (Kearney & Harrison, 1928), and few of these have been characterized. Cotton breeders have long aimed to develop cotton cultivars with fuzzless seeds and a high lint percentage to capitalize on their ginning advantages, but to achieve that goal, it is essential to identify the genes underlying the regulation of fuzz and lint development and to understand their biological roles and genetic interactions.

Cotton is an allotetraploid with At and Dt subgenomes. The N1 and the n2 genes have been localized to chromosomes 12 (A12) and 26 (D12) (a pair of homoeologous chromosomes), respectively (Endrizzi & Ramsay, 1980; Samora et al. 1994). The n1 locus has not yet been mapped, but is reported to be unlinked to n1 (Turley & Kloth, 2002). Commercial Gb cultivars produce some of the highest quality lint fibres of any cotton species, but all appear to contain the n2 gene making them almost universally fuzzless, although there is considerable environmental effect on the amount of fuzz (Kearney & Harrison, 1928). It is not known if all Gb cultivars also carry the n1 locus, but this is highly likely. A recent study using a map-based cloning strategy has identified the dominant N1 gene to be a defective allele of the At-subgenome homoeolog of MYB25-like (i.e. MYB25-like_At) that generates small interfering RNAs from its 3′ end due to production of an overlapping antisense transcript that post-transcriptionally silences both homoeologs of this gene (Wan et al., 2016). MYB25-like is a master regulator involved in fibre initiation and development as silencing MYB25-like results in cotton seeds lacking both fuzz and lint fibres (Walford et al., 2011). However, the identities of the n2 and n3 genes are as yet unknown.

High-throughput genotyping and next-generation sequencing technologies are revolutionizing the speed and ability to fine-map and clone genes underlying agronomically important traits, which are usually controlled by multiple genes with complex interactions (Schneeberger, 2014; Zhu et al., 2014a). In cotton, a large number of single nucleotide polymorphism (SNP) markers have been reported based on whole genome re-sequencing of diverse cotton cultivars and transcriptome sequencing (Byers et al., 2012; Zhu et al., 2014b; Hulse-Kemp et al., 2015a; Wang et al., 2015; Fang et al., 2017). A cotton SNP array containing ~63 000 SNPs, mainly from US and Australian cotton cultivars, was recently developed (Hulse-Kemp et al., 2015b) and has been widely used by the cotton community for a diversity of studies (Ellis et al., 2016; Li et al., 2016; Wang et al., 2016; Gapare et al., 2017; Hinze et al., 2017; Huang et al., 2017a), including identification of the gene responsible for okra leaf shape (Zhu et al., 2016). One of the applications of next-generation sequencing in identification of genes underlying specific phenotypes is mapping-by-sequencing (MBS) where pools of segregating progeny are bulked according to their mutant phenotype and their genome sequenced to identify genomic regions inherited predominantly from the parent displaying the phenotype (Zhu et al., 2017). This approach has been applied in cotton to map genes related to the development and properties of fibres (Thyssen et al., 2014, 2015, 2016, 2017; Islam et al., 2016) and branching architectures (Chen et al., 2015). In model plant species, such as Arabidopsis, it is possible to narrow down the resolution of MBS to the single gene level as demonstrated recently by the cloning of an imprunted gene involved in endosperm cellularization (Huang et al., 2017b), but this is still difficult in allotetraploid species such as cotton, and therefore final identification of the causative genes usually requires other additional approaches once a genomic region containing the gene is identified.

In this study, we aimed to uncover the genetic basis of the fuzzless trait from G. barbadense and to explore the possibility of breeding G. hirsutum cottons with fuzzless seeds and without the lint percentage penalty normally associated with this trait that has so far prevented its adoption in commercial breeding programmes. To this end, we developed and used near isogenic lines (NILs) for identification of genetic loci tightly linked to the fuzzless trait using the Cotton SNP63K array and the MBS approach. We found that the fuzzless seed trait in Pima S-7, a Gb cultivar, is controlled by multiple
recessive loci, including a locus containing MYB25-like_Dt. Our results indicate that lint development is associated with the expression levels of both MYB25-like_At and MYB25-like_Dt at ~0 dpa, while fuzz development is mainly determined by the expression level of MYB25-like_Dt at ~3 dpa.

Methods and materials

Plant materials, development of nearly isogenic lines, and segregating populations

One G. barbadense (Pima S-7, fuzzless) and four G. hirsutum acces-
sions (Sicala 40, Sicala V-2, T586, and Xu142f) were used in genetic and gene expression analyses. Sicala 40 and Sicala V-2 are normal fuzz commercial cultivars producing copious amounts of lint, T586 is a lint bearing cotton genetic standard line containing the dominant fuzzless gene Nf (Endrizzi & Taylor, 1968), while Xu142f is a fibreless mutant known to carry nF amongst other fibre mutations (Zhang & Pan, 1991; Du et al., 2001). In addition, 134 Gb accessions with variable fuzz phenotypes were selected from the National Mid-
term Genetic Bank of Cotton at the Institute of Cotton Research (ICR) of the Chinese Academy of Agricultural Sciences, Anyang, China, and used for association analysis.

