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ORIGINAL ARTICLE

The genetic basis of flecking and its relationship to disease resistance in the IBM maize mapping population

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Abstract

Key message **In this paper, we determine the genetic architecture controlling leaf flecking in maize and investigate its relationship to disease resistance and the defense response.**

Abstract Flecking is defined as a mild, often environmentally dependent lesion phenotype observed on the leaves of several commonly used maize inbred lines. Anecdotal evidence suggests a link between flecking and enhanced broad-spectrum disease resistance. Neither the genetic basis underlying flecking nor its possible relationship to disease resistance has been systematically evaluated. The commonly used maize inbred Mo17 has a mild flecking phenotype. The IBM-advanced intercross mapping population, derived from a cross between Mo17 and another commonly used inbred B73, has been used for mapping a number of traits in maize including several related to disease resistance. In this study, flecking was assessed in the IBM population over 6 environments. Several quantitative trait loci for flecking were identified, with the strongest one located on chromosome 6. Low but moderately significant correlations were observed between stronger flecking and higher disease resistance with respect to two diseases, southern leaf blight and northern leaf blight and between stronger flecking and a stronger defense response.

Introduction

Disease lesion mimics are one of the most common classes of mutations in maize and other plant systems. These mutations cause the spontaneous formation of lesions (patches of dead cells) on leaves and other organs (Fig. 1a, Johal 2007). The lesion phenotypes vary in size, shape, and frequency depending on the specific lesion mimic mutation and on various other factors such as genetic background, temperature, light, and growth conditions (Hoisington et al. 1982). Their name derives from the fact that many of the phenotypes resemble lesions caused by disease or by the so-called hypersensitive defense response (HR), a rapid localized cell death at the point of pathogen penetration (Mur et al. 2007).

In recent years, several of the genes underlying these mutations have been identified in maize and other systems and it is becoming clear that they are caused by defects in a number of pathways, some of which are not involved in defense response (Johal 2007). Consequently, these mutations are perhaps better referred to as lesioned mutants and this is how they will be referred to for the remainder of this report.

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Vijay Vontimitta and Bode A Olukolu contributed equally to this work.

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Examples of mutations in defense response pathways causing lesioned phenotypes include mutations in the *Rp1* disease resistance gene in maize (Hu et al. 1996) and the *mlo* mutant in barley (Büschges et al. 1997; Piffanelli et al. 2004). Other pathways associated with lesioned phenotypes include (but are not limited to) redox regulation (Gray et al. 1997), programmed cell death (Brodersen et al. 2002), and in various membrane-associated functions (Jambunathan et al. 2001; Johal 2007).

Most lesioned mutants that have been characterized, in maize and other species, are the result of chemical or radioactivity-based mutagenesis and confer rather extreme phenotypes (Fig. 1a). It is apparent, however, that several maize inbred lines derived from a variety of breeding programs display a mild, often environmentally dependent lesion phenotype that we have termed flecking. Flecking is similar qualitatively, if not quantitatively, to the phenotype of some lesioned mutants (Fig. 1b). Although largely uncharacterized, this flecking phenotype has been anecdotally associated with increased disease resistance (M. Goodman, W. Dolezel, pers. com.). The flecking phenotype is largely recessive, tending not to be expressed in F_1 hybrids between flecking and non-flecking lines (M. Goodman pers. com. and unpublished data).

One line displaying flecking is the well-studied line Mo17 (Fig. 2a). The IBM (Intermated B73 × Mo17) population is an advanced intercross line (AIL) maize mapping population developed from a cross between the B73 and Mo17 inbred lines followed by selfing and four generations of random mating following the formation of the F_2 generation and prior to the development of inbred lines (Lee et al. 2002). Compared to more conventional recombinant inbred line (RIL) mapping populations, which do not have this F_2 intercrossing step, AIL populations capture more recombination events and expand the genetic map approximately four-fold (Lee et al. 2002), leading to improved mapping accuracy (Balint-Kurti et al. 2007). The IBM population consists of a relatively large number of lines (302) which have been densely genotyped with more than 2000 molecular markers (Coe et al. 2002). In this study, we have used the IBM population to map loci associated with the Mo17-derived flecking phenotype. We have also assessed to association between flecking in this population and various disease resistance-related traits.

