Stay-green QTLs in temperate elite maize

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Abstract

The ability to stay-green (SG) in later stages of the crop cycle is a valuable trait for plants cultivated in standard or stressful environments. Few QTLs studies for SG have been conducted in temperate maize, apart from some experiments carried out with Chinese lines. The aim of our study was to identify QTLs related to SG in temperate maize using PHG39, an important SG line in private breeding. We developed two large F_2 mapping populations by crossing PHG39 to the no stay-green (NSG) lines B73 (Corn Belt Dent) and EA1070 (European Flint). Samples of individuals of the extreme tails (high and low) of the populations for visual score were genotyped. We found an association between markers and SG in three regions at bins 1.04-1.09, 5.02 and 10.04-10.06. The association was strong for some markers in chromosome 1, for example, for *bnlg1556* the frequency of the SG allele was 0.75 and 0.34 in the high and the low tail, respectively. Furthermore, for this marker the homozygotes with the SG allele had 4 times more chlorophyll than the homozygotes with the NSG allele two months after flowering. Some alleles most likely conferred SG because they increased the maximum chlorophyll content at flowering while other alleles did by diminishing the rate of senescence. The SG conferred by some alleles could be functional as

some favourable alleles for SG were also favourable for kernel weight. Regardless of the physiological basis of the SG, the significant markers detected could be useful for marker assisted selection.

Key words: chlorophyll content; fluorescence; photosynthesis; senescence; *Zea mays*

Introduction

Stay green (SG) is a general term used for a genotype with an heritable foliar delayed senescence compared to a reference genotype (Thomas and Howarth 2000). The term is also used for the trait itself. SG is considered a valuable trait for plants cultivated in standard environmental conditions, and also for plants facing drought and nitrogen limitation. Thus, numerous studies have associated SG and its genetic loci with responses to drought in sorghum (Thomas and Ougham 2014). Furthermore, the generic physiological mechanisms underlying the SG trait suggest that similar SG QTLs could enhance drought adaptation in other major cereals as maize (Borrell et al 2014). SG can be cosmetic or functional. In cosmetic SG phenotypes, only the green colour is maintained while plants have lost their photosynthetic capacity. A functional SG refers to a phenotype which stays green and keeps up with its capacity of photosynthetic activity. Diverse ways for plants to SG have been described (Thomas and Smart 1993; Thomas and Howarth 2000): a delay in the start of senescence (Type A), a slow rate of senescence (Type B), a lesion in pigment breakdown (Type C), killing the leaf –frozen, boiling, etc- (Type D) and a high concentration of chlorophyll at flowering (Type E).

Studies carried out to identify SG related QTLs have used either tropical (Câmara 2007; Belícuas et al. 2014) or temperate maize (Beavis et al. 1994; Zheng et al. 2009; Wang et al.

2012). Beavis et al. (1994) used a mapping population derived from two lines that are not stay-green, which limits the possibility of detection of relevant QTLs for this trait. On the other hand, Zheng et al. (2009) and Wang et al. (2012) used a mapping population derived from the cross of a SG to a non stay-green (NSG) line. Wang et al. (2012) used a SG line (A150-3-2) of unknown pedigree which was bred in their laboratory, while Zheng et al. (2009) used the SG line Qi-319 derived from the Pioneer hybrid "P78599". The lines derived from this hybrid do not belong to the main heterotic groups found in temperate areas: Lancaster, Reid (Iowa Stiff Stalk Synthetic or Iodent), and European Flint in America and Europe, and Tangsipingtou and Ludahonggu in China (Yu et al. 2007). Mikel and Dudley (2006) surveyed the lineage of corn inbred lines protected by US Patent or the US Plant Variety Protection (PVP) Act from 1980 and 2004 and found that much of the modern temperate germplasm derived from only seven inbred lines. One of the seven lines is PHG39, a Corn Belt line of Reid origin which was developed by Pioneer Hi-Bred International (PH) from the public inbreds B37, B14, B96, I205, and IDT. The importance of PHG39 in modern germplasm was also emphasized by Mikel (2008) who analyzed the diversity among 47 widely grown hybrids (24 from Holden's Foundation Seeds and 23 from PH) and found that 18 of the 23 hybrids from PH had a common PHG39 derivative parent. Mikel (2011), analyzing the genetic contribution of progenitors to 1132 corn inbreds registered from 1984 to 2008, found that PHG39 contributed to 2–4% of the lines. This line presents an excellent SG when compared to other standard lines as, for example B73, according to our observations and the PVP certificate (PVP number 8300115). Furthermore, several lines derived from PHG39 also present the SG characteristic, for example, PHW52 (PVP 8800215), PHEG9 (PVP 9400090), PHHB9 (PVP 9300108), PHP38 (PVP 9000250), PHT47 (PVP 9200091) and PHPR5 (PVP 9200088), suggesting that the lines inherited a favourable allele (or alleles) for SG from PHG39.

