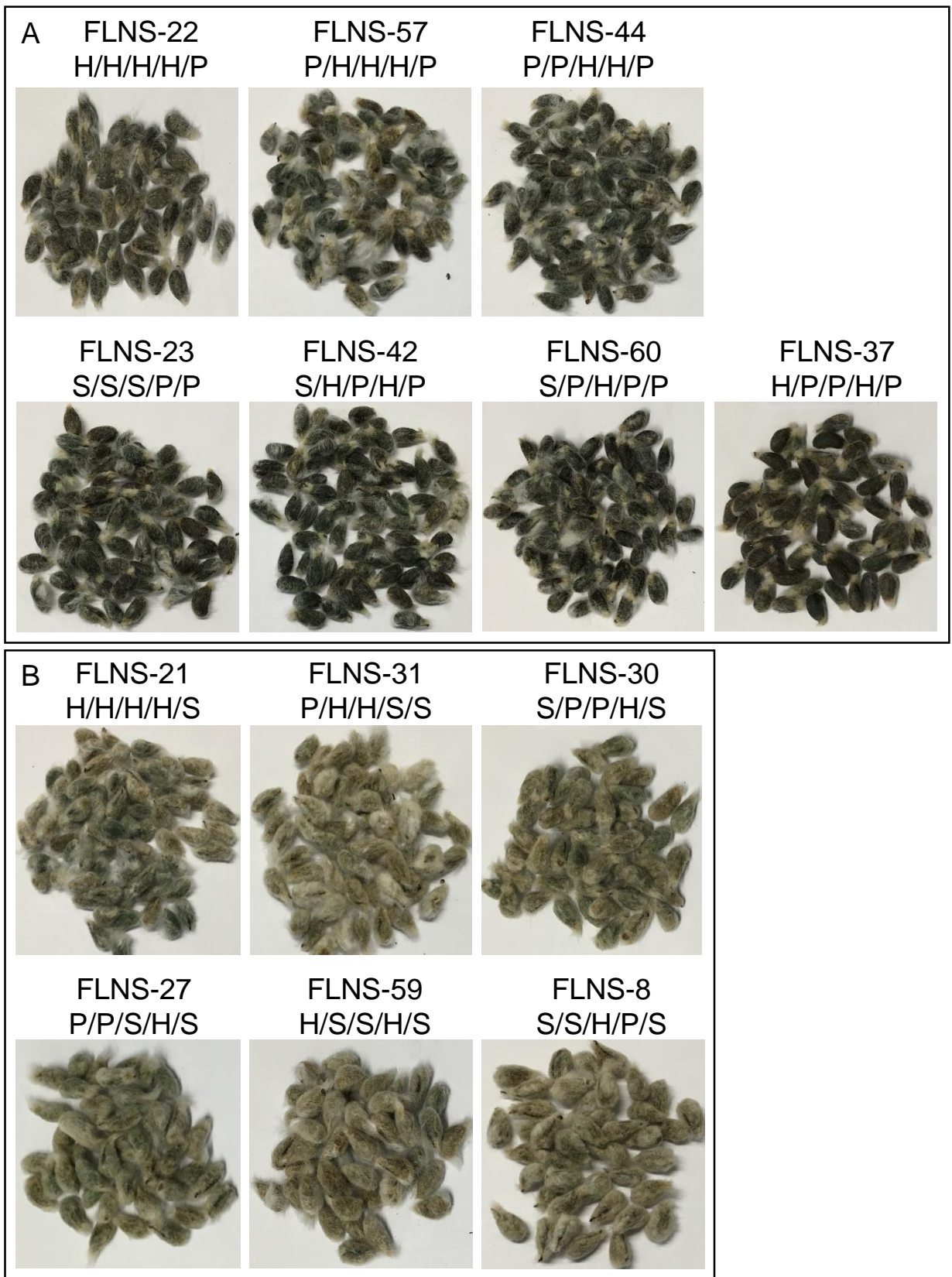
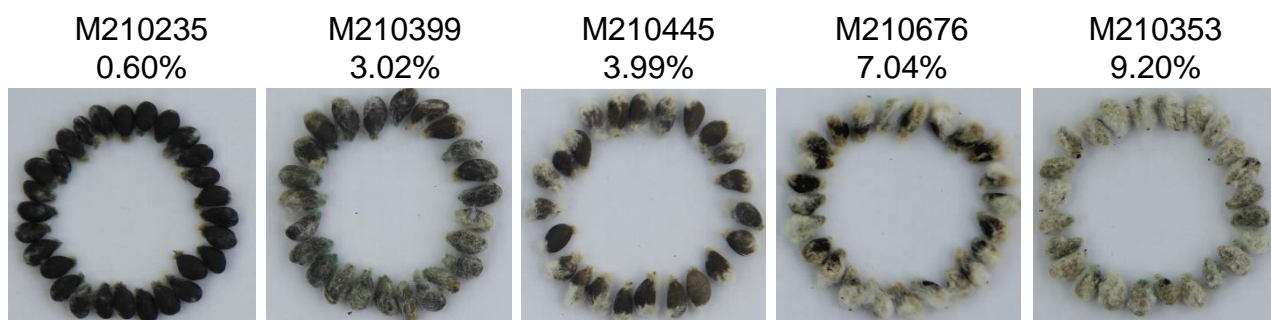


**Figure S1** Schematic of the development of the near isogenic lines (NILs) used in SNP genotyping and mapping-by-sequencing. (A) The BC<sub>1</sub>F<sub>1</sub> was selfed and fuzzless BC<sub>1</sub>F<sub>2</sub> was used in further backcrosses, which were repeated three more times. After several generations of selfing, BC<sub>4</sub>F<sub>4</sub> and BC<sub>4</sub>F<sub>5</sub> NILs showing normal and reduced fuzz (including fuzzless and intermediate) were genotyped using the Cotton SNP63K array. BC<sub>4</sub>F<sub>5</sub> progeny derived from BC<sub>4</sub>F<sub>4</sub> NILs homozygous for normal fuzz (NFN4) or fuzzless (FLN1) phenotype were used for the collection of outer integuments for gene expression analysis. BC<sub>4</sub>F<sub>6</sub> progeny derived from fuzzless and fuzzy seeded NILs were used in mapping-by-sequencing. (B) Fuzz phenotype of BC<sub>4</sub>F<sub>4</sub> NILs with fuzzless (FLN1), intermediate (IFN3) and normal fuzz (NFN4) seeds, and their representative BC<sub>4</sub>F<sub>5</sub> progeny.



**Figure S5** Representative fuzz phenotype of the FLNS  $F_2:F_3$  families with a genotype of homozygous Pima S-7 (A) or Sicala 40 (B) for Locus V.  $F_2:F_3$  family homozygous for Pima S-7 had less fuzz than  $F_2:F_3$  family homozygous for Sicala 40. The genotypes of loci I/II/III/IV are also shown for each  $F_2:F_3$  family. S: homozygous Sicala 40; P: homozygous Pima S-7; H: heterozygous.



