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ORIGINAL PAPER

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# The genetic basis of stay-green in rice analyzed in a population of doubled haploid lines derived from an *indica* by *japonica* cross

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**Abstract** Delayed leaf-senescence, or stay-green, has been regarded as a desired characteristic for the production of a number of crops including rice. In this study, we analyzed the genetic basis of stay-green using a population of 190 doubled haploid lines from the cross between an *indica* parent Zhenshan 97 and a stay-green *japonica* parent Wuyujing 2. The population was tested in replicated field trials in 2 consecutive years, and six traits were defined to evaluate the stay-green characteristics. A genetic linkage map with 179 SSR (simple sequence repeat) marker loci was constructed. The software QTLMapper, based on a mixed linear model approach, was applied to detect QTLs, epistatic effects and their environmental interactions for these traits. A total of 46 main-effect QTLs was detected for the six traits that can be localized to 25 chromosomal regions. The individual effects of all the QTLs were small. Fifty digenic interactions were resolved that involved 66 loci distributed on all 12 chromosomes. Environmental interactions were detected for 18 of the main-effect QTLs and 14 of the epistatic interactions. Collectively, the epistatic effects and QTL by year interactions accounted for large proportions of the phenotypic variations. The results also showed that most of the stay-green traits were negatively correlated with yield and its component traits. The implications of the results in crop improvement were discussed.

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## Introduction

Senescence in plants is the final developmental stage. It is internally programmed, as well as affected by external signals such as drought, temperature, insect or disease invasions, and other biotic and abiotic stresses (Buchanan-Wollaston 1997, Nooden et al. 1997). A typical feature of leaf senescence is the loss of chlorophyll and progressive decline in photosynthetic capability. Premature senescence would result in the deterioration of the quality of vegetables, ornamental plants and turf grasses, and also lead to poor grain quality and the yield loss of crop plants.

Stay-green is a term for the delayed yellowing, during later plant development (Thomas and Howarth 2000) compared with a reference genotype. Due to its importance, it has been studied in many plants such as sorghum (Walulu 1994), soybean (Pierce et al. 1984), maize (Gentinetta et al. 1986), durum wheat (Spano et al. 2003) and *Phaselous vulgaris* (Fang et al. 1998). Stay-green has usually been considered to be associated with the retention of the high photosynthetic capacity and yield increment (Gentinetta et al. 1986, Thomas and Howarth 2000). Rosenow et al. (1983) reported a stay-green sorghum variety B35 that showed post-flowering drought resistance, which contributed to an improvement in high and stable yield-production under drought-prone conditions. Stay-green plants of some other species also showed increased resistance to pest and disease invasion, better quality forages for animals, high chlorophyll content and extended pigment source for food industry, as well as the attractive ornamental period (Ambler et al. 1987; Thomas and Smart 1993; Xu et al. 2000b). Therefore, understanding the mechanism of stay-green would be very important for the improvement of plants, including the most important crop species.

It has been known that stay-green is also regulated by genetic factors in addition to the environment (Pierce et al. 1984; Walulu et al. 1994). Genetic analyses of stay-green have been conducted using various germplasms showing different types of stay-green. In soybean, three

stay-green mutants were found and analyzed. A cytoplasmic gene, *cytG*, two recessive alleles, *d1* and *d2*, and a dominant gene, *G*, regulated the greenness in foliages, pod walls, seed coats and embryos (Guiamet et al. 1990). The *cytG* product hindered the conversion of chlorophyll b to chlorophyll a resulting in higher stability of chlorophyll b during the degradation process (Guiamet et al. 1991), and the *d1d2* homozygote showed a significant delay of soluble protein degradation during senescence (Guiamet and Giannibelli 1996). The stay-green phenotypes of pea and *Festuca pratensis* were found to be induced by the lesion of phaeophobide, a oxygenase (PaO) during chlorophyll catabolism (Thomas 1987; Vicentini et al. 1995). Physiological, cytological, biochemical and genetic analyses of a non-yellowing mutant found in *F. pratensis* were also conducted (Thomas 1977, 1982, 1987, 1997; Thomas and Matile 1988; Thomas et al. 1999), which revealed that the stay-green character was regulated by a single recessive allele of the nuclear locus *sid* (Thomas 1987).

With the development of high-density molecular linkage maps (Causse et al. 1994; Hurushima et al. 1998; Temnykh et al. 2000, 2001) and analytical methods (Lander and Botstein 1989; Zeng 1994; Wang et al. 1999), the genetic bases for a large number of quantitatively inherited traits in many plant species, including yield and the agronomic performance of the most important crop species such as tomato, maize and rice (Paterson et al. 1988; Xu 1997; Xing et al. 2002) have been well elucidated. Genetic mapping of the QTL (quantitative trait locus) conferring stay-green was also undertaken in a number of plant species. Thomas (1997) mapped the single recessive nuclear allele *sid* for stay-green in *F. pratensis*, in an interval between two AFLP markers. Thorogood et al. (1999) observed up to six QTLs for leaf senescence in a *Lolium* population. In *Arabidopsis*, a recessive gene *fiw*, which exhibited premature cessation of inflorescence growth and early leaf senescence, was mapped on chromosome 4 (Nakamura et al 2000). Xu et al. (2000a) detected three stay-green QTL regions (*stg1*, *stg2*, *stg3*) in a recombinant inbred-line population of sorghum by using a restriction fragment length polymorphism (RFLP) map. Further analysis found that these regions contained the genes for key photosynthetic enzymes, heat shock proteins and an abscisic acid responsive gene, which attached the importance of these regions to yield and drought- and heat-stresses. In rice, Cha et al. (2002) mapped a recessive mutant gene *sgr(t)* for stay-green to chromosome 9 between RFLP markers RG662 and C985.

In rice, many *japonica* genotypes, including a number of high yielding cultivars, have good stay-green characteristics at maturity, while premature leaf senescence appears to be a common problem in a number of high yielding *indica* cultivars and hybrids. It has been speculated that delaying the senescence at the terminal stage of maturity may lead to increased yield and improved grain quality. For this purpose we developed a population of doubled haploid (DH) lines from a cross between a high yielding *japonica* cultivar Wuyujing 2 showing good stay-green and good maturity characters at harvest and Zhenshan 97, an *indica* line that is the female parent for a number of widely cultivated hybrids. The objectives of the study reported in this paper were to determine the genetic basis of stay-green in this population, and to identify QTLs that may be useful for the improvement of the hybrid performance in breeding programs.

