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Identification of molecular markers linked to drought tolerance using bulked segregant analysis in Kenyan maize (*Zea mays* L.) landraces

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Maize, drought tolerance, anthesis-silking interval, QTL analysis, SSR, MAS

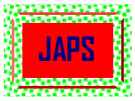
1 SUMMARY

***Introduction*: Drought is an important climatic phenomenon, which is the second most severe constraint to maize production in developing countries, after low soil fertility. Maize (*Zea mays* L.) is particularly sensitive to water stress at reproductive stages, and breeding to improve drought tolerance has been a challenge. When drought occurs just before or during flowering, it results in delayed silking and a consequent increase in the length of anthesis-silking interval (ASI). Selection for a reduced ASI has shown correlated response to improved grain yields under drought. However, conventional selection has been limited by the difficulty of managing uniform experimental conditions to eliminate environmental effects and their possible interactions with the genotype. *Objectives*: Simple sequence repeat (SSR) molecular markers were used to identify genomic regions responsible for the expression of ASI in F2 population derived from a cross of drought susceptible and tolerant landraces using bulked segregant analysis (BSA).**

***Methodology*: An F_2 population of 203 individuals was developed from a cross between drought susceptible (KCB) and drought tolerant (GBK032357) maize landraces. The population was screened under drought to categorize into drought tolerant and drought susceptible phenotypes. Based on the BSA procedure, the DNA from 10 most drought tolerant and 10 most drought susceptible F_2 plants based on ASI values were used to make DNA pools. These DNA pools and DNA from parents were assayed at 109 loci.**

***Results:* A confirmation of polymorphic candidate markers on these 20 individuals revealed four genomic regions associated with ASI. These were regions near markers *p-umc2189*, *p-bnlg1179* and *p-bnlg1014* on chromosome 1 and *p-umc1542* on chromosome 2. The candidate QTLs accounted for about 65% of the observed variation for ASI. Significant phenotypic correlations among flowering parameters, grain yield and yield components were observed. Overlaps between the corresponding candidate QTLs were also observed. For instance, markers, p-*umc2189* and *p-bnlg1014* showed significant association with female flowering time (FFT) whereas markers *p-bnlg1179* and *p-umc1542* showed significant association with both kernel number (KN) and grain yield (GY). This finding implied pleiotropism between loci for ASI and FFT, KN and GY.**

***Conclusion and application of results*: The four candidate markers (*p-umc2189, p-bnlg1179* and *p-bnlg1014* and *p-umc1542*) identified in this study may be useful in developing molecular marker assisted selection (MAS) strategies to transfer drought tolerance traits into the elite varieties. The use of MAS will significantly expedite the breeding process by reducing length of**



the selection cycles required in conventional breeding. However, the candidate QTLs should be validated in other environments to establish their stability.

2 INTRODUCTION

Maize (*Zea mays* L.) is essential for global food security. Despite its being a C_4 plant with metabolism that ought to confer a high photosynthetic rate and relatively lower transpiration rate, maize has been found to be one of the most sensitive species to water deficit (drought). This sensitivity has been attributed to reproductive development, which determines sink strength especially over the period from about one week before to one week after flowering (Tardieu and Simonneau, 1998). Drought tolerance is a quantitative trait whose performance is regulated by many gene loci and hence subject to multiple genotype x environment (GXE) and gene x gene interactions (epistasis) (Campos *et al.,* 2004). The difficulty in genetic manipulation of such traits as grain yield under drought stress is related to their complexity in expression, which according to Ribaut *et al.* (2002), necessitates that several regions or QTLs be manipulated simultaneously. This is because the effects of individual regions cannot be easily identified. Under such conditions, selection for secondary traits, which are correlated to grain yield and having relatively high heritability, have been shown to increase the selection efficiency (Ribaut *et al.,* 1996).

In maize, drought tolerance is closely associated with a short anthesis-silking interval (ASI), reduced kernel barrenness, increased growth rates of the ovaries and the stay green characteristic (Chapman and Edmeades, 1999). This trait is relatively simple to measure in the field and has been shown to have a significantly high negative correlation with grain yield under drought (Ribaut *et al.*, 1996; Banziger *et al.,* 2000). Breeding efforts have therefore been geared towards selecting for reduced ASI (Bolanos and Edmeades, 1996; Ribaut *et al.,* 2004), a strategy that resulted in increased yield under water deficits in a CIMMYT program by 3% per cycle for eight cycles of selection (Bolanos and Edmeades, 1993). These efforts, according to Bruce *et al.* (2002) have resulted in a decrease in sensitivity to water deficits at flowering time in new hybrids than in older varieties. Despite the fact that several of these new hybrids have been released, most farmers in Kenya still cultivate the local landrace populations (Munyiri, 2008). The landrace material is assumed to have high genetic variation and to be well adapted to the natural and anthropological environments where they have evolved. They are believed to contain locally adapted alleles and thus represent an irreplaceable bank of highly co-adapted genotypes which form essential components of sustainable agriculture (Perez-Velasquez *et al.,* 1995; Lucchin *et al..,* 2003).

Since obtaining uniform field experimental conditions has been shown to be quite difficult, the use of molecular markers to assist selection could offer an alternative. These markers are environmentally stable and abundant in plant genomes thus are potentially more powerful tools for characterization, identification, assessment of germplasm diversity and selection (Lee, 1995). The main objective of this study was to identify genomic segments responsible for the expression of ASI in an F_2 population derived from a cross between drought susceptible and drought tolerant Kenyan maize landraces based on SSR markers using Bulked segregant analysis (BSA).