**Figure S6** Seed fuzz phenotype of representative *G. barbadense* accessions.

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Xu142fl_At      MQQSPSPCKVGLKGGPWTPEEDQKLLSYIQEHGGGSRWGLPAKAGLQRCGKSCRLRWINY 60
Xu142_At       MQQSPSPCKVGLKGGPWTPEEDQKLLSYIQEHGGGSRWGLPAKAGLQRCGKSCRLRWINY 60
TM-1_At        MQQSPSPCKVGLKGGPWTPEEDQKLLSYIQEHGGGSRWGLPAKAGLQRCGKSCRLRWINY 60
Pima S-7_At    MQQSPSPCKVGLKGGPWTPEEDQKLLSYIQEHGGGSRWGLPAKAGLQRCGKSCRLRWINY 60
Xu142fl_Dt     MQQSPSPCKVGLKGGPWTPEEDQKLLSYIQEHGGGSRWGLPAKAGLQRCGKSCRLRWINY 60
Xu142_Dt       MQQSPSPCKVGLKGGPWTPEEDQKLLSYIQEHGGGSRWGLPAKAGLQRCGKSCRLRWINY 60
TM-1_Dt        MQQSPSPCKVGLKGGPWTPEEDQKLLSYIQEHGGGSRWGLPAKAGLQRCGKSCRLRWINY 60
Pima S-7_Dt    MQQSPSPCKVGLKGGPWTPEEDQKLLSYIQEHGGGSRWGLPAKAGLQRCGKSCRLRWINY 60
*****

Xu142fl_At     LRPDIKRGKFFSSQEERTIIQLHALLGNRWSAIAAHLPKRTDNEIKNYWNTQLKKRLTTIG 120
Xu142_At       LRPDIKRGKFFSSQEERTIIQLHALLGNRWSAIAAHLPKRTDNEIKNYWNTQLKKRLTTIG 120
TM-1_At        LRPDIKRGKFFSSQEERTIIQLHALLGNRWSAIAAHLPKRTDNEIKNYWNTQLKKRLTTIG 120
Pima S-7_At    LRPDIKRGKFFSSQEERTIIQLHALLGNRWSAIAAHLPKRTDNEIKNYWNTQLKKRLTTIG 120
Xu142fl_Dt     LRPDIKRGKFFSSQEERTIIQLHALLGNRWSAIAAHLPKRTDNEIKNYWNTQLKKRLTKIG 120
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TM-1_Dt        LRPDIKRGKFFSSQEERTIIQLHALLGNRWSAIAAHLPKRTDNEIKNYWNTQLKKRLTKIG 120
