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QTL analysis for sugar-regulated leaf senescence supports flowering-dependent and -independent senescence pathways

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Summary

- The aim of this work was to determine the genetic basis of sugar-regulated senescence and to explore the relationship with other traits, including flowering and nitrogen-use efficiency.
- Quantitative trait loci (QTLs) for senescence were mapped in the Arabidopsis Bay-0 × Shahdara recombinant-inbred line (RIL) population after growth on glucose-containing medium, which accelerates senescence. The extent of whole-rosette senescence was determined by imaging the maximum quantum yield of photosystem II (F_v/F_m).
- A major QTL on the top of chromosome 4 colocalized with *FRI*, a major determinant of flowering. This QTL interacted epistatically with a QTL on chromosome 5, where the floral repressor *FLC* localizes. Vernalization accelerated senescence in late-flowering lines with functional *FRI* and *FLC* alleles. Comparison with previous results using the Bay-0 × Shahdara population showed that rapid rosette senescence on glucose-containing medium was correlated with early flowering and high sugar content in compost-grown plants. In addition, correlation was found between the expression of flowering and senescence-associated genes in Arabidopsis accessions. However, an additional QTL on chromosome 3 was not linked to flowering, but to nitrogen-use efficiency.
- The results show that whole-rosette senescence is genetically linked to the vernalization-dependent control of flowering, but is also controlled by flowering-independent pathways.

Introduction

Leaf senescence is an important nutrient recycling process. Nitrogen, especially, is exported out of the senescing leaves (Masclaux-Daubresse *et al.*, 2008) and can be used in other parts of the plants (e.g. for the production of new leaves or seeds). In monocarpic plants, such as Arabidopsis (*Arabidopsis thaliana*), whole-plant senescence is linked to reproduction. Surprisingly, sink demand of the inflorescence (as shown in sterile mutants or after removal of the inflorescence) does not control senescence of individual Arabidopsis leaves (Hensel *et al.*, 1993; Noodén & Penney, 2001). In addition, no effect on senescence was found in the late-flowering *constans* mutant (Hensel *et al.*, 1993). However, in other mutants with altered flowering senescence was also affected, such as in *terminal flower2* (Kim *et al.*, 2004), *axe1* (Wu *et al.*, 2008) and *de-etiolated2* (Chory *et al.*, 1991). The effect of altered senescence on flowering is also variable. For example, Miao *et al.* (2004) describe delayed flowering and senescence in RNAi plants with reduced expression of the senescence-induced transcription factor gene *WRKY53*, whereas Guo & Gan (2006) found no differences in development other than delayed senescence in mutants of the senescence-induced transcription factor *AtNAP*. This suggests interaction between senescence and flowering regulation, but also independent pathways for the regulation of these two life-history traits.

*These two authors contributed equally to this work.

In natural accessions of Arabidopsis, senescence of the largest leaves and of the whole rosette is correlated with flowering time, indicating a naturally evolved link between floral initiation and plant longevity (Levey & Wingler, 2005). Using a larger collection of accessions Balazadeh *et al.* (2008) confirmed these findings, but also showed that the correlation between whole-plant senescence and flowering is tighter than that between chlorophyll content of the largest leaves and flowering. Analysis of senescence regulation is complicated by the observation that senescence of leaves formed later during development and contributing to overall rosette longevity can be regulated in the opposite manner to senescence of the six first leaves (Diaz *et al.*, 2005; Masclaux-Daubresse *et al.*, 2007). For example, ^{15}N labelling experiments have shown that senescence of the first leaves mainly recycles nitrogen to the leaves that emerge later during development, thereby contributing to overall rosette longevity rather than reproduction (Diaz *et al.*, 2008). It is therefore important to differentiate between sequential senescence, which is responsible for nutrient recycling from the first leaves to the subsequent vegetative growth, and monocarpic senescence, which links senescence of the major rosette leaves to floral initiation. The mechanisms that determine the link between monocarpic senescence and floral initiation are unknown.

Most accessions of Arabidopsis normally used for senescence studies are early-flowering summer annuals. Their rapid life-cycle makes it possible to perform experiments in a short period of time. However, these accessions do not reflect the large variation in flowering time found in nature (Shindo *et al.*, 2005). Similar to winter crops, flowering of late-flowering, winter annual Arabidopsis accessions can be promoted by a prolonged period of cold temperature in a process called vernalization. The genes involved and the molecular mechanisms underlying vernalization have been explored in some detail (Sung & Amasino, 2005; Bäurle & Dean, 2006). Vernalization dependence mainly results from interaction of two genes, *FRIGIDA* (*FRI*) and *Flowering Locus C* (*FLC*). *FRI* activates expression of *FLC*, which is a MADS-box transcription factor that inhibits flowering. The combination of functional *FLC* and *FRI* alleles thus confers late flowering, whereas plants with either a nonfunctional *FRI* or *FLC* (or both) are summer annual and flower early in the absence of vernalization. In the cold, *FLC* expression is progressively silenced through histone modification, releasing the repression of flowering.

The genetic basis of senescence regulation can be determined with the help of quantitative trait locus (QTL) analysis. In addition to mapping the genomic regions that are responsible for a trait and the possible identification of candidate genes, QTL analysis provides the opportunity to compare whether different traits have a common genetic basis. For Arabidopsis, several QTLs were mapped for longevity of the sixth leaf and post-bolting rosette longevity at low and high nitrogen (Luquez *et al.*, 2006), with some QTLs colocalizing. Diaz *et al.* (2006) mapped QTLs for leaf yellowing and redness (owing to anthocyanin accumulation) under short days and low nitrogen supply for the Bay-0 × Shahdara recombinant inbred line (RIL) population. None of the QTLs for yellowing and redness colocalized, suggesting an independent genetic control of these two senescence-related traits. However, colocalization of QTLs for redness and flowering time was found, with redness being positively correlated with flowering time.

Recombinant-inbred lines of the Bay-0 × Shahdara population show large variation in senescence when cultivated on low nitrogen agar medium with addition of glucose, whereas variation in the absence of glucose is small (Diaz *et al.*, 2005). The variation in senescence found on glucose-containing agar medium was confirmed under more natural conditions in compost (Masclaux-Daubresse *et al.*, 2007), suggesting that this system is suitable for the analysis of the genetic basis of senescence regulation. Nevertheless, the role of sugar signalling in senescence regulation is still controversial (van Doorn, 2008). However, recent gene expression studies have provided evidence for a role of sugar accumulation rather than starvation in the initiation and/or acceleration of leaf senescence (Pourtau *et al.*, 2006; Wingler & Roitsch, 2008; Wingler *et al.*, 2009).

Based on these findings, QTL analysis in the present study was performed with plants grown on glucose-containing medium. Chlorophyll fluorescence imaging was used to determine the senescence-dependent decline in maximum quantum yield of photosystem II (F_v/F_m) of the whole leaf rosette to obtain a functional characterisation of the senescence process. Measurements were nondestructive, making it possible to monitor the extent as well as the rate of senescence. The aim was to compare the QTLs for F_v/F_m with QTLs for leaf yellowing of the first six leaves (Diaz *et al.*, 2006), flowering date (Loudet *et al.*, 2002), nitrogen-use efficiency (Loudet *et al.*, 2003) and carbohydrate content (Calenge *et al.*, 2006) in the Bay-0 × Shahdara population. As colocalization was found with flowering QTLs, specifically *FRI*, the effect of vernalization on flowering and senescence of lines with different combinations of functional and nonfunctional *FRI* and *FLC* alleles was also determined.

