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# Evaluation of QTLs for Shoot Fly (*Atherigona soccata*) Resistance Component Traits of Seedling Leaf Blade Glossiness and Trichome Density on Sorghum (*Sorghum bicolor*) Chromosome SBI-10L

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**Abstract** Shoot fly is a major insect pest of sorghum damaging early crop growth, establishment and productivity. Host plant resistance is an efficient approach to minimize yield losses due to shoot fly infestation. Seedling leaf blade glossiness and trichome density are morphological traits associated with shoot fly resistance. Our objective was to identify and evaluate QTLs for glossiness and trichome density using- i) 1894 $F_2$s, ii) a sub-set of 369 $F_2$-recombinants, and iii) their derived 369 $F_{2:3}$ progenies, from a cross involving introgression lines RSG04008-6 (susceptible)×J2614-11 (resistant). The QTLs were mapped to a 37–72 centimorgan (cM) or 5–15 Mb interval on the long arm of sorghum chromosome 10 (SBI-10L) with flanking markers *Xgap001* and *Xtxp141*. One QTL each for glossiness (*QGls10*) and trichome density (*QTd10*) were mapped in marker interval *Xgap001-Xnhsbm1044* and *Xisep0630-Xtxp141*, confirming their loose linkage, for which phenotypic variation accounted for ranged from 2.29 to 11.37 % and LOD values ranged from 2.03 to 24.13, respectively. Average physical map positions for glossiness and trichome density QTLs on SBI-10 from earlier studies were 4 and 2 Mb, which in the present study were reduced to 2 Mb and 800 kb, respectively. Candidate genes *Glossy15* (Sb10g025053) and ethylene zinc finger protein (Sb10g027550) falling in support intervals for glossiness and trichome density QTLs, respectively, are discussed. Also we identified a sub-set of recombinant population that will facilitate further fine mapping of the leaf blade glossiness and trichome density QTLs on SBI-10.

**Keywords** Shoot fly · $F_2$ · $F_{2:3}$ · Leaf blade glossiness · Trichome density · QTLs

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## Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most important cereal crop globally. It is grown predominantly in semi-arid tropical conditions and used for food, feed, fodder and fuel (FAOSTAT 2010). Shoot fly, *Atherigona soccata* (Rondani) is one of the major insect pests of sorghum grown in Africa, Asia and Mediterranean Europe. In peninsular India, sorghum is cultivated during rainy and post-rainy seasons where shoot fly attacks the crop and damages early stages of crop growth, adversely affecting establishment and productivity (Sharma et al. 2003). Shoot fly infests sorghum seedlings from 7 days after emergence (DAE) to 30 DAE. The female shoot fly has just 30-days' life span and lays white, elongated cigar-shaped eggs singly on the abaxial (lower) surface of leaf blades parallel to the midrib (Dhillon et al. 2006). Eggs hatch into maggots following 1–2 days of incubation, and each larva/maggot enters the central leaf whorl of the seedling on which it hatched. The larva reaches and cuts the seedling growing point, and feeds on the decaying tissue,

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resulting in drying of the central whorl causing a typical 'dead heart' symptom.

Among several components of integrated pest management practices used to minimize losses due to shoot fly infestation of sorghum, host plant resistance (HPR) and timely sowing remains the most preferred options as they are cost-effective, eco-friendly and easily adopted by farmers (Kumar et al. 2008). HPR to shoot fly is mediated by a number of morphological, biochemical and genetic factors. Shoot fly morphological component traits including seedling leaf blade glossiness (Maiti et al. 1984), seedling leaf blade trichome density (Maiti and Bidinger 1979), seedling vigor and leaf sheath pigmentation are positively associated with shoot fly resistance (SFR) (Tarumoto 2005). Further, these SFR component traits have been mapped, putative Quantitative Trait Loci (QTLs) identified for individual traits, and subsequently validated by marker-assisted backcrossing (MABC)-based introgression into genetic backgrounds highly susceptible to shoot fly (Usha Kiranmayee et al. 2015b). Using a sorghum recombinant inbred line (RIL) population derived from cross (BTx623×IS18551), Sajjanar (2002) and, Folkertsma et al. (2003) mapped SFR QTLs on SBI-01, SBI-03, SBI-05, SBI-07, SBI-09 and SBI-10. Similarly, using a (296B×IS18551)-based RIL population, Deshpande (2005); Mehtre (2006) and Satish et al. (2009, 2012) mapped SFR QTLs on SBI-01, SBI-03, SBI-04, SBI-05, SBI-06, SBI-09, SBI-07, and SBI-10. Aruna et al. (2011) mapped SFR QTLs on SBI-01, SBI-02, SBI-03, SBI-04, SBI-06, SBI-07, SBI-09, and SBI-10 using shoot fly resistance source IS2122. In a RIL population based on a reciprocal cross (IS18551×296B), Apotikar et al. (2011) found SFR QTLs on SBI-01 and SBI-03. Five putative QTLs for SFR component traits from IS18551 were then validated by MABC into the genetic backgrounds of elite shoot fly-susceptible hybrid seed parent maintainer lines 296B and BTx623 (Jyothi 2010). Probable candidate genes underlying the target QTLs for seedling leaf blade glossiness and trichome density have been reported by Satish et al. (2009, 2012) and Aruna et al. (2011). In the present study we attempted to refine QTL intervals for trichome density and glossiness QTLs on SBI-10 by comparing whole sorghum genome sequence (Paterson et al. 2009) annotation and a sequence-based physical map integrated with sorghum linkage maps (Ramu et al. 2010), with genetic and physical maps from different QTL mapping studies integrated based on whole genome sequence information (Mace and Jordan 2011). We also tried to compared earlier shoot fly resistance QTL mapping studies on sorghum chromosome SBI-10 with the present study based on genetic and physical maps.

Identification of genes, pathways and mechanisms involved in sorghum phenotypes for seedling leaf blade glossiness and trichome density have not yet been completed in sorghum. Most such studies have been done in model species like *Oryza sativa* (rice), *Arabidopsis* and *Zea mays* (maize). Wax deficient mutant loci in maize, *Brassica napus* and sorghum are defined as '*glossy*' loci, where as in *Arabidopsis thalina* and *Hordeum vulgare* (barley) they were named as *ceriferum* (cer) mutant loci (Kunst and Samuels 2003). In *Arabidopsis* many studies have reported shine (*shn*) mutants, which were isolated and characterized, determining that the *shn* gene encodes AP2/EREBP (ethylene responsive element binding protein) transcriptional factors that act in up and down regulation of lipid biosynthesis (Aharoni et al. 2004). More than 30 '*glossy*' loci have been identified and a few were cloned (*gl1*, *gl2*, *gl3*, *gl4*, *gl8*, *gl13* and *gl15*) in maize (Li et al. 2013) and their functional roles in glossiness have been reported. Similarly, for trichome density many studies have reported that WRKY and MYB transcription factors play important roles (Eulgem et al. 2000; Johnson et al. 2002; Ishida et al. 2007; Liang et al. 2014).

In order to understand the genomic regions responsible for seedling leaf blade glossiness and trichome density, several QTL mapping and validation studies have laid the foundation for using favourable alleles at the SFR QTLs in MABC programs. The reference genome sequence for sorghum is that of elite, shoot-fly susceptible sorghum maintainer line BTx623 (Paterson et al. 2009), which was one of the susceptible recurrent parents into which favorable SFR alleles from resistance source IS18551 were backcrossed for validation. When resistance QTL introgression lines (ILs) were field evaluated for shoot fly resistance performance (Jyothi 2010), one of the ILs viz., J2614-11 was identified as one of the better performing SFR introgression lines. Thus we used J2614-11 as the resistant parent in the present study, which focused on linked SFR component trait QTLs mapped to the long arm of chromosome SBI-10 (SBI-10L).

In the present study we have conducted experiments to re-evaluate the presence of QTLs for seedling leaf blade glossiness and trichome density as components of SFR on sorghum chromosome SBI-10L. We used a cross of non-glossy, shoot fly-susceptible, *rabi* adapted stay-green introgression line (RSG04008-6) with a drought-sensitive, glossy, shoot fly-resistant introgression line having high trichome density (J2614-11), to produce a high-resolution mapping population of 1,894 $F_2$ individuals. We further selected a sub-set of recombinant $F_2$-derived $F_3$ ($F_{2:3}$) progenies for refining the QTL interval of seedling leaf blade glossiness and trichome density QTLs on SBI-10L. The results of this study will contribute to fine mapping and cloning of genes underlying the confirmed QTLs.

