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Association of *Lr 34* gene complex with spot blotch disease resistance at molecular level in wheat (*Triticum aestivum* L.)

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Abstract

Leaf rust and spot blotch are among most important wheat diseases causing substantial yield losses in several parts of the world. The studies at phenotypic level suggested that, leaf tip necrosis (LTN) not only associated with multi fungal resistance gene *Lr34* but also confer spot blotch resistance. This LTN – spot blotch association has not been tested at molecular level and hardly validated in different genetic backgrounds. A total of 87 near isogenic lines (NILs) segregating for *Lr34* gene were evaluated for spot blotch resistance and genotyped with the molecular markers linked to QTL *QSb.bhu-7D*. A set of 147 advanced breeding lines was also evaluated for spot blotch besides being genotyped with markers belonging to *Lr34* genic region. Out of 14 markers located on chromosome 7D, four markers segregated in NILs. The genotypic and phenotypic results indicated that the markers reportedly linked with spot blotch differentiate *Lr34*+ and *Lr34*- lines and *vice versa*. This supports the hypothesis that *Lr34, Yr18* and *QSb.bhu-7D* lies in the same gene region. Hence, the linked markers may be used to select both for *Lr34* and spot blotch resistant lines.

Key words: Spot blotch, *Lr34*, leaf tip necrosis, SSR, AUDPC

Introduction

Even after several decades of leaf rust resistance breeding, it is still number one disease in several parts of the word. The rapid evolution in the pathogen population and change in the environmental conditions are important reasons. On the other hand, spot blotch is one of the prominent diseases, causing significant yield loss in warmer and humid regions of the world such as Eastern India, Bangladesh, the Terai of Nepal, Latin America, China and Africa (Gupta et al. 2018). It affects nearly 9 mha area of the North-Eastern Plains Zone (NEPZ) of India (Joshi et al. 2007). *Bipolaris sorokiniana* [*Cochliobolus sativus* (Ito & Kurib.) Drechsl. ex Dast.] [Anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem] is the causative organism for this destructive disease. Saari (1998) reported up to 16% yield loss in Nepal and 15% in Bangladesh, while Mehta (1994) reported up to 100% yield loss in Latin America under the most severe conditions. Several markers and quantitative trait loci (QTLs) for spot blotch resistance have been mapped in wheat (Gupta et al. 2018). Recently, Kumar et al. (2015a, b) dissected a QTL on chromosome 5B into a single Mendelian gene (*Sb2*) using Yangmai#6 as the source of resistance. The 7BS and 7DL chromosomal region also carry spot blotch resistance QTLs (Singh et al. 2016, Kumar et al. 2010). Lillemo et al. (2013), mapped the *Sb1* gene on the chromosome 7D in the same region where the QTL for spot blotch detected.

The *Lr34* gene is one of the most relevant gene in breeding disease resistant wheat and studied widely. *Lr34* is used in breeding programs since decades and has not been overcome by new pathotypes. This locus has contributed durable resistance to leaf rust (*Puccinia triticina*), stripe rust/yellow rust (*P. striiformis*) and powdery mildew (*Blumeria graminis*), making *Lr34* complex an unique resource for breeding. It is also

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used as a model for understanding the molecular basis of durable resistance. The map based cloning of *Lr34* gene region revealed presence of multiple genes in a gene complex, stripe/yellow rust (*Yr18*), powdery mildew (*Pm38*) and for leaf tip necrosis (*Ltn*) on chromosome 7D (Bossolini et al. 2006, Krattinger et al. 2009, Lagudah et al. 2009).

Flag leaves of many wheat cultivars possesses necrotic tip, a morphological marker of *Lr 34* (Singh 1992a). The report of Joshi et al. (2004) revealed that leaf tip necrosis (LTN) is associated with spot blotch resistance. The *Lr34* gene mapped on the short arm of chromosome 7D has been cloned (Krattinger et al. 2009, Lagudah et al. 2009). Using a bi-parental mapping population (Chirya#3 × Sonalika, F_8), the QTL for spot blotch resistance was also mapped on the short arm of chromosome 7D by Kumar et al. (2010). The *Lr34* genomic region consists of 24 exons. The amino acid sequence predicted from this gene and it belongs to the pleiotropic drug resistance subfamily of ABC transporters. ABC transporter gene comprised of five ORFs and 23 introns produce specific protein responsible for necrosis and produced in the leaf tip. Since the morphological marker LTN is reportedly associated with both, *Lr34* and spot blotch resistance, it is interesting to investigate whether the ABC transporter gene which is part of *Lr34* (Krattinger et al. 2011), has effects on spot blotch resistance. Therefore, the markers mapped close to spot blotch resistance on chromosome 7DS were used for genotyping of Jupateco NILs segregating for *Lr34* gene, to study the segregation and establish relation between *Lr34* and spot blotch resistance at molecular level in different genetic backgrounds.