An F2 population derived from Pima S-7 × Sicala 40 (PS; 169 plants) was used in segregation and fuzz percentage analysis. Pima S-7 and two genetically related Gh cultivars, Sicala V-2 and Sicala 40, were used in the development of a set of NILs each showing fuzzless or fuzzy seeded phenotypes that were selected from a breeder’s population aimed at generating a fuzzless seed in an elite commer-
cial Gh background. After four backcrosses, homozygous fuzzless

DNA extraction, SNP genotyping

DNA extraction, SNP genotyping using the Cotton SNP63K array or KASP (Kompetitive Allele Specific PCR) were performed as previ-
ously described (Zhu et al., 2016). For calculation of SNP frequencies

Bulk segregant analysis and mapping-by-sequencing

DNA was extracted from 24 randomly selected BC2F2 progeny derived from NILs segregating for the fuzz phenotype and bulked to form the Recessive Fuzzless Bulked (RFB1) DNA pool. DNA was extracted from 15 BC2F3 progeny derived from fuzzless NILs and bulked to form the RFB2 DNA pool. Two barcoded DNA sequencing-libraries were created using RFB1 and RFB2, and sequenced in a single lane using the Illumina HiSeq2500 platform at the Australian Genome Research Facility (Melbourne, Australia). Approximately 50 Gb of 100-bp paired-end reads were generated.

After quality check using FastQC, the clean reads of the two bulks were separately mapped to the Sicala 40 genome sequence, which was generated by mapping re-sequenced Sicala 40 reads to the TM-1 (G. hirsutum) reference genome (Zhang et al., 2015) using CLC Genomics Workbench (version 7.5.1) with the following parameter settings: mismatch cost, 2; insertion and deletion cost, 3; length fraction, 0.5; similarity fraction, 0.95; and non-specifically matched reads ignored. Variants (including SNPs and indels) between RFB1 or RFB2 and Sicala 40 were identified using the ‘basic variant detection’ module implemented in CLC Genomics Workbench with the following settings: ploidy, 2; default read quality filters (i.e. neighbour-

Gene expression analysis using quantitative real-time PCR

Two types of tissues, whole ovules and ovule outer integuments (a 3–4-cell layer including the epidermis that produces the lint and fuzz fibres) were used in gene expression analysis. Whole ovules of differ-
ent developmental stages were collected from normal fuzz (NFL) or fuzzless (FLN) NILs and used for dissection of outer integuments based on the SNP array, Pima S-7, Sicala 40 and heterozygous alleles were designated 1, 0 and 0.5, respectively. For MYB25-like_Dt, a KASP marker was designed using a SNP located within its coding region (see Supplementary Fig. S2A). The MYB25-like_At alleles of Pima S-7 and Xu142f were distinguished using species-specific SNPs (Gb vs Gh) and a non-synonymous SNP within the coding region of the Xu142f MYB25-like_At (Supplementary Fig. S2B), respectively. KASP oligos are shown in Supplementary Table S1.

Phenotyping

Fuzzy, intermediate, and fuzzless seed phenotypes were scored based on visual inspection. The fuzz phenotype of the PS F2 population and the 134 Gb accessions was measured quantitatively using fuzz percentage analyses. Gene expression analysis was performed using Pima S-7, Sicala 40, T586, Xu142f, and different BC2F2 NILs that had normal fuzz or were fuzzless. The contributing effects of MYB25-like and MYB25-like loci on fuzz and lint development were evaluated using two more F2 populations: PX (92 plants) from Pima S-7 × Xu142f and FLNX (87 plants) from FLN1-10 × Xu142f. The reason for use of Xu142f was that both of its MYB25-like homoeologues are very lowly expressed and therefore potentially dysfunctional. All cotton plants, except the 134 Gb accessions, which were planted in the field of ICR’s Experiment Station in Xinjiang, China (2015), were grown in a glasshouse (Canberra, Australia) at 28 ± 2 °C with natural lighting.

DNA sample preparation and SNP genotyping

DNA extraction, SNP genotyping using the Cotton SNP63K array or KASP (Kompetitive Allele Specific PCR) were performed as previ-
ously described (Zhu et al., 2016). For calculation of SNP frequencies

based on the SNP array, Pima S-7, Sicala 40 and heterozygous alleles were designated 1, 0 and 0.5, respectively. For MYB25-like_Dt, a KASP marker was designed using a SNP located within its coding region (see Supplementary Fig. S2A). The MYB25-like_At alleles of Pima S-7 and Xu142f were distinguished using species-specific SNPs (Gb vs Gh) and a non-synonymous SNP within the coding region of the Xu142f MYB25-like_At (Supplementary Fig. S2B), respectively. KASP oligos are shown in Supplementary Table S1. All primer pairs had a similar
PCR efficiency (89.9–99.2%) determined by LinRegPCR (http://www.hartfaalcentrum.nl/index.php?main/files&sub=LinRegPCR).