74 The aim of our study was to identify QTLs related to SG in temperate maize using the
75 PHG39 lite line
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Materials and Methods

Two F_2 populations were used for mapping QTLs: (PHG39×EA1070)F_2 (Pop1) and (PHG39×B73)F_2 (Pop2) derived from a cross between a SG line (PHG39) and a NSG line (EA1070 or B73). EA1070 is a European Flint line derived from the Spanish landrace, Hembrilla de Novillas (Aragon, Northeast of Spain), and B73 is a Corn Belt Dent line derived from Iowa Stiff Stalk Synthetic. The number of individual F_2 plants was 544 in Pop1 and 2500 in Pop2. The field trials were conducted on a sandy loam soil at the Misión Biológica de Galicia (MBG) research station (Pontevedra-Spain) in 2012 (Pop1) and 2013 (Pop2). The plant density of both trials was 60,000 plants/ha (0.8×0.21 m). In Pop2 the parents were repeated at regular intervals and used as checks. Applications of 300 kg/ha of mineral fertilizer (22-8-10) and 82 units of N using Nitramon® (27% N and 3.5% magnesium oxide) were done. To control weeds, 350 L of herbicide (with 1.5 L of Camix® each) were applied twice.

The SG expression of individual plants was estimated two months after flowering with a visual score in a scale from 1 (dead leaves) to 5 (completely healthy leaves). A measurement of the chlorophyll content was made with a chlorophyll content meter (CCM200 Opti-Sciences, USA) for every single plant at flowering (Chlo1) and one (Chlo2) and two (Chlo3) months after flowering. The flowering data of each individual plant was taken and the dates of chlorophyll content measurement were chosen for each plant according to its flowering time. For each plant, two measurements were done, one on each side of the ear leaf midrib at 10 cm from the ligule.

The photosystem II maximum quantum yield (F_V/F_M) was measured with a chlorophyll fluorometer (OS-30p, Opti Sciences Inc., USA) in dark-adapted leaves of plants in Pop2 in the same leaf where chlorophyll content was taken. A first measurement (F_v/F_{M1}) was done on

five random plants per row one month after flowering when all plants were green and no symptoms of senescence were observed. A second measurement (F_v/F_{M2}) was done two months after flowering in all plants that stayed green. Similarly to chlorophyll content, the dates of F_V/F_M measurement were chosen for each individual plant according to its flowering time. At grain maturity, ears were harvested, threshed, dried in a stove for one week at 80 °C and then weighted.

Leaf samples for genotyping were collected 3 weeks after plant emergence, then lyophilized (Christ® Beta 2.8 Lo plus) and conserved in a freezer at -80 °C. After the visual scoring, individuals of the high and low tails of the F_2 visual score distributions were selected for DNA extraction and genotyping. DNA extraction followed, with some modifications, the procedure described by Lui and Whittier (1994). DNA purification followed the phenol/chloroform/isoamyl protocol described by Moore and Dowhan (2002). PCRs were carried out in a Bio-Rad MyCycler Thermal Cycler (**BioRad** Laboratories, Hercules, CA). PCR fragments were separated in an acrylamide gel (6% acrylamide/bis-acrylamide 19:1) in the presence of ethidium bromide. Gel images were digitalized with a system VisiDoc-it™ "Imaging sYstem 6.4" LCD and Stand UVP lamp (Upland, Ca, USA) and analyzed using Quantity One 4.6.6 (BioRad Laboratories, Hercules, CA).

279 Simple Sequence Repeats (SSRs) were analysed to asses variability in the parental lines of the F_2 population, resulting in 218 polymorphic markers randomly distributed along the maize genome which were used to genotype the selected F_2 samples.

The high number of individuals of Pop2 has a favourable effect on the precision of QTL location by increasing the number of recombinants, but a negative effect in the precision of phenotypic data as more spatial heterogeneity is expected in a larger trial. A spatial analysis

was done to adjust the phenotypic data for localized effects within the trial following the method described by Zas (2006).

Extreme phenotypes for visual score were used in the QTL analysis to increase the power of detection because much of the linkage information resides in individuals with extreme phenotypes (Lynch and Walsh 1998) The number of individual selected in the high and low tail of Pop1 were respectively 24 and 36, while the number of individual selected in the high and low tail of Pop2 were77 and 280 respectively. We assumed that the distribution of the allele number in the high tail of the F_2 distribution followed a binomial distribution with 0.5 probability. At each marker and for the allele at highest frequency, we determined the binomial probabilities of all possible samples with equal or higher frequency than the observed. The accumulated probability was calculated and the null hypothesis (the marker is not associated to the trait) was rejected when this probability was lower than experimentwise threshold of 0.05, after Bonferroni correction (Weir 1996). The shift of allele frequency from the expected 0.5 in the whole F_2 population due to unknown factors (segregation distortion) can generate false positives in our analysis. To avoid this type of error we calculated the allele frequencies in the low tail for the significant markers in the high tail and those markers having high frequency of the same allele in both tails were not considered associated to the trait. For the regions where SG QTLs were detected a maize-sorghum synteny analysis was made using SyMAP (Soderlund et al. 2006).