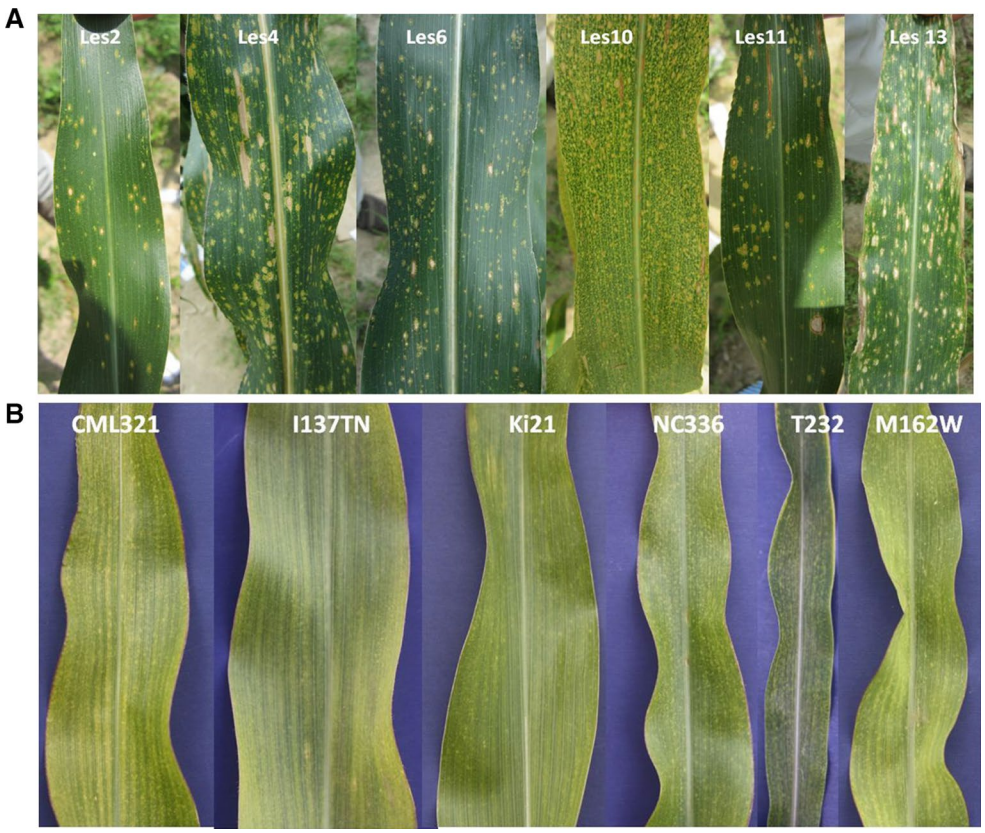

Fig. 1 **a** Picture of leaves on the inbred Mo20W into which specific indicated dominant *Les* mutant genes have been introgressed. **b** Examples of specific indicated *inbred lines* showing a mild 'flecking' phenotype. *Pictures* taken in Clayton NC 12 weeks after planting. Seed of the *Les lines* was a gift from *T. Kazic*