## Materials and methods

### Materials and field planting

The experimental population consisted of 190 DH lines derived by anther culture of the $F_1$ from a cross between Zhenshan 97 and Wuyujing 2. The DH lines and the two parents were planted in the rice-growing seasons of 2001 and 2002 in the experimental farm of Huazhong Agricultural University, Wuhan, China. The planting time was June 12 in 2001 and May 17 in 2002, to make the experimental conditions, especially temperature and photoperiod, of the 2 years very different during the rice-growing periods. The field planting followed a randomized complete block design with two replications within a year. In each block, 20 plants from each line were planted in a two-row plot with a distance of 16.5 cm between plants within a row, and 26.4 cm between the rows. Field management followed essentially the normal agricultural practices. Irrigation of the field was maintained to avoid drought stress to the late maturing lines. The lines were harvested individually at maturity to prevent yield loss from over-ripening.

### Trait measurements

The degrees of greenness of the flag and second leaves from five plants in the middle of a row were measured at the day of heading and also 30 days after, using a Minolta Chlorophyll Meter SPAD-502 (Minolta Camera Co., Japan). To ensure that the measurements were taken in the right day for the right tiller, tillers were tagged at the day of heading. The SPAD readings of the flag and second leaves measured at the day of heading were designated as dgf and dgs for the flag and second leaves, and the SPAD values at 30 days after heading were used as measurements for the retention-degrees of greenness, designated as rdgf and rdgs, respectively. The ratios of rdgf to dgf and rdgs to dgs were used as indexes for the relative retention of greenness, designated as rrgf and rrgs, respectively.

Another measurement of stay-green was an independent visual estimation of the retention of the green-area (rga) for leaves at 30 days after heading on a 1 to 5 scale. A rating of 1 indicated complete or nearly complete leaf death, while rating 5 corresponded to a complete green leaf. Similarly, rgaf and rgas were used as designations for the first and second leaf, respectively.

Yield and its component traits examined, included the grain yield per plant (g), the number of tillers per plant, the number of grains per panicle, 1,000-grain weight (g) and seed-setting rate, taken from eight plants in the middle of the rows. Seed-setting rate was scored as the number of grains divided by the total number of spikelets from the reproductive tillers of a plant. The measurements for the other four traits were essentially as described previously by Yu et al. (1997).

### DNA markers and assays

Exactly 8 g of leaf tissue was harvested from each line, and ground to a fine powder under liquid nitrogen. Total cellular DNA was extracted using a CTAB method slightly modified from that by Murray and Thompson (1980).

SSR (simple sequence repeat) markers were used for map construction. The markers of the RM-series were designed according to Temnykh et al. (2000, 2001), and those of the

MRG-series were according to the rice genome sequences of Monsanto Company (McCouch et al. 2002). The markers polymorphic between the parents were used to assay the entire population of 190 lines.

The protocol included 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and a final extension for 5 min at 72°C in a thermocycler (MJ Research, USA). PCR was performed with 50–100 ng of genomic DNA, 0.2 μM of each primer and 1 unit of *Taq* DNA polymerase in a 20-μl reaction volume. PCR products were resolved on a sequencing gel containing 6% polyacrylamide and 7 M urea. The amplified DNA bands were visualized using a silver-staining method (Bassam et al. 1991).

### Linkage map construction and QTL assays

A molecular marker linkage map was constructed using Mapmaker 3.0 (Lincoln et al. 1992). For each trait, the phenotypic means of the plots were subjected to the analysis using QTLMapper 1.0 (Wang et al. 1999) that tested the QTL main effects, epistasis and their environmental interactions, treating the years as two different environments. In the analysis, the likelihood ratio (LR) and *t*-test were combined to test the significance of the single-locus QTL additive effects, epistatic effects and the QTL by environment (QE) effects. The LR and *t* values corresponding to $P$=0.005 were used as the threshold for claiming the putative main-effect, epistatic QTLs or QEs. The peak points of the LR in the linkage map were taken as the putative positions of the QTLs. When a QTL was involved in more than one epistasis, its position and additive effect were taken from the point showing the largest effect. The relative contribution of a genetic component was calculated as the proportion of phenotypic variance explained by that component in the selected model.

## Results

### Measurements of the stay-green traits

Table 1 presents a number of descriptive statistics of the stay-green traits for the two parents and the DH population. Large differences were found for these traits between the two parents, and the DH population exhibited approximately normal distributions for all the stay-green traits. The trait measurements were quite similar in the 2 years and the heritabilities were generally high. Also, the stay-green traits were highly correlated with each other in both years (Table 2).

**Table 2** Coefficients of pairwise correlations of the stay-green traits observed in 2001 (upper) and 2002 (lower). All the correlations are significantly different from zero at the $P \leq 0.001$ level ($r_{0.001}$=0.23)

| Item | rdgf | rrgf | rdgs | rrgs | rgaf |
|---|---|---|---|---|---|
| rrgf | 0.92<br>0.88 | | | | |
| rdgs | 0.80<br>0.84 | 0.69<br>0.78 | | | |
| rrgs | 0.76<br>0.69 | 0.74<br>0.79 | 0.94<br>0.90 | | |
| rgaf | 0.57<br>0.66 | 0.60<br>0.60 | 0.38<br>0.56 | 0.39<br>0.49 | |
| rgas | 0.52<br>0.70 | 0.54<br>0.67 | 0.48<br>0.77 | 0.50<br>0.69 | 0.80<br>0.75 |

### Linkage map

A survey of 293 SSR primer pairs identified 213 loci polymorphic between the parents, and 179 SSR loci, with a good coverage of the 12 chromosomes according to the map of Temnykh et al. (2000, 2001), were selected to assay the entire population. Mapmaker analysis at LOD (logarithm of odds) 3.0 clustered these loci into 12 groups, based on which a map was constructed. The map spanned 1,849.4 cM with an average interval of 9.4 cM between adjacent marker loci. The marker orders in the linkage map corresponded well with that of Temnykh et al. (2000, 2001).