3 MATERIALS AND METHODS

3.1 Development of F_2 population: Parental genotypes (KCB/GBK 032357) for this study were selected based on results from a previous study (Munyiri, 2008) which sought to morphologically characterize 64 Kenyan maize landraces for drought tolerance. This work was carried out at the Kenya

Agricultural Research Institute-(KARI)-Masongaleni farm, in Kibwezi District, Eastern province. The farm is located at 2^0 21.6' south and 38^0 7.3' east, at an elevation of approximately 650m asl. The area falls under the lowland zones of Kenya. The land is semi-arid under Kenya's agro-ecological zone VI and receives 400mm of rainfall per year with an annual evapo-transpiration potential of between 1650 to 2300mm. Average maximum temperature is 24 to 30^0C described as fairly hot to very hot (KARI, 2004). The parents were selected from the two tails of the drought tolerance continuum based on ASI. KCB is a common open pollinated variety (OPV) cultivated in the dry mid-altitude ecologies whereas GBK032357 is a landrace from Kwale, a coastal district in Kenya. KCB was selected for having an average ASI of 12 days hence considered to be drought susceptible while GBK032357 had an average ASI of 3 days thus considered drought tolerant. Seed from the plant that best expressed the trait of interest from each population was harvested and stored separately for development of the mapping population. Paired rows for the parents in three replications were planted and crosses made through sibbing in August 2006 with synchronization between anthesis and silking being achieved through temporal separation. The crossing (June-Oct. 2006), F_1 selfing (Jan-March 2007) and F_2 screening (June-Oct. 2007) were done in the same location with the parents and F_1 being raised exclusively under irrigation.

3.2 Screening of the F_2 population: The aim of this screening was to categorize the segregating plants into drought tolerant (T) or susceptible (S) groups based on ASI and other parameters. Ten rows of 21 hills each were planted to the F_2 population alongside a row each of the original progenitors (P_1, P_2) and F_1 as internal checks. The experiment was not replicated because the population under screening (F_2) was segregating. The recommended agronomic practices were applied and the experiment was raised exclusively under irrigation. Plants were protected from stem borer using Bulldock granules at knee-high stage. Fresh leaf samples were taken from each plant at four-leaf stage and stored at -80°C while awaiting completion of field phenotyping. Irrigation was stopped at one week to flowering and resumed one week after the silking of the last plant for 3 times.

3.3 Field measurements: All parameters were measured on individual plant basis since the plants were segregating. Male flowering time (MFT) was recorded as the number of days from emergence to the first anther extrusion from tassel whereas female flowering time (FFT) was recorded as the number of days from emergence to the first visible silk. Anthesis- silking interval was calculated as the difference between the FFT and the MFT per plant. Leaf rolling was assessed on a scale of 1-5 (1 = no rolling, 5 = extreme rolling) beginning 4 days after stopping of irrigation, thrice at 4 days intervals. Leaf senescence was recorded as the percentage of leaf area with premature leaf deaths over the total leaf area on a scale of 1-10 (1= 10% senescence; 10= 100% senescence). Plant height was measured in cm from the base to the first flag leaf per plant. Tassel size was recorded on a scale of 1-5 (1 = 2-3 branches, 5 = more than 15 branches) per plant. Number of ears per plant was recorded as the number of cobs with at least one grain. Seed set was recorded as the number of kernels/ear per plant whereas yield was recorded as the weight in grams of the total number of kernels per plant.

3.4 Analysis of field data: Simple Pearson correlation coefficients were calculated for all the genotypes using the PROC MIXED procedure in SAS. Distribution of the measured traits was checked using histograms.

3.5 Simple sequence repeats (microsatellites) (SSR) analysis

3.5.1 Genomic DNA extraction: Maize genomic DNA was extracted from parental samples and 203 F_2 individual samples using a modified CTAB procedure as described by Hoisington *et al.* (1994). The DNA samples were purified and checked for quality and quantity in a 1.5% agarose gel using unmethylated and uncut lambda DNA standards. The samples were then standardized to 10ng/µl.

3.5.2 Polymerase chain reaction (PCR) amplification for BSA: Ten most tolerant and 10 most susceptible F_2 individuals were selected from the F_2 population based on results for ASI from the field. DNA from these individuals was then used to constitute the two bulks for bulked segregant analysis (BSA). The two DNA bulks were constructed by mixing equimolar amounts (10 µl) of genomic DNA from these individuals. These DNA bulks were then assayed for SSR polymorphisms alongside parental DNA (P_1, P_2, B_1 and B_2) at 109 loci. A total PCR reaction volume of 10 µl was used containing 1X PCR reaction buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM of each primer set 0.5

U Taq DNA polymerase (SIGMA) and adjusted to 10 µl using double distilled water (ddH_2O). Amplifications were carried out in a MyGenie 96 thermocycler (BIONEER) with the following temperature profiles: 1 initial denaturation step at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 sec, 55 °C for 1 min and 72 °C for 1 min, 30 sec, and a final extension cycle at 72 °C for 10 min. The amplified products were separated by electrophoresis in a 2.0% agarose in 1X TBE buffer (89 mM Tris, 89 mM Boric acid and 2.5 mM EDTA pH 8.0), containing 0.15 µg/µl of Ethidium Bromide. The gels were visualized under ultraviolet (UV) light and images captured using a digital camera and transferred to a computer for analysis.