Results

The fuzzless trait in G. barbadense is controlled by multiple recessive genetic loci

F₂ seeds of 169 F₂ plants derived from Pima S-7 (Gb) × Sicala 40 (Gh; hereafter named PS) showed a continuous gradation of the fuzz phenotype, from Pima S-7-like (fuzzless) through to Sicala 40-like (normal fuzz) (Fig. 1). Of the 169 F₂:F₃ families, seeds of only three families (e.g. PS-38; Fig. 1) looked similar to the naked seeds of Pima S-7, fitting a model with three recessive genes controlling fuzz formation (χ²,63,1=0.0497, P=0.8236). However, for the FLNS F₂ population created using Sicala 40 and a fuzzless NIL (FLN1-10; Fig. 1; Supplementary Fig. S1), five out of the 60 F₂:F₃ families showed a Pima S-7-like naked seed phenotype (e.g. FLNS-37; Fig. 1), fitting a model with two recessive genes (χ²,15,1=0.4444, P=0.5050). We noticed that the three fuzzless F₂:F₃ families observed in the PS population were not completely fuzzless and had a little more fuzz than Pima S-7, and we also observed transgressive segregants (with more fuzz than Sicala 40) in the PS but not in the FLNS F₂ populations. These results indicate that (i) fuzz development is complex with at least three recessive genes being involved in the regulation of the fuzzless trait in Pima S-7, and (ii) Pima S-7 must contain additional epistatic modifier(s) affecting the function and/or the interaction of the three loci responsible for its fuzzless phenotype that have not been identified here due to the small population sizes used.

Identification of genetic loci, including the MYB25-like_Dt containing locus, associated with low seed fuzz

We used NILs with different fuzz phenotypes to identify genetic loci controlling fuzz development (see Supplementary Fig. S1). Eight normal fuzz and 20 reduced fuzz (fuzzless or intermediate) BC₃:F₂ or BC₃:F₃ NILs were used in the SNP array analysis. Of the ~63 000 SNPs, 5426 were polymorphic between Pima S-7 and Sicala 40, and these were distributed across all 26 chromosomes, although chromosomes A02, A04, and D04 had less than 20 SNPs each (Supplementary Table S2). The Pima S-7 allele frequency of the polymorphic SNPs was plotted for each chromosome based on their genomic coordinates relative to the TM-1 genome. For the pool of the 20 reduced fuzz NILs, each candidate locus associated with low fuzz would have a Pima S-7 allele frequency close to 1 because the fuzzless NILs should be homozygous for the Pima S-7 allele and the NILs close to being fuzzless could be still heterozygous for the Pima S-7 allele. For the pool of the eight normal fuzz NILs, each candidate locus would have a Pima S-7 allele frequency <0.5 because these NILs could contain heterozygous or null Pima S-7 alleles. Three regions on chromosomes A12, D05, and D12 met these criteria (Supplementary Table S3; Fig. 2E; Supplementary Fig. S3). The A12 locus contained only three SNP markers and all of them were mapped to the D12 interval based on previous interspecific mapping results (Hulse-Kemp et al., 2015b) and so can be excluded as their positions were incorrectly assigned based on blasting. We further created and sequenced two bulked DNA libraries, RFB1 (progeny of normal fuzz NILs) and RFB2 (progeny of fuzzless NILs). Genome-wide SNP identification was performed for RFB1 and RFB2 using Sicala 40 as a reference. Candidate loci associated with low fuzz seeds would be expected to have a Pima S-7 allele frequency of ~0.5 and 1 in RFB1 and RFB2, respectively. Nine regions on seven different chromosomes met these criteria (Supplementary Table S3; Fig. 2A–D; Supplementary Fig. S4). The D12 locus was identified in both MBS and SNP array analyses, and so represents the best candidate for a major locus contributing to fuzzless seeds.

Because the fuzzless NILs used were self-pollinated for a few generations, some of the ten candidate regions identified could thus be false positives due to fixation of Pima S-7 alleles not related to the fuzzless phenotype. To refine these candidate loci we designed KASP marker assays (between 1 and 3 assays for each region) across all the ten candidates and genotyped the PS F₂ population with those markers and also measured fuzz percentage for each F₂ individual. For each SNP marker, F₂ plants were grouped based on their genotypes (i.e. homozygous Pima S-7 or Sicala 40, and heterozygous) and the average fuzz percentage was compared for each genotype. Of the ten candidate regions, five (regions 2, 4, 5, 9, and 11) were found to be positively or negatively correlated with the amount of seed fuzz. They were designated loci I–V (Supplementary Table S3; Fig. 3). For loci II–V, plants with a genotype of homozygous Pima S-7 regions had a significantly lower fuzz percentage than those with a genotype of homozygous Sicala 40, but for locus I (region 2), plants with a genotype of homozygous Pima S-7 had a significantly higher fuzz percentage than those with a genotype of homozygous Sicala 40 (Fig. 3A), suggesting Pima S-7 contained an enhancer rather than an inhibitor of fuzz development within that locus. For locus V (region 11), the fuzz percentage of heterozygous plants was intermediate and significantly different from either homozygous Pima S-7 or Sicala 40 (Fig. 3A), suggesting a major and additive effect of locus V on fuzz percentage, whereas the other loci were closer to one or other of the homozygous parental genotypes. This was supported by the fuzz phenotypes of the segregants within the FLNS population, as its F₂:F₃ families with a homozygous Pima S-7 region at locus V always had less fuzz than those with a homozygous Sicala 40 region (see Supplementary Fig. S5), and this trend was not observed for the other loci.