**Table 1** Descriptive statistics of the stay-green traits in parents and the DH population observed in 2001 (upper) and 2002 (lower)

| Trait[a] | Parent [b] | | Population | | | | |
|---|---|---|---|---|---|---|---|
| | Zhenshan 97 | Wuyujing 2 | Range | Mean | Kurtosis | Skewness | $h^2$ [c] |
| rdgf | 27.8±2.4<br>31.4±2.4 | 47.2±4.2<br>45.0±2.1 | 13.1–51.0<br>15.1–56.5 | 32.0<br>38.3 | –0.59<br>–0.35 | –0.05<br>–0.28 | 72.6 |
| rrgf | 0.6±0.07<br>0.7±0.1 | 0.9±0.0<br>0.9±0.1 | 0.3–1.0<br>0.4–1.1 | 0.7<br>0.8 | –0.58<br>0.01 | –0.13<br>–0.41 | 79.0 |
| rdgs | 32.8±2.5<br>29.0±2.1 | 47.1±1.9<br>45.1±2.5 | 14.4–49.6<br>10.5–52.6 | 32.0<br>36.7 | –0.46<br>0.27 | –0.20<br>–0.42 | 78.5 |
| rrgs | 0.7±0.0<br>0.6±0.1 | 0.9±0.0<br>1.0±0.1 | 0.3–0.9<br>0.2–1.1 | 0.7<br>0.8 | –0.47<br>1.30 | –0.46<br>–0.59 | 75.2 |
| rgaf | 1.0±0.0<br>1.0±0.0 | 5.0±0.0<br>5.0±0.0 | 1.0–5.0<br>1.0–5.0 | 3.8<br>3.6 | –0.67<br>1.16 | –0.77<br>–1.01 | 61.2 |
| rgas | 1.0±0.0<br>1.0±0.0 | 5.0±0.0<br>5.0±0.0 | 1.0–5.0<br>1.0–5.0 | 3.2<br>3.4 | –0.57<br>0.92 | –0.41<br>–0.80 | 69.7 |

[a] Abbreviations as described in Materials and methods

[b] The numbers in each of the cells are the mean ± standard deviation for the parent. All the differences between the two parents within the same year are statistically significant at the 0.01 probability level

[c] Heritability (%) calculated as $h^2=\sigma_g^2/(\sigma_g^2+\sigma_{gy}^2/n+\sigma_e^2/nr)$, where $\sigma_g^2$ is the genotypic variance, $\sigma_{gy}^2$ is the variance due to genotype by year interaction, $\sigma_e^2$ is the error variance

QTLs for stay-green traits

*Retention degree of greenness of the flag leaf*

The analysis identified 11 main-effect QTLs for the retention degree of greenness of flag leaf (rdgf) on the basis of combined data from 2001 and 2002 (Table 3). Three QTLs were detected on chromosome 2 (*rdgf2a*, *rdgf2b* and *rdgf2c*), two QTLs on chromosome 8 (*rdgf8a* and *rdgf8b*) and one on each of chromosomes 3 (*rdgf3*), 4 (*rdgf4*), 6 (*rdgf6*), 7 (*rdgf7*), 9 (*rdgf9*) and 10 (*rdgf10*). Alleles from Zhenshan 97 at *rdgf2a*, *rdgf8a* and *rdgf8b* had positive effects on rdgf while, at the remaining eight QTLs, Wuyujing 2 genotypes increased rdgf. The additive effects of the QTLs ranged from 0.89 to 2.20 SPAD units. Together, these QTLs explained 21.4% of the total phenotypic variation.

Ten pairs of loci were also detected as showing significant interactions (Table 3), and the loci involved in the interactions were referred to as epistatic QTLs. Five of the ten pairs involved loci that did not show significant QTL main-effects, and the remaining five pairs each involved one main-effect QTL. The estimated effects of the epistasis showed that the parental two-locus genotypes for five pairs of the loci had positive effects on the retention of greenness, while for the other five pairs of loci, the recombinant two-locus genotypes imparted positive effects. In all, the epistatic effects accounted for 15.0% of the phenotypic variation.

Three main-effect QTLs and two pairs of epistatic QTLs displayed significant interactions with years. The interactions of the main effects with the years explained 8.6% of the phenotypic variation, while the interactions of epistatic QTLs with the years explained 5.5% of the variation. In all the cases, the effects detected in 2001 were significantly more pronounced than in 2002.

*Relative retention of greenness of the flag leaf*

Four main-effect QTLs, located on chromosomes 2, 7, 10 and 12 respectively, were identified for relative retention of greenness of the flag leaf (Table 4). The Wuyujing 2 alleles at all of the QTLs contributed to increased retention of greenness. These QTLs totally accounted for 8.9% of the variation.

One pair of loci showed significant interaction explaining 1.51% of the phenotypic variation, with the recombinant two-locus genotypes showing a favorable effect on green retention (Table 4). Two main-effect QTLs and one pair of epistatic QTLs also showed significant interactions with years, which together apparently accounted for a large proportion (16.4%) of the variation.

*Retention degree of greenness of the second leaf*

A total of nine main-effect QTLs were resolved for the retention degree of greenness of the second leaf (Table 5). Zhenshan 97 alleles at four of the QTLs (*rdgs2a*, *rdgs2b*, *rdgs8a* and *rdgs8b*) showed increase effects on rdgs, while at the other five QTLs alleles from Wuyujing 2 contributed to green retention. Totally, these QTLs explained 21.5% of the phenotypic variation.

Epistatic effects were detected for 18 pairs of the loci, which together explained a large fraction (33.9%) of the phenotypic variation. Twelve pairs of the loci did not involve main-effect QTLs, and the remaining six pairs each involved one main-effect QTL. Parental genotypes for 11 of the two-locus combinations had positive effects on green retention, while the recombinant genotypes had positive effects for the remaining seven pairs.

Interactions with years were also detected for three main-effect QTLs and one pair of the epistatic QTLs.

*Relative retention of greenness of the second leaf*

Five QTLs were detected on five different chromosomes for the relative retention of greenness of the second leaf (Table 6). Zhenshan 97 alleles at one (*rrgs3*) of the QTLs contributed to increased green retention, and alleles from Wuyujing 2 increased green retention at the other four QTLs (*rrgs2*, *rrgs6*, *rrgs7* and *rrgs10*). Totally, these QTLs explained 9.6% of the phenotypic variation.

Epistatic effects were detected in three pairs of loci; only one pair of loci involved one main-effect QTL. Totally, epistatic interactions accounted for 5.0% of the phenotypic variation.

Environmental interactions were also detected for two main-effect QTLs and three pairs of the epistatic QTLs. The QEs totally explained 22.8% of the phenotypic variation.

*Retention of the green area of the flag leaf*

The analysis resolved ten QTLs for the green area retention of the flag leaf (Table 7), with the additive effects ranging from 0.12 to 0.24 of the visual units. Zhenshan 97 alleles at four of the QTLs increased green area retention while, for the remaining six QTLs, alleles from Wuyujing 2 contributed to green area retention. These QTLs explained 17.6% of the phenotypic variation.