Material and methods

Plant materials

A total of 87 Jupateco (II-12300//LERMA-ROJO-64/ II-8156/3/NORTENO-67) near isogenic lines (NILs) including parents derived from the cross of two 'Jupateco' sister lines (*Lr34*+ and *Lr34*-) named as Jup+ and Jup-developed at CIMMYT, Mexico were used (Singh 1992b). The leaf rust data for Jupateco NILs obtained from Obregon, Mexico while the spot blotch data obtained from Borlaug Institute for South Asia (BISA), Samastipur in Bihar, India. The additional set of 147 advanced breeding lines obtained from CIMMYT, Mexico also evaluated for spot blotch resistance and LTN under natural conditions.

Creation of artificial epiphytotic conditions

The NILs, were field evaluated during March following an artificially induced epiphytotic condition at the BISA, Samastipur, Bihar in the 2013/2014 and 2014/2015 crop seasons. Each line was planted in three replications as two rows of three meters long plot with 20 cm spacing. Following the protocol described by Kumar et al. (2009), susceptible cultivar Sonalika was planted after every 20th row and in alleys to promote inoculum build-up and disease spread. Sowing time was late December to coincide the post-anthesis stage with the higher temperatures conducive to the development of the disease (Chaurasia et al. 2000). The pathogen strain used to create the artificial epiphytotic was a pure culture of the aggressive isolate (isolate No. HDBHU, NCBI KJ412455). The isolate was multiplied on sorghum grains (Chand et. al. 2013). An aqueous spore suspension (10^4 per mL) sprayed on the plants during evening hours at the time of flag leaf emergence (GS47, growth stage 47 on Zadoks scale), early heading (GS53) and heading complete (GS57, Zadoks et al. 1974), following Chaurasia et al. (2000). After the inoculation, the plots irrigated to provide the humid environment required for the development of a high level of infection.

Field evaluation for yellow rust and LTN at BISA, Ludhiana

The Jupateco population was evaluated for yellow rust and LTN at BISA, Ludhiana during 2013-14 under replicated trials. Approximately 60 seeds of each line were grown in two rows of one meter with 20 cm distance between the rows. Susceptible cultivar PBW343 was planted in alleys of the plots as infector to facilitate development of stripe rust epidemic that occurs naturally in Northern part of India. Rust ratings were taken using a modified Cobb's scale of disease severity (DS) (Peterson et al. 1948). This rating scale describes the actual percentage of the flag leaf covered with rust uredinia in increments of 0, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%. The first scoring was taken when the susceptible check PBW343 has approximately 50% disease severity. All Jupateco NILs were evaluated for the presence or absence of LTN at GS69 (Fig. 1) following Singh (1992b).

Evaluation for spot blotch and LTN at BISA, Pusa, Bihar

The Jupateco population was also evaluated for spot blotch and LTN under artificial inoculation at BISA, Pusa Bihar while the advanced breeding lines evaluated

Fig. 1. Expression of leaf tip necrosis (LTN) in wheat lines; a) LTN+ and b) LTN-

for spot blotch under natural infection. The spot blotch disease severity of each line was recorded visually in percentage following Kumar et al. (2009) at GS63 (early flowering stage), GS69 (flowering complete) and GS77 (late milk stage) on 0–100 scale, where zero is immune and 100 is completely susceptible. An area under disease progress curve (AUDPC) based on disease severity recorded at above mentioned growth stages was derived using method of Roelfs et al. (1992). The Jupateco NILs and the advanced breeding lines were evaluated for the presence or absence of LTN at GS69.