For markers with a significant frequency of one allele in the high tail and a consistent frequency of the alternative allele in the low tail (higher than 0.5), the genotypic means for both homozygotes and for the heterozygote were calculated for the visual score trait using the individuals of the two tails jointly. The genotypic means for the homozygotes and the heterozygote were also calculated for Chlo1, Chlo2, Chlo3, F_V/F_{M1}, F_V/F_{M2} and kernel weight. Assuming a completely randomized design with a single factor (marker effect) and three

149 levels (the different genotypes: AA, AB and BB), an analysis of variance (ANOVA) was
150 carried out for each marker at a significance level $\alpha=0.05$. The proportion of phenotypic
151 variance explained (PVE) by a marker was estimated as the sum of squares (SS) associated to
152 marker effect in the ANOVA over the total SS. The analyses were carried out with Minitab®
153 (Minitab 17.1.0).

154

155 **Results**

156 In the F_2 distribution of Pop1, 300 plants had visual score values of 3 or 4, while 100 plants
157 had visual scores in the remaining values of the scale (Figure 1a). This distribution is different
158 to that of Pop2 where most of the individuals were concentrated in visual score 1 (Figure 1b).
159 The remaining scores in Pop2 followed a relatively symmetric distribution centered in visual
160 score 3. PHG39 had an average value close the medium value of the visual scale (3), while
161 B73 had a lower value. For Pop1, the score value had a moderate correlation with Chlo3
162 (r=0.59) and no correlation with kernel weight (Table 1). The kernel weight had a low, but
163 significant correlation with the three measurements of chlorophyll (r=0.14-0.20). For Pop2,
164 the correlation of score values with Chlo3 was 0.57, similar to the correlation in Pop1,
165 however the correlation of score value with kernel weight was significant in contrast to Pop1.
166 Kernel weight had also a significant correlation with the chlorophyll measurements in Pop2,
167 although the values were slightly higher than in Pop1. While no correlation existed between
168 F_v/F_{M1} and the other traits, F_v/F_{M2} was correlated to Chlo3 (r=0.54) and the score values
169 (r=0.62).

170 For Pop1, 10 markers had a significantly higher frequency of the allele derived from
171 PHG39 than the allele derived from EA1070 in the high tail of the distribution (Table 2). For
172 those markers, the frequency of the PHG39 allele in the high tail varied from 0.74 to 1.00.
173 The markers with highest frequency were *phi101* in which the PHG39 allele was fixed in the
174 high tail (bin 5.06, frequency=1.00, $p=1.46\times10^{-11}$) and *umc2029* (bin 1.08, frequency=0.92,
175 $p=6.53\times10^{-11}$) in which only homozygotes for the PHG39 allele and heterozygotes were found
176 in the high tail. Nine of the significant markers were located between bins 1.04-1.09 and two
177 of the markers in bins 5.02and 5.06. For Pop2, 3 markers had a significantly higher frequency
178 of the allele derived from PHG39 in the high tail of the distribution (Table 2). All the three
179 markers were located in chromosome 10 at bins 10.04 and 10.06 and had frequencies of the

PHG39 allele between 0.69 and 0.76. Therefore, for all significant markers the PHG39 allele was at higher frequency than the alternative allele (EA1070 in Pop1 and B73 in Pop2) in the high tail of the distribution and we will refer to the PHG39 allele as stay-green (SG) and the alternative alleles as no stay-green (NSG).

From the 10 markers with a significantly higher frequency of the SG allele in the high tail of the distribution in Pop1, 9 had a higher frequency of the NSG in the low tail (Table 2). Furthermore, for 6 of those 9 markers (*umc1144*, *umc1281*, *bnlg1556, phi037, umc2029* and *bnlg1660*) the binomial probability of sampling an allele frequency higher than the observed was lower than the threshold value of 0.05 (after Bonferroni correction) in the low tail. For Pop2, from the 3 markers with significantly higher frequency of the SG allele in the high tail of the distribution, *umc1077* (bin10.04) and *bnlg1028* (bin 10.06) had a higher frequency of the NSG allele in the low tail of the distribution. For *bnlg1028* the probability of sampling by chance a frequency of the NSG allele higher than the observed was lower than the threshold value of 0.05 (after Bonferroni correction) in the low tail of the distribution.

In Pop1, the visual score mean of the homozygotes with the SG allele varied between 3.0 and 4.5 across the significant markers, while the mean of the homozygotes with the NSG allele varied between 1.0 and 1.5 (Table 3). The visual scores of heterozygotes (between 1.4 and 3.5) for the markers located at chromosome 1 were intermediate to the scores of the homozygotes, although closer to the value of homozygotes with no NSG alleles. However, for *bnlg1660* located at bin 5.02 the heterozygote had a visual score mean higher than the homozygote with the SG allele, although the difference was not significant. The proportion of phenotypic variance (PVE) explained for visual score was high for most of the markers, particularly *bnlg1556* and *umc2029* (about 40 %).