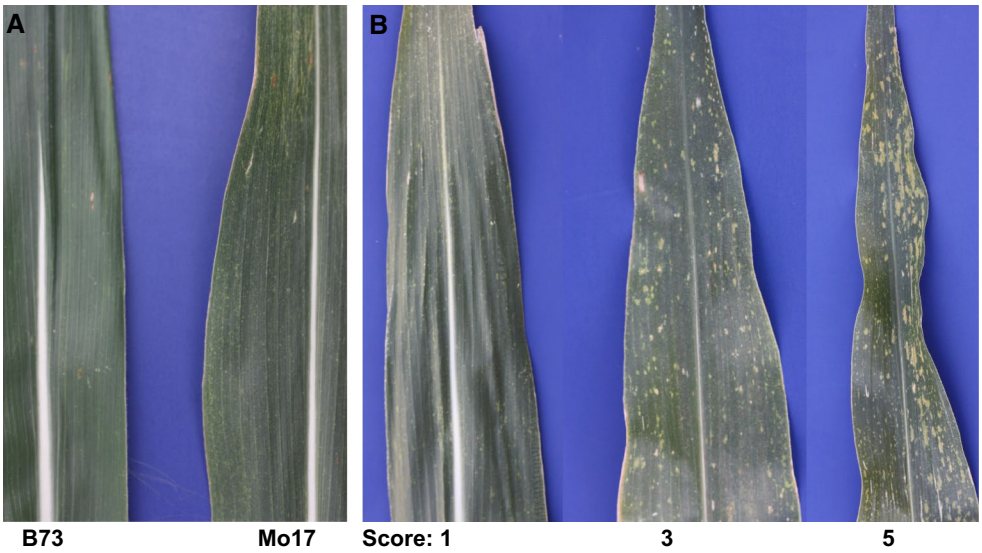

Fig. 2 **a** Leaves of the inbred lines B73 and Mo17, parents of the IBM mapping populations, displaying, respectively, no flecking and mild flecking. **b** *Lines* from the IBM mapping populations displaying increasing amounts of flecking *left* to *right*. The scores designated to each line on the 0–10 scoring scale are indicated. All *pictures* were taken on the same day using leaves from a field in West Lafayette, IN, 12 weeks after planting

Materials and methods

Plant materials

The IBM mapping population is comprised of 302 $F_{7:8}$ recombinant inbred lines (RILs) derived from the cross of maize inbred lines B73 and Mo17. This population had been intermated four times subsequent to the F_2 stage before inbred lines were derived (Lee et al. 2002). Of these 302 lines 300 were used in at least one of the 6 field trials.

Field trials and phenotypic observations

Six field trials were undertaken in Clayton NC in 2011, 2012, and 2013 (NC11, NC12, NC13), in Purdue IN in 2011 and 2012 (IN11, IN12), and in Urbana-Champaign Illinois in 2005 (IL05). In each case, the trial was planted as a single replication in a completely randomized design. Due to seed shortages, the full line set was not planted every time. Furthermore, germination or growth problems made it impossible to score some rows. The number of lines in each location for which we obtained reliable data was 268, 200, 187, 252, 280, and 288 for NC11, NC12, NC13, IN11, IN12, and IL05, respectively. 300 of the 302 IBM lines had at least one reliable observation of which 7, 10, 20, 80, 35, and 148 lines had observations from 1, 2, 3, 4, 5, and 6 environments, respectively.

Experiments were performed at the University of Illinois, Champaign-Urbana genetics farm in the summer of 2005, the North Carolina State University Central Crops Research Station located at Clayton, NC in the summers of 2011, 2012, and 2013 and at the Purdue Agronomy Center for Research and Education (ACRE) in West Lafayette, IN, in the summers of 2011 and 2012.

In Clayton, ten seeds were planted in each plot and without thinning. One plot of inbred border was planted on all sides of the experiment. Overhead irrigation was applied as needed. Standard fertilizer and herbicide regimes for central North Carolina were used. Plots were 2 m in length with a 0.6 m alley at the end of each plot. Inter-row spacing was 0.97 m. In West Lafayette, IN, and Urbana-Champaign, IL, 20 seeds were planted in each 20 ft. rows spaced 2.5 ft. apart.

The planting and scoring dates for each environment were as follows: IL05 was planted May 3rd 2005 and scored July 6th and August 2nd 2005 (64 and 91 days after planting); IN11 was planted May 17th 2011 and scored July 12th and July 25th 2011 (56 and 69 days after planting); IN12 was planted 22nd May 2012 and scored 9th July 2012 and 15th August 2012 (48 and 85 days after planting); NC11 was planted April 25th 2011 and scored June 29th 2011 and July 7th 2011 (65 and 73 days after planting); NC12 was planted May 2nd 2012 and scored June 6th 2012 and July 10 2012 (35 and 69 days after planting); while NC13 was planted April 25th 2013 and scored June 14th 2013, July 1st 2013, and July 24th 2013 (50, 67, and 90 days after planting). Scoring was generally undertaken soon after flecking became apparent and again 2–5 weeks later.