Molecular analysis

DNA from Jupateco NILs was isolated, using the CTAB method (Doyle and Doyle 1990) where 200 to 300 mg leaf tissue harvested from 15-day-old seedlings of each line. The DNA dissolved in nuclease-free water to a concentration of 5-10 ng per µl for use as a PCR template. Fourteen SSR markers (Eight reportedly linked with *Lr34* and six to spot blotch) on the chromosome 7DS were used to screen the parents (Jup+ and Jup-) (Table S1). The PCR program comprised of an initial denaturation step (92°C 3 min) followed by 45 cycles of 92°C, 1 min, 50, 55 or 60°C (depending on the SSR involved), 1 min and 72°C, 2 min. The final extension step performed at 72°C for 10 min, following Röder et al. (1998), and Ganal and Röder (2007).

Statistical analysis and genetic linkage map

Karl Pearson correlation coefficients between leaf rust, yellow rust, LTN and spot blotch in Jupateco NILs calculated using the 'cor.test' command of the R-statistical package. QTL IciMapping v4.0 (Wang et al. 2012) used for linkage group construction using all polymorphic markers in Jupateco NILs. Three general steps were involved in linkage map construction: Grouping, Ordering and Rippling. The minimum LOD of 3.0 and recombination frequency of 0.3 used for grouping keeping the window size as 5cM. The LTN (LTN+ or LTN -) used as phenotypic markers for linkage analysis. The advanced breeding lines used to study the correlation between leaf tip necrosis and spot blotch.

Results and discussion

The mean, variance and standard deviation were calculated using SAS statistical software for all the traits under investigation. The leaf rust data was made available by one of the co-authors from CIMMYT, Mexico. The means disease severity and the variance for leaf rust in Jupateco NIL population were 27.5 and 595.2, respectively with wide range of severity from 5 to 70%. The population mean and variance for stripe rust were 29.6 and 623.3, respectively with a range of 0 to 60%. Due to frequent occurrence of stripe rust in North Western Plains Zone (NWPZ) and spot blotch in North Eastern Plains Zone (NEPZ) of India, the stripe rust was recorded at Ludhiana and spot blotch at Pusa. Based on the earlier recommendations (Singh, 1992b, Joshi et al. 2004), the LTN recorded at GS69 (Zadoks 1974). Scoring of LTN beyond GS69 often confounds with leaf senescence while scoring before GS65 has the possibility of false negatives (Joshi et al. 2004). Although, we observed little variation in the degree of expression of LTN at both the locations (Ludhiana and Pusa), most of the lines behaved constantly for LTN at Ludhiana and Pusa (r = 0.98). The results are in agreement with earlier reports indicating some variation for LTN expression across the environment (Juliana et al. 2015).

Although there are several methods to evaluate lines for spot blotch disease resistance (Saari and Prescott 1975, Eyal et al. 1987), we used AUDPC method, suggested to be a more pragmatic approach (Jeger 2004). To calculate AUPDC, spot blotch data recorded visually at three different growth stages (GS63, GS69 and GS77). We observed few early and late lines (5-6 lines) also but did not observe significant difference on disease severity in the advanced breeding lines. The earlier report (Joshi et al. 2002) suggests that resistance to spot blotch is independent of days to maturity. The spot blotch AUPDC in Jupateco NILs ranged from 231 to 1036 with the mean of 660.8 ± 24.9 (Table 1). The test of normality using Shapiro-Wilk test revealed that Jupateco population fits a normal

Table 1. Descriptive statistic for stripe rust, leaf rust and spot blotch in Jupateco NILs

Trait	No of lines	Mean	Vari-ance	Std error	Range	W-test	P-value
Leaf rust	87	27.5	595.2	2.9	0-70	0.79	<0.01
Stripe rust	87	29.6	623.3	2.7	0-60	0.73	<0.01
AUDPC	87	660.8	53247.8	24.9	231-1036	0.85	<0.01

distribution for leaf rust (W =0.73, P =<0.01), stripe rust (W =0.79, P = <0.01) and spot blotch (W =0.85, P = <0.01) (Table 1). The normal distribution in the population indicates variation for spot blotch and rusts. Segregation of LTN in a qualitative fashion enabled us to record as LTN+ (LTN present) and LTN-(LTN absent) in the Jupateco population as well as in the set of advanced lines following Singh (1992b).

A high and positive correlation was found between spot blotch, LTN, leaf rust and stripe rust (Table 2). Highest positive correlation observed between LTN and leaf rust (r = 0.89; P < 0.0001) while lowest but still high positive correlation was observed between stripe rust and spot blotch (r = 0.79; P < 0.0001).