Eleven pairs of loci detected epistatic effects. Seven of the pairs did not involve any main-effect QTLs, and the other four pairs of epistatic QTLs each involved one main-effect QTL. Parental two-locus genotypes appeared to be favorable for retention of the green-leaf area for five of the pairs, while recombinant two-locus combinations had positive effects on green area retention for the remaining six pairs.

Four of the main-effect QTLs, and seven pairs of epistatic QTLs showed interactions with the years.

**Table 3** Main effects, digenic epistatic effects and environmental interactions of QTLs for the retention degree of greenness of the flag leaf, analyzed using QTLMapper 1.0 at the likelihood ratio LR-threshold of 18.6 (the LR value is equal to a chi-square value for *df*=6 at *P*=0.005) combining the data of 2001 and 2002

| Ch-Ini[a] | QTL | Flanking markers | Ch-Inj[a] | QTL | Flanking markers | LR | $a_i$ [b] | $h^2a_i$ [e] | $a_j$ [b] | $h^2a_j$ [e] | $aa_{ij}$ [c] | $h^2aa_{ij}$ [e] | $ae_i$ [d] | $h^2ae_i$ [e] | $ae_j$ [d] | $h^2ae_j$ [e] | $aae_{ij}$ [d] | $h^2aae_{ij}$ [e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1–20 | | RM104–RM14 | 8–5 | *rdgf8a* | RM126–RM310 | 104.6 | | | 2.12 | 4.05 | −0.76 | 0.53 | | | −1.63 | 4.83 | | |
| 2–12 | *rdgf2a* | RM29–RM341 | 6–1 | | RM170–RM190 | 42.2 | 1.68 | 2.55 | | | 0.81 | 0.60 | | | | | | |
| 2–16 | *rdgf2b* | RM263–RM221 | 10–9 | | RM228–RM333 | 33.6 | −0.89 | 0.72 | | | 1.04 | 0.98 | | | | | −1.30 | 3.05 |
| 2–18 | *rdgf2c* | RM6–RM240 | 9–3 | *rdgf9* | MRG2533–RM257 | 41.2 | −1.54 | 2.14 | −1.07 | 1.04 | | | | | −0.98 | 1.73 | | |
| 3–1 | | RM81B–RM132 | 5–10 | | RM108A–RM274 | 29.1 | | | | | −1.35 | 1.65 | | | | | | |
| 3–1 | | RM81B–RM132 | 6–5 | | RM111–RM276 | 30.2 | | | | | 1.38 | 1.72 | | | | | | |
| 3–6 | | RM218–RM251 | 6–12 | *rdgf6* | RM275–RM30 | 43.1 | | | −2.20 | 4.39 | | | | | | | | |
| 3–7 | | RM251–MRG2803 | 11–9 | | RM206–RM254 | 22.3 | | | | | 1.43 | 1.85 | | | | | | |
| 3–9 | | RM282–RM473A | 6–7 | | RM121–RM136 | 23.2 | | | | | 1.42 | 1.81 | | | | | | |
| 3–17 | | RM293–RM143 | 9–3 | | MRG2533–RM257 | 63.9 | | | | | | | | | | | | |
| 3–17 | *rdgf3* | RM293–RM143 | 12–1 | | RM19–RM117 | 62.4 | −2.10 | 4.00 | | | | | | | | | | |
| 4–2 | *rdgf4* | MRG5943–RM261 | 12–10 | | RM235–MRG227 | 47.9 | −1.39 | 1.75 | | | −1.56 | 2.21 | | | | | | |
| 6–13 | | RM30–RM340 | 7–4 | *rdgf7* | RM82–RM125 | 74.3 | | | −0.91 | 0.74 | −1.38 | 1.72 | | | | | | |
| 8–4 | | RM25–RM126 | 8–8 | *rdgf8b* | RM331–RM342A | 36.5 | | | 0.92 | 0.76 | | | | | | | | |
| 8–9 | | RM342A–RM284 | 10–6 | *rdgf10* | RM269b–RM304 | 43.8 | | | −1.16 | 1.21 | | | | | −1.06 | 2.03 | | |
| 10–5 | | RM271–RM269b | 12–2 | | RM117–RM155 | 40.3 | | | | | −1.46 | 1.92 | | | | | −1.16 | 2.44 |

Overall contributions. Additive: $h^2a$=21.4%; Epistasis: $h^2aa$=15.0%; QE interactions: $h^2ae$=8.6%; $h^2aae$=5.5%

[a] Ch-Ini and Ch-Inj represent the chromosome number-interval of the points being tested in the analysis

[b] $a_i$ and $a_j$ are the additive effects of testing points i and j, respectively. A positive value indicates the Zhenshan 97 genotype having a positive effect on the trait

[c] $aa_{ij}$ is the effect of additive by additive interaction between points i and j; a positive value indicates that the parental two-locus genotypes have a positive effect on the trait and the recombinants had negative effects

[d] $ae_i$, $ae_j$ and $aae_{ij}$ are the effects of interactions of locus i, j and epistasis with the environment, respectively; a positive value means that the effect in 2001 is larger than that in 2002

[e] $h^2a_i$, $h^2a_j$, $h^2aa_{ij}$, $h^2ae_i$, $h^2ae_j$ and $h^2aae_{ij}$ are the percentages of the phenotypic variations explained by $a_i$, $a_j$, $aa_{ij}$, $ae_i$ and $ae_j$ and $aae_{ij}$, respectively

**Table 4** Main effects, digenic epistatic effects and environmental interactions of QTLs for relative retention of greenness of the flag leaf, analyzed using QTLMapper 1.0 at a likelihood ratio LR-threshold of 18.6 (the LR value is equal to a chi-square value for *df*=6 at *P*=0.005) combining the data of 2001 and 2002

| Ch-Ini[a] | QTL | Flanking markers | Ch-Inj[a] | QTL | Flanking markers | LR | $a_i$ [b] | $h^2a_i$ [e] | $a_j$ [b] | $h^2a_j$ [e] | $aa_{ij}$ [c] | $h^2aa_{ij}$ [e] | $ae_i$ [d] | $h^2ae_i$ [e] | $ae_j$ [d] | $h^2ae_j$ [e] | $aae_{ij}$ [d] | $h^2aae_{ij}$ [e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2–2 | | RM154–RM211 | 4–9 | | RM241–RM303 | 28.2 | | | | | −0.04 | 1.51 | | | | | 0.05 | 5.28 |
| 2–16 | *rrgf2* | RM263–RM221 | 5–12 | | RM87–RM334 | 39.0 | −0.07 | 4.65 | | | | | 0.06 | 7.40 | | | | |
| 4–1 | | RM335–MRG5943 | 7–6 | *rrgf7* | RM2–RM11 | 27.5 | | | −0.03 | 1.20 | | | | | −0.04 | 3.70 | | |
| 7–5 | | RM125–RM2 | 12–4 | *rrgf12* | RM277–RM309 | 18.8 | | | −0.03 | 1.06 | | | | | | | | |
| 10–7 | *rrgf10* | RM304–RM147 | 12–2 | | RM117–RM155 | 22.4 | −0.04 | 2.03 | | | | | | | | | | |