## Results

### Development of High-Resolution Population

*Parental Polymorphism and Confirming $F_1$s*

Introgression line J2614-11 was the donor parent for seedling leaf blade glossiness and trichome density in the cross RSG04008-6×J2614-11. Parents were clearly differentiated visually for both glossiness and trichome density. In order to confirm their allelic composition, nine polymorphic SSR markers were assessed in pairs of parents (RSG04008-6 and J2614-11) and grandparents (R16 and E36-1 for RSG04008-6; BTx623 and IS18551 for J2614-11) across the target genomic region (marker interval *Xgap001-Xtxp141*). Marker alleles for each parent - grandparent pair of E36-1 - RSG04008, and J2614-11 - IS18551 were monomorphic across this SBI-10 target region, but these marker alleles were polymorphic between the two pairs of parents and the two donor grandparents, confirming that the introgressed parental target regions under study were derived from their respective grandparent donors. A total of seven plant×plant crosses were executed and seed from a single plant×plant cross was sown with one seed per hill. From a single plant×plant cross involving RSG04008-6×J2614-11 during *rabi* season 2010 (with plant no. U1000019) twelve putative $F_1$ seeds were produced. All 12 putative $F_1$ plants were screened for heterozygosity with a total of 9 polymorphic co-dominant SSR markers distributed across the target interval (*Xgap001-Xtxp141*). High quality grade 1 (Kanyika et al. 2015) marker allele profiles were obtained for all markers (Fig. S1). Eleven were confirmed to be true $F_1$s having heterozygous parental alleles, whereas one plant was homozygous for the seed-parent alleles, and was discarded.

*Developing $F_2$s and Selection of Informative $F_{2:3}$-Progenies*

All 11 of the true $F_1$s were selfed to produce 11 $F_2$ seed lots. Out of these eleven, one seed lot derived from a single $F_1$ plant (U110055) with 1,958 seeds was selected for advancement during late *rabi* season 2011/12, and used as a high-resolution recombinant mapping population.

A total of 1,894 $F_2$ individuals (surviving after sowing) from the high resolution cross (HRC), along with its parental introgression lines RSG04008-6 and J2614-11, were genotyped with 5 SSR markers covering the target SFR QTL region on sorghum chromosome SBI-10L. The five markers were selected in particular for genotyping the population because the introgression line parent J2614-11 was bred using *Xgap001* and *Xtxp141* as flanking markers for transfer of a two-component shoot fly resistance QTL by MABC from donor IS18551 into recurrent parent BTx623 background. We genotyped the complete $F_2$ population of 1,894 individuals with 5 SSR markers (Table S1) of which two were flanking markers *Xgap001* and *Xtxp141* (Sajjanar 2002; Deshpande 2005; Sharma et al. 2005; Dhillon et al. 2006; Mehtre 2006; Jyothi 2010; Ramu et al. 2010); markers *Xnhsbm1044* (Satish et al. 2009) and *Xisep0630* (Ramu et al. 2010) were reported to be associated with trichome density that conferred shoot fly resistance in sorghum population 296B×IS18551 (Satish et al. 2009); and *Xiabt340* was a marker located between *Xgap001* and *Xtxp141* which was not previously associated with either of the two shoot fly resistance component target QTLs. We selected 369 homozygous and nearly homozygous recombinant $F_2$ plants based on these 5 SSR markers. Individuals showing complete homozygosity for- i) RSG04008 alleles, or ii) J2614 alleles, or iii) complete heterozygosity across this region were not given preference. The 369 selected informative $F_2$ recombinant individuals were genotyped with 3 additional markers to extend the flanking regions for detection of the exact location of the target QTL regions and selection of a sub-set for fine mapping (Table S1). All 369 selected informative recombinant $F_2$ individuals were selfed to produce $F_3$ seed during the late *rabi* season 2011/12 sowing.

### Trait Variation and Correlation of Seedling Leaf Blade Glossiness and Trichome Density Scores

The parental introgression lines differ significantly with each other for both glossiness and trichome density scores (Fig. 1 and Table 1). Among the $F_2$ population and their recombinant $F_3$ progenies, glossiness scores ranged from 0.09 to 4.95 and

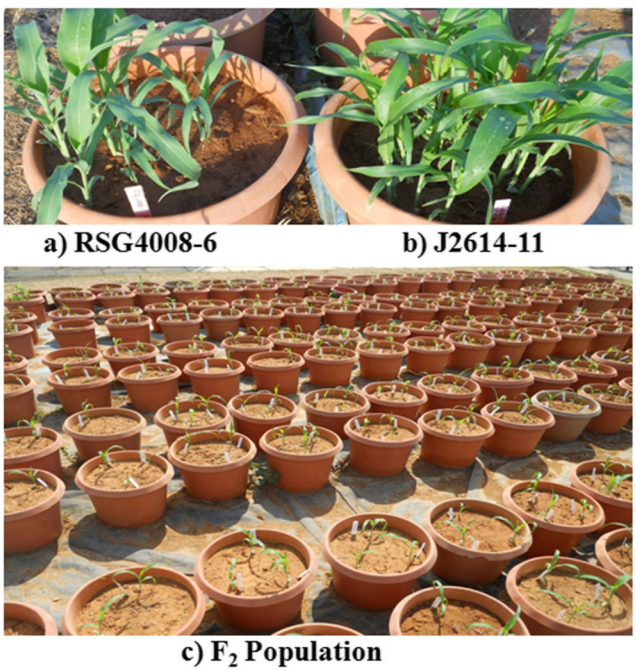


**Fig. 1** **a** RSG4008-6 parent showing non-glossy leaves **b** J2614-11 parent showing glossy leaves **c** F2 population sown in pots

**Table 1** Descriptive statistics and correlations of seedling leaf blade glossiness score and trichome density score in the complete $F_2$ population and $F_3$ progenies derived from 369 selected informative recombinant $F_2$ individuals

| Trait | P1 (RSG) | P2 (J2614) | Min | Max | Mean ± SE | $h^2$ (%) | CV (%) | Correlations | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Gls | Td |
| $F_2$Gls | 5.00 | 1.00 | 1.00 | 4.96 | 1.94±0.09 | 97.75 | 13.7 | 1 | |
| $F_2$Td | 2.00 | 4.00 | 0.00 | 5.08 | 2.66±0.11 | 89.22 | 11.9 | −0.0097* | 1 |
| $F_3$Gls | 5.00 | 1.00 | 1.02 | 4.94 | 2.10±0.24 | 97.99 | 11.9 | 1 | |
| $F_3$Td | 2.00 | 5.00 | 0.27 | 4.90 | 3.63±0.25 | 91.95 | 11.7 | −0.0065* | 1 |

*correlation significant at $P<0.05$, $F_2$ 1,894 individuals, $F_3$ selfed progeny of 369 selected informative recombinant $F_2$ individuals, $h^2$ heritability, *CV* coefficient of variation, *Gls* glossiness, *Td* trichome density

trichome density scores ranged from 0.00 to 5.00. In both the $F_2$ population and its derived $F_3$ progenies, glossiness and trichome density scores were negatively correlated with each other, indicating that a high trichome density score was associated with a low glossiness score and therefore that high trichome density is associated with a high degree of glossiness. Heritability estimates were very high (≥0.90) for both glossiness and trichome density scores (Table 1). The statistical *Z* test results showed (significant $P<0.05$) genetic variation for glossiness score (*Gls*) and trichome density (*Td*) indicating that data is suitable for QTL mapping.

*Seedling Leaf Blade Glossiness*

We scored 1,894 individual $F_2$ plants for the morphological component traits of shoot fly resistance. The trait glossiness was scored visually and the results were divided into two categories- (i) glossy and (ii) non-glossy (Satish et al. 2009). The complex glossiness trait was characterized by narrow, erect, pale, shiny green leaves and 1,457 $F_2$ individuals (76.92 %) exhibited glossy leaves (Fig. 1a and b). A total of 437 $F_2$ individuals (23.08 %) with non-glossy leaves were characterized by broad, dull, droopy leaves. For this trait, the phenotypes of $F_2$ individuals followed Mendelian genetics and segregated in a 3:1 ratio ($\chi^2=0.99$; Fig. 2a), with the glossy phenotype of shoot fly-resistant introgression line parent J2614-11 being dominant.

*Seedling Leaf Blade Trichome Density*

We observed substantial variation in trichome density score in the $F_2$ population (Fig. 2b). Very low trichome density scores ranging from 0.0 to 1.0 were observed in 285 $F_2$ individuals (15.05 %), and low trichome density scores between 1.0 and 2.0 were observed for 260 $F_2$ individuals (13.72 %). Likewise, under categories medium trichome density score 124 individuals (6.54 %), high trichome density score 863 individuals (45.56 %) and very high trichome density score 362 individuals (19.11 %) were noted (Fig. 2b.).