3.5.3 Confirmation of candidate markers: Polymorphic markers identified from BSA were marked for confirmation. DNA of the 10 F_2 individuals making up each DNA bulk was assayed alongside parental DNA at those loci found to be polymorphic from BSA. The PCR protocol adopted for BSA was followed but using 22 samples (P_1, P_2, 10T, 10S). The amplicons from this step were separated on 3% MetaPhor agarose gels. Gel documentation was done using a digital camera. A 100bp DNA ladder was included in the gels as standard molecular weight. Data was scored as presence (1) or absence (0) of a DNA band. Missing data was recorded as (-). The total number of amplified bands was scored though only the data from intensely stained, unambiguous polymorphic bands was used for analysis. Scoring was done three times to minimize scoring errors.

3.5.4 Quantitative Trait Analysis: A one-way ANOVA using single markers as treatments was used to test for association between markers and quantitative traits in the F_2 individuals selected for bulked segregant analysis. A probability level of $P<0.05$ value based on the F-test significance, was used as the threshold for considering the likely presence of a QTL near a marker. The proportion of the total phenotypic variance attributable to each QTL was estimated via linear regression. The total percentage of phenotypic variance explained for each trait by all QTLs was calculated with a multiple regression analysis using the trait as dependent variable and the previously identified markers, linked to a QTL, as treatments according to Crouzillat *et al.* (2000). Recombination frequencies were calculated for all the markers with significant associations with the trait.

4 RESULTS AND DISCUSSION

4.1 Field trait analyses: The various secondary traits measured in the F_2 population under drought stress showed variations in their relationships with each other and with the primary trait, the grain yield (GY) (Table 1). Male flowering time (MFT) and female flowering time (FFT) were highly significant and positively correlated under drought ($r = 0.69^{**}$). This trend was also consistent in the other genotypes (data not shown). These results confirm previous findings of Ribaut *et al.* (1996) ($r > 0.70$) and Munyiri (2008) (0.87^{**}). ASI was highly significant and positively correlated with FFT ($r = 0.84^{**}$). This result confirms previous observations that drought causes major delays in silking by slowing ear development (Bolanos and Edmeades, 1993; Ribaut *et al.*, 1996; Campos *et al.*, 2004). However, most previous studies report that ASI has only slight effects on MFT. The significant positive correlation between ASI and MFT ($r = 0.2^{*}$) in this study could be attributed to non-uniform response to drought by the segregating F_2 plants under varying water deficit intensities over time. Results from this experiment also indicate that GY under drought stress at flowering has a strong dependency on kernel number, ear number per plant and ASI. All these had significant correlations with grain yield (Table 1). These findings confirm the results obtained by Bolanos and Edmeades (1996), who reported highly significant and positive correlation ($r = 0.8^{**}$) for kernel number with grain yield and relatively high correlations ($r = -0.4$ to -0.7) for ASI with yield in tropical maize. The significantly negative correlation between FFT and grain yield ($r = -0.31$) and its high positive correlation to ASI demonstrate that ASI is determined largely by variation in FFT under drought stress. Frova *et al.* (1999) suggested that the high negative correlation between ASI and GY under drought stress indicated that the variation in GY under drought is due to variation in ear setting processes that are related to biomass partitioning at flowering. According to Bruce *et al.* (2002) and Monneveux (2006), the ovule abortion rate was positively correlated with ASI; hence, ASI determined the kernel set. MFT, leaf senesce, plant height, and tassel size showed non-significant correlations with grain yield (Table 1)

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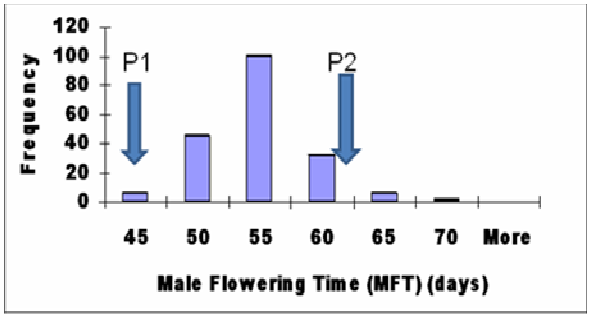
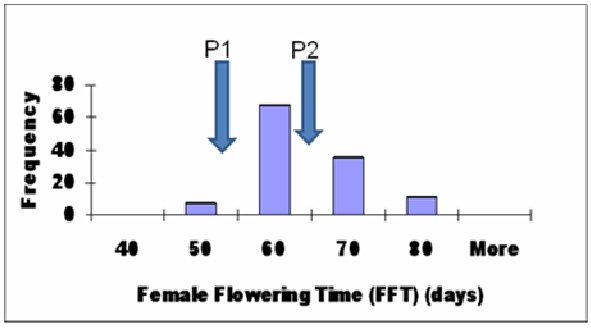
Table 1: Linear correlation between various traits measured in an F_2 maize population under severe drought stress in Masonagaleni, Kenya.

	MFT	FFT	ASI	LR	LS	PHT	TS	EPP	KN	GY
MFT	1.00	0.69**	0.20*	0.19**	0.09 ns	-0.10 ns	0.08 ns	-0.10 ns	0.07 ns	0.04 ns
FFT		1.00	0.84**	0.19*	0.06 ns	-0.01 ns	0.39**	-0.36**	-0.30**	-0.31**
ASI			1.00	0.29**	0.02 ns	-0.03 ns	0.40**	-0.41**	-0.47**	-0.45**
LR				1.00	0.43**	-0.25**	0.11 ns	-0.13 ns	-0.16 ns	-0.24*
LS					1.00	-0.16*	0.04 ns	-0.18 ns	-0.003 ns	-0.02 ns
PHT						1.00	0.05 ns	-0.07 ns	-0.05 ns	-0.04 ns
TS							1.00	-0.24*	0.19 ns	-0.18 ns
EPP								1.00	0.60**	0.54**
KN									1.00	0.91**
GY										1.00

Key:*, ** Significant at 5% and 1% probability levels respectively
MFT=male flowering time; FFT=female flowering time; LR=leaf rolling; LS= leaf senescence; PHT= plant height; TS=tassel size; EPP= ears per plant; KN= kernel number; GY= grain yield.