Overall contributions. Additive: $h^2a$=8.9%; Epistasis: $h^2aa$=1.5%; QE interactions: $h^2ae$=11.1%; $h^2aae$=5.3%

[a–e] See footnotes in Table 3

**Table 5** Main effects, digenic epistatic effects and environmental interactions of QTLs for the retention degree of greenness of the second leaf, analyzed using QTLMapper 1.0 at a likelihood ratio LR-threshold of 18.6 (the LR value is equal to a chi-square value for *df*=6 at *P*=0.005) combining the data of 2001 and 2002

| Ch-Ini[a] | QTL | Flanking markers | Ch-Inj[a] | QTL | Flanking markers | LR | $a_i$ [b] | $h^2a_i$ [e] | $a_j$ [b] | $h^2a_j$ [e] | $aa_{ij}$ [c] | $h^2aa_{ij}$ [e] | $ae_i$ [d] | $h^2ae_i$ [e] | $ae_j$ [d] | $h^2ae_j$ [e] | $aae_{ij}$ [d] | $h^2aae_{ij}$ [e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1–11 | | RM294B–RM306 | 3–16 | | RM520–RM293 | 37.1 | | | | | −0.93 | 0.90 | | | | | | |
| 1–19 | | RM315–RM104 | 3–5 | | MRG0002–RM218 | 38.6 | | | | | 1.63 | 2.75 | | | | | | |
| 1–20 | | RM104–RM14 | 6–10 | | RM3–RM162 | 47.8 | | | | | −1.51 | 2.37 | | | | | | |
| 2–7 | | RM53–RM145 | 8–10 | | RM284–RM210 | 32.7 | | | | | 2.12 | 4.68 | | | | | | |
| 2–8 | *rdgs2a* | RM145–RM322 | 2–16 | | RM263–RM221 | 33.4 | 0.90 | 0.84 | | | −0.93 | 0.90 | | | | | | |
| 2–11 | | RM301–RM29 | 3–9 | *rdgs3a* | RM282–RM473A | 33.1 | | | −0.67 | 0.46 | 0.68 | 0.48 | | | | | | |
| 2–12 | *rdgs2b* | RM29–RM341 | 9–6 | | RM321–MRG2912 | 25.3 | 1.36 | 1.92 | | | | | | | | | | |
| 2–15 | | RM262–RM263 | 3–17 | *rdgs3b* | RM293–RM143 | 65.7 | | | −1.58 | 2.58 | 1.03 | 1.10 | | | | | | |
| 2–21 | | RM108b–RM266 | 7–13 | *rdgs7* | RM118–RM248 | 34.4 | | | −1.09 | 1.24 | 2.04 | 4.32 | | | −0.14 | 0.02 | | |
| 3–5 | | MRG0002–RM218 | 8–14 | | RM264–RM281 | 34.4 | | | | | −1.61 | 2.70 | | | | | | |
| 3–8 | | MRG2803–RM282 | 10–8 | *rdgs10* | RM147–RM228 | 47.3 | | | −2.01 | 4.21 | | | | | 0.70 | 0.52 | | |
| 3–16 | | RM520–RM293 | 7–7 | | RM11–RM346 | 56.0 | | | | | −1.26 | 1.66 | | | | | 1.30 | 3.49 |
| 3–17 | | RM293–RM143 | 4–6 | | RM177–RM273 | 25.6 | | | | | 1.27 | 1.68 | | | | | | |
| 3–20 | | RM570–RM85 | 8–1 | | RM337–RM152 | 28.6 | | | | | 1.10 | 1.26 | | | | | | |
| 4–13 | | RM348–RM131 | 11–1 | | RM286–RM20B | 38.5 | | | | | 1.23 | 1.57 | | | | | | |
| 4–13 | | RM348–RM131 | 5–4 | | RM169–RM146 | 33.3 | | | | | 1.21 | 1.53 | | | | | | |
| 4–14 | | RM131–RM280 | 6–13 | | RM30–RM340 | 36.6 | | | | | 1.33 | 1.85 | | | | | | |
| 6–7 | | RM121–RM136 | 8–4 | *rdgs8b* | RM25–RM126 | 50.1 | | | 1.90 | 3.77 | | | | | −0.32 | 0.10 | | |
| 6–12 | *rdgs6* | RM275–RM30 | 7–12 | | RM18–RM118 | 48.2 | −1.52 | 2.39 | | | −1.16 | 1.41 | | | | | | |
| 8–8 | *rdgs8a* | RM331–RM342A | 10–4 | | RM184–RM271 | 51.7 | 0.93 | 0.90 | | | −1.37 | 1.96 | | | | | | |
| 9–3 | | MRG2533–RM257 | 10–4 | | RM184–RM271 | 28.6 | | | | | 0.98 | 1.00 | | | | | | |

Overall contributions. Additive: $h^2a$=21.5%; Epistasis: $h^2aa$=33.9%; QE interactions: $h^2ae$=0.6%; $h^2aae$=3.5%
[a–e] See footnotes in Table 3

**Table 6** Main effects, digenic epistatic effects and environmental interactions of QTLs for relative retention of greenness of the second leaf, analyzed using QTLMapper 1.0 at a likelihood ratio (LR) threshold of 18.6 (the LR value is equal to a chi-square value for *df*=6 at *P*=0.005) combining the data of 2001 and 2002