Pima S-7_Dt    LRPDIKRGKFFSSQEERTIIQLHALLGNRWSAIAAHLPKRTDNEIKNYWNTQLKKRLTKIG 120
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TM-1_At        IDPATHRPKTDTLGSTPKDAANLSHMAQWESARLEAEARLVRESKRVSNPQNQFRFTSS 180
Pima S-7_At    IDPATHRPKTDTLGSTPKDAANLSHMAQWESARLEAEARLVRESKRVSNPQNQFRFTSS 180
Xu142fl_Dt     IDPATHRPKTDTLGSTPKDVANFVSHMAQWESARLEAEARLVRESKRVSNPQNQFRFTSS 180
Xu142_Dt       IDPATHRPKTDTLGSTPKDVANFVSHMAQWESARLEAEARLVRESKRVSNPQNQFRFTSS 180
TM-1_Dt        IDPATHRPKTDTLGSTPKDVANLSHMAQWESARLEAEARLVRESKRVSNPQNQFRFTSS 180
Pima S-7_Dt    IDPATHRPKTDTLGSTPKDVANLSHMAQWESARLEAEARLVRESKRVSNPQNQFRFTSS 180
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Xu142fl_At     SAPPLVSKIDVGLAHATKPQCLDVLKAWQRVVTGLFTFNTDNLQSPSTSSFTENTLPIS 240
Xu142_At       SAPPLVSKIDVGLAHATKPQCLDVLKAWQRVVTGLFTFNTDNLQSPSTSSFTENTLPIS 240
TM-1_At        SAPPLVSKIDVGLAHATKPQCLDVLKAWQRVVTGLFTFNTDNLQSPSTSSFTENTLPIS 240
Pima S-7_At    SAPPLVSKIDVGLAHATKPQCLDVLKAWQRVVTGLFTFNTDNLQSPSTSSFTENTLPIS 240
Xu142fl_Dt     SAPPLVSKIDVGLAHATKPQCLDVLKAWQRVVTGLFTFNTDNLQSPSTSSFTENRLPIS 240
Xu142_Dt       SAPPLVSKIDVGLAHATKPQCLDVLKAWQRVVTGLFTFNTDNLQSPSTSSFTENRLPIS 240
TM-1_Dt        SAPPLVSKIDVGLAHATKPQCLDVLKAWQRVVTGLFTFNTDNLQSPSTSSFTENRLPIS 240
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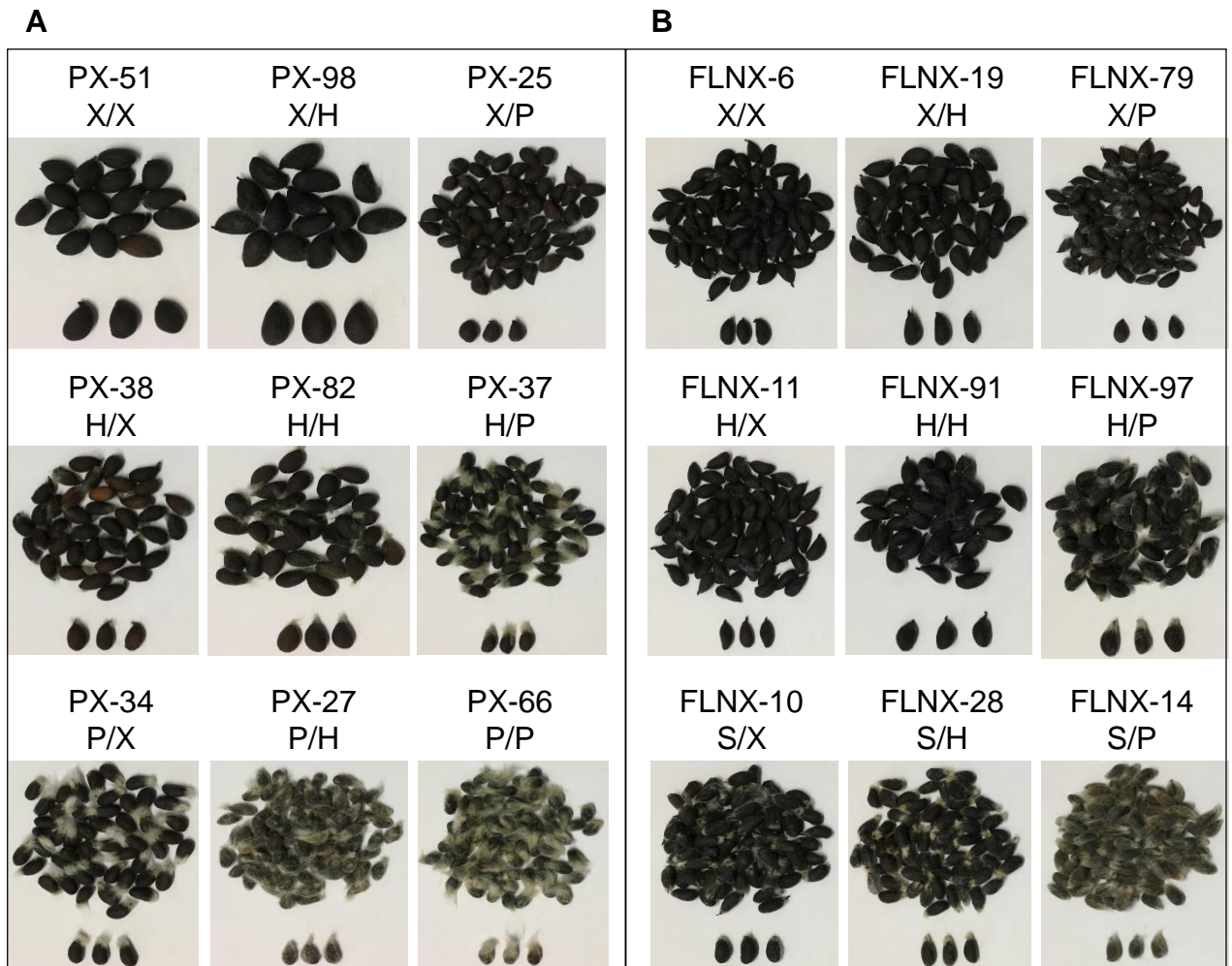