## Genetic Linkage Map

The entire $F_2$ population of 1,894 individuals were genotyped with 5 linked SSRs (*Xgap001*, *Xnhsbm1044*, *Xiabt340*, *Xisep0630*, *Xtxp141*) spanning the introgression target region for SBI-10, resulting in a map distance of 37 cM (Fig. 3a). Based on marker arrangement, genotyping data were categorized into different classes having homozygotes of RSG04008-6, homozygotes of J2614-11, heterozygotes and near-homozygotes with different recombinations. Based on genotyping data across this target region, 369 informative recombinant $F_2$ individuals were selected for advancement to the $F_3$ generation. The selected informative recombinant $F_2$ individuals, were genotyped with three additional markers (*Xisep0621* and *Xisp10262* above *Xgap001*, and *Xisep1011* below *Xtxp141* on SBI-10L) to more

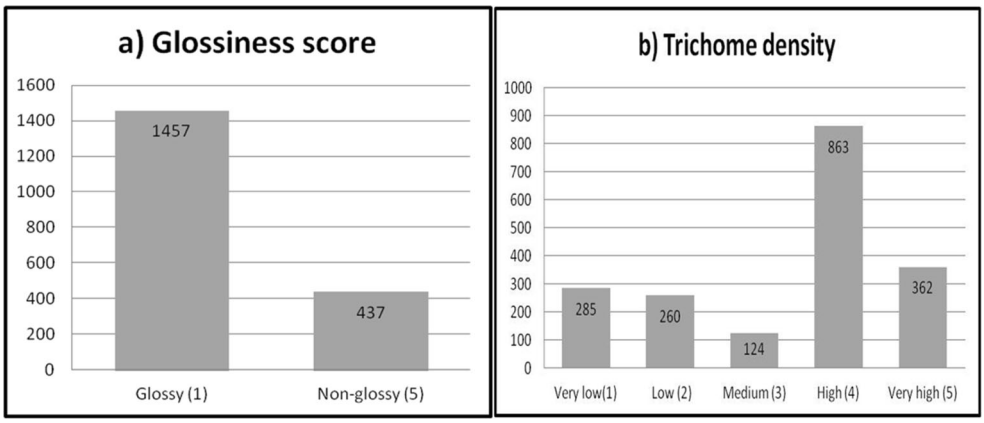


**Fig. 2** Trait segregation among 1,894 $F_2$ individuals for **a** Glossiness score, and **b** trichome density score

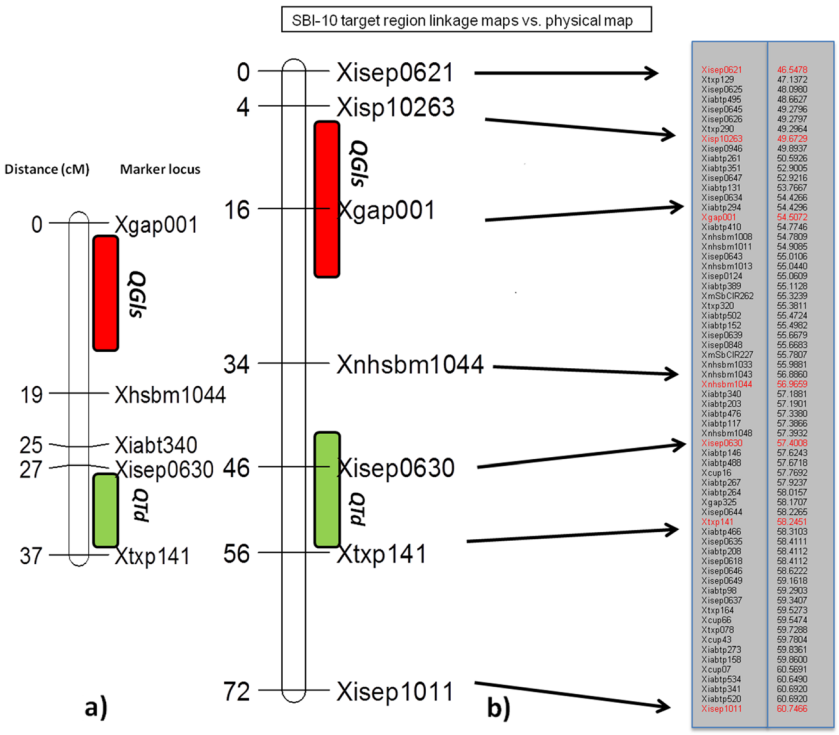


**Fig. 3** Genetic linkage maps of target region **a** with 5 SSRs on F2 population of 1,894 individuals **b** with 7 SSRs on 369 selected informative recombinant F2 individuals, and linkage map vs. physical map

fully encompass the '*Gls*' and '*Td*' genomic regions. The linkage map constructed for these selected recombinant $F_2$ individuals had an expanded total length of 72 cM with 7 SSRs (*Xgap001*, *Xnhsbm1044*, *Xisep0630*, *Xtxp141*, *Xisep0621*, *Xisp10262* and *Xisep1011*) instead of eight and linkage map marker order was similar to the physical map (Fig. 3b). Marker *Xiabt340* was excluded from the linkage map of the recombinant sub-set as it had a large portion of missing data in the selected 369 recombinants when compared to the full population of 1, 894 $F_2$ individuals.

## QTLs Detected in Complete $F_2$ Population

Composite interval mapping (CIM) analysis identified two QTLs for shoot fly resistance component traits on SBI-10, one each for leaf glossiness and trichome density in the $F_2$ population of 1,894 individuals (Table 2). The QTL for seedling glossiness score (*QGls10*) was mapped at LOD 24 (Fig. 4a) between markers *Xgap001* and *Xnhsbm1044* with an $R^2$ value of 6.23 % (indicating it is a relatively minor QTL but mapped with high confidence), with homozygosity for the J2614 allele from grandparent IS18551 reducing glossiness score (but increasing glossiness) by circa 1.36 units compared to the RSG04008 allele from grandparent E36-1. The seedling leaf blade trichome density score QTL (*QTd10*) was mapped between *Xisep630* and *Xtxp141* at LOD 8.11 with an $R^2$ value of 2.88 % (indicating unexpectedly that it too is a minor QTL) (Fig. 4b). The glossiness QTL and trichome density QTL were found within the intervals of 0–10 cM and 25–37 cM, respectively, on the map of the *Xgap001-Xtxp141* interval on SBI-10L of the $F_2$ high resolution mapping population (Table 2). $F_2$ QTL mapping resulted in incomplete confidence intervals for both '*QGls*' and '*QTd*' within the *Xgap001* and *Xtxp141* marker interval. In order to more exactly locate flanking genomic

**Table 2** Shoot fly resistance component trait QTLs detected on SBI-10 using QTL Cartographer with data from a large $F_2$ population of 1,894 individuals derived from cross RSG04008-6×J2614-11

| QTL | Pos (cM) | Marker interval | Supp. IV (cM) | LOD | R^2 % | Add[a] | Dom* |
|---|---|---|---|---|---|---|---|
| *QGls10* | 1.0 | *Xgap001-Xnhsbm1044* | 0–10 | 24.13 | 6.23 | −0.69 | 0.01 |
| *QTd10* | 31.6 | *Xisep0630-Xtxp141* | 25–37 | 8.11 | 2.88 | 0.31 | −0.09 |

*QTL* Quantitative trait locus, *Pos* Position of QTL in cM, *LOD* Logarithm of odds, *R^2%* Percentage of phenotypic variance, *Add* Additive, *Dom* Dominance

[a] Genetic effects for the identified QTLs as per description in the QTL Cartographer Ver. 1.17 manual (page no.13. For trait *Gls* in this study, the lower trait value (1-glossy) is preferred over higher trait value (5-non-glossy), the negative additive effects for *QGls10* are contributed by J2614-11. For *QTd10*, the positive additive genetic effects are contributed by J2614-11

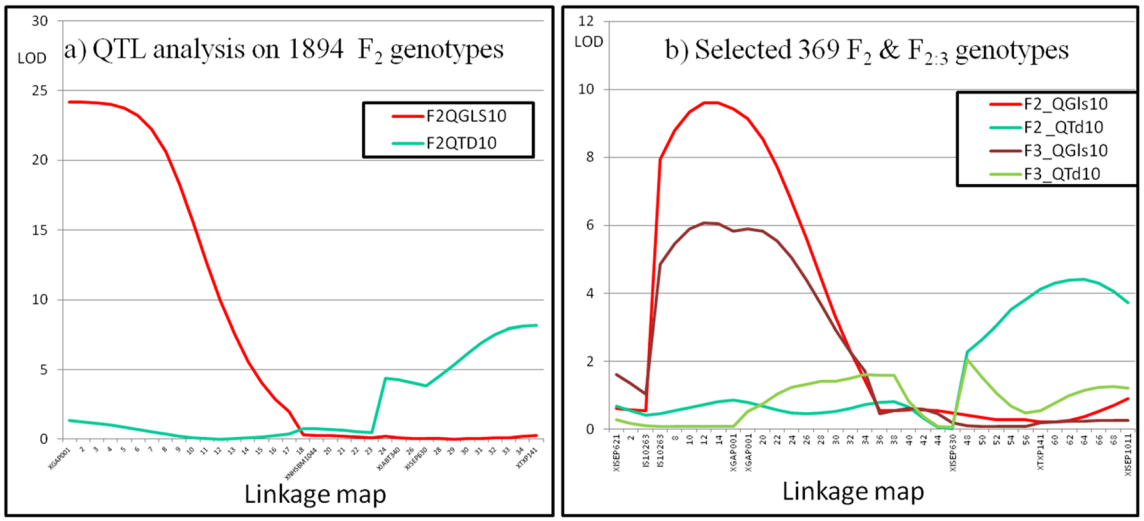


**Fig. 4** **a** Map for glossiness score and trichome density score QTLs on SBI-10L among 1,894 $F_2$ individuals evaluated in *rabi* season of 2010–2011, **b** QTL confirmation among 369 selected informative recombinant $F_2$ individuals evaluated in *rabi* season of 2010–2011 and their derived $F_{2:3}$ progenies evaluated in a late *kharif* season 2012 sowing

regions contributing to the observed variation, three more polymorphic markers (*Xisep0621*, *Xisp10263* and *Xisep1011*) were added to the linkage map for the sub-set of selected informative $F_2$ individuals.