Several explanations have been given for the behaviour of phenotypic traits in maize under flowering water stress. According to Monneveux *et al.* (2006), severe drought stress just before flowering reduces stomatal conductance thereby reducing assimilate partitioning to the tassel which results in delayed anthesis (MFT). Mugo *et al.* (1998) and Frova *et al.* (1999) noted that delays in silking were due to less partitioning of assimilates into the ear and that this is closely associated with barrenness. According to Tollenaar and Wu (1999), grain abortion occurred during the first 2-3 weeks after silking and got worse as the photosynthetic canopy is reduced by stress leading to reduced assimilates into the ear. Bolanos and Edmeades (1993) also noted that drought at or immediately after flowering accelerated leaf senescence thus reducing leaf area, which leads to reduced intercepted radiation and photosynthesis that results in a reduction in photo-assimilate flux to the spikelets. Banziger *et al.* (2000) suggested that genotypes with small tassels allowed allocation of the scarce assimilates during drought to the developing ears, also supporting this inference. According to Boyer and Westgate (2004), water deficit in later stages of development reduces kernel size rather than kernel number and this is largely determined by the available photosynthetic assimilates to the ear. Therefore, results from this study imply that grain yield loss under drought is mainly as a result of reduced photosynthesis that results in reduced assimilates. All the measured traits revealed a large quantitative variability and a fairly normal frequency distribution under drought conditions (Figure 1). The two parents were differentiated for all traits. Thus, F_2 population was subjected to QTL analysis for drought tolerance.





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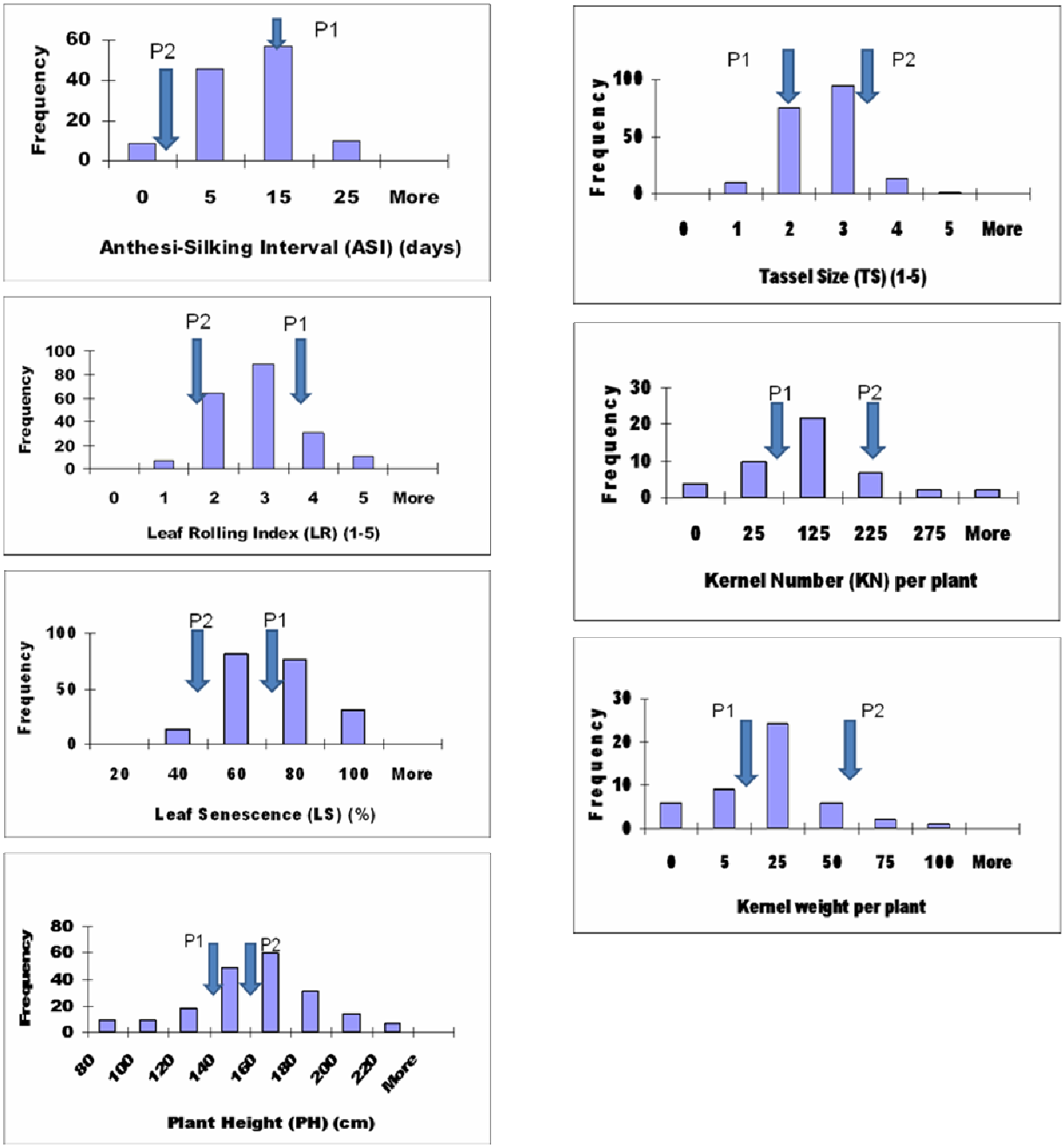