By checking the co-segregation of markers on the individual plants used with the Cotton SNP63K array analysis and the overlap between the SNP array and MBS analyses, we were able to deduce the intervals containing the candidate gene(s) for the five individual loci that contained between 20 and >300 annotated genes. For example, the size of the overlapping interval of locus V is ~1.4 Mbp containing 84 annotated genes (see Supplementary Tables S3 and S4). Fine mapping and/or other strategies will be required to pinpoint the actual causal gene(s) within these loci. However, one of the locus V genes is MYB25-like_Dt (Gh_D12G1628), a homoeolog of...
MYB25-like_At that has been shown to be critical for fuzz development (Wan et al., 2016). MYB25-like_Dt is thus the best candidate at this locus.

We also found that the average fuzz percentage of the 134 Gb accessions used in this study was low at 2.68% (with a range of 0.29–9.20%), and generally considerably less than the 10–12% found with normal fuzz Gh cultivars. Although the majority of these Gb accessions were fuzzless or close to fuzzless, a few were normal fuzzy seeded (e.g. M210353 in Supplementary Fig. S6). Despite their variable fuzz phenotypes, all of these Gb accessions had a homozygous Pima S-7 genotype in the five loci we have identified, suggesting that there could be additional modifiers of those five fuzzless loci in the rare fuzzy seeded Gb accessions, but this will have to await a more detailed genetic and molecular analysis to ensure that these accessions have not been misclassified or are partial hybrids with Gh.

Expression of MYB25-like_Dt is down-regulated in outer integuments of fuzzless NILs during fuzz initiation

In 0–9 dpa ovule outer integuments of NILs with normal fuzz (NFN) or fuzzless (FLN) seeds, MYB25-like_At and MYB25-like_Dt had a similar expression profile to each other, but were differentially expressed in both seed phenotypes, with approximately 70–80% of the total expression of MYB25-like contributed by its At homoeolog (Fig. 4). In NFN, the expression level of MYB25-like_Dt dropped steadily from 0 to 5 dpa, and then remained relatively low thereafter. The fuzzless FLN had a lower expression level of MYB25-like_Dt from 0 dpa and declined more rapidly than in the normal fuzz NFN NILs (Fig. 4C), such that there was little expression at 3 dpa, just prior to when fuzz fibres would normally initiate. Although the significantly reduced fuzz percentage observed in F2 plants homozygous for the Pima S-7 allele of MYB25-like_Dt (i.e. MYB25-like_DtPimaS7/PimaS7; Fig. 3A) could be contributed by other gene(s) within the same linkage block as MYB25-like_Dt, this expression profile of MYB25-like_Dt, together with the previously reported role of its At homoeolog in fuzz development (Wan et al., 2016), provides additional support for MYB25-like_Dt being one of the best candidates for a gene involved in the reduced fuzz of Pima S-7.

The protein coding sequences of MYB25-like_Dt between the reference sequence of TM-1 (fuzzy seeded) and Pima S-7 differ at three nucleotides with two of them giving rise to
changed amino acids in Pima S-7 (see Supplementary Fig. S2A). In both cases, however, the changed bases are all found in either the Dt-subgenome or the At-subgenome homoeologs from other cultivars including TM-1, Sicala 40, and Xu142 (Supplementary Figs S2A and S7) that have fuzzy seeds, so these SNPs are unlikely to lead to a loss of function of \( \text{MYB25-like}_\text{Dt} \) in Pima S-7. The core promoters (−1 to −250 bp) of \( \text{MYB25-like}_\text{Dt} \) and \( \text{MYB25-like}_\text{At} \) in Pima S-7, Sicala 40, and TM-1 were very similar, but the remaining upstream sequences of their promoters were quite different. Whether any of these differences is the cause of their differential expression remains to be investigated by fusing different lengths of promoter to reporter genes. The promoter of \( \text{MYB25-like}_\text{Dt} \) in Pima S-7, compared with that in Sicala 40 and TM-1, had seven differences, including three SNPs, two small indels (1 bp deletion or insertion), and two deletions (>20 bp) (Supplementary Fig. S8).