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TM-1_At        SVGFIDSVFGNSNNSCCGNWECVEKSSQVAELQERLDNSMGLHDIDLSSDVFVQGSY 300
Pima S-7_At    SVGFIDSVFGNSNNSCCGNWECVEKSSQVAELQERLDNSMGLHDIDLSSDVFVQGSY 300
Xu142fl_Dt     SVGFIDSVFGNSNNSCCGNWECVEKSSQVAELQERLDNSMGLHDIDLSSDVFVQGSY 300
Xu142_Dt       SVGFIDSVFGNSNNSCCGNWECVEKSSQVAELQERLDNSMGLHDIDLSSDVFVQGSY 300
TM-1_Dt        SVGFIDSVFGNSNNSCCGNWECVEKSSQVAELQERLDNSMGLHDIDLSSDVFVQGSY 300
Pima S-7_Dt    SVGFIDSVFGNSNNSCCGNWECVEKSSQVAELQERLDNSMGLHDIDLSSDVFVQGSY 300
*****

Xu142fl_At     RAENMMEGYSDTLMVCDSDGHPKLSLMEPRQNFVNGTASNASSFEENKNYWNNILNLFANAS 360
Xu142_At       RAENMMEGYSDTLMVCDSDGHPKLSLMEPRQNFVNGTASNASSFEENKNYWNNILNLFANAS 360
TM-1_At        RAENMMEGYSDTLMVCDSDGHPKLSLMEPRQNFVNGTASNASSFEENKNYWNNILNLFANAS 360
Pima S-7_At    RAENMMEGYSDTLMVCDSDGHPKLSLMEPRQNFVNGTASNASSFEENKNYWNNILNLFANAS 360
Xu142fl_Dt     RAENMMEGYSDTLMVCDSDGHPKLSLMEPRQNFVNGTASNASSFEENKNYWNNILNLFANAS 360
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TM-1_Dt        RAENMMEGYSDTLMVCDSDGHPKLSLMEPRQNFVNGTASNASSFEENKNYWNNILNLFANAS 360
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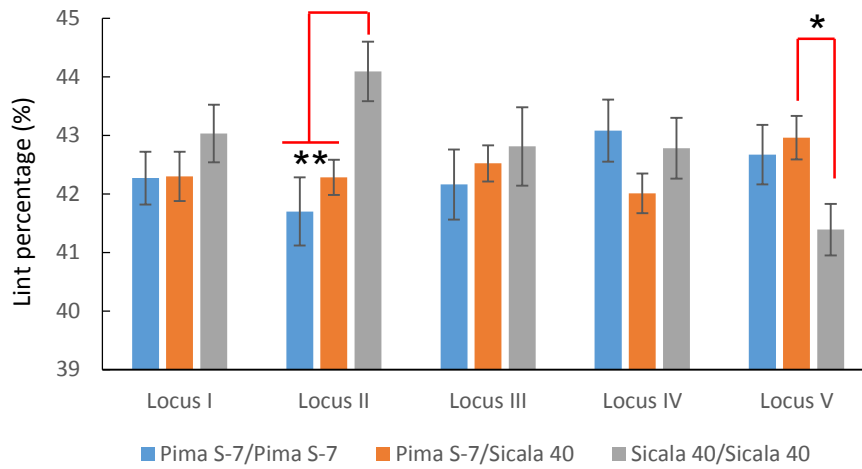
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Xu142_Dt       PSGSSVF 367
TM-1_Dt        PSGSSVF 367
Pima S-7_Dt    PSGSSVF 367