Figure 1: Frequency distribution of the traits measured in the field under water stress at flowering in the F_2 population used in the co-segregation QTL analysis. P_1 = Parent 1; P_2 = Parent 2

4.2 Bulked segregant analysis for markers linked to ASI: In this study, only 27.5% (30 primer pairs) of the 109 screened primer pairs showed polymorphism between the parents and/or the DNA bulks selected for anthesis-silking interval (ASI) (Table 2). Such primer pairs were taken as candidate markers for further analysis e.g. primers *p-umc1358, p-umc2225* among others (Figure 2). The parents used in the study were open pollinated landraces hence the possibility of expressing genetic diversity for the trait among the selected F_2 individuals was high. A lot of polymorphism would therefore be expected. However, the low level of polymorphism realized in this study could be attributed to the use of BSA, when applied to QTL identification; it only detected the loci with large genetic effects (Wang and Patterson, 1994; Grattapaglia, *et al.*, 1996). The results as obtained

here showed that effects attributable to each candidate marker ranged from 25-39%, and their total effect was 65%, supporting the fact that BSA is successful in tagging QTL of very large effects.

Table 2: List of primers showing polymorphisms between parental genotypes and/or F_2 progeny DNA bulks (BSA)

No.	Linkage Group	Primer Name	SSR Primer Sequences (F5'-3'//R5'-3')
1	1	*p-umc1566**	ATCTCGTCTACCTAACCCACCCTC//CAGGTGAAGAATCTGGTGAGGTC
2	1	*p-umc1292**	GAAGTGAATGTGGGAACATGGTTC//TCACGGTTCAGACAGATACAGC TC
3	1	*p-bnlg1124**	TCTTCATCTCTCTCTATCAAACTGACA//TGGCACATCCACAAGAACAT
4	1	*p-bnlg1179**	GCGATTCAGTCCGCAGTAGT//GTACTGAACAAACCGGTGGGC
5	1	*p-bnlg1014**	CACGCTGTTTCAGACAGGAA//CGCCTGTGATTGCACTACAC
6	1	*p-umc2225**	TCGGCTGACATAATAAAACCATAGC//ATGCGAATTTTACCGGGTTTTT
7	1	*p-bnlg1429**	CTCCTCGCAAGGATCTTCAC//AGCACCGTTTCTCGTGAGAT
8	1	*p-umc2226**	TGCTGTGCAGTTCTTGCTTCTTAC//AGCTTCACGCTCTTCTAGACCAAA
9	1	*p-bnlg109**	GCCAGCTGATGTCTGATGAACAGCACA//GATCGGGCCAGATTTCTCAA GTCGTCA
10	1	*p-umc1397**	GTTACACTTGCAGACAAACAACCG//GTCATGATCCGGGAGTAAATCT
11	1	*p-bnlg2180**	ACAAGGGCGTACCAACCAC//TGACCAGAGGCTTCCATACC
12	1	*p-umc1611**	TACTACCAGCAGCTTGCTTCAACA//CTTCTTGTTCTTCACGCAGTTGTC
13	1	*p-umc2234**	GACGGACTATAGAGGGCGATGAG//CAAGATCGTTAGGTTCTAGGCGT C
14	1	*p-umc1358**	AGAACCTCCCGCTTGACGAC//ACCTCAACCTCGACCTCTGCAT
15	1	*p-mmc0041**	AGGACTTAGAGAGGAAACGAA//TTTATCCTTGCAGTTGC
16	1	*p-umc2189**	CGTAAGTACAGTACACCAATGGGC//ACACCGACTACAAGCCTCTCAAC T
17	1	*p-umc1331**	TTATGAACGTGGTCGTGACTATGG//ATATCTGTCCCTCTCCCACCATC
18	2	*p-umc1542**	TAAAGCTATGATGGCACTTGCAGA//CATATTTGCCTTTGCCCTTTTGTA
19	2	*p-umc1824**	ATCGTGCTTAAGCGGTTATAGGAAT//TGCACATGCTTTGTATAAGATG CC
20	2	*p-umc1776**	AAGGCTCGTGGCATACCTGTAGT//GCTGTACGTACGGGTGCAATG
21	2	*p-bnlg2248**	CCACCACATCCGTTACATCA//ACTTTGACACCGGCGAATAC
22	1	*p-umc1922**	CTTTTCCGCTGCTCTTCCTTTT//TTCCCTTGTTCCAGATCCTCAATA
23	1	*p-umc2252**	GTCTTTGACCCCTTCCTCTTCTTG//CACTGCACTGCAAGGTACATACG
24	1	*p-umc2372**	CACCAGGCGTAGTGAGACAGC//ACCCCTTGCGTTCTCTTCTGTT
25	1	*p-mmc0271**	CGTAATGCGTAGCAACATAG//CAACATCCTTTCCACCG
26	1	*p-bnlg2077**	GACCAGAGGATGGGGAAATT//GTAGGCACATGCACATGAGG
27	1	*p-phi435417**	CTGACGCCACTGTTGCTTG//AAAAGTAGCCAATCTGCCACG
28	1	*phi339017**	ACTGCTGTTGGGGTAGGG//GCAGCTTGAGCAGGAAGC
29	2	*phi127**	ATATGCATTGCCTGGAACTGGAAGGA//AATTCAAACACGCCTCCCGAG TGT
30	5	*phi331888**	TTGCGCAAGTTTGTAGCTG//ACTGAACCGCATGCCAAC

Linkage group=chromosome number; *Polymorphic at BSA step

It is however acknowledged that interval mapping detects more QTLs with smaller effects, which could not be possible with BSA due to the possibility of sampling effects in the bulk

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composition (Grattapaglia, *et al.*, 1996; Ribaut *et al.*, 1996). The low proportion of polymorphic markers observed in this study could also be attributed partly to the possibilities of past introgression events or a common genealogy between the parents. KCB is an improved open pollinated cultivar for the dry mid-altitude areas especially in Eastern Province of Kenya whereas GBK032357 is a landrace cultivar from Kwale, a neighbouring coastal district. Both cultivars are adapted to almost similar environments, following selection in similar environments and continued changes resulting from farmer management (Carvalho *et al.* 2002; Perales 2003).