Expression levels of MYB25-like_Dt but not of MYB25-like_At predominantly determine the fuzz phenotypes

We compared the expression levels of the two \( \text{MYB25-like} \) homoeologs in 0–5 dpa whole ovules of the linted-fuzzless T586 (N1) and fibreless Xu142/f lines with those of Pima S-7 and Sicala 40 (Fig. 5). At 0 dpa when lint fibres have started to initiate, the total expression level of \( \text{MYB25-like} \) was highest in Sicala 40 and lowest in Xu142/f with a ranking of Sicala 40>Pima S-7>T586>Xu142/f (Fig. 5A), which is consistent with their rankings in final lint percentage (Sicala 40: 41.58%; Pima S-7: 34.51%; T586: 11.37%; Xu142/f: 0), suggesting that the expression level of \( \text{MYB25-like} \) at ~0 dpa is correlated...
with lint fibre initiation and development. In 1–5 dpa ovules, Pima S-7 had a more lowly expressed MYB25-like Dt than Sicala 40, but a significantly more highly expressed MYB25-like At (Fig. 5B, C) despite being fuzzless, supporting the association between the expression of MYB25-like Dt, but not MYB25-like At, and the poor fuzz development in Pima S-7. From 0 to 5 dpa, the expression levels of both MYB25-like homoeologs were consistently lower in Xu142 fl and T586 than in Sicala 40 and Pima S-7. Around the time of fuzz initiation at 3 dpa, Xu142 fl had the lowest expression of both homoeologs in any of the genotypes, but particularly the Dt homoeolog. In addition, there is a non-conservative substitution at position 314 within the conserved MYB DNA binding domain of MYB25-like At in Xu142 fl (see Supplementary Fig. S2B) that is likely to negatively impact on the activity of this transcription factor, supporting the assertion previously reported (Walford et al., 2011) that this fibreless mutant line has two dysfunctional MYB25-like homoeologs.

To investigate the effect of different MYB25-like homoeologs from Gh and Gb on lint and fuzz development, we generated two other segregating F2 populations, PX from Pima S-7 x Xu142 fl and FLNX from FLN1-10 (MYB25-like DtPima S7/Pima S7 locus in an essentially Sicala 40 genetic background) x Xu142 fl and examined lint and fuzz phenotypes of the F2 plants with different allele combinations determined by the presence of SNPs specific to each of the MYB25-like homoeologs and their parental origins (Table 1; Supplementary Fig. S9). The difference in these two populations was in the maternal allele of MYB25-like At, which was from Pima S-7 in PX and from Sicala 40 in FLNX, both of which should be highly expressed and as demonstrated below, fully functional (Fig. 5B). For each F2 population, individual plants were classified into 9 types (1–9 and 10–18 for PX and FLNX, respectively) based on the homozygosity or heterozygosity of parental origin for the At and Dt homoeolog of MYB25-like.

A number of observations were clear from this analysis:

(i) Fuzzless-lintless seeds (all lacking a micropylar tuft) (types 1 and 10, Table 1) were only produced when the two homozygous defective homoeologs, MYB25-like AtXu142 fl/Xu142 fl and MYB25-like DtXu142 fl/Xu142 fl, were combined together, as in the original Xu142 fl fibreless parent.
Replacing a single copy of *MYB25-like_Dt* with the Pima S-7 Dt allele (types 2 and 11) or the more highly expressed At allele from Pima S-7 (type 4) was sufficient to allow lint, but not fuzz, to develop on the seeds. This suggests that *MYB25-like_DtPimaS7* and *MYB25-like_At PimaS7* both produce fully functional MYB proteins able to activate lint fibre initiation. However, although *MYB25-like_AtPimaS7* is highly expressed from 0 through to 5 dpa (Fig. 5B), a single copy (type 4) was unable to rescue fuzz fibre production (Table 1), although two copies (type 7) allowed the development of some intermediate and normal fuzzy seeds in some plants, suggesting that fuzz and lint development are both dependent on gene dosage and hence total expression of *MYB25-like* homoeoalleles at specific times during development. Interestingly, plants in the PX population with homozygous Pima S-7 alleles for both homoeologs (type 9) were not all fuzzless like the Pima S-7 parent, attesting to the influence of some of the other identified fuzzless loci. All plants in the two populations have a lowly expressed *MYB25-like_Dt* allele, i.e. *MYB25-like_DtXu142fl* or *MYB25-like_Dt PimaS7*, and regardless of whether their *MYB25-like_At* allele was functional or mutant, produced mostly fuzzless or reduced fuzz seeds, suggesting it is the expression level of the Dt homoeolog that is critical for fuzz development, but either homoeolog can support lint fibre development provided that it expresses a functional protein during lint initiation.

As Pima S-7 and Xu142*fl* are both believed to carry alleles of *n₁* causing their fuzzless seeds, this provides support for *MYB25-like_Dt* being a good candidate for *N₂*.

(ii) The Gb allele *MYB25-like_At PimaS7* was less effective than that from Gh, *MYB25-like_At Sicala40*, in suppressing fuzz development in the presence of mutated Dt homoeologs, producing fewer fuzzless and more intermediate and fuzzy seeded F₂ segregants (comparing types 7–9 with types 16–18), perhaps because of its higher expression during fuzz initiation (Fig. 5B), or because of other modifier genes brought in with the different genetic backgrounds.

(iii) Lint percentage (and hence lint fibre development) was largely determined by the functionality and expression (gene dosage) of the At homoeolog of *MYB25-like*, but could also be influenced by the Dt homoeolog. The negative effects of the defective *MYB25-like_Dt* on lint percentage was less severe in the predominantly Gh background of the FLNX plants than in the Gb background of the PX plants that had lower lint percentages with all allele combinations other than in those with fibreless seeds (Table 1).