For most of the significant markers located in chromosome 1 the chlorophyll content was similar at flowering and decreased at a similar rate during the first month in the SG and NSG homozygotes. However the NSG homozygotes had a sharp decrease in chlorophyll content compared to the SG homozygotes during the second month (Figure 2) which resulted in significantly lower chlorophyll content two months after flowering (Table 3). For the significant marker *bnlg1660* (5.02) the rate of chlorophyll decay during the first and second month was similar in the SG and NSG homozygotes (Figure 3). However, the homozygote with the SG allele had higher chlorophyll content at flowering than the NSG homozygotes (50 vs 40, approximately). Thus, the SG homozygote still had relatively high chlorophyll content two months after flowering, although the differences between genotypes were not significant (Table 3). For Chlo3, the proportion of phenotypic variance explained for the markers varied, approximately, between 20 and 40%, for most of the markers in Pop1, except *bnlg1556* which explained almost 60 % of the phenotypic variance. For Chlo3 the observed distribution of the homozygotes for the SG allele at *bnlg1556* did not overlap with the distribution of the homozygotes for the NSG allele, except for one homozygote (Fig 4).

In Pop1, for *umc1281* the SG homozygote had higher kernel weight than the NSG homozygote, while the kernel weight of the heterozygote was between the two homozygotes. For the remaining markers with significant allele frequencies in Pop1 there were not differences between genotypes for kernel weight. The marker *umc2083* had a high frequency (0.69) of the PHG39 allele in the high tail, although below the 0.05 threshold level ($P=3.9\times10^{-3}$). For this marker the homozygote with the PHG39 allele had significantly higher kernel weight than the homozygote with the alternative allele, while the heterozygote had higher value, although no significant different, than the best homozygote (data not shown).

In Pop2 the visual score means of the homozygotes with the SG allele were 4.3 and 3.5 for *bnlg1028* and *umc1077*, respectively, while the visual score means of the homozygotes with

228 the NSG allele were respectively 3.2 and 2.1 (Table 3). The visual scores of the heterozygotes
229 were intermediate between both homozygotes although closer to the SG homozygote. For
230 *blng1028* the differences between genotypes were not significant for any trait, but for
231 *umc1077* the SG homozygote had higher Chlo3 than the NSG. For *umc1077*, similarly to
232 *bnlg1660*, the chlorophyll content was higher in the SG homozygote at flowering, although
233 the decay in chlorophyll content after flowering was similar in both homozygotes (Figure 3).
234 For *umc1077* there were significant differences between genotypes for kernel weight: the
235 heterozygote had higher value than the NSG homozygote. For the two significant markers, the
236 average value of F_V/F_M one month after flowering (F_V/F_{M1}) was 0.8 for the two homozygotes
237 and for the heterozygote. The value of F_V/F_M two months after flowering (F_V/F_{M2}) was greatly
238 reduced, although the reduction was larger for the NSG homozygotes (data not shown). Thus,
239 F_V/F_{M2} was approximately twice in the SG homozygotes than in the NSG (0.26 vs 0.13 in
240 *umc1077* and 0.54 vs 0.30 in *blng1028*), although the differences were not statistically
241 significant.

242

243 **Discussion**

244 The distribution of visual score 2 months after flowering in Pop2 was very similar to the
245 distribution of the green leaf area measured at ripening stage found by Wang et al. (2012) and
246 two months after flowering found by Zheng et al. (2009). However, in Pop1 most individuals
247 of the F_2 population were concentrated at higher scores as in the distribution of Wang et al.
248 (2012) and Zheng et al. (2009) when the green leaf area was measured 1 month after
249 flowering. The environmental conditions during the crop season can determine the rate of
250 senescence, and some particular conditions not determined in our experiment could have
251 favoured a slow senescence in Pop1.

We found a low, but significant, correlation between kernel weight and chlorophyll content two months after flowering. A similar correlation have been found by other authors (Beavis et al. 1994; Zheng et al. 2009) confirming that SG can be regarded as a desirable trait for crop production. Ma and Dwyer (1998) also found that low chlorophyll contents during the maize grain-filling period may lead to considerable reductions in the dry weight. An important amount of the seasonal dry matter is fixed during grain filling period (Lee and Tollenaar 2007) and the elongation of the period with the photosynthetic apparatus active will suppose more accumulation of dry matter. Other authors (Badu-Apraku et al. 2012; Worku et al. 2012) found higher correlation between senescence and grain yield in soils with low N indicating that the SG is particularly useful under those conditions. There was a moderate correlation between chlorophyll content and photosystem II maximum efficiency two months after flowering indicating that, at least partially, the SG is functional, i.e. not only some plants are greener at latest stages, but also their photosynthetic machinery is working more efficiently. We found also a moderate correlation between visual score and two photosynthesis related traits: chlorophyll content and F_v/F_M. The visual score allows a quick and cheap screening of large number of genotypes that could be useful in breeding for photosynthetic traits.