| Ch-Ini[a] | QTL | Flanking markers | Ch-Inj[a] | QTL | Flanking markers | LR | $a_i$ [b] | $h^2a_i$ [e] | $a_j$ [b] | $h^2a_j$ [e] | $aa_{ij}$ [c] | $h^2aa_{ij}$ [e] | $ae_i$ [d] | $h^2ae_i$ [e] | $ae_j$ [d] | $h^2ae_j$ [e] | $aae_{ij}$ [d] | $h^2aae_{ij}$ [e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2–3 | | RM211–RM233A | 4–9 | | RM241–RM303 | 38.9 | | | | | −0.04 | 2.10 | | | | | 0.05 | 7.29 |
| 2–16 | *rrgs2* | RM263–RM221 | 8–1 | | RM337–RM152 | 33.1 | −0.05 | 2.64 | | | | | 0.05 | 5.52 | | | | |
| 3–3 | *rrgs3* | RM175–RM36 | 7–7 | *rrgs7* | RM11–RM346 | 31.3 | 0.03 | 1.28 | −0.04 | 2.20 | | | | | | | | |
| 3–8 | | MRG2803–RM282 | 7–2 | | RM427–RM298 | 21.9 | | | | | −0.03 | 1.28 | | | | | 0.04 | 4.20 |
| 3–17 | | RM293–RM143 | 6–3 | *rrgs6* | RM510–RM314 | 31.9 | | | −0.04 | 1.80 | | | | | 0.04 | 4.20 | | |
| 7–6 | | RM2–RM11 | 9–1 | | RM245–RM215 | 20.7 | | | | | | | | | | | | |
| 10–1 | *rrgs10* | RM222–RM216 | 12–1 | | RM19–RM117 | 39.3 | −0.04 | 1.71 | | | 0.04 | 1.53 | | | | | −0.04 | 1.62 |

Overall contributions. Additive: $h^2a$=9.6%; Epistasis: $h^2aa$=5.0%; QE interactions: $h^2ae$=9.7%; $h^2aae$=13.1%
[a–e] See footnotes in Table 3

**Table 7** Main effects, digenic epistatic effects and environmental interactions of QTLs for retention of the green area of the flag leaf, analyzed using QTLMapper 1.0 at the likelihood ratio (LR) threshold of 18.6 (the LR value is equal to a chi-square value for *df*=6 at *P*=0.005) combining the data of 2001 and 2002

| Ch-Ini[a] | QTL | Flanking markers | Ch-Inj[a] | QTL | Flanking markers | LR | $a_i$ [b] | $h^2a_i$ [e] | $a_j$ [b] | $h^2a_j$ [e] | $aa_{ij}$ [c] | $h^2aa_{ij}$ [e] | $ae_i$ [d] | $h^2ae_i$ [e] | $ae_j$ [d] | $h^2ae_j$ [e] | $aae_{ij}$ [d] | $h^2aae_{ij}$ [e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1–1 | | MRG167–RM84 | 2–5 | | RM279–RM555 | 27.4 | | | | | −0.16 | 1.38 | | | | | −0.14 | 2.24 |
| 1–1 | | MRG167–RM84 | 2–17 | | RM221–RM6 | 33.4 | | | | | −0.22 | 2.63 | | | | | −0.14 | 2.27 |
| 1–6 | | RM312–RM23 | 7–7 | *rgaf7* | RM11–RM346 | 41.6 | | | −0.24 | 3.11 | | | | | −0.17 | 3.32 | | |
| 2–8 | | RM145–RM322 | 2–16 | | RM263–RM221 | 44.4 | | | | | −0.17 | 1.56 | | | | | −0.13 | 0.95 |
| 2–8 | *rgaf2a* | RM145–RM322 | 3–17 | *rgaf3* | RM293–RM143 | 59.0 | 0.18 | 1.74 | −0.17 | 1.56 | | | −0.13 | 0.92 | | | | |
| 2–15 | | RM262–RM263 | 9–1 | | RM245–RM215 | 38.5 | | | | | 0.26 | 3.60 | | | | | | |
| 2–15 | | RM262–RM263 | 10–6 | *rgaf10* | RM269b–RM304 | 33.4 | | | −0.12 | 0.78 | 0.14 | 1.04 | | | | | | |
| 2–18 | | RM6–RM240 | 4–14 | *rgaf4b* | RM131–RM280 | 60.3 | | | 0.17 | 1.58 | 0.31 | 5.40 | | | 0.16 | 1.35 | | |
| 2–21 | *rgaf2b* | RM108b–RM266 | 3–3 | | RM175–RM36 | 27.2 | 0.18 | 1.80 | | | −0.20 | 2.31 | | | | | −0.13 | 1.93 |
| 3–2 | | RM132–RM175 | 11–7 | | RM229–RM21 | 36.6 | | | | | −0.15 | 1.23 | | | | | 0.20 | 4.35 |
| 3–9 | | RM282–RM473A | 6–12 | *rgaf6b* | RM275–RM30 | 36.2 | | | −0.12 | 0.75 | 0.19 | 2.04 | | | | | | |
| 4–2 | *rgaf4a* | MRG5943–RM261 | 8–4 | *rgaf8* | RM25–RM126 | 56.7 | −0.20 | 2.24 | 0.19 | 2.06 | | | | | −0.13 | 0.87 | | |
| 5–4 | | RM169–RM146 | 12–4 | | RM277–RM309 | 28.2 | | | | | 0.15 | 1.26 | | | | | 0.14 | 1.06 |
| 6–9 | *rgaf6a* | RM150–RM3 | 8–13 | | RM149–RM264 | 23.9 | −0.23 | 2.91 | | | | | | | | | | |
| 6–10 | | RM3–RM162 | 10–1 | | RM222–RM216 | 46.6 | | | | | −0.20 | 2.28 | | | | | −0.17 | 3.13 |

Overall contributions. Additive: $h^2a$=17.6%; Epistasis: $h^2aa$=25.2%; QE interactions: $h^2ae$=5.5%; $h^2aae$=16.2%
[a–e] See footnotes in Table 3

**Table 8** Main effects, digenic epistatic effects and environmental interactions of QTLs for retention of the green area of the second leaf analyzed using QTL Mapper 1.0 at a likelihood ratio (LR) threshold of 18.6 (the LR value is equal to a chi-square value for *df*=6 at *P*=0.005) combining the data of 2001 and 2002