**Breeding fuzzless cottons without a penalty on lint fibre production is possible**

Fuzzless seeds have historically been associated with an undesirable lint percentage (Ware, *et al.* 1944), most likely due to both fuzz and lint fibres being regulated by differential
expression of the same genes. The $N_1$ gene, i.e. the dysfunctional MYB25-like $At$, has a strong negative effect on lint development (Turley et al., 2007), and therefore our ability to breed cottons with both $N_1$ and a high lint percentage is quite poor. Most commercial Gb cultivars have a relatively high lint percentage (~34%), so their fuzzless seed trait should have smaller deleterious effects on lint development. To know whether it is possible to combine the fuzzless seed trait of Gb with the high lint percentage of Gh, we created a BC$5F_2$ population (FLNS from FLN1-10 × Sicala 40) and measured lint percentages of individual plants. These plants segregated for all five fuzzless associated loci, and contained homozygous MYB25-like $At$ alleles from Sicala 40, i.e. MYB25-like $At$Sicala40/Sicala40, which is expressed at a similar level to MYB25-like $At$PimaS7/PimaS7 at 0 dpa. Overall, the average lint percentage of the BC$5F_2$ plants with a genotype of MYB25-like $Dt$Sicala40/Sicala40 (normal fuzz) and MYB25-like $Dt$PimaS7/PimaS7 (fuzzless) were similar (41.96% and 41.71%, respectively), and as high as that of the elite Gh cultivar Sicala 40 (41.58%). These results suggest that Sicala 40 contains alleles that are able to compensate for any negative effects of MYB25-like $Dt$PimaS7 on lint percentage. Individually, the Pima S-7 allele of loci V and II had a positive and negative effect on lint percentage, respectively, whereas the other three loci had no significant effect on lint percentage (see Supplementary Fig. S10).

**Discussion**

Identification and characterization of genes contributing to initiation of lint and fuzz fibres is essential for understanding the biological processes underlying fibre development and for improvement of lint yield through traditional breeding or transgenic approaches. Several fibreless or fuzzless mutants, such as Xu142fl, SL1-7-1, $N_1$, and $n_2$, have been reported and characterized (Zhang & Pan, 1991; Percy & Kohel, 1999; Turley & Kloth, 2008). They are valuable genetic resources for uncovering genes related to lint and fuzz fibre development. Transcriptome analyses using some of these fibre mutants have identified a large number of genes with a potential role in fibre development (Wu et al., 2006; Padmalatha et al., 2012; Wan et al., 2014); however, only a few of those genes (GhMYB25-like, GhVIN1, and GhJAZ2) have been shown to be essential for fibre initiation and development (Walford et al., 2011; Wang et al., 2014; Hu et al., 2016). Using a forward genetics approach, Wan et al. (2016) identified the dominant fuzzless $N_1$ gene to be a dysfunctional allele of MYB25-like $At$ that generates siRNAs and showed that suppression of
MYB25-like_At by virus-induced gene silencing phenocopied the fuzzless phenotype. In this study, we identified five loci associated with the fuzzless seed trait from Pima S-7 with the MYB25-likeDt-containing locus having a more prominent effect on fuzz development than the other loci (Fig. 3A; Supplementary Fig. S5).

We propose that MYB25-likeDt is a candidate for the N2 gene based on a number of pieces of evidence. First, N1 and n1 have previously been assigned to the two homoeologous chromosomes A12 and D12, respectively (Endrizzi & Ramsay, 1980). N1 has been shown to be localized to an interval containing MYB25-likeAt (Wan et al., 2016), and here we show that there is a major locus associated with the fuzzless seed phenotype of Gb carrying n1 that maps to an interval containing its homoeolog, MYB25-likeDt. While there are several other loci that associated with fuzzless seeds in different genetic populations segregating for n2, only the locus containing MYB25-likeDt is on D12, known to contain n2. Second, in a highly introgressed population (FLNS) that is predominantly in a Sicala 40 background, those lines carrying the allele of MYB25-likeDt from Pima S-7 all have much reduced fuzz relative to lines carrying the corresponding wild-type allele from Sicala 40, regardless of the combinations of loci for the other four fuzzless associated regions from Gb (see Supplementary Fig. S5). Third, expression of MYB25-likeDt when fuzz fibres begin to initiate at 3 dpa is consistently much lower in NILs and other lines producing fuzzless seeds compared with those with fuzzy seeds, whereas the expression of MYB25-likeAt in Pima S-7 is even higher than in the fuzzy seeded cultivar Sicala 40 and so is not indispensible for fuzz development. MYB25-likeDt from Pima S-7 does not appear to contain any obvious deleterious mutations within its coding region, and a single copy of this gene is able to restore lint development in lines containing otherwise defective homoeoalleles of MYB25-like from Xu142fl.

The Pima S-7 MYB25-likeDt locus must thus be sufficiently expressed at 0 dpa and produce a functional protein to be able to activate transcription of its downstream gene(s), at least within the lint fibre pathway. There are a number of upstream sequence differences between the MYB25-likeDt alleles from Pima S-7 and Sicala 40 (Supplementary Fig. S8) that may be responsible for the low expression of the Pima S-7 allele during fuzz initiation in lines carrying n2, but further experiments will be required to verify that they are the causal mutations that convert N2 into n2.