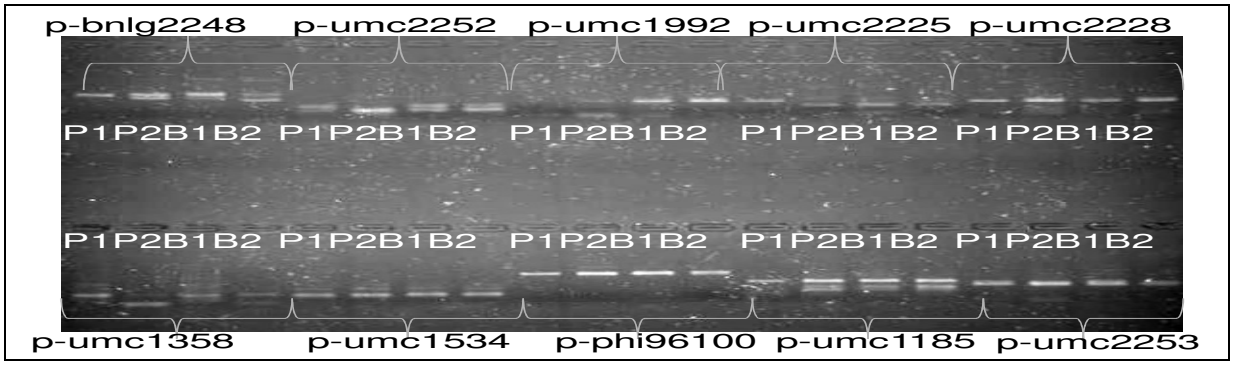

Figure 2: PCR products generated by 10 primers with P_1, P_2, B_1 and B_2 respectively, during bulked segregant analysis. P_1=drought susceptible parent, P_2= drought tolerant parent, B_1= drought susceptible bulk, B_2= drought tolerant bulk.

4.3 ASI candidate QTL analysis: Grattapaglia *et al.* (1996) noted that with a large sample size, bulked segregant analysis (BSA) followed by genotyping of a random sample of individuals would offer a quick and less expensive way for identification of genomic regions controlling quantitative traits of interest. The 30 primers used for confirmation on P_1, P_2 and the 20 F_2 individuals used to constitute the two bulks gave a total of 71 scorable bands (alleles) with band sizes ranging from 70- 350 bp (Figure 3). The results showed that four out of the 71 F tests were significant and these regions have been taken as candidate QTLs conferring the expression of ASI. These were regions near markers *p-umc2189*, *p-umc1542*, *p-bnlg1179* and *p-bnlg1014*.

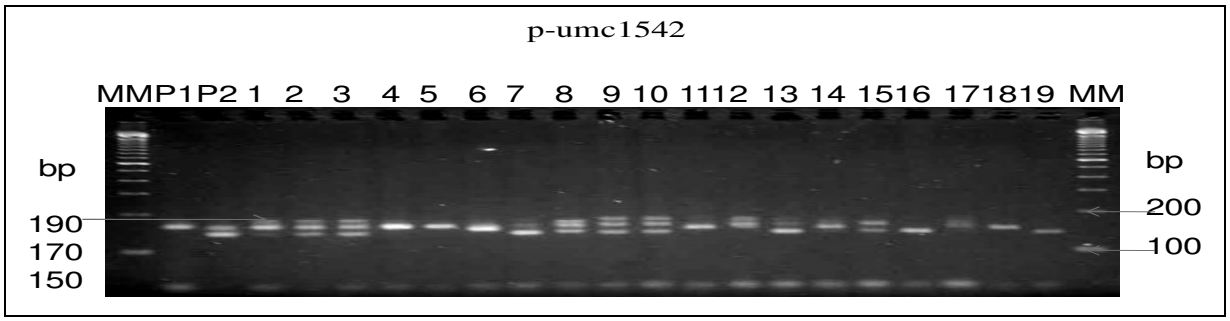

Figure 3: PCR products generated by marker *p-umc1542* on parents, 10 drought tolerant and 9 drought susceptible F_2 individuals separated on MetaPhor agarose (3%).
MM= 100 bp DNA ladder, P_1= parent 1 (drought susceptible), P_2= parent 2 (drought tolerant), 1-10= F_2 plants constituting the drought tolerant bulk, 11-19= F_2 individuals constituting the drought susceptible bulk used in BSA. Right bp= the fragment sizes of the two standard marker bands flanking bands generated by marker p-umc1542. Left bp= the estimated allele sizes of the three scored bands.

Table 3: Candidate QTL data for ASI from F_2 progeny of maize lines KCB x GBK 032357.