### *Mapping of Glossiness and Trichome Density Using the $F_2$ Total Population with Physical Map Positions*

A moderately large segregating population with 1,894 $F_2$ indiviuals was developed from cross RSG04008-6×J2614-11 and was used in further mapping of the SBI-10 '*QGls*' and '*QTd*' region using physical positions. By aligning genotype and phenotype of all the 1,894 $F_2$s (Table S1) recombinant haplotypes were identified and their phenotypic variant locations were tabulated in Table 3. Marker *Xgap001* showed clear allelic association with glossy phenotype value. In case of trichome density, the marker interval *Xisep630-Xtxp141* might contain trichome coding region/genes, but the evidence is not clear.

### *QTLs Detected Among Selected $F_2$ Individuals and Their $F_{2:3}$ Progenies*

A sub-set of 369 $F_2$ recombinants and their $F_{2:3}$ progenies were also utilized for QTL analysis. At LOD 5.95 the leaf blade glossiness score QTL (*QGls10*) was mapped between *Xisp10263* and *Xgap001* with $R^2$ of 11.37 % for the selected $F_2$ recombinant individuals and $R^2$ of 6.60 % for their $F_{2:3}$ progenies, with LOD values of 9.67 and 5.95, respectively (Table 4). This shows that there was indeed a glossiness QTL in the target region of sorghum chromosome SBI-10L. Further, it was expressed in both the post-rainy season (*rabi*, wherein the $F_2$ population was evaluated) and the rainy season (*kharif*, wherein the $F_{2:3}$ progenies were evaluated). The trichome density QTL (*QTd10*) for the selected 369 $F_2$ recombinants mapped between *Xtxp141* and *Xisep1011*, whereas that for their derived $F_{2:3}$ progenies mapped between *Xisep0630* and *Xtxp141*, within the same support interval. The portion of phenotypic variation accounted for by this trichome density QTL for the 369 selected informative $F_{2:3}$ progenies was just 2.29 % and for the selected recombinant subset of the $F_2$ population was 3.70 % with LOD values of 2.32 and 4.40, respectively (Fig. 4b, Table 4). The sub-set of 369 $F_2$ and $F_3$ progenies were sown in different environmental conditions and the $F_2$ has many heterozygous loci might have showed effects on QTL detection.

### *Mapping of QGls and QTd in $F_{2:3}$ Selected Recombinants*

From the complete $F_2$ population, 369 recombinants were selected and further genotyped with *Xisep0621*, *Xisp10263* and *Xisep1011*, and the combined marker data was used for locating QTL-flanking markers for both glossiness and trichome density. Genotype marker data and their $F_3$ phenotye data were aligned for recombinant identification. Various recombination groups and their mean values were aligned and the $F_2$ data results were combined with $F_3$ results, as well as QTL mapping results. This clearly indicates a glossiness QTL region near *Xgap001* i.e., 54–55 Mb (interval *Xisp10263-Xgap001*). There is weaker evidence for a trichome density QTL region at 57–58 Mb (*Xisep0630-Xtxp141*). Nearly 2.0–2.5 Mb regions were reduced to 800 Kb regions for each QTL (Table 5).

**Table 3** Genotype and phenotype of homozygous recombinants selected from the $F_2$ population

| Physical pos (Mb) | 54.51 | 56.97 | 57.19 | 57.40 | 58.25 | BLUPs | | |
|---|---|---|---|---|---|---|---|---|
| $F_2$ recombinant | *Xgap001* | *Xnhsbm1044* | *Xiabt340* | *Xisep0630* | *Xtxp141* | Glossiness | Trichome density | No. |
| RSG04008-6 | A | A | A | A | A | 4.00 | 2.00 | 60 |
| Rec1 | A | A | B | A | A | 4.91 | 0.00 | 1 |
| Rec2 | A | A | – | B | B | 4.96 | 3.27 | 2 |
| Rec3 | A | B | – | B | B | 4.93 | 4.91 | 2 |
| Rec4 | A | B | – | A | B | 4.93 | 3.10 | 1 |
| Rec5 | A | B | – | B | A | 4.96 | 1.46 | 1 |
| Rec6 | A | A | – | B | A | 4.93 | 1.75 | 4 |
| Rec7 | B | A | A | A | A | 1.05 | 3.00 | 16 |
| Rec8 | B | A | – | B | B | 1.03 | 3.19 | 11 |
| Rec9 | B | B | – | A | B | 1.02 | 4.00 | 6 |
| Rec10 | B | B | – | B | A | 1.02 | 1.29 | 1 |
| J2614-11 | B | B | B | B | B | 1.26 | 3.00 | 114 |

A and B are homozygotes for marker genotypes of RSG04008-6 and J2614-11, respectively

No. is the number of recombinants with the same genotype

*BLUPs* Best linear unbiased predicted means

### Preliminary Identification of Candidate Genes

Annotation of the sorghum genome sequence was utilized along with the UNIPROT database to identify probable candidate genes and their functional roles in controlling glossiness and trichome density of sorghum seedling leaves. In the total 15 Mb target region nearly 780 predicted genes are present (Table S2), of which probable candidate genes for the target traits based on their function are listed in Table 6. The *Xgap001-Xnhsbm1044* interval on SBI-10 L is about 2 Mb long, and contains 179 predicted genes. In the trichome density marker interval *Xisep0630-Xtxp141* 94 predicted genes are present (Table S2). Based on functional annotation *Glossy15*/AP2 transcription factor (Sb10g025053), MYB transcription factor (Sb10g024950), Calcium lipid binding domain (Sb10g025040) and cytochrome P450 (Sb10g025110) were the most likely candidate genes for the glossiness QTL as they are involved in different wax synthesis and transport mechanisms directly and indirectly. In case of the trichome density QTL, MYB transcription factor (Sb10g027280), ethylene zinc finger protein (Sb10g027550), Armadillo repeat protein (Sb10g027680), F-box domain (Sb10g027730), EF hand Ca+2 binding protein (Sb10g027610) and a key transcription factor WRKY (Sb10g025600) reported in many *Arabidopsis* trichome initiation studies all appear to be good candidates and have been previously reported in model plant *Arabidopsis* which shows further study is necessary to decode these glossy and trichome density regions.

## Discussion

For breeding shoot fly resistance, pyramiding resistance component traits appears to be the best way to develop commercially usable levels of host plant resistance, which with timely sowing (to avoid high population pressure of pest) provides

**Table 4** $F_2$ and $F_{2:3}$-based QTL mapping on SBI-10 results obtained using PlabQTL with data from the selected 369 recombinant $F_2$ individuals

| QTL | Pos (cM) | Marker interval | Supp. IV (cM) | LOD | R^2 % | Add* | Dom[a] |
|---|---|---|---|---|---|---|---|
| *$F_2$QGls10* | 14 | *Xisp10263-Xgap001* | 6–20 | 9.67 | 11.37 | 0.69 | −0.01 |
| *$F_3$QGls10* | 12 | *Xisp10263-Xgap001* | 6–20 | 5.95 | 6.60 | 0.70 | 0.36 |
| *$F_2$QTd10* | 58 | *Xtxp141-Xisep1011* | 48–70 | 4.40 | 3.70 | −0.32 | 0.56 |
| *$F_3$QTd10* | 48 | *Xisep0630-Xtxp141* | 46–54 | 2.32 | 2.29 | 0.03 | 0.01 |

*QTL* Quantitative trait loci, *Pos* Position of QTL in cM, *LOD* logarithm of odds, *R^2%* Percentage of phenotypic variance, *Add* Additive, *Dom* Dominance

[a] genetic effects for the identified QTLs as per description in the PLABQTL manual (page no. 13); the additive effect is half the difference between the genotypic values of the two homozygotes