Table 2. Correlation coefficients between spot blotch, stripe rust, leaf rust and LTN in Jupateco NILs

Disease	LTN	Leaf rust	Stripe rust
Spot blotch	0.81(P<0.01)	0.86(P<0.01)	0.79(P<0.01)
Stripe Rust	0.87(P<0.01)	0.82(P<0.01)	-
Leaf rust	0.89(P<0.01)	-	-

To study the effect of LTN on spot blotch resistance, we divided the advanced breeding lines and Jupateco population in two groups (LTN+ and LTN-) separately based on the presence or absence of LTN recorded at GS69. Both the groups in advanced breeding lines showed nearly continuous distribution for spot blotch resistance (Fig. 2) which may be ascribed due to presence of different combinations of spot blotch resistance QTLs/genes (Kumar et al. 2009, 2010). Out of 147 advanced breeding lines, only 30 lines were positive for LTN (Fig. 2). Among the LTN+ group, three lines showed AUDPC higher than the mean of population (353.3 ±13.9) while among the LTN- group (117 lines), 54 lines showed AUDPC less than the mean of the population (Table 3). The mean AUDPC values of LTN+ group and LTN- group in advanced breeding lines were 251.0±22.3 and 379.4±15.6, respectively indicating significant effect of LTN over resistance (Table 3). Similarly, the mean AUDPC values of 43 LTN+ and 44 LTN- lines in Jupateco population were 422.3±14.8 and 773.9±39.6, respectively also indicating significant effect of LTN.

Table 3. AUDPC value and LTN of advanced breeding lines evaluated at Pusa

	LTN+	LTN”
Breeding lines	30 (251.0±22.3)	117 (379.4±15.6)
AUDPC <353.3	27	54
AUDPC >353.3	3*	63

*Number of lines with high AUDPC but have LTN

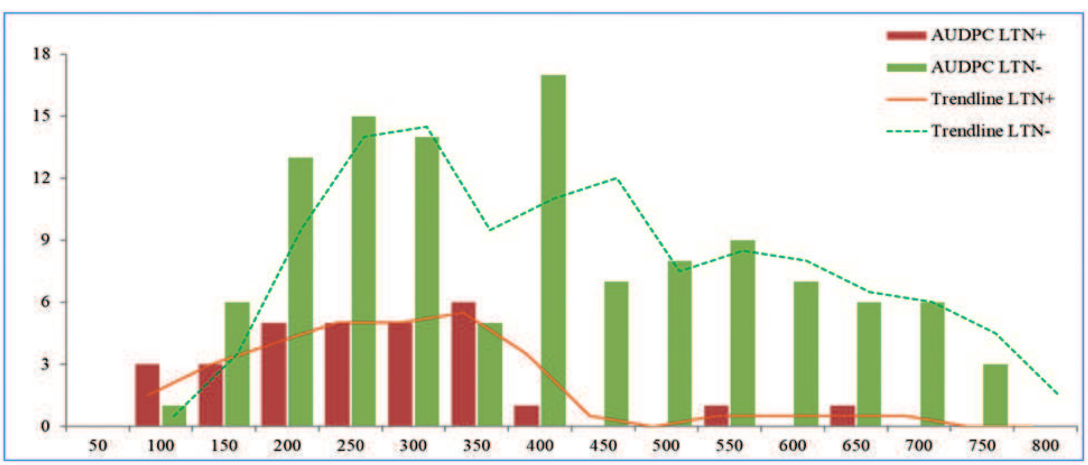

Fig. 2. Distribution of LTN+ and LTN- advanced breeding lines for AUDPC values

It was interesting to note that all advanced breeding lines, except a few (three lines), carrying leaf tip necrosis did not show resistance to spot blotch. However, all the spot blotch disease resistant lines do not necessarily possess leaf tip necrosis. Since there are several QTLs/genes located on other chromosomes as well, the resistance in LTN- lines might be due to those QTLs/genes. The other possibility could be the crossing over between *Ltn* and *Sb1* genes. Since the map based cloning of *Lr34* gene region revealed presence of multiple genes in a gene complex (*Lr34*, *Yr18*, *Pm38* and *Ltn*) on chromosome 7D (Bossolini et al. 2006; Krattinger et al. 2009; Lagudah et al. 2009), the second possibility seems to be very rare. The former possibility is also supported by the QLTs mapping on different chromosomes (Gupta et al. 2018).