We considered a marker associated to SG and one of its alleles favourable for the trait when its frequency is higher than the alternative allele in the high tail of the F_2 distribution and lower than the alternative allele in the low tail. We tested the significance using the binomial as the null distribution. Using this approach we detected 3 regions associated to SG in chromosomes 1, 5 and 10. In all regions the favourable allele derived from the SG line PHG39. Three of the 8 QTLs found by Beavis et al. (1994) were located on chromosomes 1 and 2. Câmara (2007) mapped 23 QTLs on chromosomes 1, 2 and 5. Zheng et al. (2009) and Wang et al. (2012) found each 14 QTLs located mainly on chromosomes 1, 2, 4 and 5, while Belícuas et al. (2014) mapped 9 out of 17 QTLs on chromosomes 1 (4 QTLs) and 2 (5 QTLs).

277 Belícuas et al. (2014), based on results from previous experiments and their own results,
278 suggested that the QTLs underlying stay green traits are not evenly distributed on the genome,
279 but clustered in few chromosomes, particularly on chromosomes 1, 2 and 5. Our results
280 confirm the hypothesis of Belícuas et al. (2014) since we only detected QTLs in three
281 chromosomes and two of them (1 and 5) match with the clusters reported by those authors.

282 The cluster in chromosome 1 does not span over the whole chromosome, but is concentrated
283 around the region 1.06-1.08 where we and other authors (Zheng et al. 2009; Wang et al. 2012;
284 Belícuas et al. 2014) detected the strongest association between markers and SG traits. Thus,
285 for *umc1281*, *bnlg1556*, *phi037* and *umc2029,* located in bins 1.06-1.08, the allele frequencies
286 in the high and low tails of the distribution were both significant. It stood out *umc2029* with a
287 frequency of the PHG39 allele of 0.92 in the high tail as the probability of obtaining such
288 frequency or higher by chance is very low (6.5×10^{-11}). The frequencies were less extreme in
289 the low tail (a maximum value of 0.66) than in the high tail (maximum value of 0.92)
290 probably due to the inclusion of some SG genotypes in the low tail when a biotic or abiotic
291 stress reduced their cycle length. Anyway, the frequencies detected for some markers in the
292 low tail were beyond what would be expected only by chance. The QTLs that we detected in
293 bin 10.04-10.06, although not consistently found in several experiments, were in the same
294 region than QTLs found by Messmer et al. (2011) for leaf chlorophyll, plant senescence and
295 root capacitance. High chlorophyll fluorescence genes were also located in bin 10.06 as well
296 as genes related to plant colour and ADP-glucose pyrophosphorylase which is related to the
297 photosynthetic activity (Smidansky et al. 2007).

298 The marker *phi101* which was polymorphic for the parents of Pop1 was fixed for the
299 PHG39 allele in the two tails of the F_2 distribution indicating that the marker presented
300 segregation distortion. The segregation distortion can be caused by different factors, including
301 gametophytic factors and natural selection. Lu et al. (2002) found 18 chromosomal regions on

302 the 10 maize chromosomes associated with segregation distortion which attests the
303 importance of assessing the frequencies of alleles in both tails in selective genotyping before
304 concluding whether or not a marker is associated to a trait.

305 The choice of the tails of the distribution for selective genotyping was based on visual
306 rating but not on chlorophyll content two months after flowering (Chlo3) which was
307 objectively measured in a single leaf by a chlorophyll content meter. The markers *umc1281*,
308 *bnlg1556*, *phi037* and *umc2029* in chromosome 1 which had a significant association with
309 visual rating had also a significant association with Chlo3. The association between those
310 markers and Chlo3 was revealed by large differences between homozygotes (about 20-30 vs
311 7) and a large proportion of phenotypic variance explained by the markers. The agreement
312 between the results obtained with both traits was expected as the visual rating was done based
313 on colour which depends mainly on the chlorophyll content.

314 For *umc1281*, *bnlg1556*, *phi037* and *umc2029* in Pop1 the chlorophyll content at flowering
315 was similar in the SG and NSG homozygotes, but the rate of chlorophyll decay was sharper in
316 the NSG homozygotes. Therefore, it seems that the favourable allele contributes to SG by
317 initiating the senescence on schedule, but subsequently proceeding more slowly which
318 corresponds to Type B of SG as defined by Thomas and Howarth (2000). On the other hand,
319 *bnlg1660* (bin 5.02) and *umc1077* (bin 10.04) in Pop1 and Pop2 respectively, contribute to the
320 SG by increasing the chlorophyll content at flowering, although they have no effect on the
321 rate of decay of the chlorophyll. This SG corresponds to the Type E of Thomas and Howarth
322 (2000). The alleles that confer the Type B and E of SG can be combined in one genotype to
323 obtain a strong expression of the SG trait.

324 One of the markers in region 1.06-1.08, *bnlg1556*, explained about 60% of the phenotypic
325 variance for Chlo3 and for this trait the distribution of both homozygotes did not overlap
326 except for one individual as if *bnlg1556* behaves as a Mendelian gene. Apart from the bias

caused by genotyping only the tails of the distribution, the marker seems to be linked to a gene of considerable effect on SG. In the same region, the effects associated to markers ranged from moderate in temperate germplasm (Zheng et al. 2009; Wang et al. 2012) to large in tropical germplasm (Belícuas et al. 2014). There could be allelic variation in a gene located in that region and, depending on the particular allele that is carried by the genotype the expression of the character can be different. In addition, the region where this QTL is located, 222-244 Mb of chromosome 1, present a strong synteny with a region of chromosome 1 of sorghum (16-32 Mb) where a stay-green QTL was consistently found across genetic backgrounds and environments (Reddy et al. 2014). The QTL detected could be used to improve the efficiency of selection methods for SG. The SG is measured after flowering and pollen control is not possible which reduces the efficiency of selection. Conversely, marker assisted selection can be made before flowering, increasing the selection efficiency, and saving field space and labour; furthermore, marker assisted selection can be made outside the target environment.