**Table 5** $F_{2:3}$ phenotyping data alignment with $F_2$ genotype haplotypes for mapping '*Gls*' and '*Td*'

| a) Haplotypes for glossiness | | | | | | | b) Haplotypes for trichome density | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| dist in (cM) | 0 | 4 | 16 | 34 | | | dist in cM | – | 46 | 56 | 72 | | |
| physical pos (Mb) | 46.54 | 49.67 | 54.51 | 56.97 | Pheno BLUP | | physical pos | 57.19 | 57.40 | 58.25 | 60.74 | Pheno BLUP | |
| $F_2$ recombinant | *Xisep0621* | *Xisp10263* | *Xgap001* | *Xnhsbm1044* | Glossiness | No. of rec | $F_2$ rec | *Xiabt340* | *Xisep0630* | *Xtxp141* | *Xisep1011* | Trichome density | No. of rec |
| RSG04008-6 | A | A | A | A | 4.94 | 21 | RSG04008-6 | A | A | A | A | 2.12 | 24 |
| Rec1 | A | A | A | B | 4.94 | 8 | Rec1 | – | A | A | B | 2.12 | 1 |
| Rec2 | A | A | B | A | 1.02 | 3 | Rec2 | A | A | B | B | 4.90 | 1 |
| Rec3 | A | A | B | B | 3.00 | 2 | Rec3 | – | A | B | B | 3.20 | 6 |
| Rec4 | A | H | B | A | 1.02 | 2 | Rec4 | – | B | A | A | 3.50 | 2 |
| Rec5 | B | B | B | A | 1.02 | 3 | Rec5 | – | B | B | A | 4.90 | 2 |
| J2614-11 | B | B | B | B | 1.02 | 10 | J2614-11 | B | B | B | B | 3.51 | 51 |

**Table 6** Candidate genes in mapped intervals of seedling leaf blade glossiness and trichome density QTLs on sorghum chromosome SBI-10L

| Marker interval: Trait | Sorghum gene ID | Description | Functional role | Reference |
|---|---|---|---|---|
| *Xgap001-Xnhsbm1044* (2 Mb; *Gls*) | Sb10g025040 | C2 calcium lipid-binding domain | C2 CaLB binds to membrane lipids and mediate signal transduction | De Silva et al. (2011) |
| | Sb10g025110 | Cytochrome P450 | Oxidoreductase activity in wax/cutin biosynthesis | Li-Beisson et al. (2009) |
| | Sb10g025053 | glossy15/AP2/EF/ EREBP transcription factor | Controls juvenile epidermal leaf trait and epicuticular wax synthesis and cutin deposition in maize | Foerster et al. (2015) |
| | Sb10g024950 | MYB transcription factor and DNA binding domain | Over expression of MYB transcriptional factor alters WIN1/SHN1 encodes AP2/EREBP family that encodes glossy | Cominelli et al. (2008) |
| *Xisep0630-Xtxp141* (800 kb;*Td*) | Sb10g025600 | WRKY40 transcription factor | Transparent Testa Glabra2 (TTG2) encodes WRKY transcription factor and control trichome out growth | Ishida et al. (2007) |
| | Sb10g026780 | Speckle-type POZ protein | Expressed in Arabidopsis trichomes | Jakoby et al. (2008) |
| | Sb10g027280 | MYB transcription factor | WD40-HLH-MYB complex regulates trichome development in Arabidopsis | Liang et al. (2014) |
| | Sb10g027550 | C2H2 Zinc finger protein | C2H2 zinc finger protein regulates trichome cell initiation in arabidopsis | Zhou et al. (2013) |
| | Sb10g027610 | EF-hand Ca2+−binding protein CCD1 | Interacts with a microtubule motor protein and regulates trichomemorphogenesis | Reddy et al. (2004) |
| | Sb10g027680 | Armadillo repeat protein | Sequence-specific DNA binding functional transcriptional regulator for plant development activity | Patra et al. (2013) |
| | Sb10g027730 | F-box domain | Acts as transcriptional factors in developmental and degradation process | Coates (2008) |

the most eco-friendly method for management of this pest. Combined effects of glossiness and trichome density reduce the severity of shoot fly infestation and plants with high levels of expression for both traits show better resistance to this insect pest. These morphological traits are well studied (Sharma et al. 2005; Dhillon et al. 2005, 2006; Kumar et al. 2008, 2011), genetically mapped (Sajjanar 2002; Folkertsma et al. 2003; Deshpande 2005; Mehtre 2006; Satish et al. 2009, 2012; Aruna et al. 2011; Apotikar et al. 2011) and further introgressed (Jyothi et al. 2010) into two cultivated varieties in order to deploy insect pest resistance in combination with other economically important traits like high grain and stover yields and quality. Previously these SBI-10 QTLs for trichomes and glossiness were detected in many studies, as summarized in Table 7. The average seedling leaf blade glossiness and trichome density QTLs detected were nearly 15 cM (4 Mb) in size for each trait. In the present study the size of the QTLs was reduced to 2 Mb and 800 kb for '*QGls10*' and '*QTd10*', respectively, which signifies the present study. Except for Aruna et al. (2011) (IS2122) all other QTL mapping studies, IS18551 was the donor for shoot fly resistance, but the mapping populations used varied in population size, type (segregating and recombinant inbred lines), environment and location. In the present study introgression line J2614-11 derived its SFR traits from IS18551. Regions of the sorghum genome contributing to insect resistance are mostly syntenic to maize genomic regions contributing to insect resistance, suggesting such regions were highly conserved. The glossiness QTL and possible trichome density QTL identified in the present study were detected earlier by Sajjanar (2002); Deshpande (2005); Mehtre (2006); Jyothi (2010); Aruna et al. (2011) and Satish et al. (2009, 2012). However, the present work shows evaluation of '*Gls*' and '*Td*' QTLs in the SBI-10 over different environments (late *rabi* 2011/12 and *kharif* 2012), across two seed generations ($F_2$ and $F_{2:3}$), different population sizes (1894 and 369), different mapping methods (QTL Cartographer for $F_2$ and PLAB QTL for the selected sub-set of $F_2$ and its derived $F_{2:3}$) and mapping approaches (traditional CIM and fine mapping) resulted in consistent QTLs.

In the present study, an initial linkage map of 37 cM length was constructed using five SSR markers on an $F_2$ population of 1,894 individuals derived from cross RSG04008-6×J2614-11. In previous studies this target region was reported to be above 45 cM interval but now it is 37 cM (5 Mb), which indicates a reduction in map length most likely due to population type and size. After adding three additional flanking markers and reducing the population size to 369 (selected recombinants) the map length increased to 72 cM (15 Mb), partly due to double crossovers as the recombination frequencies were converted to map distance based on the Kosambi mapping function (Kosambi 1943), but largely due to the addition of flanking markers on both ends of the mapped interval. When marker order was compared with physical map, the arrangement was the same (Fig. 3b). Mace and Jordan (2011) integrated different sorghum QTL mapping studies onto the physical map resulting in QTL clusters a in sorghum. Similarly we have compared all the shoot fly resistance QTL mapping studies in sorghum to delimit the glossy and trichome density QTL sizes on SBI-10L. The present study results are in agreement with earlier studies which shows *Xgap001 – Xnhsbm1044* and *Xisep0630 – Xtxt141* intervals need to be further studied in detail by utilizing high throughput marker genotyping or single nucleotide polymorphisms.

Due to large $F_2$ population, many recombination events have been found within the introgressed genomic segment originally introduced to BTx623-background from IS18551 by marker-assisted backcrossing (MABC) that affects the shoot fly reaction phenotype. The background of the parents vary for the introgressed segment and the $F_2$ progeny with increased number of recombinations may affect the QTL detection power when compared to recombinant inbred lines. QTL analysis can also be affected by the size of the early-generation ($F_2$ & $F_3$) and large populations can result in detection of large numbers of QTLs including minor effect QTLs (Vales et al. 2005).

In both the seasons, a single glossiness QTL (*QGls10*) was mapped near SSR locus *Xgap001*. In addition, in the post-rainy season (late *rabi* 2011/12) evaluation of the $F_2$ population, a QTL for leaf blade trichome density (*QTd10*) was mapped near *Xtxp141*. During rainy season (*kharif*) 2012 '*QTd10*' was mapped near to *Xisep0630*; but the QTL intervals for both the seasons were overlapping. However, $F_2$ and $F_3$ QTL mapping results, based on post-rainy and rainy season evaluations, respectively, were found similar for glossiness. Leaf glossiness characterized by deposition of less wax, or alteration in quantity and quality of epicuticular wax accumulation on leaves which may be controlling the leaf smoothness of the surface of the cuticle and could be responsible for leaf blade erectness (Li et al. 2013). A single gene may not be soley responsible for the glossy phenotype as other genomic regions influence the up- and/or down-regulation of wax synthesis, and at least four glossiness QTLs have been detected in prior studies that considered the whole sorghum genome. However, key transcription factors responsible for glossy phenotypes were consistently reported in the mapped QTL region between *Xisp10263*, *Xgap001* and *Xnhsbm1044*. This target glossy QTL (*QGls10*) was detected in both screening environments and also reported in previous studies (Sajjanar 2002; Folkertsma et al. 2003; Deshpande 2005; Mehtre 2006; Satish et al. 2009, 2012; Jyothi 2010; Apotikar et al. 2011, and Aruna et al. 2011). We looked into the genomic recombination events by traditional fine mapping, *Xgap001* was showing clear association with glossiness, and *glossy15* (Sb10g025053) gene is just 237 kb away from *Xgap001* within the mapped QTL region. Thus *glossy15* (Sb10g025053) could be a likely candidate gene for '*QGls10*' as it is known to control