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**Figure S7** Alignment of the amino acid sequences of *MYB25-like* from Xu142, Xu142*fl*, TM-1 and Pima S-7. The conserved MYB-binding and SANT domains are highlighted in yellow and green, respectively. The amino acid that is different between Xu142<sub>At</sub> and Xu142*fl*<sub>At</sub> in the SANT domain is shown in red. The two amino acids that are different between TM-1<sub>Dt</sub> and Pima S-7<sub>Dt</sub> are highlighted in red.



**Figure S9** Distribution of fuzz phenotypes ( $F_3$  seeds) in the  $F_2$  populations PX and FLNX by *MYB25-like* homoeoalleles. (A) Representative fuzz phenotypes of the PX  $F_2$  population derived from Pima S-7 x Xu142*fl*. (B) Representative fuzz phenotypes of the FLNX  $F_2$  population derived from FLN1-10 x Xu142*fl*. The genotype of *MYB25-like\_At/MYB25-like\_Dt* of each  $F_2$  plant are shown below the plant ID. X: homozygous Xu142*fl*; P: homozygous Pima S-7; S: homozygous Sicala 40; H: heterozygous.



**Figure S10** Effects of fuzz associated loci on lint percentage of the FLNS  $F_2:F_3$  families.  $F_2$  plants were genotyped using the markers shown in Fig. 3A. For each locus,  $F_2$  plants were grouped based on their genotypes, *i.e.* homozygous Pima S-7 (Pima S-7/Pima S-7) or Sicala 40 (Sicala 40/Sicala 40), or heterozygous (Pima S-7/Sicala 40) individuals. Error bars represent standard errors. \* and \*\* denote a significant difference at  $p < 0.05$  and  $p < 0.01$ , respectively, determined by a Student's *t*-test.