In each case ratings were taken on a 0-10 scale with 0 being no flecking and 10 being dead plants (Fig. 2b).

Average values from the two ratings in each environment were used for analysis. For NC13, since three ratings were taken, a standardized area under disease progress curve (sAUDPC) value was used (see Balint-Kurti et al. 2007).

Statistical analysis

QTL analyses were performed using the Icimapping (Inclusive Composite Interval Mapping) software (Li et al. 2008) v4.0 (accessible from http://www.isbreeding.net/software/). The ICIM-ADD method was used with a step interval of 0.5 map units and a p value for entering variables in stepwise regression of phenotype on marker variables (PIN) of 0.001. 95 % confidence thresholds for calling QTL were calculated using 1000 permutations.

The Windows QTL cartographer software package (Department of Statistics, North Carolina State University, Raleigh, NC) was also used to detect the QTL. Composite interval mapping was used with a walk speed of 0.5 cM, window size 10 cM. The Window QTL cartographer Model 6 was used with 5 control markers and threshold values determined by permutation analysis with a significance level of 0.05. Results derived using QTL cartographer were almost identical to those determined using Icimapping and consequently only the Icimapping results are presented.

Publicly available genotypic data for 1345 markers spaced over the genome was used for the QTL analysis of the IBM population. Map distances are based on the IBM2 map (http://www.maizegdb.org/). Since the units of distance in the IBM population are not, strictly speaking, centiMorgans (cM), IBM map units (Imu) are used as a measure of genetic distance. Broadly speaking, 1 cM $\approx$ 4 Imu (Falque 2005; Lee et al. 2002; Winkler et al. 2003).

All correlation analyses were performed using the PROC CORR procedure of SAS. Heritability was estimated for each trait using the PROC MIXED procedure of SAS (SAS Institute, Cary, NC), as described previously (Holland et al. 2003). The PROC MIXED procedure was also used to estimate variance components and levels of significance attributable to line as fixed effect and the environment as random effect. Since only single replications were conducted in each environment, any line by environment interactions are absorbed into the residual term.

Data transformation to produce normally distributed dataset

Following the PROC MIXED procedure, the residual diagnostics revealed the data were not normally distributed. Since most of the flecking scores approximate count data, Poisson distribution was specified in a generalized linear mixed model (GLMM) with line and environment fitted as random effects using Proc GLIMMIX in SAS v9.3 (SAS Institute, 2000–2004). An overdispersion parameter was also included in the model. The ratio of generalized Chi-square statistic with the residual degrees of freedom was examined (<1) as the usual residual dispersion estimate to protect against overdispersion problem in model fit. Best linear unbiased predictors (BLUPs) of the line random effect were consequently used as input phenotype data for QTL analysis.

Results

Phentoypic observations

The parents of the IBM population displayed different flecking phenotpyes. Mo17 displayed a mild flecking phenotype in each environment, scoring 1–2 on our 0–10 scale, while B73 showed no flecking in any of the environments tested (Fig. 2). The phenotypic distributions of the population were skewed towards low flecking in all the environments. In every environment except IN11, flecking was not observed on most lines (score of 0). Transgressive segregation was observed in each environment with several lines showing mild to strong flecking (Fig. 3).