The QTLs detected in Pop1 were not detected in Pop2 and vice versa. The mapping populations differed both in the NSG parental line and in the environment where they were evaluated. In a multi-environment mixed model analysis, Malosetti et al. (2008) distinguished between QTLs with consistent effects across environments and QTLs whose effects are environment-specific. Those authors did not find in maize QTLs for complex traits, as grain yield, with consistent effects across environments. Other authors also found an important QTL×environment interaction in maize (Moreau et al. 2004; Boer et al. 2007). On the other hand, several studies have reported a poor QTL congruency in different biparental mapping populations evaluated in similar environments indicating that the genetic background has a relevant role in QTL detection (Beavis et al. 1991; Lu et al. 2002; Mihaljevic et al. 2004; Li et al. 2009). The specificity with respect to the environment and the genetic background of the

352 QTLs detected in our study needed further research which would facilitate their use in
353 breeding.

354 The maintenance of chlorophyll content and the green colour does not guarantee the
355 photosynthetic capacity as in some genotypes the senescence is proceeding normally beneath
356 the cosmetic surface of retained pigmentation (Thomas and Howarth, 2000). We checked, in
357 Pop2, if the SG allele that conferred better visual rating also conferred better physiological
358 function measured as photosystem II maximal quantum yield (F_v/F_M). For the two markers
359 associated to SG in Pop2, the values of F_v/F_M two months after flowering for the SG
360 homozygotes were approximately twice the values of the NSG homozygotes, although the
361 differences were not significant.

362 We found a relationship between SG and kernel weight at phenotypic level that we
363 checked at molecular level by seeking if the significant markers for SG have also an effect on
364 kernel weight. We found that for *umc1281* (bin 1.06), one of the significant markers for SG
365 green, the SG homozygotes had three times more kernel weight than the NSG homozygotes,
366 indicating that a QTL for kernel weight is co-localized with the QTL for SG. A gene involved
367 in SG in the genomic region of the detected SG QTL could have a pleiotropic effect on kernel
368 weight. Alternatively, in the region there could be linked genes affecting both traits.
369 Independently of the genetic explanation of the association of the marker with the two traits,
370 *umc1281* could be used in molecular breeding to improve SG without a negative effect on
371 grain yield. Zheng et al. (2009) also found QTLs for grain yield overlapping to those of SG.

372 As conclusion, we obtained strong evidence of association between markers and SG which
373 allowed the identification of genomic regions responsible for SG of PHG39, a line of great
374 relevance in private breeding. Some alleles probably conferred SG because they increased the
375 maximum chlorophyll content at flowering while other alleles because they diminished the

rate of senescence. The SG conferred by some alleles could be functional which is consistent with the fact that some favourable alleles for SG were also favourable for kernel weight. However, further evidence is needed to support this hypothesis. Regardless of the physiological basis of the SG, the significant markers detected in this study could be useful for marker assisted selection.

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485 **Table 1** Correlation coefficients between visual score (a scale from 1=dead leaves

486 to 5=completely healthy leaves), chlorophyll content at flowering (Chlo1), 1 month after

487 flowering (Chlo2) and 2 months after flowering (Chlo3), kernel weight (g) and F_v/F_M 1 month

488 after flowering (F_v/F_{M1}) and 2 months after flowering (F_v/F_{M2}) in (PGH39×EA1070)F_2 (Pop1)

489 and (PGH39×B73)F_2 (Pop2).

	Visual score	Chlo1	Chlo2	Chlo3	Kernel weight	F_v/F_{M1}
Pop1						
Chlo1	0.08					
Chlo2	0.07	0.53**				
Chlo3	0.59**	0.32**	0.29**			
Kernel weight	0.05	0.17**	0.20**	0.14*		
Pop2						
Chlo1	-0.01					
Chlo2	0.10**	0.62**				
Chlo3	0.57**	0.09**	0.176**			
Kernel weight	0.27**	0.35**	0.44**	0.21**		
F_v/F_{M1}	-0.01	0.15	0.07	-0.11	-0.02	
F_v/F_{M2}	0.62**	-0.00	0.11**	0.54**	0.20**	-0.05

490 *, ** significant at 5 and 1 % of probability, respectively.

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Table 2 Observed allele frequency and expected probability of equal or higher allele frequency than the observed in a sample of individuals from the high tail and a sample of individuals from the low tail of the F_2 distribution for visual score[a] in (PGH39×EA1070)F_2 (Pop1) and (PGH39×B73)F_2 (Pop2). Only markers with probability in the high tail below the experiment wise threshold of 0.05 are shown (comparison wise threshold of 6.02×10^{-4} and 6.85×10^{-4} in Pop1 and Pop2, respectively).