**Table 7** Summary of sorghum shoot fly resistance mapping studies detecting seedling glossiness (Gls) and trichome density (TD) QTLs on SBI-10

| Reference | Trait of shoot fly resistance on SBI-10L | Marker interval | QTL size in cM | Closest marker | Physical map positions (Mb) | Size of QTL in Mb or kb | Pedigree | Population |
|---|---|---|---|---|---|---|---|---|
| Sajjanar 2002 | Gls[a] | *Xgap001-Xtxp141* | 34 cM | *Xgap001* | 54.50–58.24 | 3.74 Mb | BTx623/IS18551 | 252 RIL |
| Sajjanar 2002 | TD[a] | *Xgap001-Xtxp141* | 34 cM | *Xtxp141* | 54.50–58.24 | 3.74 Mb | BTx623/IS18551 | 252 RIL |
| Deshpande 2005 | TD | *Xgap001-Xcup67* | 22 cM | – | 54.50–12.27 | 42 Mb | 296B/IS18551 | 213 RIL |
| Mehtre 2006 | TD | *Xgap001-Xcup67* | 22 cM | – | 54.50–12.27 | 42 Mb | 296B/IS18551 | 213 RIL |
| Satish et al. 2009 | Gls[a] | *Xgap001-Xnhsbm1043* | 10 cM | – | 54.50–56.88 | 2.38 Mb | 296B/IS18551 | 168 RIL |
| Satish et al. 2009 | TD | *Xgap001-Xnhsbm1043* | 10 cM | – | 54.50–56.88 | 2.38 Mb | 296B/IS18551 | 168 RIL |
| Satish et al. 2009 | TD[a] | *Xnhsbm1013-Xnhsbm1048* | 11 cM | – | 55.04–57.39 | 2.35 Mb | 296B/IS18551 | 168 RIL |
| Aruna et al. 2011 | Gls[c] | *Xtxp320-Xcup16* | 17 cM | – | 55.38–57.76 | 2.38 Mb | 27B/IS2122 | 210 RIL |
| Aruna et al. 2011 | TD[a] | *Xtxp320-Xcup16* | 17 cM | – | 55.38–57.76 | 2.38 Mb | 27B/IS2122 | 210 RIL |
| Aruna et al. 2011 | TD | *Xgap001-Xtxp320* | 4 cM | – | 54.50–55.38 | 880 kb | 27B/IS2122 | 210 RIL |
| Satish et al. 2012 | Gls[a] | *Xgap001-Xnhsbm1011* | 5 cM | *Xgap001* | 54.50–54.90 | 400 kb | 296B/IS18551 | 168 RIL |
| Satish et al. 2012 | Gls[b] | *SvpepcA-XnhsbmSFC4* | 5.9 cM | – | 47.13–46.50 | 630 kb | 296B/IS18551 | 168 RIL |
| Satish et al. 2012 | Gls[c] | *XnhsbmSFC34-Xnhsbm1039* | 8 cM | – | 57.83–58.24 | 410 kb | 296B/IS18551 | 168 RIL |
| Satish et al. 2012 | TD | *Xgap001-Xnhsbm1011* | 5 cM | *Xgap001* | 54.50–54.90 | 400 kb | 296B/IS18551 | 168 RIL |
| Satish et al. 2012 | TD[a] | *XnhsbmSFC34-Xnhsbm1039* | 8 cM | – | 57.83–58.24 | 410 kb | 296B/IS18551 | 168 RIL |
| Present study | Gls[a] | *Xgap001-Xnhsbm1044* | 10 cM | *Xgap001* | 54.50–56.96 | 2.46 Mb | RSG04008-6/J2614-11 | 1894 $F_2$ |
| Present study | TD[a] | *Xisep0630-Xtxp141* | 8 cM | – | 57.40–58.24 | 800 kb | RSG04008-6/J2614-11 | 1894 $F_2$ |
| Present study | Gls[a] | *XIsp10263-Xgap001* | 14 cM | *Xgap001* | 49.67–54.50 | 4.83 Mb | RSG04008-6/J2614-11 | 369 $F_{2:3}$ |
| Present study | TD[a] | *Xtxp141-Xisep1011* | 8 cM | *Xtxp141* | 58.24–60.74 | 2.5 Mb | RSG04008-6/J2614-11 | 369 $F_{2:3}$ |

*Gls* Glossiness, *TD* Trichome density, J2614-11=IS18551 introgression line in BTx623 background, [a] co-localization of QTL, [b]/[c] New QTLs

transcriptional regulation of *glossy* phenotype expression. This suggests that '*QGls10*' needs to be studied further using a fine-mapping approach with higher density markers in this region, and other possible candidate genes in the target interval.

The seedling leaf blade trichome density '*QTd10*' was better expressed in the post-rainy season (*rabi*, characterized by lower temperatures and shorter photoperiods) than in the rainy season, but in both $F_2$ and $F_{2:3}$ segregating populations it was detected in the same support interval. In order to see the recombination events in the support interval the '*QTd10*' QTL was highly associated with *Xtxp141* and *Xisep0630*. Trichome density is largely dependent on the environmental factors and is a complicated trait to measure. More precise microscopic field observations of trichome density may resolve the location of its controlling genomic regions – but these were not practical for the large number individuals observed in the full $F_2$ population. Presence of '*QTd10*' within the same support interval (*Xisep0630-Xtxp141*) across generations and seasons showed the consistency of the QTLs in sorghum molecular mapping of component traits for shoot fly resistance..

### $F_2$ and $F_{2:3}$ QTL Mapping on Selected 369 Individuals

A consistent QTL was detected in two different seasons with two different generations, confirming the presence of a QTL region for seedling leaf blade glossiness that needs to be finely mapped in this population with a larger number of polymorphic molecular markers. We conclude that one QTL for glossiness score (with the glossy allele originating from donor parent IS18551) is present in the SBI-10L target region. QTLs for trichome density mapped differently in the post-rainy and rainy seasons, but within a support intervals sharing a common marker, *Xtxp141*. To clearly differentiate these $F_2$ and $F_{2:3}$ '*QTd10*' QTLs, increased marker density and more efficient phenotyping is required. Fine mapping of these QTLs will improve our understanding of the molecular basis of seedling leaf blade glossiness and trichome density traits (important morphological component traits contributing to sorghum shoot fly resistance). As the glossiness and trichome density QTLs were consistent in both the $F_2$ and $F_3$ generations but showed deviation in the $F_2$ population sub-set (Fig. 4b).

In $F_2$ sub-set rate of recombination has increased due to selected recombinants with heterozygous nature, which will increase the recombination fraction and this could affect the QTL detection power and may increase the rate of false discovery rate (FDR) QTLs. Sometimes missing marker data and segregation distortion in early generation population like $F_2$ may lead to disturbance in estimation of QTL position and its effects. As $F_2$ selected informative recombinants are highly distorted from the normal Mendelian segregation and increased heterozygosity may increase the dominance effect of the detected QTL, which may be due to over dominance effect or the pseudo over-dominance effect of the QTL. Segregating populations ($F_2$ and $F_{2:3}$) have heterozygous variant regions which complicate the gene action during linkage repulsion phase of two dominant alleles results in over dominance or pseudo over dominance.

When both the loci are dominant which may result in over dominance as in case of trichome density both the parents are contributing low to medium and medium to high trichome density so overall trichome density was more in the total. The statistical analysis methods, experimental designs and the phenotyping techniques variation could also affect the dominance and over dominance effects of the detected QTL (Schnable and Springer 2013). QTLs from resistant parent express dominance or over dominance; but if they segregate in the next generation they may not be detected due to less trait variation or other genomic regions might have more influence in phenotype expression. This may also be due to environment effect on trichome density levels leading to less phenotypic variation which cannot separate the genomic regions responsible for the phenotypic variation in the target QTL region detected on SBI-10L.

### Major Component Traits of Shoot Fly Resistance

#### *Glossiness*

Leaf glossiness trait has multiple functions in biotic (shoot fly resistance) and abiotic stresses (drought, salinity, high temperature). Glossiness is visually observed as erect, narrow, pale green and shiny leaf appearance termed as the glossy trait but, all the characteristics may not be controlled by same gene. Cuticular waxes on leaf could be the reason for the glossy phenotype. Water sprinkling method on leaves differentiates non-glossy leaves from glossy leaves by adherence and non-adherence of water droplets, respectively (Tarumoto 1980). Scanning electronic microscopic observations show increased number of wax crystals on leaf surface of non-glossy leaves compared to glossy leaves (Tarumoto et al. 1981).