Marker	No. of progeny	p-value	Linkage group	Effect	R^2 (%) Single marker	R^2 (%) combined
p-bnlg1179	20	0.0338*	1	0.379163	28.3	
p-bnlg1014	20	0.0101*	1	-3.70469	38.7	
p-umc2189	20	0.0107*	1	-3.52095	34.3	65.3
p-umc1542	20	0.0250*	2	-4.25868	26.2	

** significant at 1% probability level; * significant at 5% probability level; No. of progeny = the number of F_2 individuals used in analysis; R^2 (%) single marker = the total phenotypic variance explained by each marker individually found through linear regression; R^2 (%) = the total phenotypic variance explained by all the markers together found through multiple regression; Effects = the genetic characteristics of the ASI candidate QTLs found through multiple regression- a positive value means that it increases the numeric value of the trait and vice versa.

Table 4: Co-segregation of markers *p-umc1542*, *p-umc2189*, *p-bnlg1179* and *p-bnlg1014* with ASI.

Marker	Trait	No. of progeny	present	absent	RF (%)
p-umc 1542	Short ASI	20	7	3	26
	Long ASI	19	2	7	
p-umc 2189	Short ASI	8	6	2	22
	Long ASI	10	2	8	
p-bnlg 1179	Short ASI	9	6	3	25
	Long ASI	7	1	6	
p-bnlg 1014	Short ASI	7	4	3	18
	Long ASI	9	0	9	

ASI = anthesis-silking interval; Short ASI= drought tolerant; Long ASI = drought susceptible; No. of progeny = number of F_2 individuals with scorable bands; present = the number of individuals per group (tolerant and susceptible) showing the allele of interest; absent = the number of individuals from each group without the allele of interest; RF = recombination frequency.

Table 5: Associations between the ASI candidate markers with female flowering time, kernel number and grain yield.

Trait	Marker	No. of progeny	R^2 (%)	p-value	Linkage group
FFT	*p-umc 2189*	20	26.3	0.029*	1
	p-bnlg 1014	20	31.9	0.023*	1
	combined		39.6	0.014*	1
KN	*p-bnlg 1179*	20	33.2	0.019*	1
	p-umc 1542	20	23.5	0.035*	2
	combined		36.1	0.022*	1&2
GY	*p-bnlg 1179*	20	26.1	0.043*	1
	p-umc 1542	20	33.3	0.009**	2
	combined		40.6	0.012*	1&2

** significant at 1% probability level; * significant at 5% probability level; FFT = female flowering time; KN = kernel number; GY = grain yield; combined = the total effects of the candidate QTLs for each trait found through multiple regression

The entire candidate QTLs together explained 65% of the total observed variation in ASI (Table 3). According to the maize genome data base, these candidate markers can be mapped on chromosomes 1 and 2 (*p-umc2189*, *p-bnlg1179* and *p-bnlg1014* on chromosome 1 and *p-umc1542* on chromosome 2). Flowering parameters have been studied under both well watered and water stress conditions in maize (Ribaut *et al.,* 1996; Li *et al.,* 2003; Guo *et al.,* 2008;) and QTLs for several traits identified. Guo *et al.* (2008) found four QTLs for ASI on chromosomes 3, 6 and 7 under well-watered conditions and two

QTLs on chromosomes 5 and 6 under water stress. He also reported common QTLs for ASI on chromosomes 1, 2, 6, 8 and 10 under both water regimes. Zhang *et al.* (2003) reported two QTLs for ASI on chromosomes 2 and 6 under water stress regime whereas Ribaut *et al.* (1996) reported six QTLs for ASI on chromosomes 1, 2, 5, 6, 8 and 10 under water stress conditions. Since QTLs for ASI have been previously mapped on chromosome 1 by Ribaut *et al.*, (1996) and Guo *et al.*, (2008) and chromosome 2 by Ribaut *et al.*, (1996) and Zhang *et al.*, (2003), the results from the current study seem to confirm the observation by Ribaut *et al.* (1996) that ASI QTLs are relatively consistent in the maize genome. Another important observation from this study is that three out of four identified candidate QTLs are located on linkage group 1 (chromosome 1) (Table 2), thus confirming the observation by Grattapaglia *et al.* (1996) that several of the significant markers associated with trait expression were linked on the same linkage group. The presence of the three candidate QTLs on the same chromosome in this study therefore implies that these markers could be linked and possibly inherited together. They could offer a powerful tool for indirect selection for grain yield in maize improvement under drought conditions