Materials and Methods

Plant material

The Arabidopsis (*A. thaliana* L.) Bay-0 × Shahdara RIL population has been fully described in a previous publication (Loudet *et al.*, 2002) and on http://dbsgap.versailles.inra.fr/vnat/Documentation/33/DOC.html. The F_8 seeds obtained from the last generation of single seed descent for 169 lines (core population) were used. Near-isogenic lines (NILs) were developed as heterogeneous inbred family (HIF) (Tuinstra *et al.*, 1997; Loudet *et al.*,

2005). At the F_6 stage, lines RIL312, RIL143 were homozygous everywhere on their genomes, except for a small region including few cM-intervals defined by the markers MSAT4.8 and NGA8 on chromosome 4. We planted 30 F_7 seeds from this line and genotyped the plants individually (as in Loudet *et al.*, 2002), selecting plants fixed for the Bay-0 allele (named HIF312-Bay and HIF143-Bay, respectively) and other plants fixed for the Shahdara allele (named HIF312-Sha and HIF143-Sha, respectively) at the two segregating markers. The F_8 seeds from these plants were then used for phenotyping. The same procedure was applied to RIL157, which is segregating for a small region including the interval defined by markers NGA225 and MSAT5.14 on chromosome 5, and for RIL404, which is heterozygous for at least the region located between the markers MSAT3.32 and MSAT318406 on chromosome 3. Additional heterozygosity for RIL404 was also found for marker K9I9 at the bottom of chromosome 5.

Phenotyping for QTL mapping and for the confirmation of QTLs

To analyse the effect of nitrogen limitation and glucose supply on senescence, the RILs (for QTL mapping) and NILs (for confirmation of QTLs) were grown on agar (1% w : v) plates with low nitrogen supply (LN; 4.7 mM) with or without addition of 2% glucose (Wingler *et al.*, 2004). Seeds suspended in 0.7% low-melting agarose were pipetted onto the agar plates in a row *c.* 2 cm from the edge. The plates were positioned vertically in a growth chamber in stacks of *c.* 20 plates (with the row of seeds in horizontal orientation near the top of the plates) and were illuminated for 16 h d^{-1} at a photon flux density of 100 μmol m^{-2} s^{-1}. The temperature was 21°C during the day and 18°C at night. For each RIL, four plates were used as four independent blocks, and two independent experiments were performed. Maximum quantum yield of photosystem II (F_v/F_m) was analysed using a FluorCam 700MF kinetic imaging fluorometer (Photon Systems Instruments, Brno, Czech Republic) as described previously (Wingler *et al.*, 2004). For each plant an F_v/F_m value for the whole rosette was determined reflecting the average F_v/F_m of most of the rosette leaves, but not of those old leaves that were covered by other leaves. Data for flowering (Loudet *et al.*, 2002), for leaf yellowing and redness on compost (Diaz *et al.*, 2006) and for sugar content (Calenge *et al.*, 2006) in the RIL population were used for comparison. All published phenotypes can be found at http://dbsgap.versailles.inra.fr/vnat/Documentation/33/DOC.html.

Statistical analysis and QTL mapping

Replicate and genotype effects were studied by analysis of variance with the PROC GLM procedure of the SAS Institute (1991; Carey, NC, USA) software. The genetic variance was estimated by using the PROC VARCOMP procedure of the SAS software, in which the genetic effect was assumed to be random. Heritability was estimated as $h^2 = \sigma^2 g/(\sigma^2 g + [\sigma^2 e/r])$, with $\sigma^2 g$ being the genetic variance, $\sigma^2 e$ the residual variance, and r the number of replicates. The original set of markers (38 microsatellite markers) and the genetic map obtained with MAPMAKER 3.0, as previously described (Loudet *et al.*, 2002; http://www.inra.fr/qtlat), were used in this study. First QTL analyses were performed with PLAB-QTL software (Utz & Melchinger, 1996), using a classical composite interval mapping strategy (Jansen, 1993; Zeng, 1994). Empirical threshold value for the LOD scores were determined by computing 10 000 permutations (Churchill & Doerge, 1994), using the 'permute' command of the PLABQTL software. QTL positions were determined at the local maxima of the LOD-curve plot in the region under consideration. Confidence intervals were set as the map interval corresponding to a 1-LOD decline on either side of the LOD peak. The proportion of phenotypic variance explained by a single QTL was obtained by the square of the partial correlation coefficient (R^2). Estimates of the additive effects of the QTL were computed by fitting a model including all putative QTLs for a given trait. Further QTL analyses were performed using the R package R/QTLBIM (Yandell *et al.*, 2007; Yi *et al.*, 2007). Bayes factor profiles were used to estimate the number of QTLs and the QTL effects together.

Determination of natural senescence and vernalization response

To determine natural senescence and vernalization response seeds were stratified for 3 d at 4°C in 0.1% (w : v) agar and then pipetted onto compost (Levington Multi-Purpose Compost; The Scotts Company, Godalming, UK). Plants were then either directly transferred to the standard growth conditions as described for the QTL analysis above, or vernalized first. For the vernalization treatment, plants were left at room temperature for 1 d to initiate germination. Plants were then incubated for 48 d at 4°C under 10 h illumination per day at a photon flux density of 25 μmol m^{-2} s^{-1}. After this treatment they were transferred into the standard growth conditions together with the nonvernalized plants. To compare vernalized and nonvernalized plants, early development was monitored closely. The dates were adjusted by matching the number of leaves during early development to allow comparison of the same developmental stages in vernalized and nonvernalized plants. The F_v/F_m was determined by imaging chlorophyll fluorescence in the whole rosette as described for plants grown on agar plates. In addition, relative chlorophyll content was measured nondestructively in the three largest leaves of each plant using a

chlorophyll meter (N-tester; Hydro-Agri, Immingham, UK).

Analysis of the relationship between the expression of flowering and senescence-associated genes

To determine the relationship between the expression of flowering and senescence-associated genes, 16 Arabidopsis accessions were grown in compost in a glasshouse supplemented with light at an intensity of 180–200 μmol m^{-2} s^{-1} for 16 h d^{-1}. Fifteen of these accessions (Ct-1, Oy-0, Sha, Blh-1, Bur-0, Jea, Cvi-0, Ge-0, Alc-0, St-0, Can-0, Pyl-1, N13, Mt-0 and Mh-1) belong to a genetically diverse set identified by McKhann *et al.* (2004). In addition, Bay-0 was included to replace Ita-0. The largest leaves were harvested on days 32 and 42. RNA was extracted in TRIzol (Invitrogen) and treated with Turbo DNase (Ambion) according to the manufacturer's instructions. 0.6 μg of RNA was reverse transcribed using the random hexamer protocol of the SuperScript III reverse transcriptase kit (Invitrogen). For each RNA sample, reactions were also run without reverse transcriptase (negative control) to check for DNA contamination. Real-time PCR was performed using an Applied Biosystems 7500 Real Time PCR System with Power SYBR Green (Applied Biosystems, Foster City, CA, USA) for detection. The PCR conditions consisted of an initial denaturing stage of 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct values) were determined using the SDS v1.2 software (Applied Biosystems). The ΔCt values were calculated by subtracting the Ct value for the reference gene (*UBC9*, At4g27960; Czechowski *et al.*, 2005) from that of the gene of interest. To compare accessions, ΔCt values for the early flowering accession Pyl-1 were subtracted from ΔCt values for each accession and each of the two time-points. Expression of the following genes was analysed: *SAG12* (At5g45890), *PAP2* (At1g66390), *GLN1;4* (At5g16570), *FRI* (At4g00650), *FLC* (At5g10140), *FT* (At1g65480) and *SOC1* (At2g45660). Primer sequences are given in the Supporting Information Table S1. If Ct values were not reached within 40 cycles in at least two out of three technical replicates, expression was considered too low for analysis and data were discarded. For correlation analysis, Pearson correlation coefficients were determined for the combined data from both time-points.

Results

Phenotypic variation, heritability, QTL mapping and interactions

F_v/F_m was measured on days 16, 29, 34, 36 and 40 after planting to characterise the senescence process in the Bay-0 × Shahdara RIL population. Imaging of F_v/F_m in whole plants was used to determine whole-rosette senescence, which during later stages of development reflects monocarpic senescence. For days 29 to 40, the heritability was high (from 0.56 to 0.87) demonstrating that the F_v/F_m during senescence was mainly genetically determined. Because the F_v/F_m values decreased linearly during the interval between days 29 and 40, the slope of the decline ($\Delta F_v/F_m$) representing the rate of senescence was calculated for each RIL.