#### *Candidate Genes for Glossiness*

Seedling leaf blade glossiness variation was observed between the two mapping population parents and QTL analysis conducted on 1,894 $F_2$ high resolution population and 369 $F_3$ selected genotypes resulted in identification of very similar QTLs, which we consider to be a single entity viz., '*QGls10*'. In both phenotyping generations, this glossiness QTL was mapped near to SSR marker *Xgap001*. The mapped QTL region was searched for candidate genes and several wax synthesis-related genes were found. One of the candidate genes related to wax synthesis and deposition of wax present in the QTL region has a C2 calcium lipid-binding domain (Sb10g025040), which is involved in plant stress signal

transduction, and this C2 domain was able to bind membrane lipid ceramides (de Silva et al. 2011). These wax-deficient mutant loci in maize, brassica and sorghum are defined as '*glossy*' loci and in *Arabidopsis* and barley are named as *ceriferum* (*cer*) mutant loci (Kunst and Samuels 2003, 2009).

One of the candidate genes, *glossy15* (Sb10g025053), encodes an *APEPETAL2* (AP2) -like transcription factor involved in the transition from juvenile leaf epidermis characteristics to adult leaf epidermis characteristics, and is expressed after second leaf growth stage (Moose and Sisco 1994, 1996). AP2/ERF transcriptional factors are reported to be involved in wax biosynthesis (Tiwari et al. 2012). Recently Go et al. (2014) reported AP2/ERF (Sb10g025053) acts as a bi-functional transcriptional factor that down regulates the wax biosynthesis pathway by interacting with promoter regions of wax synthesis proteins. MYB transcription factor present in the mapped glossy QTL region (Sb10g024950) has been reported to be involved in activation of AP2/ERF transcription factors involved in wax biosynthesis (Cominelli et al. 2008)

### *Trichome Density*

Trichomes are non-glandular, cellular appendages that protrude above the epidermis (Maiti and Gibson 1983). Trichomes act as physical barriers between the insect pests and the leaf blade epidermis that inhibit egg laying and/or larval movement, which leads to reduction in 'dead heart' formation. Trichome density is genetically controlled and negatively correlated with oviposition- and dead heart incidence-based (Maiti and Gibson 1983; Dhillon et al. 2005) measures of susceptibility sorghum shoot fly.

### *Candidate Genes for Trichome Density*

An MYB transcription factor gene homolog (Sb10g027280) is present in the trichome density QTL region-. Liang et al. (2014) showed that in *Arabidopsis* a WD40 + HLH + MYB transcriptional factor complex regulates the trichome initiation process programmed by cell development. This complex recognizes the specific DNA motifs in gene regulatory regions to activate or repress transcription, mostly by interacting with other proteins like Armadillo repeats, Speckle-type POZ-like proteins, F-box domain proteins, WRKY proteins, MYB transcription factors, ethylene zinc finger proteins, EF-hand $Ca^{2+}$-binding proteins, and thumatin-like proteins. In *Arabidopsis thaliana*, *TRANSPARENT TESTA GLABRA2* (*TTG2*) encodes a WRKY transcription factor and is expressed in young leaves, trichomes, seed coats, and root cells which are not involved in root hair production. During epidermal cell differentiation, MYB transcription factors and HLH transcription factors regulate *TTG2*, which modulates *Glabra2* expression in trichomes (Eulgem et al. 2000; Johnson et al. 2002; Ishida et al. 2007). One additional WRKY transcription factor gene homolog (Sb10g025600) is present in the target trichome density QTL region; this is one of its probable candidate genes. An ethylene zinc finger protein gene homologous with Sb10g027550 has a key role in regulating trichome development in *Arabidopsis*. ZFP5 and ZFP6, the zinc finger proteins, necessary for gibberellic acid and cytokinin signalling to regulate trichome cell differentiation (Zhou et al. 2013). An Armadillo repeat protein gene that appears to be homologous to Sb10g027680 regulates both the gene expressions and cell-cell adhesion. Patra et al. (2013) demonstrated that *ubiquitin protein ligase3* (*upl3*) N-terminal domain has Armidallo repeats that interact with the C-terminal domain of *Glabra3*/*Enhanced Glabra3* for trichome development in *Arabidopsis*. An F-box domain protein homologous to Sb10g027730 also has Armadillo repeats that may act as transcriptional factors and involved in the degradation process plant developmental processes (Coates 2008). An EF-hand $Ca^{2+}$-binding protein gene homolog (Sb10g027680) is also one of the candidate genes underlying the putative *QTd10* trichome density QTL. Kinesin-like calmodulin (KIC) is a EF-hand $Ca^{2+}$-binding protein that interacts with a microtubule motor protein and regulates trichome morphogenesis. Over expression of KIC inactivates kinesin-like calmodulin binding protein (KCBP) by disrupting its interaction with microtubules and its participation in trichome morphological complex resulting in trichomes with less branches/no branches (Reddy et al. 2004). Jakoby et al. (2008) mentioned Speckle-type POZ proteins (homologous to Sb10g026780) were also expressed in trichomes.

In case of the glossiness QTL the increase in score value indicates non-glossiness and lower scores are more preferred for the trait (glossy). Glossiness is also inherited from resistant parent where the moderately large $F_2$ population had more dominance effect due to large population size and high scores which could influence the dominance nature of the detected QTL. In both generations, a glossiness QTL was detected within the same support interval. This confirms that a single glossiness QTL is located in the target marker interval. Further, fine mapping and focused gene expression studies can be carried out utilizing this high resolution cross. This should reveal which of the underlying candidate genes is responsible for the observed variation and its functional role. In contrast, the putative QTL for trichome density on the lower surface of seedling leaf blades, thought to have been introgressed from grandparent IS18551 into BTx623-background line J2614 by Jyothi et al. (2010), was detected in the full $F_2$ population and the sub-set of 369 informative recombinants selected from this under lower-temperature, short-day length post-rainy conditions, but was not clearly detected in the derived $F_3$ progenies when these were evaluated in the rainy season. This warrants considerable further study – starting with phenotyping of the same $F_3$ progenies

for lower leaf blade trichome density during the post-rainy season using available remnant seed. Expression of this QTL only under post-rainy conditions vs. rainy season conditions would warrant considerable further study to understand environmental regulation of this QTL for this trait. Based on $F_2$ genotyping data of 7 co-dominant SSR markers and $F_{2:3}$ phenotyping data, we have selected a further reduced sub-set of 182 most informative recombinants, and selfed their corresponding $F_3$ progenies to produce $F_4$ seeds which can go for replicated field trials and can be used for further study to restrict the genomic region that appears to contribute to the control of sorghum seedling leaf blade glossiness and lower surface trichome density (Usha Kiranmayee et al. 2015a).

### Implications of This Study in Breeding Program

As both the parental lines are introgression lines, but having different genetic backgrounds, the background noise for the interested traits has not been reduced substantially. None-theless, we could identify genotypes having combinations of RSG04008-6 stay-green (drought tolerance) trait with glossines and trichome density. Selfing until homozygosity of the pyramided genotype should lead to development of a multiple resistance trait donor for use in breeding and crossing programs.

## Materials and Methods

### Parents

Parent J2614-11 (glossy, highly trichomed) is a single plant selection from a shoot fly resistant introgression line derived from IS18551 alleles introduced by MABC into BTx623 background having validated donor alleles for seedling leaf blade glossiness and trichome density on SBI-10 (Fig. 1b) (Jyothi 2010). RSG04008-6 (non-glossy, less trichomed) is a single-plant selection from a high yielding drought tolerant but shoot fly susceptible introgression line (IL) with E36-1 alleles for stay-green-associated drought tolerance in highly senescent R16 background (Fig. 1a) (Kassahun 2006).

### Plant Material: Development of the $F_2$ Population and Recombinant $F_{2:3}$ Progenies

At ICRISAT-Patancheru, a manually emasculated and pollinated plant×plant cross was made between RSG04008-6 (susceptible) and J2614-11 (resistant) during post-rainy 2010 to produce $F_1$ seeds. Morphologically and genotypically confirmed $F_1$ plants were self-pollinated using selfing bags to produce $F_2$ seed lots. A moderately-large, high-resolution mapping population of 1,894 $F_2$ individuals derived from a single selfed $F_1$ plant (U110055), was grown in three batches in plastic pots during late post-rainy (late *rabi*) 2011 (Feb - Jun 2012) with triply-repeated parents for each $F_2$ sowing. The parental population were thinned to 3 plants per plot per sowing. Plants were labelled individually (with plant number starting from U120001 to U121931) for $F_2$ progenies, while parents were tagged with their names (Fig. 4c). DNA samples of a emerged $F_2$ plants and parents (9 repeats in total for each parent) were isolated and genotyped initially with 5 simple sequence repeat (SSR) markers namely *Xgap001*, *Xnhsbm1044*, *Xiabt340*, *Xisep630* and *Xtxp141* on SBI-10L where QTLs for seedling leaf blade glossiness and trichome density from donor parent IS18551 had previously been mapped, and then introgressed into BTx623 background to produce parent J2614-11 (Jyothi 2010). By careful examination of the genotyping data across the target QTL region a subset of homozygous and nearly homozygous recombinant $F_2$ plants was selected. All the selected sub-set of recombinant $F_2$ individuals were advanced by selfing to the $F_3$ generation (Fig. 5). All 1,894 individual $F_2$ plants were scored for both of the target traits, i.e., seedling leaf blade glossiness abbreviated as '*Gls*' and seedling leaf blade trichome density abbreviated as '*Td*'. The selected subset of recombinant $F_3$ progenies were sown in the field during rainy season (*kharif*) 2012 (July), in a single plot of 4 m per entry with 10–12 plants per plot after thinning, with two replications of parent. Phenotyping in $F_3$ generations was similar to $F_2$ generation.