Marker	Bin	Origin of allele at highest frequency	Frequency	Probability	Origin of allele at highest frequency	Frequency	Probability
		High tail			Low tail		
Pop1							
umc1144	1.04	PHG39	0.80	5.30×10^{-5}	EA1070	0.61	8.55×10^{-4}*
umc1396	1.06	PHG39	0.74	4.68×10^{-4}	EA1070	0.58	4.89×10^{-2}
umc1281	1.06	PHG39	0.75	2.05×10^{-4}	EA1070	0.64	8.55×10^{-4}*
bnlg1556	1.07	PHG39	0.75	3.59×10^{-4}	EA1070	0.66	2.67×10^{-4}*
umc1147	1.07	PHG39	0.83	1.02×10^{-6}	EA1070	0.55	1.54×10^{-1}
phi037	1.08	PHG39	0.79	1.79×10^{-5}	EA1070	0.60	1.46×10^{-2}*
umc2029	1.08	PHG39	0.92	6.53×10^{-11}	EA1070	0.60	1.46×10^{-2}*
umc1512	1.09	PHG39	0.82	2.81×10^{-6}	EA1070	0.53	2.68×10^{-1}
bnlg1660	5.02	PHG39	0.84	1.22×10^{-5}	EA1070	0.62	1.05×10^{-2}*
phi101	5.06	PHG39	1.00	1.46×10^{-11}	PHG39	1.00	5.82×10^{-11}*
Pop2							
umc1077	10.04	PHG39	0.76	7.92×10^{-7}	B73	0.55	1.09×10^{-1}
umc1045	10.06	PHG39	0.70	1.33×10^{-4}	PHG39	0.51	4.31×10^{-1}
bnlg1028	10.06	PHG39	0.69	3.14×10^{-4}	B73	0.65	1.37×10^{-2}*

[a] Visual score was recorded using a scale from 1 (dead leaves) to 5 (completely healthy leaves)

* Probability in the low tail below the experiment wise threshold of 0.05 (comparison wise threshold of 4.55×10^{-2} and 1.67×10^{-2} in Pop1 and Pop2, respectively)

501 **Table 3** Means[a] of homozygotes and heterozygotes for visual score[b], chlorophyll content and kernel weight for those markers with significant

502 frequency in the high tail of the F_2 distribution for (PGH39×EA1070)F_2 (Pop1) and (PGH39×B73)F_2 (Pop2).

Marker	Bin	Visual Score				Chlo1				Chlo3								Kernel weight (g)			
		A[c]	AB	B	PVE[d] %	A	AB	B	PVE %	A	AB	B	PVE %	A	AB	B	PVE %	A	AB	B	PVE %
Pop 1																					
umc1144	1.04	4.5[a]	2.3[ab]	1.5[b]	33.8	44.6[a]	49.3[a]	45.9[a]	4.3	34.3[a]	39.5[a]	39.5[a]	5.2	24.6[a]	13.6[ab]	8.1[b]	19.3	48.7[a]	53.1[a]	38.3[a]	3.0
umc1396	1.06	3.0[a]	1.7[b]	1.4[b]	14.1	48.7[a]	45.6[a]	48.9[a]	2.0	36.8[a]	37.7[a]	35.1[a]	1.1	25.3[a]	13.1[b]	9.4[b]	23.4	46.9[a]	46.3[a]	55.0[a]	0.8
umc1281	1.06	3.6[a]	2.0[b]	1.1[b]	23.5	45.7[a]	46.1[a]	49.9[a]	2.5	36.2[a]	38.0[a]	33.7[a]	3.0	28.8[a]	15.6[b]	6.6[c]	33.2	63.1[a]	48.6[ab]	21.2[b]	21.2
bnlg1556	1.07	4.4[a]	1.7[b]	1.3[b]	39.4	50.5[a]	44.6[a]	47.1[a]	4.3	43.2[a]	33.4[b]	35.6[ab]	13.3	33.3[a]	12.0[b]	7.6[b]	57.8	52.2[a]	47.9[a]	36.7[a]	2.6
umc1147	1.07	3.4[a]	1.5[b]	1.3[b]	26.0	44.6[a]	49.4[a]	47.7[a]	3.4	38.4[a]	35.6[a]	34.5[a]	2.4	26.5[a]	11.4[b]	6.4[b]	41.2	50.7[a]	41.3[a]	53.8[a]	3.1
phi037	1.08	3.5[a]	1.6[b]	1.3[b]	25.5	45.5[a]	47.2[a]	48.5[a]	1.0	38.1[a]	36.2[a]	35.0[a]	1.3	27.7[a]	14.1[b]	6.1[b]	41.0	50.7[a]	42.0[a]	53.8[a]	2.8
umc2029	1.08	3.7[a]	1.4[b]	1.0[b]	42.2	44.6[a]	50.0[a]	47.7[a]	4.4	37.1[a]	36.9[a]	34.9[a]	71.0	22.8[a]	12.0[b]	7.6[b]	23.7	51.1[a]	40.0[a]	62.5[a]	4.9
umc1512	1.09	3.1[a]	1.8[b]	1.0[b]	19.2	44.0[a]	49.9[a]	45.0[a]	6.0	35.4[a]	39.2[a]	30.3[a]	9.9	24.2[a]	14.3[b]	4.4[b]	26.8	54.5[a]	45.4[a]	42.0[a]	3.2
bnlg1660	5.02	3.3[a]	3.5[b]	1.0[a]	15.1	47.3[a]	48.7[a]	37.0[a]	9.9	37.0[a]	46.2[a]	27.1[a]	23.8	19.1[a]	27.1[a]	3.0[a]	14.3	58.8[a]	49.0[a]		2.3
Pop 2																					
umc1077	10.04	3.5[a]	2.9[b]	2.1[c]	14.5	47.3[a]	43.5[a]	38.6[b]	12.2	38.3[a]	34.0[a]	32.48[a]	4.3	12.5[a]	8.1[ab]	4.9[b]	5.2	103.6[ab]	119.8[a]	98.3[b]	6.5
bnlg1028	10.0	4.3[a]	3.9[a]	3.2[a]	7.9	45.2[a]	45.7[a]	45.9[a]	0.1	35.4[a]	34.3[a]	34.99[a]	0.2	12.3[a]	12.6[a]	10.2[a]	0.9	112.6[a]	119.1[a]	107.5[a]	1.4