| Ch-Ini[a] | QTL | Flanking markers | Ch-Inj[a] | QTL | Flanking markers | LR | $a_i$ [b] | $h^2a_i$ [e] | $a_j$ [b] | $h^2a_j$ [e] | $aa_{ij}$ [c] | $h^2aa_{ij}$ [e] | $ae_i$ [d] | $h^2ae_i$ [e] | $ae_j$ [d] | $h^2ae_j$ [e] | $aae_{ij}$ [d] | $h^2aae_{ij}$ [e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1–20 | | RM104–RM14 | 6–9 | *rgas6a* | RM150–RM3 | 27.1 | | | −0.13 | 1.44 | −0.19 | 3.13 | | | 0.08 | 0.50 | | |
| 3–2 | | RM132–RM175 | 11–7 | | RM229–RM21 | 20.2 | | | | | −0.18 | 2.77 | | | | | | |
| 3–5 | | MRG0002–RM218 | 3–20 | *rgas3* | RM570–RM85 | 27.0 | | | −0.28 | 6.86 | 0.15 | 2.04 | | | | | | |
| 3–9 | | RM282–RM473A | 6–9 | | RM150–RM3 | 18.8 | | | | | 0.17 | 2.43 | | | | | | |
| 3–13 | | RM16–RM135 | 7–13 | *rgas7a* | RM118–RM248 | 29.3 | | | −0.17 | 2.46 | | | | | −0.20 | 7.08 | | |
| 4–5 | | RM142–RM177 | 7–3 | *rgas7b* | RM298–RM82 | 26.5 | | | −0.11 | 1.00 | 0.19 | 3.37 | | | −0.04 | 0.16 | | |
| 5–11 | | RM274–RM87 | 6–12 | *rgas6b* | RM275–RM30 | 21.0 | | | −0.20 | 3.68 | | | | | −0.01 | 0.01 | | |
| 6–1 | | RM170–RM190 | 12–2 | | RM117–RM155 | 20.5 | | | | | −0.22 | 4.37 | | | | | | |
| 8–13 | | RM149–RM264 | 10–5 | *rgas10* | RM271–RM269b | 22.2 | | | −0.20 | 3.47 | | | | | | | | |
| 10–4 | | RM184–RM271 | 11–1 | *rgas11* | RM286–RM20B | 23.4 | | | −0.15 | 2.01 | −0.21 | 3.76 | | | | | | |

Overall contributions. Additive: $h^2a$=22.0%; Epistasis: $h^2aa$=21.8%; QE interactions: $h^2ae$=7.5%; $h^2aae$=0.0%
[a–e] See footnotes in Table 3

**Table 9** Correlations of the stay-green traits with yield and yield-component traits observed in 2001 (upper) and 2002 (lower)

| Stay-green trait | Yield | Tillers/plant | Grains/panicle | Seed setting (%) | Grain weight |
|---|---|---|---|---|---|
| rdgf | –0.33** | –0.02 | –0.26** | –0.23** | –0.16* |
| | –0.35** | –0.03 | –0.36** | –0.28** | –0.10 |
| rrdgf | –0.31** | 0.02 | –0.26** | –0.32** | –0.17* |
| | –0.28** | 0.01 | –0.32** | –0.32** | 0.01 |
| rdgs | –0.35** | 0.02 | –0.25** | –0.16* | –0.22** |
| | –0.30** | 0.00 | –0.32** | –0.22** | –0.12 |
| rrdgs | –0.29** | 0.04 | –0.21** | –0.17* | –0.21** |
| | –0.23** | 0.01 | –0.27** | –0.25** | 0.00 |
| rgaf | –0.11 | –0.21** | –0.05 | –0.20* | 0.02 |
| | –0.23** | –0.04 | –0.18* | –0.14 | –0.04 |
| rgas | –0.11 | –0.21** | –0.03 | –0.09 | –0.01 |
| | –0.23** | 0.00 | –0.26** | –0.20* | –0.01 |

*, ** Significantly different from 0 at probabilities 0.05 and 0.01 levels

Overall, these environmental interactions explained a total of 21.7% of the phenotypic variation.

*Retention of the green area of the second leaf*

A total of seven main-effect QTLs were detected for retention of the green area of the second leaf (Table 8). The Wuyujing 2 alleles at all of the loci showed positive effects on retention of the green area. Altogether, these QTLs explained 22.0% of the phenotypic variation.

Seven pairs of loci showed epistatic effects on green area retention of the second leaf, with positive effects conferred by both parental and recombinant two-locus genotypes.

Interactions with years occurred in four of the main-effect QTLs totally explaining 7.5% of the phenotypic variation. But interactions with years were not detected for the epistatic QTLs.

Relationship between stay-green traits and yield

All the stay-green traits were negatively correlated with yield, and most of the negative correlations were highly significant (Table 9). Among the component traits (tillers per plant, grains per panicle and grain weight) of yield, the number of grains per panicle was also negatively correlated with most of the stay-green traits, presumably because of the negative correlations between the stay-green traits and seed-setting rate. Negative correlations were also detected between the other two component traits and some of the stay-green traits (Table 9).

## Discussion

We used six traits to describe various aspects of stay-green, that may well reflect the degree of delayed leaf senescence. Using the software QTLMapper 1.0, the analyses resolved a total of 46 main-effect QTLs, and 50 epistatic effects involving a total of 66 loci located on all of the 12 chromosomes. The analyses also revealed that 18 of the main-effect QTLs and 14 pairs of epistatic QTLs had significant interactions with years.

Compared to the results of the analyses of yield and yield component traits using the same software (Xing et al. 2002), the QTLs for stay-green seemed to have several distinct characteristics. First, the relative effects of the main-effect QTLs were small as evaluated by the amount of variation explained by the QTLs; only one QTL explained 6.86% of the phenotypic variation and all the remaining QTLs explained less than 5.0% of the variation. This indicates that there were no major QTLs underlying the genetic basis of the stay-green traits. Second, the effects of digenic interactions of the stay-green traits appeared to be more pronounced than those of yield traits, as demonstrated by both the numbers of significant interactions and the amounts of variation explained by the epistatic interactions. Moreover, the amounts of variation explained by epistasis for three of the six stay-green traits analyzed were equal to, or larger than, that explained by the main-effect QTLs. Thus, the genetic basis of stay-green appeared to be complex. Third, the effects of QTL by year interactions detected for stay-green were also much more pronounced than those resolved in yield and yield component traits, in which only small amounts of interactions were detected between main-effect QTLs and years, and the overall effects of QTL by year interactions were trivial (Xing et al. 2002). Whereas in the stay-green traits, the overall effects of QEs, including the interactions of main-effect QTLs as well as the epistatic QTLs with years, were large. In some cases, the amount of variation explained by QEs were on the same order of magnitude or even larger than the QTL main-effects and epistatic effects (Tables 4, 6 and 7). This suggests that stay-green traits are more sensitive to environmental changes.