In a first step, QTLs were determined using the PLABQTL software (Utz & Melchinger, 1996) to map loci with main additive effect on F_v/F_m values (Table 1). This program allowed mapping of significant QTLs at days 29, 34, 36 and 40. For these four dates, a main QTL for F_v/F_m was mapped on the top of chromosome 4 (F_v/F_m-29.1, F_v/F_m-34.1, F_v/F_m-36.1 and F_v/F_m-40.1), explaining 12.1–17.5% of the phenotypic variation. A minor QTL was detected on the bottom of chromosome 3 on days 29 and 34 (F_v/F_m-29.2 and F_v/F_m-34.2). For all QTLs detected, the decreasing allele came from Bay-0, meaning that the Bay-0 allele resulted in lower F_v/F_m values and therefore in earlier whole-rosette senescence. The experiment was repeated and the main QTL on chromosome 4 confirmed independently. No QTL interaction was detected using PLABQTL.

In order to check for interactions between the QTLs detected and to detect additional QTLs with weak effects, QTL epistasis was investigated using the R/QTLBIM software. This software is built on top of R/QTL and provides Bayesian analysis of multiple interacting QTL models (Yandell *et al.*, 2007; Yi *et al.*, 2007). Using this method, the main QTL for F_v/F_m (F_v/F_m-29.1, F_v/F_m-34.1, F_v/F_m-36.1 and F_v/F_m-40.1) was still detected on the top of chromosome 4, and numerous minor QTLs were mapped in other areas (see Tables 1, S2). This analysis also highlighted interactions at days 34 and 40 between the QTL for F_v/F_m on the top chromosome 4 (F_v/F_m-34.1 and F_v/F_m-40.1) and a minor QTL on the top of chromosome 5 (F_v/F_m-34.3 and F_v/F_m-40.2).

QTL detection for the rate for senescence (i.e. the decline in F_v/F_m, $\Delta F_v/F_m$) was carried out using the same software (Table 1). PLABQTL yielded two main QTLs, one of them on the top of chromosome 4 ($\Delta F_v/F_m$.1) colocalizing with QTLs for F_v/F_m (F_v/F_m-29.1, F_v/F_m-34.1, F_v/F_m-36.1 and F_v/F_m-40.1) and one on chromosome 2 ($\Delta F_v/F_m$.2). Interestingly, QTL $\Delta F_v/F_m$.1 interacted with a minor QTL on the top of chromosome 5 ($\Delta F_v/F_m$.3) detected by R/QTLBIM.

QTL comparisons

Confidence intervals of QTLs located on the top of chromosome 4 (F_v/F_m-29.1, F_v/F_m-34.1, F_v/F_m-36.1 and F_v/F_m-40.1) overlapped suggesting that the same QTL was detected at different dates and colocalized with the QTL

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Table 1 Quantitative trait locus (QTL) analysis of senescence on glucose-containing medium

Software	Trait	QTL	Chromosome	Position (cM)	Confidence interval (cM)	LOD	Variation explained (%)	Additive effect	Decreasing allele
PLABQTL	F_v/F_m-29	F_v/F_m-29.1	Chrom4	2	0–8	4.76	12.1	−0.01	Bay-0
		F_v/F_m-29.2	Chrom3	66	52–68	2.97	7.7	−0.008	Bay-0
	F_v/F_m-34	F_v/F_m-34.1	Chrom4	10	2–18	5.23	13.6	−0.024	Bay-0
		F_v/F_m-34.2	Chrom3	54	44–62	2.55	6.9	−0.015	Bay-0
	F_v/F_m-36	F_v/F_m-36.1	Chrom4	10	2–18	4.13	17.5	−0.033	Bay-0
	F_v/F_m-40	F_v/F_m-40.1	Chrom4	16	4–28	2.32	15.6	−0.027	Bay-0
	$\Delta F_v/F_m$	$\Delta F_v/F_m$.1	Chrom4	10	0–20	2.72	7.5	−0.003	Bay-0
		$\Delta F_v/F_m$.2	Chrom2	32	20–40	2.54	7	−0.002	Bay-0
R/QTLBIM	F_v/F_m-16	F_v/F_m-16.1	Chrom4	0.0		5.9	21.8		
	F_v/F_m-29	F_v/F_m-29.1	Chrom4	0.0		5.3	11.0		
		F_v/F_m-29.2	Chrom3	62.1		4.7	9.8		
	F_v/F_m-34	F_v/F_m-34.1	Chrom4	7.8		7.6	16.2		
		F_v/F_m-34.2	Chrom3	68.6		3.8	7.7		
		F_v/F_m-34.3	Chrom5	0.0		3.2	6.4		
		F_v/F_m-34.1*F_v/F_m-34.3				2.9	5.9		
	F_v/F_m-36	F_v/F_m-36.1	Chrom4	6.8		2.7	8.9		
	F_v/F_m-40	F_v/F_m-40.1	Chrom4	7.1		3.0	11.3		
		F_v/F_m-40.2	Chrom5	0.0		3.1	11.9		
		F_v/F_m-40.1*F_v/F_m-40.2				2.9	11.1		
	$\Delta F_v/F_m$	$\Delta F_v/F_m$.1	Chrom4	9.9		6.5	10.7		
		$\Delta F_v/F_m$.2	Chrom2	29.4		7.2	12.1		
		$\Delta F_v/F_m$.3	Chrom5	0.0		7.2	12.1		
		$\Delta F_v/F_m$.1*$\Delta F_v/F_m$.3				4.1	6.6		

The traits determined were F_v/F_m on days 16 to 40 (F_v/F_m-16, F_v/F_m-29, F_v/F_m-34, F_v/F_m-36, F_v/F_m-40) and the slope of the decline in F_v/F_m ($\Delta F_v/F_m$). The major QTLs mapped using the PLABQTL and R/QTLBIM software are presented. The complete list of the QTLs detected is presented in the Supporting Information, Table S2. For each trait measured, several QTLs were detected and named according the trait, day and rank of significance.

$\Delta F_v/F_m$.1 (Table 1). These QTLs mapped in the same region in which a QTL for flowering date (SD1 and LD1) has been detected by Loudet *et al.* (2002) and colocalize most likely with the *FRI* gene (At4g00650). A second QTL for flowering date (SD2 and LD2) also detected by Loudet *et al.* (2002) at the position of *FLC* (At5g10140) colocalized with the minor QTL on the top of chromosome 5 (F_v/F_m-34.3, F_v/F_m-40.2 and $\Delta F_v/F_m$.3). Furthermore, an interaction was detected between this minor QTL and the QTL on chromosome 4. A QTL for yellowness (YP3.4; Diaz *et al.*, 2006) colocalized with the QTLs F_v/F_m-29.2 and F_v/F_m-34.2 on chromosome 3. These loci on the bottom of chromosome 3 and on the top of chromosomes 4 and 5 are named hereafter PSII-3, PSII-4 and PSII-5, respectively (Fig. 1). Dependent on plant age, PSII-4 was responsible for between 9% and 22% of the variation in F_v/F_m, while PSII-3 explained between 7% and 10% of the variation found (Table 1).

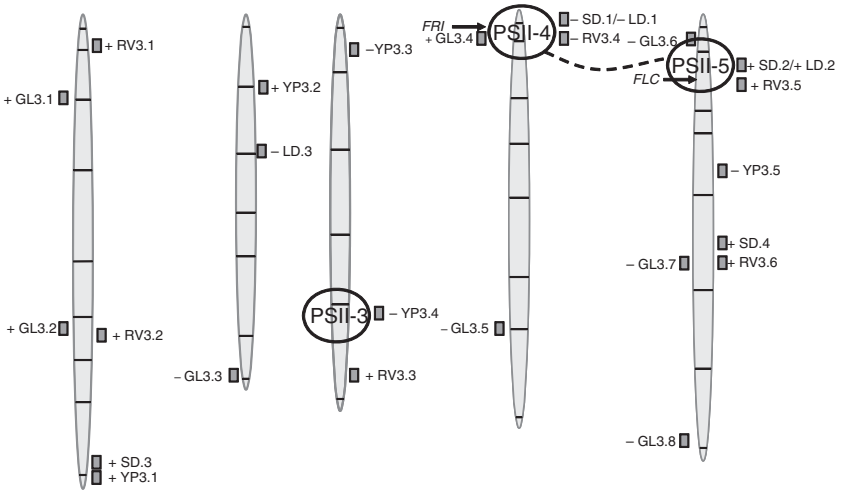

Fig. 1 Quantitative trait locus (QTL) locations (PSII-3, PSII-4 and PSII-5) on the genetic map. The dotted line indicates QTL interaction between PSII-4 and PSII-5. The QTLs for leaf yellowness and leaf redness (YP and RV; Diaz *et al.*, 2006), for flowering date under short days and long days (SD and LD; Loudet *et al.*, 2002) and for glucose content (GL; Calenge *et al.*, 2006) are also indicated. Minus (–) indicates that the Bay-0 allele has a decreasing effect, while plus (+) indicates an increasing effect on the trait. Estimated positions of *FRI* and *FLC* genes are indicated by black arrows.