In each environment, scoring was generally undertaken soon after flecking became apparent and again 2–5 weeks later. The correlations between scoring dates were generally highly significant; the correlations for IL05, IN11, IN12, and NC11 were 0.88.0.63, 0.8, and 0.78, respectively. For NC13, three ratings were taken. The correlation between rating 1 and 2 was 0.56 and between ratings 2 and 3 was 0.64. The correlation between scores for NC12 was not significant. Correlations between environments were moderate to high (Pearson correlation coefficients of between 0.37 and 0.66) with all pairwise correlations highly significant at $p < 0.0001$ (Table 1). Heritability on a plot basis was 0.56 (standard error = 0.03) and on a family mean basis was 0.71 (standard error = 0.02). Line effect was a significant contributor to phenotypic variance while environment was not significant (Table 2).

QTL analysis

Since the environmental effect was not significant (Table 2) and since correlations between environments were reasonably high (Table 1), we used the least square means for flecking over all the environments to identify QTL for the overall flecking phenotype (Table 3). We also calculated the QTL for flecking in each individual environment (Table S1). The QTL identified with the highest log of odds (LOD = 7.34), highest contribution to phenotypic variance (7.6 %), and highest additive effect (−0.36) was on chromosome 6 at 88 Imu. A flecking QTL was also detected

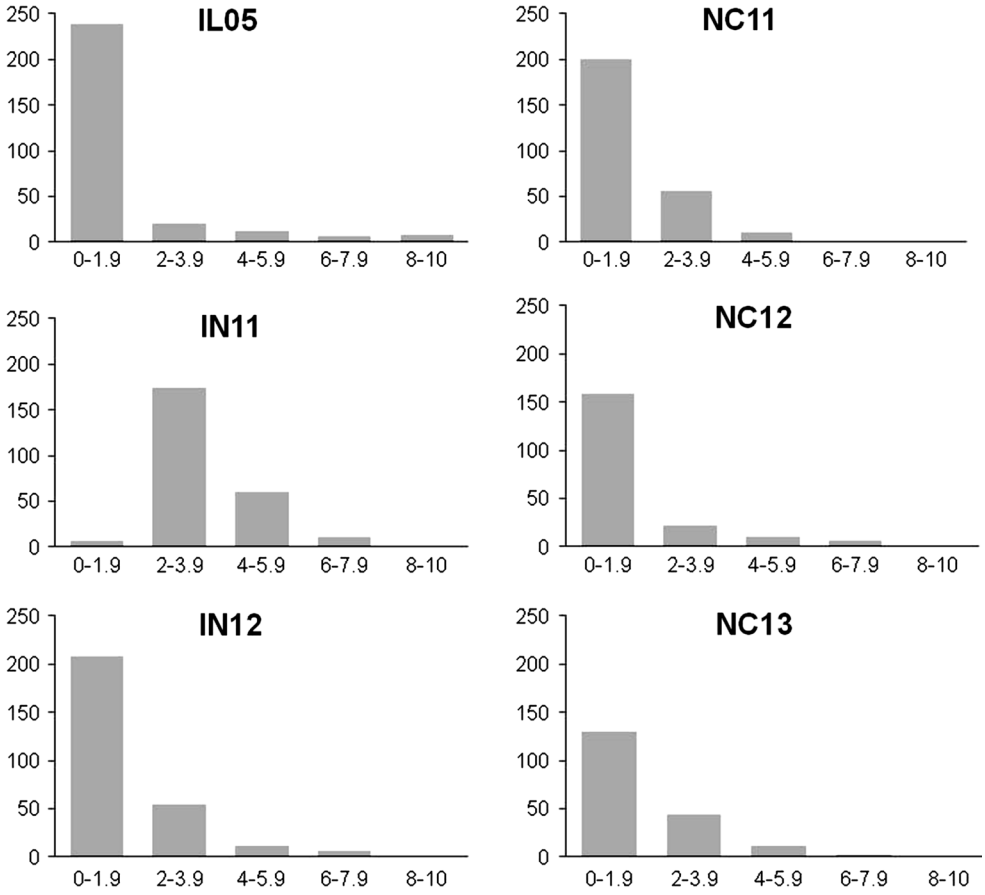

Fig. 3 Distribution of phenotypic scores in each environment. The number of individuals in each phenotypic class is indicated on the y-axis. Phenotypic classes are indicated on the x-axis. The *parental lines* B73 and Mo17 scored 0 and 1–2, respectively, in each environment