	6																				

[a] For each trait, within the same row means followed by the same letter are not statistically different at 5 % of probability.

[b] Visual score was recorded using a scale from 1 (dead leaves) to 5 (completely healthy leaves)

[c] A stands for homozygotes with the allele of PHG39 (SG/SG), B for homozygotes with the alternative allele (NSG/NSG) and AB for heterozygotes (SG/NSG)

[d] PVE: proportion of variance explained

508 a

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511 b

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514 **Fig. 1** F_2 distribution for visual score estimated two months after flowering (using a scale
515 from 1=completely healthy leaves to 5=death leaves) in (PGH39×EA1070)F_2 (Pop1) (a) and
516 (PGH39×B73)F_2 (Pop2) (b). The average values of the parental lines are indicated in Pop2.

517

518 **Fig. 2** For Pop1 average decrease in the chlorophyll content for the homozygotes with the
519 SG and the NSG allele for two significant markers (*umc1281* and *phi037*) located in
520 chromosome 1. The pattern shown in this figure for two markers was similar for the other
521 significant markers of chromosome 1 in Pop1.

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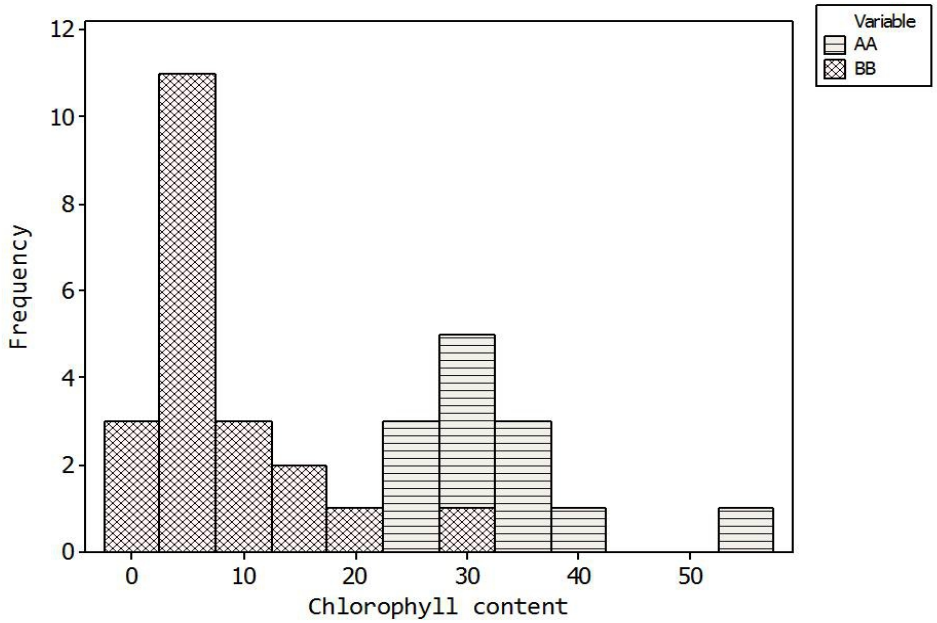
524

525 **Fig. 3** Average decrease in the chlorophyll content for the homozygotes with the SG and the

526 NSG allele for *bnlg1660* (bin 5.02) and *umc1077* (bin 10.04) in Pop1 and Pop2, respectively.



527

528 **Fig. 4** Chlorophyll content two months after silking (Chlo3) of homozygote plants for the
529 marker *bnlg1556* in both phenotypic tails in 2012. Homozygotes AA have the PHG39 (SG)
530 allele and homozygotes BB have the alternative allele (NSG) in Pop1

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