4.4 Co-segregation of the candidate QTLs with ASI in F_2 progeny of KCB x GBK 032357: The population used in this analysis was a subset of the F_2 population derived from the two tails hence a segregation ratio of 1:1 was expected for each marker because the SSRs are co-dominant markers. Any deviations from this in the two subsets (tolerant and susceptible) were treated as recombinants. The candidate QTLs identified from the study i.e. regions near markers *p-umc2189, p-umc1542, p-bnlg1179* and *p-bnlg1014* had 22, 26, 25 and 18%, respectively, recombination frequencies for ASI, (Table 4). Because genetic linkage is based on meiotic recombination, a lower recombination frequency indicates close linkage between the marker and the genomic region conferring the trait. Therefore in this case, crossing –over during meiosis is limited, hence the marker is co-inherited with the trait of interest. Unlike in other QTL studies (Li *et al.,* 2003; Zhang *et al.* 2003; Guo *et al.* 2008), the parents used to develop the F_2 population in this study were landraces therefore the possibility of genetic variability in the population cannot be ruled out. The relatively high recombination frequency observed in the candidate markers could be attributed to this fact. The absence of the marker of interest in some of the individuals constituting the same bulk in this study may not necessarily mean the presence of recombination as treated in this case. This could imply that not all individuals in a bulk population derived their trait of interest from the same loci and especially so for a quantitave trait like ASI. This suggests that the allele shared between both tolerant and susceptible individuals could not have been phenotypically expressed in the susceptible individuals because some other genes necessary for expression could be lacking in the group. This indicates pleiotropic and epistatic interactions among loci responsible for ASI. Grattapaglia *et al.* (1996) noted that multiple alleles as well as multiple QTLs could be expected from a genetically heterogeneous population as compared to homogeneous populations. The presence of candidate markers with relatively close association with ASI regardless of possible heterogeneity of the population as observed in this study agrees with the observation of Ribaut *et al.* (1996) that ASI QTLs are relatively consistent in the maize genome. Only a small percentage of the total scorable markers (5.6%) were found to co-segregate with ASI. Several non-parental alleles that appeared to segregate in the F_2 populations were also observed, a phenomenon that could be attributed to the heterogeneity of the parental population (Michelmore *et al.*, 1991). Because the original parents in this study were landraces (OPVs), the two parents included in the analysis may not necessarily be the real parents of the 20 F_2 individuals analyzed. Landraces are generally heterogeneous hence there could be several alleles in the initial population that conferred a reduced ASI. The fact that only 5.6% of the markers that had appeared polymorphic (Table 2) significantly co-segregated with the trait of interest could imply "false positives" due to sampling (Grattapaglia *et al.,* 1996) However, the results reported in the present study may not be all that conclusive as they are based on a subset of the F_2 population selected from the two tails of the segregation continuum. Map positions of polymorphic loci and the localization of QTLs for the trait cannot be established at this level, and so is the definite determination of allelic effects. These would require genetic analysis and linkage mapping of the data from the whole F_2 population with the selected candidate markers using the available computer programs such as the MAPMAKER and

MAPMAKER/QTL (Ribaut *et al.,* 1996; Grattapaglia *et al.,* 1996; Guo *et al.,* 2008), among others. Mendelian segregation ratios for the candidate markers also need to be established using assays on the whole F_2 population.

4.5 Phenotypic Correlation and Pleiotropism: Two (*p-umc2189* and *p-bnlg1014*) of the four candidate markers associated with ASI that are located on chromosome 1 showed significant association with FFT (Table 5), implying a possible co-localization of QTLs for the two traits (ASI and FFT). The two markers together account for 39 % of the total observed variation. The other two markers, (*p-bnlg1179* and *p-umlc1542*) which are located on chromosome 1 and 2, respectively, also showed significant association with both KN and GY (Table 5). These two accounted for 36.1% of the total observed variation in kernel number (KN) and 40.6% of the observed variation in grain yield (GY). The co-location of QTLs for several traits has been reported in previous QTL studies. Ribaut *et al.* (1996) found four common QTLs between ASI and FFT on chromosomes 1, 2, 5 and 8. Guo *et al.* (2008) also reported co-localization of QTLs for ASI and grain yield (GY) on chromosome 10 while Xiao *et al.* (2005) reported two co-localized QTLs for KN and GY on chromosome 1. The co-location of QTLs for different traits implies the likely presence of pleiotropic or close linkage between the QTLs that control the traits (Lebreton *et al.,* 1995; Agrama and Moussa, 1996; Tuberosa *et al.,* 2002). Paterson *et al.* (1991) suggested that the co-localization of QTLs for several traits is associated with a correlation in the phenotypic data. In this study, the phenotypic correlation between ASI and FFT was positive and highly significant (r= 0.84**). ASI was also significantly and negatively correlated with KN (r= -0.47**) and GY (r= -0.45**) thus verifying the expected association between the level of phenotypic correlation and the linkage of QTLs. Although pleiotropic gene action may be the most likely explanation for the potential co-localization of these candidate QTLs, the current data are insufficient to establish, with certainty, the presence of pleiotropic effects. This is more so considering the fact that ASI is calculated as the difference between FFT and MFT data thus making it possible to correlate with FFT and/or MFT. The data used in this study was based on a subset of the entire F_2 population, which may necessitate further confirmation of these associations on the entire F_2 population. Furthermore, these observations were made by regressing data from markers found by pooling DNA based on ASI phenotypic data, on the phenotypic data of the other measured traits such as FFT, KN and GY. Therefore these results are just indicative of the possibility of co-localization of genomic regions responsible for several traits. The presence of two common candidate QTLs for ASI and both KN and GY in this study, coupled with the high negative correlation between ASI and GY and other phenotypic yield components suggest that GY can be improved by using ASI for indirect selection . These results also confirm the genetic basis of the phenotypic findings by Andrade *et al.* (1999) and Xiao *et al.* (2005) that EPP and KN directly influence the GY; GY in turn is more correlated with KN than EPP thereby demonstrating the fact that KN is the most important yield component for maize than EPP and 100 Kernel weight.

5 CONCLUSION

Understanding the genetic basis of drought tolerance in maize is fundamental to enable the breeder to develop new methods of selection. It is generally recognized that complex traits can be dissected as Mendelian factors with molecular markers. It is therefore necessary to identify the QTLs for yield components and morphological traits in more populations, different locations and under different stress regimes. In this study, only a few candidate QTLs with sharp effects were detected possibly because of the BSA procedure, which has been shown to identify only QTLs with large genetic effects. A clustering of QTLs for drought related traits in specific chromosomal regions was also apparent. Three of the four candidate QTLs for ASI are located on chromosome 1. A more common goal of quantitative trait mapping is the use of QTL in marker-assisted selection (MAS) to transfer genomic regions associated with drought tolerance from a tolerant source to an elite but drought-susceptible genotype, thus augmenting and enhancing the outcomes of phenotypic selection. These candidate QTLs have potential use in molecular MAS with the expectation that shortening the ASI using MAS should result in improvements that will render tolerance to drought stress.

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