There are reports suggesting strong association between spot blotch resistance and LTN (Joshi et al. 2004, Lillemo et al. 2013). The *Lr34* gene mapped on short arm of chromosome 7D was also reported to be associated with LTN (Lillemo et al. 2013). Therefore, we used the markers from short arm of chromosome 7D only. Out of 14 SSR markers tested, five (one linked to spot blotch and four linked to *Lr34*) were polymorphic between the parents and used for the genotyping Jupateco NILs. Interestingly, among five polymorphic markers, *Xswm10* and *cslv34* reportedly linked with *Lr34* (Krattinger et al. 2009) on chromosome 7DS were also associated with spot blotch resistance in the tested material. The *Xswm10* amplified a fragment of 192bp in 'Jup+' and 198bp in 'Jup-' (Supplementary Fig. S1) while Krattinger et al. (2009) reported that *Xswm10* produced 208bp and 214bp fragments in *Lr34*+ and *Lr34*-genotypes respectively. Although two markers (*Xswm10* and *cslv34*) linked with spot blotch and *Lr34*. However due to allelic variation, it is worthwhile to validate the loci identified in one genotype into different genetic backgrounds for effective use in breeding. Our findings supported by the results of Brent et al. (2012), who also reported allelic variation (194bp, 208bp, 210bp, 212bp, 214bp) for the molecular marker *Xswm10* linked to the *Lr34/Yr18* coding region. The marker *Xswm10* produced fragments of 192 and 198bp in Jupateco population (Supplementary Fig. S1) while the other marker *csLV34* produced 175bp and 255bp fragments in 'Jup+' and 'Jup-', respectively Fig. 3).

QTL IciMapping v4.0 (Wang et al. 2012) used for marker analysis and map construction. The linkage map of gene region is comprised of 5 marker loci and a leaf tip necrosis (*LTN*) as phenotypic marker spanning 9.5 cM with an average interval of 1.6 cM (Fig. 4). The order of loci on the map was in agreement

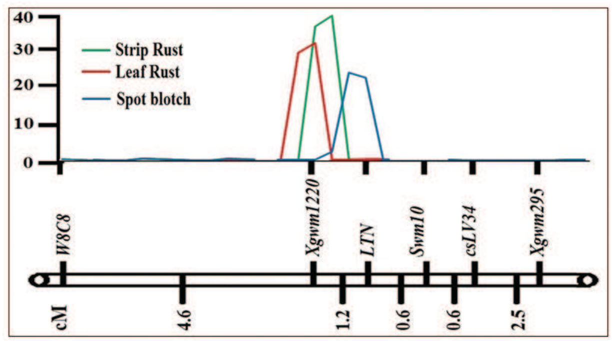

Fig. 4. Genetic Linkage map and LOD curve obtained by ICIM method of Ici Mapping v4.0 for the QTL located on 7DS based on disease severity for spot blotch, leaf rust and stripe rust in "'Jup (R) × Jup (S)" cross

Table 4. Flanking markers, LOD values and phenotyping variance for spot blotch, leaf rust and stripe rust resistance QTL in "Jup + × Jup -" cross

Year	Left marker	Right marker	LOD	PVE (%)
Stripe rust	*Xgwm1220*	*LTN**	35.04	93.90
Leaf rust	*Xgwm1220*	*LTN*	28.5	89.37
Spot blotch	*LTN*	*Xswm10*	22.86	81.07

*LTN was used as phenotypic marker in linkage analysis

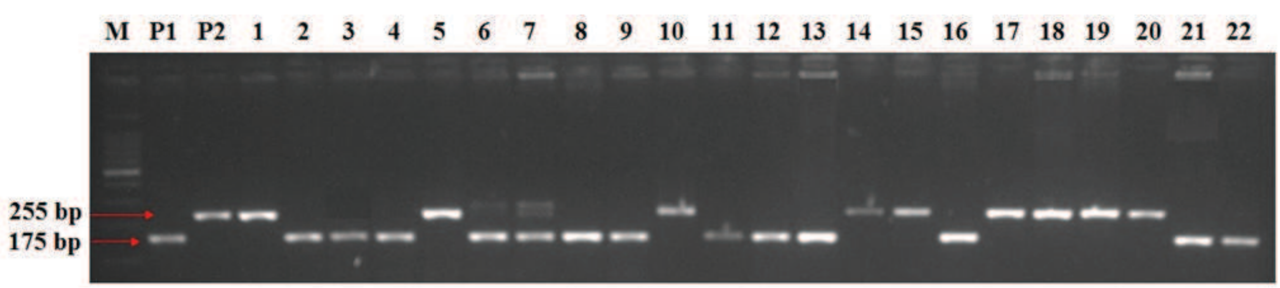

Fig. 3. Segregation of the *Lr34* linked marker *csLV34* in the near isogenic lines (F_{12}) of cross 'Jup +' × 'Jup -'; M, 100bp ladder. The line with positive and negative alleles produces 175bp and 255bp fragments respectively