Taking advantage of those dysfunctional MYB25-like homoeoalleles in Xu142fl, we used it to infer both the functionality and the dosage effects of MYB25-likeDtPimaS7 in fuzz development by comparing fuzz phenotype of F2 segregants with MYB25-likeDtXu142fl or MYB25-likeDtPimaS7PimaS7 in common MYB25-likeAt backgrounds (Table 1). This investigation allowed us to not only demonstrate a key role for MYB25-likeDt in both fuzz and lint development, but also to uncover subtle differences in expression and dosage effects between MYB25-likeDtPimaS7 and MYB25-likeDtXu142fl alleles in fuzz and lint fibre development. In addition, the loss of fuzz production by MYB25-likeDtPimaS7 could be alleviated by the relatively highly

### Table 1. Segregation of the fuzzless trait by MYB25-like homoeoallele combination in two F2 populations

| Type | MYB25-like_At | MYB25-likeDt | No. of plants (%) | Lint (%) | Significance |
|------|---------------|---------------|-------------------|---------|-------------|
|      | Normal | Intermediate | Fuzzless | Tufted | Not tufted |          |
| The PX F2 population (Pima S-7 x Xu142fl) | | | | | | |
| 1 | Xu142fl(M)/Xu142fl(M) | Xu142fl(M)/Xu142fl(M) | 0 (0) | 0 (0) | 5 (5.43)c | 0 (0) | 5 (5.43) | 0.77 | a | A |
| 2 | Xu142fl(M)/Xu142fl(M) | Xu142fl(M)/Pima S-7(M) | 0 (0) | 0 (0) | 11 (11.96)c | 0 (0) | 11 (11.96) | 17.17 | b | B |
| 3 | Xu142fl(M)/Pima S-7(M) | Pima S-7(M)/Pima S-7(M) | 0 (0) | 0 (0) | 3 (3.26) | 0 (0) | 3 (3.26) | 22.44 | bc | BC |
| 4 | Xu142fl(M)/Pima S-7(N) | Pima S-7(N)/Pima S-7(M) | 0 (0) | 0 (0) | 15 (16.30) | 10 (10.87) | 5 (5.43) | 24.21 | bc | CD |
| 5 | Xu142fl(M)/Pima S-7(N) | Pima S-7(M)/Pima S-7(M) | 0 (0) | 1 (1.09) | 24 (26.09) | 23 (23.00) | 2 (2.17) | 27.26 | d | CD |
| 6 | Xu142fl(M)/Pima S-7(N) | Pima S-7(M)/Pima S-7(M) | 1 (1.09) | 0 (0) | 6 (6.52) | 5 (5.43) | 2 (2.17) | 28.94 | de | D |
| 7 | Pima S-7(N)/Pima S-7(N) | Xu142fl(M)/Xu142fl(M) | 1 (1.09) | 1 (1.09) | 5 (5.43) | 7 (7.61) | 0 (0) | 29.61 | de | D |
| 8 | Pima S-7(N)/Pima S-7(N) | Xu142fl(M)/Pima S-7(M) | 3 (3.26) | 7 (7.61) | 2 (2.17) | 11 (11.96) | 1 (1.09) | 29.99 | de | D |
| 9 | Pima S-7(N)/Pima S-7(N) | Pima S-7(M)/Pima S-7(M) | 3 (3.26) | 2 (2.17) | 2 (2.17) | 7 (7.61) | 0 (0) | 31.03 | e | D |
| The FLNX F2 population (FLN1-10 x Xu142fl) | | | | | | |
| 10 | Xu142fl(M)/Xu142fl(M) | Xu142fl(M)/Xu142fl(M) | 0 (0) | 0 (0) | 5 (5.49) | 0 (0) | 5 (5.49) | 0 | a | A |
| 11 | Xu142fl(M)/Xu142fl(M) | Pima S-7(M)/Pima S-7(M) | 0 (0) | 0 (0) | 4 (4.40) | 2 (2.20) | 2 (2.20) | 36.50 | cd | CD |
| 12 | Xu142fl(M)/Xu142fl(M) | Pima S-7(M)/Pima S-7(M) | 0 (0) | 0 (0) | 12 (12.09) | 0 (0) | 11 (12.09) | 31.26 | b | B |
| 13 | Xu142fl(M)/Sicala 40(N) | Xu142fl(M)/Xu142fl(M) | 0 (0) | 0 (0) | 25 (27.47) | 13 (14.29) | 12 (13.19) | 36.72 | cd | CD |
| 14 | Sicala 40(N)/Sicala 40(N) | Xu142fl(M)/Xu142fl(M) | 0 (0) | 1 (1.10) | 4 (4.40) | 5 (5.49) | 0 (0) | 39.30 | d | D |
| 15 | Sicala 40(N)/Sicala 40(N) | X142fl(M)/Pima S-7(M) | 0 (0) | 0 (0) | 8 (8.79) | 5 (5.49) | 3 (3.30) | 34.74 | c | BC |
| 16 | Sicala 40(N)/Sicala 40(N) | X142fl(M)/Pima S-7(M) | 0 (0) | 1 (1.10) | 6 (6.59) | 7 (7.69) | 0 (0) | 36.58 | cd | CD |
| 17 | Sicala 40(N)/Sicala 40(N) | Pima S-7(M)/Pima S-7(M) | 0 (0) | 7 (7.69) | 5 (5.49) | 11 (12.09) | 1 (1.10) | 37.22 | cd | CD |