In addition to colocalization with flowering time QTLs, the $\Delta F_v/F_m$ values showed a weak but significant (for $\alpha = 0.05$) positive correlation with the flowering date in short and long days noted by Loudet *et al.* (2002) for RILs of the Bay-0 × Shahdara population (correlation coefficient $r = 0.23$ and $r = 0.27$, respectively). This indicates that RILs that senesced faster (resulting in a more negative slope) flowered earlier. Moreover, there was a negative correlation with the sugar content measured by Calenge *et al.* (2006) (correlation coefficient $r = -0.26$), suggesting that RILs that contained more sugar senesced faster.

Confirmation of the QTL at PSII-3 using NILs

Near-isogenic lines from the heterogeneous inbred family (HIF) 404 were used to confirm the QTL on chromosome 3 (PSII-3). These NILs were raised through self-fertilization of the recombinant-inbred line RIL404, which was heterozygous for at least the region located between the markers MSAT3.32 and MSAT318406 on chromosome 3. HIF404-Bay has the Bay-0 section of chromosome 3, whereas HIF404-Sha has the Shahdara genotype in this region. Our QTL analysis suggested that the Bay-0 allele at this locus decreases F_v/F_m, indicating accelerated senescence (Table 1). On low nitrogen medium with glucose HIF404-Bay showed an earlier senescence-dependent decline in F_v/F_m than HIF404-Sha (Fig. 2), thus supporting the QTL for glucose-induced senescence. However, F_v/F_m dropped temporarily (between days 11 and 19) in HIF404-Sha before recovering when new leaves were formed. This is in agreement with the finding that the Bay-0 allele at this locus confers late senescence in the six first leaves (Diaz *et al.*, 2006).

Confirmation of the QTL at PSII-4 using near-isogenic lines

To confirm the QTL on chromosome 4 (PSII-4) NILs from two HIFs were used. These NILs originated from RIL312 and RIL143, which were heterozygous for at least the region located between the markers MSAT4.8 and NGA8 (Supporting Information Fig. S1). Owing to the interaction between PSII-4 and PSII-5, HIF312 and HIF143 were chosen as they differ in the top of chromosome 5 at the location of PSII-5. While HIF312 has a Bay-0 section of this chromosome, HIF143 has the Shahdara genotype at PSII-5.

For HIF312, F_v/F_m on glucose-containing medium declined earlier in the line with the Bay-0 allele at PSII-4 (HIF312-Bay) than the line with the Shahdara allele (HIF312-Sha) (Fig. 3a), confirming the QTL at PSII-4 and the negative effect of the Bay-0 allele on F_v/F_m. No senescence was observed during the duration of the experiment for plants growing without glucose and the F_v/F_m of HIF312-Bay and HIF312-Sha was similar. For HIF143, only a small difference was observed between HIF143-Bay and HIF143-Sha on glucose-containing medium at the end of the experiment (Fig. 3b), but again the Bay-0 chromosome region was associated with lower F_v/F_m. For HIF312-Bay and HIF312-Sha differences in senescence on glucose-containing medium were visible as leaf yellowing (Fig. 4a). The difference in senescence was confirmed for plants grown on compost (Fig. 4b). HIF312-Sha was also characterized by late flowering. Again, results for HIF143-Bay and HIF143-Sha were not as clear but, in addition to slightly earlier flowering, HIF143-Bay showed a reduction in rosette size. These findings demonstrate that the QTL at PSII-4 had a larger effect in combination with the Bay-0 allele than with the Shahdara allele at PSII-5, confirming the epistatic interaction found between these two QTL.

Effect of vernalization on senescence in near-isogenic lines for the QTL at PSII-4

Owing to the late flowering of HIF312-Sha (Fig. 4) and the colocalization of the senescence QTL determined here with *FRI* and *FLC* (Fig. 1) the question arose whether the senescence phenotype was related to differences in floral initiation. Of the parental lines, Shahdara has a functional

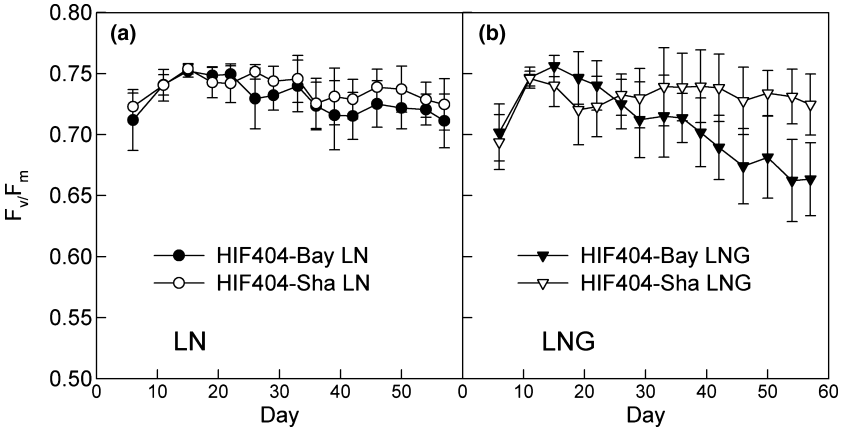

Fig. 2 Senescence-related decline in F_v/F_m in near-isogenic lines for PSII-3. Near-isogenic lines from the heterogeneous inbred family HIF404 with the Bay-0 (closed symbols) or Shahdara (open symbols) genomic regions for the quantitative trait locus (QTL) at PSII-3 were grown on low-nitrogen medium without glucose (LN; a) or with addition of 2% glucose (LNG; b). Data are means of at least five plants for LN and 25 plants for LNG ± SD.

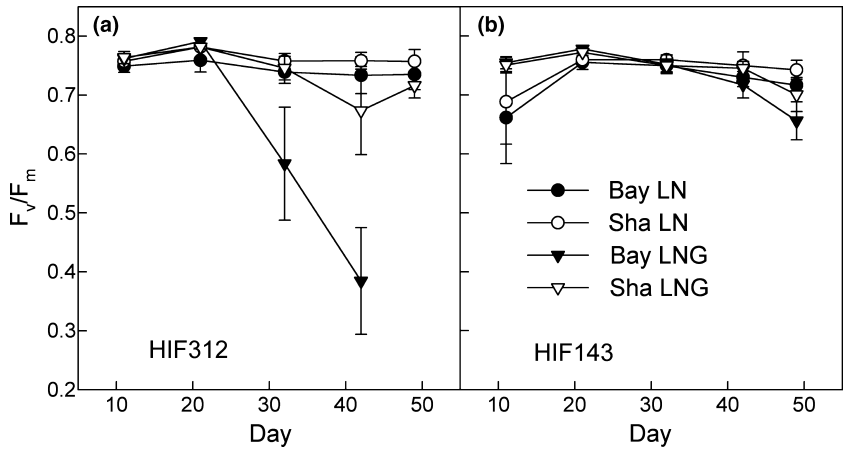

Fig. 3 Senescence-related decline in F_v/F_m in near-isogenic lines for PSII-4. Near-isogenic lines from the heterogeneous inbred families HIF312 (a) and HIF143 (b) with the Bay-0 (closed symbols) or Shahdara (open symbols) genomic regions for the quantitative trait locus (QTL) at PSII-4 (see the Supporting Information, Fig. S1 for chromosome maps of HIF312 and HIF143) were grown on low-nitrogen medium without (LN; circles) or with addition of 2% glucose (LNG; triangles). Data are means of at least 13 plants ± SD.

allele of *FRI* and a weak, but probably partially functional (Michaels *et al.*, 2003) allele of the floral repressor *FLC*, resulting in early flowering (Loudet *et al.*, 2002). In Bay-0, by contrast, *FLC* is functional, whereas *FRI* is not, resulting in low expression of *FLC* and, again, early flowering. However, combination of the Shahdara *FRI* and the Bay-0 *FLC* alleles results in a late-flowering phenotype, as seen in, for example, HIF312-Sha (see the Supporting Information Fig. S1). By contrast, HIF143-Sha combines the Shahdara *FRI* and *FLC* alleles, resulting in later flowering than in HIF312-Bay, but earlier flowering than in HIF312-Sha.