Table 1 Pearson correlation coefficient between average scores for each environment. All correlations are significant at $p < 0.0001$

	IL05	IN11	IN12	NC11	NC12
IN11	0.53 *n = 246*				
IN12	0.63 *n = 273*	0.66 *n = 250*			
NC11	0.50 *n = 259*	0.47 *n = 237*	0.66 *n = 260*		
NC12	0.52 *n = 188*	0.37 *n = 171*	0.42 *n = 184*	0.42 *n = 176*	
NC13	0.59 *n = 179*	0.49 *n = 164*	0.64 *n = 177*	0.61 *n = 172*	0.46 *n = 184*

Below each correlation the number of pairwise comparisons used to determine each value is indicated in italics

Table 2 Variance component estimates and standard errors for flecking in the intermated B73/Mo17 (IBM) population

	Flecking trait	*p* value
Environment	0.81 (0.51)	ns
Line	1.28 (0.13)	$p < 0.0001$
Residual	1.07 (0.04)	$p < 0.0001$

within 12 Imu (=~3 cM) of this position in three individual environments (IL05, NC11, and IN12) with another QTL detected on chromosome 6 at 128 Imu in NC13 (Table 3, S1). In NC12, a peak was detected on chromosome 6 at 110 Imu and with a LOD of 2.4, which was slightly under the threshold for declaring QTL.

Seven other QTL were identified for overall flecking. Of these, two QTL on chromosome 4 at 295.8 Imu and on chromosome 5 at 368.3 Imu were associated with more than 5 % of the overall phenotypic variance (Table 3). These QTL were also detected in some of the individual environments. The chromosome 4 QTL was detected in IL05 (Table S1), while QTL peaks were detected at the same map position in NC12 and NC13 but with LOD values slightly below (<0.5 points) the threshold for declaring a QTL. The chromosome 5 QTL was detected in IL05 and IN11 (Table S1) while peaks were detected at the same positon in NC11 and IN12 again with LOD values just below the threshold for declaring a QTL.

At five of the eight QTL, including the three strongest described above, the Mo17 allele conferred higher levels of flecking (signified by a negative additive effect). The B73 allele conferred higher levels of flecking for QTL on chromosomes 1, 4, and 8 (Table 3).

Table 3 Parameters associated with the quantitative trait loci identified for the over-environment least square means for the leaf flecking trait

Chm[a]	Peak[b]	Bin[c]	LOD[d]	PVE[e]	Add[f]	2-LOD Imu[g]	2-LOD markers[h]
1	471.5	1.05	2.69	2.67	0.22	469.4–473.8	MMP124-UMC1601
1	655.5	1.07	3.15	3.34	−0.24	653.4–658.6	umc1358-bnlg1556
2	50.8	2.02	4.04	4.06	−0.26	47.4–55.6	umc1165-npi254a
4	295.8	4.05	6.29	6.69	−0.34	292.8–297.8	bnl15.45-psr128
4	644.3	4.09	3.27	3.25	0.24	624.8–651.8	umc1573-npi593a
5	368.3	5.04	5.44	5.54	−0.31	359.8–371.8	myb3-csu308
6	88	6.01	7.34	7.59	−0.36	86–97	cdo545-uck1
8	205	8.03	3.50	3.50	0.25	203–206	rz244a-umz1157

[a] Chromosome on which the QTL is located

[b] The positions of peak likelihood for each QTL. All values are in IBM map units (Imu Balint-Kurti et al. 2007) and are based on the IBM2 map (Coe et al. 2002)

[c] Chromosome bin location of QTL peak on one of the ten chromosomes of the maize genome. Bins divide the genetic map into 100 approximately equal segments. The segments are designated with the chromosome number followed by a two digit decimal (e.g., 1.00, 1.01, 1.02, and so on) (Davis et al. 1999)

[d] The log of odds (LOD) value at the position of peak likelihood of the QTL

[e] An estimate of the proportion of phenotypic variance (%) explained by the detected QTL