a M and N represent mutated and normal alleles, respectively; see Supplementary Table S1 for markers used in distinguishing different alleles.
b Four were completely lintless and one was close to lintless.
c Four were close to lintless.
d Weight of lint as a percentage of total seed cotton weight.
e Values with the same letter were not significantly different determined by Student’s t-test.
expressed \(MYB25\)-like\(\_At^{PimaS7}\) allele that was more effective than the less expressed \(MYB25\)-like\(\_At^{Sicala40}\) allele (Table 1; Fig. 5B).

Based on our results, we proposed a working model for the role of \(MYB25\)-like homoeologs in lint and fuzz development (Fig. 6). According to this model, transferring a homozygous allele with low expression at \(~3\) dpa, such as \(MYB25\)-like\(\_Dt^{PimaS7/PimaS7}\), into a \(Gh\) background by backcrossing such that there is a high level of total \(MYB25\)-like at \(~0\) dpa to enhance lint fibre development should allow the breeding of elite commercial cotton lines with fuzzless seeds and without any penalty on lint yield, provided that other yield components (such as seeds per boll and bolls per plant) are not affected. We believe we are well on the way to achieving that result.

Interactions between \(MYB25\)-like\(\_At\) and \(MYB25\)-like\(\_Dt\), and other loci indicate that the genetic model regulating the fuzzless seed trait from Pima S-7 is genetic background dependent. For instance, the female parents of the PS and FLNS \(F_2\) populations (Pima S-7 and FLN1-10, respectively) had the same genotype at loci I–V (i.e. homozygous Pima S-7), but their \(F_2\) populations showed a three- and two-gene-model for the fuzzless trait, respectively. In addition, the PS but not the FLNS \(F_2\) population showed transgressive segregation, and a few \(Gh\) accessions (with homozygous Pima S-7 alleles at all five loci) produce considerable amounts of fuzz (see Supplementary Fig. S6). These results suggest the presence of as yet unknown modifier(s) affecting expression of the fuzzless genes or the interaction amongst the fuzzless genes in the \(Gh\) background, consistent with the previous finding that the genetics of the Pima S-7 fuzzless phenotype is complex (Rong et al., 2005).

In conclusion, the \(Gh\) fuzzless seed trait is regulated by multiple recessive genetic loci. The role of \(MYB25\)-like\(\_Dt\) in regulating fuzz initiation and development is yet to be confirmed by specific silencing of its expression using gene editing, but the expression profile of \(MYB25\)-like\(\_Dt\) and genetic analyses of cottons with variable lint and fuzz phenotypes provided quite convincing evidence for \(MYB25\)-like\(\_Dt\) being the major fuzz gene in addition to its role in regulating lint development together with \(MYB25\)-like\(\_At\).

**Supplementary data**

Supplementary data are available at *JXB* online.

Fig. S1. Schematic of the development of the near isogenic lines (NILs) used in SNP genotyping and mapping-by-sequencing.

Fig. S2. Alignment of the coding sequences of \(MYB25\)-like.

Fig. S3. The Cotton SNP63K array based frequency of the Pima S-7 allele of the polymorphic SNPs between Pima S-7 and Sicala 40 in the NILs with normal or reduced fuzz.

Fig. S4. Distribution of the Pima S-7 allele frequency across the 26 cotton chromosomes in the NILs showing fuzzless (RFB2) and segregating fuzz phenotype (RFB1) determined by MBS.

Fig. S5. Representative fuzz phenotype of the FLNS \(F_2:F_3\) families.

Fig. S6. Seed fuzz phenotype of representative \(G.\ barbadense\) accessions.

Fig. S7. Alignment of the amino acid sequences of \(MYB25\)-like homoeoalleles from Xu142, Xu142\(fl\), TM-1, and Pima S-7.

Fig. S8. Alignments of the promoter sequences of \(MYB25\)-like from mutant and wild-type lines.

Fig. S9. Distribution of fuzz phenotypes (\(F_3\) seeds) in the \(F_2\) populations PX and FLNX by \(MYB25\)-like homoeoalleles.

Fig. S10. Effects of fuzz associated loci on lint percentage of the FLNS \(F_2:F_3\) families.

Table S1. Primers used in the study.
Table S2. Chromosomal distributions of the 5426 polymorphic SNPs between Pima S-7 and Sicala 40 based on the Cotton SNP63K array.

Table S3. Candidate regions identified based on mapping-by-sequencing (MBS) and SNP array association analysis.

Table S4. List of annotated genes in the five chromosomal regions associated with fuzz development.

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