As vernalization promotes flowering in late-flowering Arabidopsis accessions, it was tested how vernalization affects flowering and senescence in compost-grown plants of HIF143 and HIF312. For HIF312, the Shahdara genomic region clearly delayed the senescence-dependent decline in F_v/F_m and in the chlorophyll content of plants grown without vernalization in compost (Fig. 5a,c). This was accompanied by late flowering, as indicated by flowering time (not shown) and the number of rosette leaves formed until flowering (Fig. 5e). Conversely, HIF312-Bay senesced and flowered early. Vernalization abolished the differences between HIF312-Sha and HIF312-Bay, resulting in early flowering and senescence in both lines. For HIF143, the differences between nonvernalized lines with the Bay-0 and Shahdara genomic regions were smaller than in HIF312, but again the Shahdara genomic region conferred later senescence (Fig. 5b,d) and flowering (Fig. 5f), while vernalization resulted in early senescence and flowering in both lines. These findings suggest that vernalization overcomes the differences in senescence regulation and that senescence is related to flowering time control. This is also indicated by a strong positive correlation between chlorophyll content after the start of senescence and the number of leaves at flowering in nonvernalized lines (including HIF312 and HIF143, the parental lines Shahdara and Bay-0 and the late-senescing RIL310; Fig. S2).

Confirmation of the QTL at PSII-5 using NILs

If allelic variation in *FLC* is responsible for differences in senescence regulation, this effect would only be detectable in a background with a functional *FRI* to activate *FLC* expression. To confirm the QTL at PSII-5 in the presence of a strong *FRI* allele, NILs were used that were obtained from RIL157, which contained the functional *FRI* from

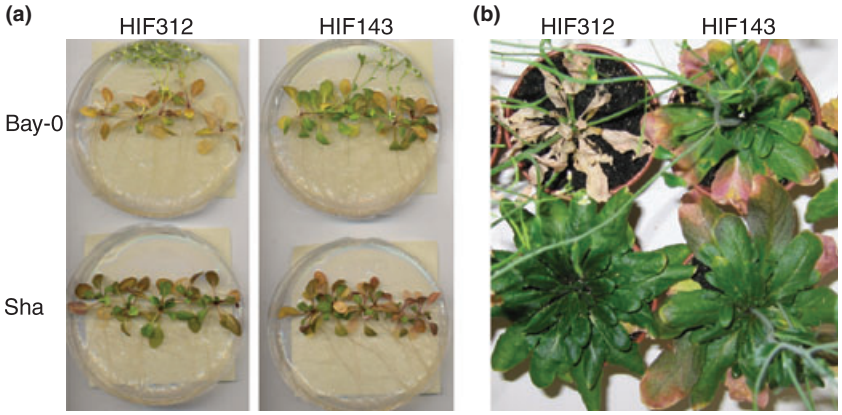

Fig. 4 Senescence phenotype of near-isogenic lines for PSII-4. Near-isogenic lines from the heterogeneous inbred families HIF143 and HIF312 with the Bay-0 or Shahdara genomic regions for the quantitative trait locus (QTL) at PSII-4 were grown for 32 d on low-nitrogen medium with 2% glucose (a) and for 43 d in compost (b).

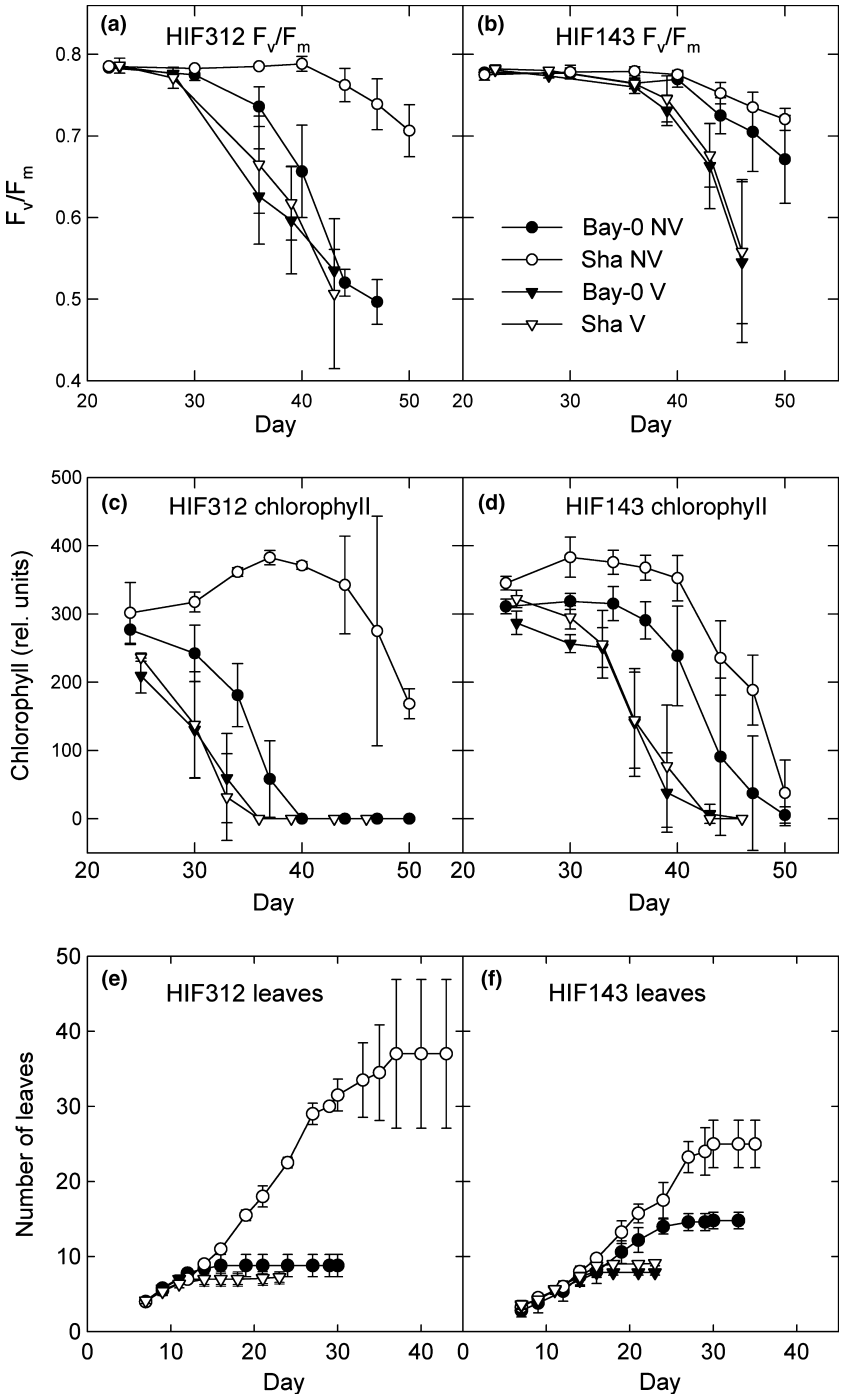

Fig. 5 Effect of vernalization on near-isogenic lines for PSII-4. Senescence-related decline in F_v/F_m (a,b) and chlorophyll (c,d), and number of primary rosette leaves until flowering (e,f) were determined in near-isogenic lines from the heterogeneous inbred families HIF312 and HIF143 with the Bay-0 (closed symbols) or Shahdara (open symbols) genomic regions for the QTL at PSII-4 (see the Supporting Information, Fig. S1 for chromosome maps of HIF143 and HIF312). Plants were grown in compost and either not vernalized (NV; circles) or vernalized at 4°C for 7 wk before the start of the experiment (V; triangles). Data are means of two to four plants ± SD.