with previously published ITMI map (Ganal and Röder 2007). All markers used in genotyping of Jupateco NILs segregated in the expected 1:1 ratio (P<0.05).

The QTL for spot blotch mapped between the phenotypic marker *LTN* and the marker *Xswm10* with an interval of 0.6cM using AUDPC with a LOD value of 22.9 (Fig. 3). The phenotypic variance estimated was up to 81.07% (Table 4). Similarly, the stripe rust and leaf rust genes mapped between *Xgwm1220* and the phenotypic marker LTN with a LOD score of 35.0 and 28.5 respectively. The marker interval mapped as 1.2 cM. Being a gene with large effect, the phenotypic variances were up to 93.9% and 89.37% for strip rust and leaf rust respectively. Although, the analysis for spot blotch was performed in a QTL fashion, but due to high phenotypic variance and LOD value, the QTL is being considered as a gene which is supported by earlier findings (Kumar et al. 2015a, Lillemo et al. 2013, Krattinger et al. 2009). Co-segregation of phenotypic as well as molecular markers, independently for spot blotch and *Lr34*, clearly indicated both are linked. Therefore, it may be concluded that *Lr34*/*Yr18*/*Pm38*/*Ltn* gene complex possess the gene for spot blotch resistance named as *Sb1* (Lillemo et al. 2013) in the Jupataco NILs as well as in advanced breeding lines. This validation of *Lr34* gene complex and *Sb1* gene in advanced breeding lines will be useful to develop not only spot blotch but also leaf rust resistance. Our results from advanced breeding lines as well as the Jupateco NILs indicate that LTN belong to the same genomic region where the gene for spot blotch and leaf rust is present, the LTN will help breeders to accelerate the selection of spot blotch and leaf rust resistant genotypes without any pathological experiments.

Authors' contribution

Conceptualization of research (UK, SK); Designing of the experiments (UK, SK, RPS, AKJ); Contribution of experimental materials (RPS, UK); Execution of field/ lab experiments and data collection (MSR, PC, GSM, SK); Analysis of data and interpretation (UK, SK, AKJ); Preparation of manuscript (SK, UK, MSR, AKJ).

Declaration

The authors declare no conflict of interest.