The analyses also revealed high phenotypic correlations between different stay-green traits. The genetic basis of such high correlations can largely be explained by the co-localization of the QTLs for these traits, either due to pleiotropic effects or tight linkage. For instance, inspection of Fig. 1 and Tables 3–8 could identify genomic

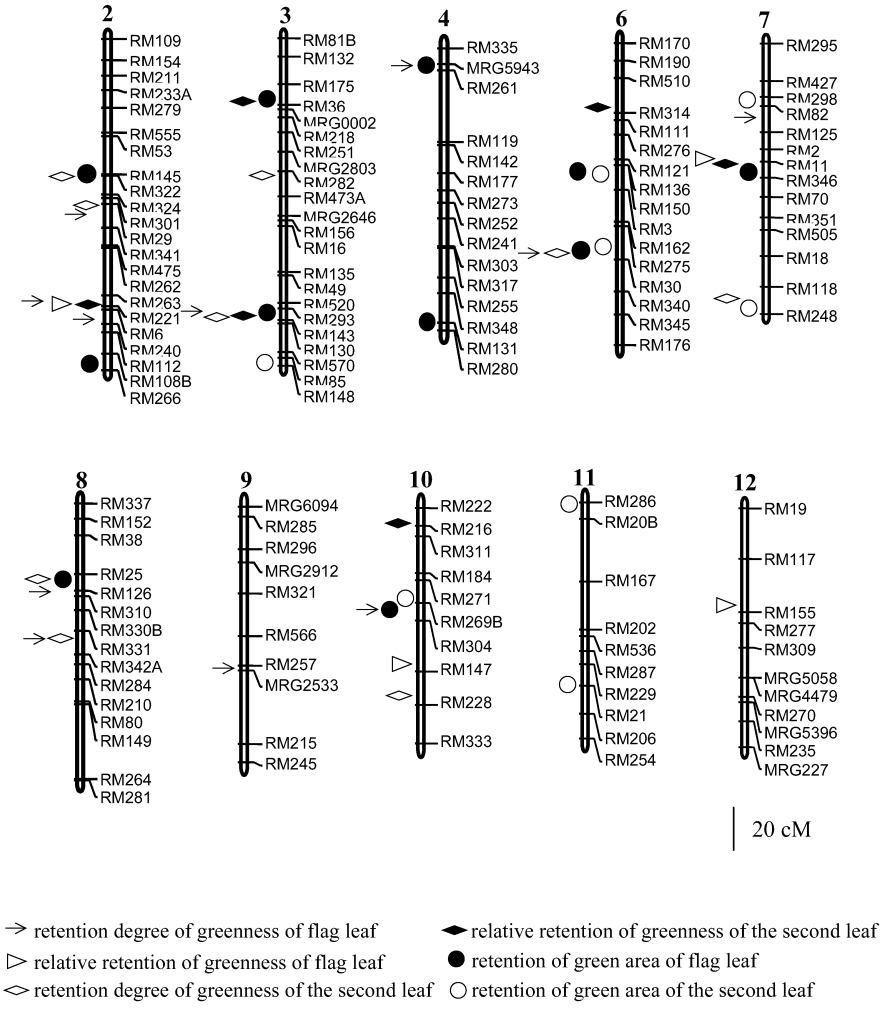


**Fig. 1** Distribution of main-effect QTLs on the molecular linkage map as detected by the QTL Mapper

regions that had effects on three to four of the six stay-green traits investigated. Examples of such genomic regions included: the interval marked by RM263 and RM221 on chromosome 2 where QTLs for rdgf, rrgf and rrgs were simultaneously detected, the region marked by RM293 and RM143 on chromosome 3 for QTLs contributing to rdgf, rdgs, rrgs and rgaf, the region bordered by RM275 and RM30 on chromosome 6 for QTLs specifying rgaf, rgas, rdgf and rdgs, the region between RM11 and RM346 on chromosome 7 for QTLs affecting rrgs, rrgf and rgaf, the region marked by RM25 and RM126 on chromosome 8 for QTLs controlling rdgs, rgaf and rdgf, and the interval between RM269B and RM304 on chromosome 10 for QTLs underlying rgaf, rgas and rdgf. There were also a number of regions where two QTLs for different traits were simultaneously detected, and examples of such QTLs can be found in Fig. 1 and Tables 3–8. In addition to co-localization, linkages of various degrees between QTLs also contributed to the observed genetic correlations among the stay-green traits. From a physiological standpoint, the stay-green traits, as defined in this study, are also functionally related with each other as several of them describe the greenness of the same leaves.

Stay-green, or delayed senescence, of the leaves has often been considered to be a favorable characteristic in crop production, especially under drought-stressed conditions (Gan and Amasino 1995, Xu et al. 2002a). It thus seems surprising to find that the stay-green traits were negatively correlated with yield and yield-component traits. Lack of correlation between stay-green and yield was also found in some previous reports (Wada and Wada 1991; Bolanos and Edmeades 1996).

Several explanations may be provided for the lack of, or negative, correlation between stay-green and yield, as observed in this study. One possible cause is that the experimental population was derived from an inter-subspecific cross and the partial sterility occurred in a fraction of the lines. Such partial sterility would result in difficulty for nutrient transport and re-location from leaves to other plant parts, especially to the developing seeds, which causes lower seed-setting rate accompanied by greener leaves due to higher chlorophyll content as a result of higher nitrogen concentration. We also performed QTL analysis for yield and yield-component traits (data not shown) and found that some of the QTLs for stay-green traits were co-localized or tightly linked to those for yield and its components, but the directions of

these QTL effects were opposite in increasing the trait performance. For example, the Zhenshan 97 allele of the QTL for rdgf around RM30 on chromosome 6 had a relatively large effect on decreasing the greenness, but a QTL was also detected in this region with the Zhenshan 97 allele showing a large effect on increasing yield. Cha et al. (2002) reported a recessive mutant (*sgr*) on chromosome 9 in rice, delaying the progress of yellowing but not functionally keeping the photosynthetic capability. Such gene actions may have partly contributed to the negative correlations between yield and stay-green as observed in this population. It may also be interesting to find out whether there is any favorable effect of stay-green on yield under stressed conditions, as in the cases reported in sorghum (Borrell et al. 2000).

It is also interesting to note that some of the QTLs for stay-green detected in this study appear to be syntenic to stay-green QTLs detected in other cereals. These may include the QTL for rrgs and rdgf around RM257 on chromosome 9 resolved in this study with the one for stay-green in the region around UMC5 on linkage group D in sorghum (Xu et al. 2000a). The QTL for rgas around RM118 on chromosome 7 detected in this study may correspond to the one around TXS1537 on linkage D2 of sorghum (Crasta et al. 1999). Such positional correspondence may imply the conserved organization of the genes in the genome and also the functions of the genes underlying the stay-green traits.

Stay-green phenotypes have now been observed in many crop species (Pierce et al. 1984; Gentinetta et al. 1986; Walulu et al. 1994; Fang et al. 1998; Spano et al. 2003). Making use of the stay-green traits to delay leaf senescence as a means to increase crop production, has remained an attractive strategy. Genetic and physiological understanding is necessary for making use of the stay-green characteristic in crop plants.

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