Shahdara, but was heterozygous in the region located between NGA225 and MSAT5.14 (Fig. S1). The line with the functional allele of *FLC* (HIF157-Bay) would be expected to show delayed senescence compared with the line with the weak allele of *FLC* (HIF157-Sha). F_v/F_m of HIF157-Bay and HIF157-Sha was monitored on medium with glucose compared with medium without sugar. For any given time-point there was no clear difference between F_v/F_m of HIF157-Bay and HIF157-Sha on glucose-containing medium (Fig. 6). Despite only small differences in F_v/F_m between the two lines for each time-point, the slope (i.e. $\Delta F_v/F_m$) between days 32 and 46 was different (Mann–Whitney Test; $\alpha = 0.05$), indicating a faster rate of senescence in HIF157-Sha than in HIF157-Bay on the medium with glucose. This confirms the QTL for $\Delta F_v/F_m$ at PSII-5 (Table 1). However, the Shahdara *FLC* is not a

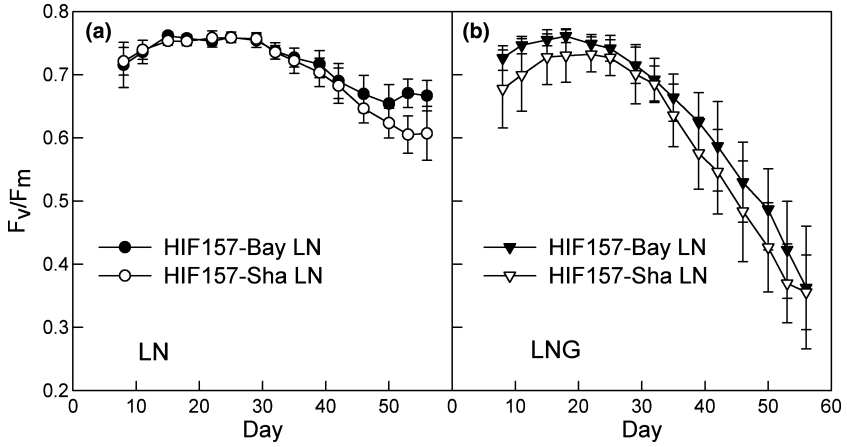

Fig. 6 Senescence-related decline in F_v/F_m in near-isogenic lines for PSII-5. Near-isogenic lines from the heterogeneous inbred family HIF157 with the Bay-0 (closed symbols) or Shahdara (open symbols) genomic regions for the quantitative trait locus (QTL) at PSII-5 (see the Supporting Information, Fig. S1 for chromosome map of HIF157) were grown on low-nitrogen medium without glucose (LN; a) or with addition of 2% glucose (LNG; b). Data are means of at least 23 plants ± SD.

null allele and may show partial activity (Michaels *et al.*, 2003), which could explain why differences between HIF157-Bay and HIF157-Sha were small.

Effect of vernalization on senescence in NILs for the QTL at PSII-5

As vernalization results in silencing of *FLC*, the impact of vernalization on the senescence-dependent decline in chlorophyll content and on the number of leaves until flowering was determined in HIF157-Bay and HIF157-Sha. The vernalization experiment was also repeated for HIF312 to confirm that the results were reproducible and that the vernalization treatment was successful. As expected, all vernalized plants flowered and senesced early, as indicated by the early decline in chlorophyll (Fig. 7a,b). For nonvernalized lines, the Shahdara *FRI* in HIF312 and the Bay-0 *FLC* in HIF157 conferred late flowering (indicated by the larger number of leaves at flowering) (Fig. 7c,d), which is consistent with the functional *FLC* from Bay-0 and the weak *FLC* from Shahdara reported in the literature. However, the contrast in flowering between HIF157-Sha and HIF157-Bay was not as pronounced as for the HIF312 lines. In addition, no difference in senescence was found between HIF157-Sha and HIF157-Bay, suggesting that *FLC* itself does not play a major role in natural senescence. Nevertheless, vernalization strongly accelerated senescence in both HIF157-Sha and HIF157-Bay.

Relationship between the expression of flowering and senescence-associated genes

We determined the expression of flowering and senescence-associated genes in 16 Arabidopsis accessions. The rationale was that this analysis would enable us to use the same leaf material to assess the state of flowering regulation and senescence response in each plant and then determine the relationship by comparing different plants. As a senescence marker, we used the cytosolic glutamine synthetase gene *GLN1;4*, whose expression increases strongly during senescence (Buchanan-Wollaston *et al.*, 2005; Pourtau *et al.*, 2006). Cycle threshold (Ct) values were determined by quantitative real-time polymerase chain reaction (qRT-PCR). After subtracting Ct values for the constitutive reference gene *UBC9* to obtain ΔCt values, differences between ΔCt for each accessions and ΔCt for the early-flowering reference accession Pyl-1 were calculated to obtain ΔΔCt values. A ΔΔCt value above zero indicates lower expression compared with Pyl-1, whereas a value below zero indicates higher expression. Expression of *GLN1;4* showed a statistically significant positive correlation with expression of the flowering genes *FT* ($r = 0.731$) and *SOC1* ($r = 0.544$), whereas a negative correlation was found between the expression of *GLN1;4* and *FLC* ($r = -0.582$; Fig. 8a–c, Table S3). As *FT* and *SOC1* promote flowering and their expression is reduced by *FLC*, these results support a positive relationship between flowering and senescence within a plant. Expression of the senescence markers *PAP2* and *SAG12* was also analysed. However, as expression of these two genes was very low in pre-senescent leaves, values could not be obtained for all ecotypes. Nevertheless, the expected negative correlation was found between *SAG12* and *FLC* expression and positive correlations were found between *PAP2* and *SOC1*, *PAP2* and *SAG12* and *PAP2* and *GLN1;4* expression (Table S3). We did not expect to find a correlation between the expression of *FRI* and senescence genes as functionality of *FRI* rather than its expression is important, and our results support this (Fig. 8d).

Discussion

Mutant and transgenic studies have resulted in contradictory findings concerning the relationship between flowering and senescence in Arabidopsis (Chory *et al.*, 1991; Hensel *et al.*, 1993; Kim *et al.*, 2004; Miao *et al.*, 2004; Guo & Gan, 2006; Wu *et al.*, 2008). However, such studies are