### Phenotyping for Traits

$F_2$ plants were tagged and scored individually for the two traits during *rabi*11/12, whereas in the $F_{2:3}$ generation the plants were segregating for traits within each family; so maximum group of plants with similar phenotype were scored for each genotype family during *kharif* 2012. Seedling leaf blade glossiness was scored visually at 12–15 days after emergence (DAE) as described in Sharma et al. (1992) where 1=shiny, pale green, pointed, narrow and erect leaves (glossy) and 5= dull, dark green, broad and droopy leaves (non-glossy) (Fig. 1a and b). Leaf blade trichome density was scored by visual appearance of trichomes on leaves as described in Bourland et al. (2003), but based on the trait variation, in the present population, scores were defined as follows: As trichomes are hairy leaf structures, leaf surface roughness indicated degree of trichome density and smooth leaf surfaces indicated absence of trichomes. Scores were given as 0=absent, 1=very low density, 2=low density, 3=medium density, 4=high density, 5=very high density.

### DNA Isolation and Genotyping

Single plant DNA was extracted from each of the 1,894 $F_2$ seedlings, and each sample of the two parents, using a modified-CTAB and phenol:chloroform:isoamyl alcohol

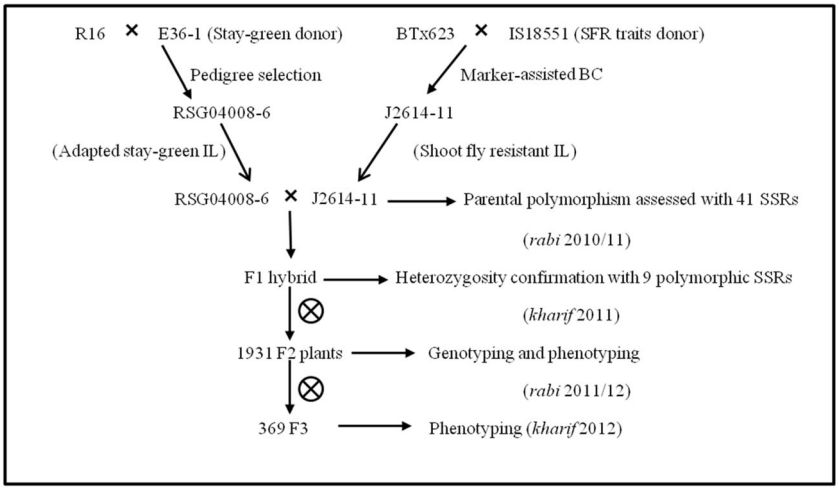


**Fig. 5** Schematic representation for developing ILs and their genetic material from ILs for mapping '*QGls10*' and '*QTd10*' on SBI-10L

(25:24:1) method as described by Mace et al. (2003). DNA was quantified on 0.8 % agarose gel and normalized to 2.5–5 ng/μl using distilled water. PCR was performed in 5.0 μl reaction volumes with 1.0 μl of normalized DNA, 2.0 mM $MgCl_2$, 0.1 mM of dNTPs, 1× PCR buffer, 0.4 pM of each primer and 0.1 U of DNA polymerase enzyme using a Gene Amp® PCR system 9700 thermal cycler (Applied Biosystems®, USA).

PCR products were resolved by capillary electrophoresis on an ABI 3730 DNA sequencer (Applied Biosystems®, USA) and the data generated was analysed with Genemapper® v4.0 software (Applied Biosystems®). This analysis provides a series of automatic fragment sizing, allele scoring, bin-building and auto panelize algorithms that helped in calling A', 'B' and 'H' allele scores ('A'=homozygous for allele of RSG04008, 'B'=homozygous for allele of J2614-11, and 'H'=heterozygous) for PCR products from each SSR primer pair. A set of 41 SSR markers mapping single-copy loci in the extended target region of sorghum chromosome SBI-10L (Table S3). For parental polymorphism assessment a sub-set of nine ([1]*Xgap001*, [2]*Xnhsbm1008*, [3]*Xnhsbm1011*, [4]*Xisep0643*, [5]*Xiabtp389*, [6]*Xnhsbm1044*, [7]*Xiabtp340*, [8]*Xisep0630*, [9]*Xtxp141*) polymorphic SSRs were identified for the initial target marker interval region of *Xgap001-Xtxp141*.

Due to the large $F_2$ population, initially a set of five SSRs distributed across the *Xgap001-Xtxp141* interval on SBI-10L were selected based on their amplification, segregation patterns, good polymorphism between parents, and clear peak patterns for GeneMapper® analysis. SSR markers *Xgap001* (Brown et al. 1996), and *Xtxp141* (Bhattramakki et al. 2000) were previously identified as flanking markers for the target region (Deshpande 2005; Sharma et al. 2005; Dhillon et al. 2006; Mehtre 2006; Jyothi 2010; Ramu et al. 2010). Three SSR markers mapping between these, viz. *Xnhsbm1044* (Satish et al. 2009), *Xisep0630* (Ramu et al. 2009), and *Xiabt340* (Ramu et al. 2010), were selected based on their physical map positions, polymorphism and distribution across this interval. To extend the flanking regions on either side of the target interval, three additional SSR markers (*Xisep0621*, *Xisp10263* and *Xisep1011*) were later added. Due to large percentage of missing data (>50 %) in 369 selected recombinants, SSR marker *Xiabt340* was dropped from linkage analysis, and a new linkage map was developed with seven SSRs (Fig. 3a and b).

## Statistical Analysis

From the $F_2$ and $F_3$ generations, observed phenotyping data was analysed using SAS software package (SAS Institute, USA). A PROC – MIXED augmented design analysis using 'entries' as random model was used to provide covariance parameter estimates and Best Linear Unbiased Predicted means (BLUPs) were derived (with *Z* estimates). Heritabilities values ($h^2$) were estimated from the covariance parameter values. The $F_2$ population was sown in 3 blocks, with each block including 600–650 individual $F_2$ plants along with three replicates of the parental checks in each block. Block effect was estimated from the means of repeated checks and adjusted for each $F_2$ phenotype value in each block in order to minimize the rate of experiment-level error. In case of the $F_2$-derived $F_3$ progenies, parents repeated twice were used as checks.

## Linkage Map Construction and QTL Analysis

### *$F_2$-population*

To tide over the resources and time required for genotyping the complete $F_2$-population, we strategically selected only five (*Xgap001*, *Xnhsbm1044*, *Xiabt340*, *Xisep0630*, *Xtxp141*) out of total nine available polymorphic SSR markers spanning the initial target region (*Xgap001-Xtxp141*). Genotyping data on 1894 $F_2$ population for these five SSRs was generated and

used as input for JoinMap V3.0 (Van Ooijen and Voorrips 2001). This data was used to select a sub-set of informative $F_2$-individuals (to develop $F_3$ progenies) capturing the recombination events in steps. The Kosambi map function was used to convert recombination fractions into centi-Morgans (cM) (Kosambi 1943). Marker order was assigned at minimal LOD 3 and segregation distortion and *chi*-square values were calculated using JoinMap V3.0 (Van Ooijen and Voorrips 2001). QTL mapping for the 1,894-entry $F_2$ population was performed using Composite Interval Mapping (CIM) implemented in QTL Cartographer Windows V2.5 (Wang et al. 2010) with default settings (window size of 10 cM, walking speed of 1 cM, control markers=5 and backward regression). Significance of each QTL interval was determined with the threshold level estimated at 1000 permutations with $P \leq 0.05$ for significant QTL detection.

### *$F_{2:3}$ population*

Genotyping data of additional three markers (*Xisep0621*, *Xisp10263* and *Xisep1011*) on selected 369 $F_2$ was used for generating a new linkage map with 7 SSR markers with JoinMapV3.0 (Van Ooijen and Voorrips 2001). The phenotyping data for the 369 selected recombinant $F_3$ progenies along with their respective $F_2$ individuals was merged with the corresponding $F_2$ genotyping data, and QTLs were positioned and their effects estimated by CIM (Zeng 1994; Jansen 1994) implemented in PLAB QTL version 1.2 (Utz and Melchinger 1996) for both target traits. LOD 2 was set as criteria for detecting QTLs at 1000 permutations. QTL Cartographer Windows V2.5 was used for $F_2$-population QTL analysis and we used PLABQTL for $F_{2:3}$ progenies.

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