[f] The additive effect of the QTL in terms of the zero to ten scale employed. A positive number indicates that the allele for increased flecking is derived from B73

[g] The genetic positions that define the two LOD interval around the position of peak likelihood for the QTL. All values are in IBM map units (Imu) and are based on the IBM2 map

[h] The closest molecular markers flanking the 2-LOD interval

Table 4 Pearson correlation coefficient between average scores for flecking and average scores for several disease-related traits measured in the IBM population in previous studies

	HR	NLB	GLS	SLB
Fleck	−0.14* *n* = 232	−0.19** *n* = 292	ns *n* = 288	−0.12* *n* = 297

A negative correlation implies that increased flecking is associated with increase resistance or with increased levels of HR

HR the strength of an autoactive hypersensitive defense response (height ratio as defined in Chintamanani et al. 2010), *NLB* resistance to northern leaf blight (Balint-Kurti et al. 2010), *GLS* resistance to gray leaf spot (Balint-Kurti et al. 2008), *SLB* resistance to southern leaf blight (Balint-Kurti et al. 2007), *n* the number of pairwise comparisons used to estimate each correlation

* $p < 0.05$

** $p < 0.001$

Correlations with other disease traits

In separate multi-environment studies, we have previously used the IBM population to map QTL associated with several disease-related traits: The severity of an autoactive hypersensitive defense response (HR, Chintamanani et al. 2010), resistance to northern leaf blight (NLB, Balint-Kurti et al. 2010), resistance to gray leaf spot (GLS, Balint-Kurti et al. 2008), and resistance to southern leaf blight (SLB, Balint-Kurti et al. 2007). Pearson correlation coefficients were calculated between overall scores for flecking and overall scores for these previously analyzed traits measured in the IBM population. Table 4 shows that low correlations of moderate significance were detected between flecking and resistance to NLB and SLB and between flecking and HR severity. The correlation between flecking and resistance to GLS was not significant at any level. The negative correlations detected are consistent with an association between increased flecking and increased levels resistance/defense response.

QTL analysis of transformed data

While QTL analysis is quite robust, the model used is based on a statistical assumption that the data are normally distributed (Lander 1989). This assumption is violated in our case (Figs. 3, S1). For this reason we transformed the data to produce a more normally distributed dataset (Figure S1) and performed additional QTL analysis to make sure that the analyses were consistent. The QTL identified were almost identical to those identified using the non-transformed data (Table S2); The four QTL detected on chmromosomes 1,2,4, and 5 with peak values at 655.5,50.8, 295.8, and 368.3 Imu, respectively, were detected at exactly the same loci with both datasets, while the QTL on chromosome 6 detected with a peak at 88 Imu with the non-transformed data was detected with a peak at 92 Imu using the transformed data. The 2-LOD confidence interval for

this QTL was almost unchanged. The QTL on chromosomes 1 (peak at 471.5 Imu), 4 (644.3 Imu), and 8 (205 Imu) detected with the non-transformed data were not detected with the transformed data.

Discussion

Several reviews (Johal 2007; Lorrain et al. 2003; Moeder and Yoshioka 2008) have described the range of lesioned mutants characterized at a molecular level in plant systems, mainly maize and Arabidopsis. A connection between many of these mutants and some disruption in plant defense response pathways is clear (e.g., Makepeace et al. 2007; Wright et al. 2013; Yin et al. 2000), although much work remains to be done to elucidate the various pathways controlling cell death. Naturally occurring mild leaf spotting, or flecking, is widespread in plants and might be considered analogous to the phenotypes conferred by many lesioned mutants, albeit substantially less severe (Fig. 1). This type of flecking has rarely been studied with respect to either its underlying genetic cause or its possible connection to disease resistance.