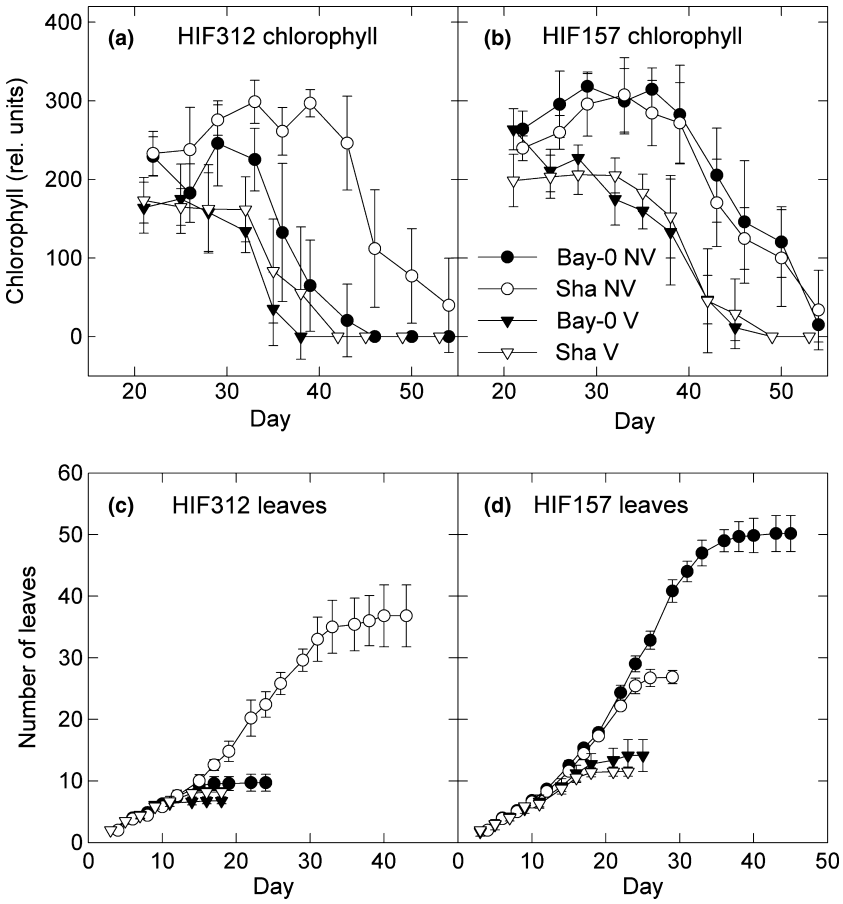

Fig. 7 Effect of vernalization on near-isogenic lines for PSII-5. Senescence-related decline in chlorophyll (a,b) and number of leaves (c,d) were determined in near-isogenic lines from the heterogeneous inbred families HIF157 and HIF312 with the Bay-0 (closed symbols) or Shahdara (open symbols) genomic regions for the quantitative trait locus (QTL) at PSII-4 (for HIF312) or at PSII-5 (for HIF157) (see the Supporting Information, Fig. S1 for chromosome maps of HIF312 and HIF157). Plants were grown in compost and either not vernalized (NV; circles) or vernalized at 4°C for 7 wk before the start of the experiment (V; triangles). Data are means of four to five plants ± SD.

limited to the role of individual genes, whereas the QTL-based approach used here does not make any prior assumptions about the genes or pathways involved. This work allowed us to dissect flowering-dependent and flowering-independent senescence pathways. The quantitative trait used was whole-rosette senescence monitored by imaging of F_v/F_m. During later stages, whole-rosette senescence determined in this way mainly reflects monocarpic senescence. Our results suggest that flowering and whole-rosette senescence are genetically linked by the vernalization pathway (Figs 4,5,7 and Fig. S2) and determined by a main QTL on the top of chromosome 4 (PSII-4) with epistatic interaction with a minor QTL on chromosome 5 (PSII-5) (Fig. 1). In addition, a flowering-independent QTL for sugar-induced senescence was linked to nitrogen-use efficiency and mapped to chromosome 3 (PSII-3).

Flowering-independent senescence regulation

PSII-3 was localized in the same position (Fig. 1) as the leaf yellowing QTL for the six first leaves (YP3.4; Diaz *et al.*, 2006), which probably also corresponds to a QTL for 6th leaf longevity and post-bolting rosette longevity mapped in the L*er* × Cvi population (Luquez *et al.*, 2006). This QTL has not been identified as a flowering QTL by Loudet *et al.* (2002) and the HIF404-Bay and HIF404-Sha lines used here (Fig. 2) both flowered late, but at the same time. This suggests that this locus affects senescence in a flowering-independent manner. Interestingly, the allelic effect was opposite here (with the Bay-0 allele accelerating rosette senescence, i.e. decreasing F_v/F_m) compared with Diaz *et al.* (2006), where the Bay-0 allele delayed yellowing of the first six leaves. However, the temporary decrease in F_v/F_m in HIF404-Sha also indicates early senescence of the first leaves, before the plants recovered and leaves formed subsequently senesced late. These findings show an involvement of PSII-3 in the opposite regulation of sequential and monocarpic senescence. The QTLs for nitrogen-use efficiency under nitrogen-limiting conditions, including QTLs for total nitrogen percentage (NP3.5) and for free amino acid content (AA3.4), also localized to this chromosome region (Loudet *et al.*, 2003). It is therefore possible that the gene underlying this PSII-3 is a gene for nitrogen metabolism, such as *ASN1*, as suggested by Diaz *et al.* (2006). The Bay-0 allele at this locus decreases total shoot nitrogen and amino acid content (Loudet *et al.*, 2003). This is consistent with a model in which sequential senescence of the first leaves during vegetative development provides nitrogen for

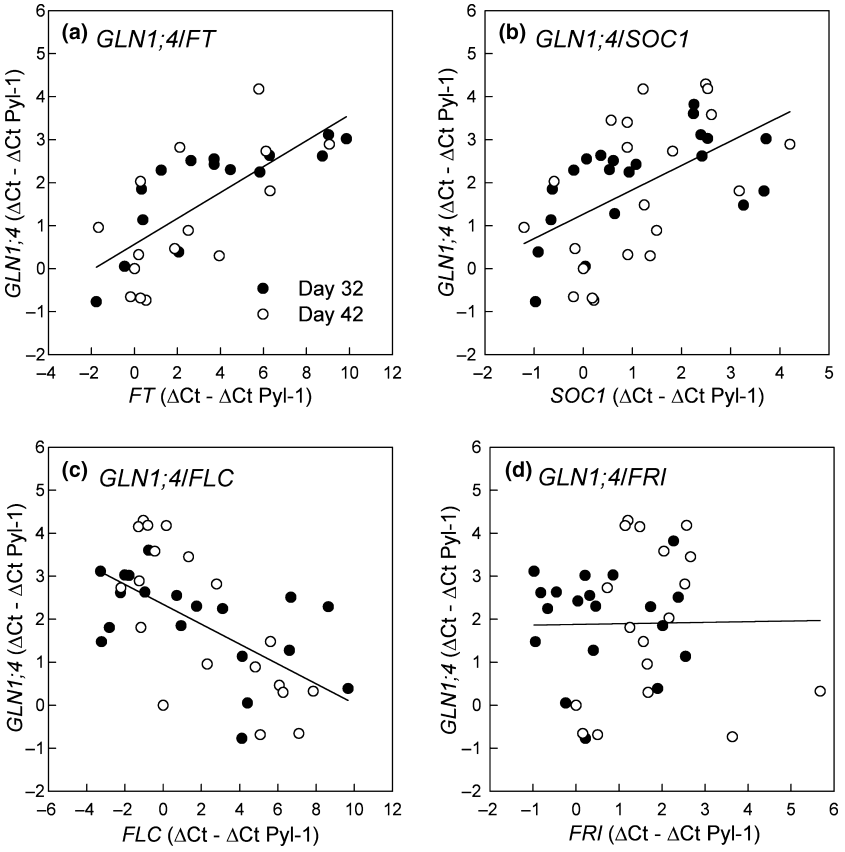

Fig. 8 Relationship between the expression of the senescence-associated gene *GLN1;4* (At5g16570) and flowering genes. Gene expression was determined by quantitative real-time polymerase chain reaction (qRT-PCR) in Arabidopsis accessions harvested at days 32 and 42. After subtracting the cycle threshold (Ct) value for the reference gene (*UBC9*) to calculate ΔCt values, the difference between the ΔCt value of each accession and that of Pyl-1 was calculated to compare the accessions. The senescence-associated genes were (a) *FT* (At1g65480), (b) *SOC1* (At2g45660), (c) *FLC* (At5g10140) and (d) *FRI* (At4g00650).

the new leaves (Diaz *et al.*, 2008) and delayed senescence of the six first leaves thus results in lower nitrogen content in rosette leaves formed later. Conversely, accelerated monocarpic senescence would lower nitrogen content of the whole leaf rosette.