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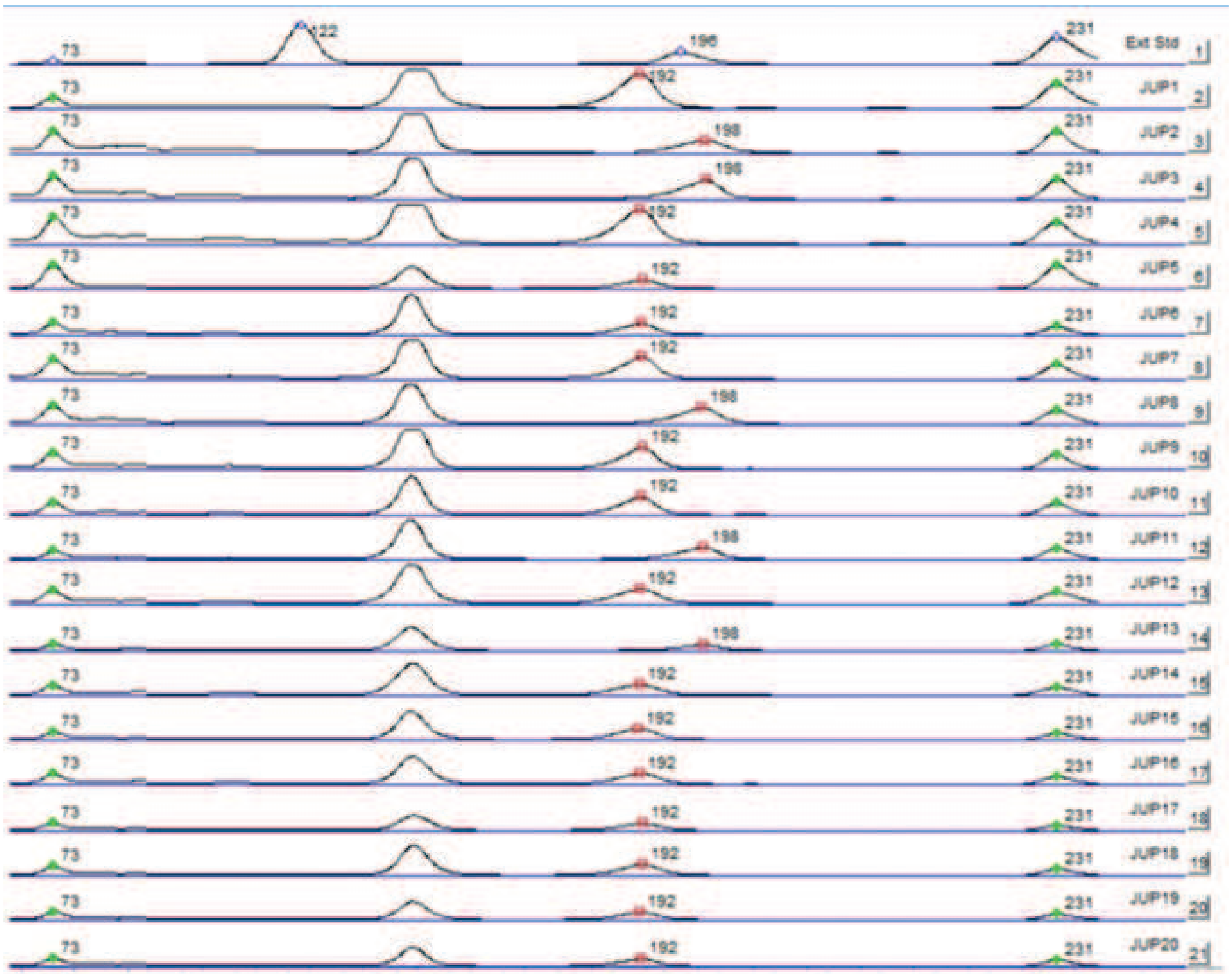
Supplementary Table S1. List of *Lr34* linked and spot blotch linked markers used to screen *Lr34* isogenic lines

Markers	Amplicon size (bp)	Forward primers (5‘-3‘)	Reverse primer (5‘-3‘)
Lr34			
csLVMS	114/117	CTCCCTCCCGTGAGTATATTC	ATCAAAATCCCATTGCCTGAC
csLV34	175/255	GTTGGTTAAGACTGGTGATGG	TGCTTGCTATTGCTGAATAGT
L34SPF/ L34DINT13R2	158/-	GGGAGCATTATTTTTTTCCATCATG	CTTTCCTGAAAATAATACAAGCA
cssfr6	136/-	CTGAGGCACTCTTTCCTGTACAAAG	GCATTCAATGAGCAATGGTTATC
cssfr7	215/-	GCGTATTGTAATGTATCGTGAGAG	CATAGGAATTTGTGTGCTGTCC
cssfr1	286/-	TTGATGAAACCAGTTTTTTTTCTA	GCCATTTAACATAATCATGATGGA
cssfr2	137/-	TTGATGAAACCAGTTTTTTTTCTA	TATGCCATTTAACATAATCATGAA
gwm1220	128/- & 139/141	Sequence not disclosed	
Spot blotch			
Xgwm111	145-149	TCTGTAGGCTCTCTCCGACTG	ACCTGATCAGATCCCACTCG
Xgwm815	142/180/187	Sequence not disclosed	
Xgwm1168	127/228/237	Sequence not disclosed	
Xswm008	123/146 & 237/246	GCTCTTGAACTTAGTCTCATCAAGG	CTCTCCCGCTGCAGTGTCTC
Xgwm437	159/161	GATCAAGACTTTTGTATCTCTC	GATGTCCAACAGTTAGCTTA
Xswm10	192/198	GCCTACTTTGACGGCATATGG	CCATCTTGACATACTTTGGCCTTCC



Supplementary Fig. S1. Segregation of the SB linked marker *Xswm10* in NILs (F_{12}) of 'Jup +' × 'Jup -' cross. The line with positive and negative alleles produces 192bp and 198bp fragments respectively