One exception is the characterization of the *Arabidopsis thaliana* accession Est-1, which develops macroscopic necrosis on fully expanded leaves with microscopic lesions being detectable at earlier developmental stages. The naturally occurring lesioned phenotype of Est-1 was determined to be due to a novel allele of the *ACCELERATED CELL DEATH 6* (*ACD6*) gene (Todesco et al. 2010). While that the Est-1 ACD6 allele causes reduced growth and fertility, it appears to be maintained in the population due to the higher levels of disease resistance it confers. Similarly, it has been hypothesized that mild flecking may be associated with increased disease resistance in maize, but this hypothesis, while widespread has not been subjected to any systematic examination.

In the present study, we have characterized the genetic basis for the flecking in the IBM mapping population, which is derived for the cross between two commonly used maize inbreds, B73 and Mo17, over six environments. The moderate correlations and the differences in QTL detected between environments (Table 1) were likely influenced by the effects of light and temperature and other environmental factors. It has been well established that fluctuation in both light and temperature can have significant effects on a variety of lesion phenotypes (Hoisington et al. 1982; Negeri et al. 2013; Neuffer and Calvert 1975; Wu and von Tiedemann 2004).

We identified nine "overall" QTL, each associated with a moderate effect on flecking. Zehr et al. (1994) identified several markers associated with a "disease lesion mimic [that] is characteristic of Mo17″ in S_2 progeny derived from the cross of inbred line Mo17 with population BS11(FR) C7, a population resulting from seven cycles of full-sib reciprocal selection for grain yield in the BS11 synthetic (Frank and Hallauer 1999). Thirty-four RFLP markers were scored with each chromosome arm being represented by at least one marker. Significant associations were detected on chromosome 3 at 787.1 Imu; chromosome 4 at 397.4 and 728.7 Imu; and chromosome 9 at 90.1 Imu. Given the very limited resolution of the Zehr et al. (1994) study, the chromosome 4 associations reported may correspond to the QTL identified in this study at 295.7 and 644.3 Imu on chromosome 4 (Table 3) while the chromosome 9 association may correspond to the QTL identified in environment IN11 on chromosome 9 at 25.5 Imu (Table S1).

In separate multi-environmental studies we previously used the IBM population to map QTL associated with resistance to several diseases—northern leaf blight (Balint-Kurti et al. 2010), gray leaf spot (Balint-Kurti et al. 2008), and southern leaf blight (Balint-Kurti et al. 2007)—and with variation in the strength of the hypersensitive defense response (Chintamanani et al. 2010). The only QTL that co-localizes to any degree between the QTL identified in these previous studies and those identified here for the overall flecking phenotype is the QTL on chromosome 4 at 292.8–297.8 Imu that is very close to QTL identified for gray leaf spot resistance at 288–292 Imu on the same chromosome which accounts for 8 % of the observed variation. However, in this case, the B73 allele which is associated with increased resistance is also associated with decreased flecking which is not the expected pattern. The strongest flecking QTL on chromosome 6 at 86–97 Imu is close to a QTL at 23–73 Imu for SLB, mapped in juvenile plants in a population derived from the same parents as the IBM population (Balint-Kurti and Carson 2006) and to major southern leaf blight resistance gene known as *rhm1* which was most recently mapped at ~56.7 Imu on chromosome 6 (Zhao et al. 2012). In general agreement with this lack of QTL colocalization, the phenotypic correlations between flecking and these other traits are low (<0.2, Table 4). Thus, in this case, using data derived, by necessity, from separate environments we show some suggestive though weak evidence that the flecking phenotype may be associated with disease resistance and the defense response. We only used a single population in this study. Flecking likely is based on perturbation of several different pathways depending on the line or population. The genetic basis of segregation in the flecking phenotype in this population may not be typical of the genetic basis of flecking in maize as a whole. Examination of these traits in more broadly based populations such as the maize association mapping (Flint-Garcia et al. 2005) or nested association mapping (NAM) (McMullen et al. 2009) populations will give a better idea if there are relationships between the genetic architectures controlling these trait populations (McMullen et al. 2009).

Author contribution statement VV, BO, BP, and PBK conducted the research, PBK, BO, and BP wrote the paper, PBK performed the analysis, GJ conceived the study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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