Flowering-dependent senescence regulation

In addition to colocalization of QTLs for sugar-regulated senescence with flowering QTLs mapped by Loudet *et al.* (2002), correlation between flowering and sugar-regulated senescence in the Bay-0 × Shahdara RIL population confirms the genetic link between these two life-history traits. The results from the QTL analysis reflect whole-rosette senescence, but were confirmed when chlorophyll content was measured in the largest leaves (Figs 5,7). We concentrated on the analysis of the largest leaves, as it was not possible to compare the same leaf positions. Because of the large difference in the number of leaves formed, leaf positions that developed into major rosette leaves in early-flowering lines were shaded and never fully expanded in late-flowering lines and thus not comparable. To confirm our result that the senescence state of major rosette leaves is associated with floral development, we showed that the expression of senescence and flowering genes was correlated in the expected manner (Fig. 8).

Whether Arabidopsis plants are early- or late-flowering depends to a large extent on interaction between *FRI* and *FLC*. In the population used here, Shahdara has a functional *FRI* (Johanson *et al.*, 2000) but a weak allele of *FLC* (Gazzani *et al.*, 2003; Michaels *et al.*, 2003), whereas the opposite is the case for Bay-0 (Caceido *et al.*, 2004). As a result, both parental lines are early flowering, but combination of functional *FRI* and *FLC* alleles in the progeny can result in late flowering. The QTL PSII-4 colocalizes with *FRI* (Fig. 1) and was confirmed using NILs from HIF312 (Figs 3–5,7). To confirm a role of *FRI*, the effect of growth on glucose-containing medium on senescence was also determined in a different background, Col-2, which contains a functional *FLC* but an inactive *FRI*. Transfer of the functional *FRI* from Sf-2 into Col-2 results in late flowering (Lee & Amasino, 1995) and in delayed senescence on glucose (Fig. S3), thus supporting our hypothesis that *FRI*, or a closely linked gene, plays an important role in the regulation of senescence. Additional evidence for an involvement of *FRI* comes from the epistatic interaction between the QTL at PSII-4 and a minor QTL at PSII-5, which is in agreement with the function of *FRI* as activator of *FLC* expression.

Colocalization was also found for PSII-4 with a QTL for leaf redness caused by anthocyanin accumulation, RV3.4 (Diaz *et al.*, 2006), a trait that is often related to plant age.

The Bay-0 allele conferred accelerated senescence here, whereas it delayed anthocyanin accumulation in Diaz *et al.* (2006). Furthermore, colocalization of RV3.5, with PSII-5 and *FLC* suggests a link between anthocyanin accumulation and flowering time control. Considering that yellowing of the six first leaves is independent of monocarpic senescence and leaf redness, it is not surprising that no QTL for yellowing of the six first leaves was found at PSII-4 in the Bay-0 × Shahdara population (Diaz *et al.*, 2006). In addition, no QTL in this position was detected in the Cvi × L*er* population (Luquez *et al.*, 2006), which was expected as neither Cvi (Gazzani *et al.*, 2003) nor L*er* (Lee & Amasino, 1995) have a functional *FRI* allele.

It has recently been shown that mutation of a histone deacetylase gene, *HDA6*, results in hyperacetylated *FLC* chromatin, increased *FLC* expression, delayed flowering and delayed senescence (Wu *et al.*, 2008). This is consistent with our finding that *FLC* expression is negatively correlated with the expression of the senescence-associated genes *GLN1;4* and *SAG12* (Fig. 8, Table S3). As *FLC* is epigenetically silenced by vernalization and the effect of vernalization on senescence has not previously been studied in Arabidopsis, we determined the effect of vernalization here (Figs 5,7). This confirmed that vernalization accelerates senescence in late-senescing lines. In agreement with the role of *HDA6* identified by Wu *et al.* (2008), this effect could be caused by silencing of *FLC*, but also by vernalization-dependent chromatin modification of other genes involved in senescence regulation.

Link between sugar signalling, senescence and flowering

Quantitative trait locus analyses were performed for plants grown on glucose-containing medium. This treatment had previously been shown to result in the activation of senescence pathways reflecting developmental senescence without resulting in enhanced stress (Pourtau *et al.*, 2006; Wingler & Roitsch, 2008). Senescence under this condition is also representative of rosette senescence under more natural conditions (as shown here and by Masclaux-Daubresse *et al.*, 2007). For the Bay-0 × Shahdara RIL population a reverse relationship between sugar content and the extent of senescence of the first leaves was found (Calenge *et al.*, 2006; Diaz *et al.*, 2006). By contrast, comparison with the results for senescence presented here suggest that RILs with higher sugar content (after growth in the absence of sugar) senesce more rapidly. The role of sugars in senescence regulation may thus differ for sequential senescence during vegetative development and monocarpic senescence. A role of sugar accumulation in monocarpic senescence is also supported by a QTL for sucrose and glucose content, most likely *FRI* (Calenge *et al.*, 2006), colocalizing with the major QTL at PSII-4 found here. The allelic effect is that the nonfunctional Bay-0 allele increases sugar content and results in earlier flowering and in earlier senescence.

Whether sugars play a role in floral initiation is not clear. We often, but not always, find that plants bolt slightly earlier in the presence than in the absence of glucose. In addition, the carbon : nitrogen ratio in the phloem sap increases during floral induction (Corbesier *et al.*, 2002) and, in the dark, sucrose promotes flowering (Roldán *et al.*, 1999). This has led to the speculation that sucrose may be the enigmatic 'florigen', but more recent work suggests that Flowering Locus T (FT) protein is a more likely candidate for the signal from the leaves that induces flowering in the shoot apex (Corbesier *et al.*, 2007). It is also possible that instead of flowering being initiated by sugars, flowering could result in increased sugar content in the leaves and thus trigger senescence. An impact of flowering on metabolism is supported by the effect of mutations resulting in altered flowering time on the expression of metabolic genes, such as amylase genes (Wilson *et al.*, 2005). We did not find any major effect of sugar supply on the expression of genes involved in flowering control (data not shown), making it more likely that floral initiation results in metabolic changes leading to senescence than that sugars initiate flowering.

Implications for fitness

Interactions between sugar signalling, senescence and flowering could also have wider implications for fitness, especially in response to temperature. We have shown that cold acclimatization reduces the sensitivity of senescence to exogenously supplied glucose (Masclaux-Daubresse *et al.*, 2007). Interestingly, glucose resulted in increased expression of cold-responsive genes in a late-senescing RIL with both functional *FRI* and *FLC* alleles. This response could be part of the mechanism responsible for reduced sugar sensitivity in this line. The consequences for fitness of a strong *FRI* allele are also dependent on temperature and determined by *FLC*, as has been demonstrated in natural accessions of Arabidopsis grown in the field (Korves *et al.*, 2007). While a functional *FRI* improved winter survival in one *FLC* genetic background, it reduced seed production in spring-germinating plants in another *FLC* background. Thus, the direct effects of *FRI* on reproductive development maximize plant fitness in interaction with environmental conditions and concurrent regulation of senescence may be an integral part of this ecological adaptation.

The importance of senescence for fecundity is supported by previous work showing that the number of fruits formed is positively correlated with plant senescence in Arabidopsis accessions (Levey & Wingler, 2005; Balazadeh *et al.*, 2008). In addition, there is a trade-off between plant weight and fecundity when different genotypes are compared (Aarsen & Clauss, 1992). These findings suggest that the link between flowering and senescence regulation determines the

allocation of resources between leaves and seeds and thus fecundity. A combination of functional *FRI* and *FLC* alleles that results in late flowering and senescence may confer higher tolerance to environmental stress because of a greater investment in structural leaf components. Conversely, early flowering and senescing accessions may recycle nutrients more rapidly resulting in a ruderal strategy with high fecundity. Ultimately, which of these two genetic predispositions proves more successful for an individual depends upon the environment it experiences.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Genotypes of the heterogeneous inbred families HIF312, HIF143 and HIF157.

Fig. S2 Correlation between chlorophyll content during senescence and the number of leaves at flowering.

Fig. S3 Senescence in a line with *FRI* from SF2 in the Col-2 background.

Table S1 Primer sequences used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Table S2 Minor quantitative trait loci (QTLs) detected using the R/QTLBIM software

Table S3 Correlation between the expression of flowering and senescence-associated genes

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