New Phytologist

Letters

From crop to model to crop: identifying the genetic basis of the staygreen mutation in the *Lolium/Festuca* forage and amenity grasses

Lolium/Festuca staygreen in context

The practical application of model systems to crop species is a topic of much current debate in the plant science community, and in this Letter we describe the progress made in determining the genetic control of staygreen in the *Lolium/Festuca* grasses, using a combination of experimental genetic analysis and publicly available genomic and transcriptomic resources. The analysis of *Lolium/Festuca* staygreen can be considered to be a useful model in this regard, as staygreen is an economically significant (and genetically recessive) trait for which molecular markers are required for efficient breeding purposes, and the gene determining staygreen plays a fundamental but, as yet, unspecified role in chlorophyll degradation. Additionally, as *Lolium/Festuca* grasses have been well characterized in terms of synteny with rice (*Oryza sativa*) (Armstead *et al.*, 2002; Jones *et al.*, 2002; Alm *et al.*, 2003; Sim *et al.*, 2005), the system is sufficiently developed to allow advantage to be taken of the available comparative genomics resources. In this Letter we will refer to the wild-type (normal yellowing) and mutant (staygreen) loci as *Y* and *y*, respectively.

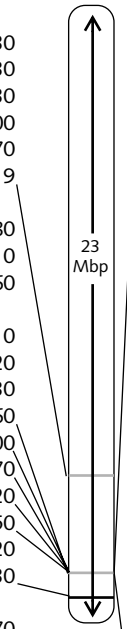
Mutation of a gene regulating the pathway of chlorophyll degradation has been shown to result in the indefinite retention of greenness in senescing leaves of the temperate grass *Festuca pratensis* (Thomas & Stoddart, 1975; Thomas, 1987; Vicentini *et al.*, 1995), a phenotype that has been incorporated into *Lolium/Festuca* amenity grasses in order to enhance their year-round utility (e.g. AberNile; Department for Environment, Food and Rural Affairs, 2006, p. 6). Staygreen in *Lolium/Festuca* and other species is an example of one of a number of distinct genetic variants that interfere with the normal expression of senescence (Thomas & Smart, 1993; Thomas & Howarth, 2000) and advanced varieties of some of the world's major crops, for example maize (*Zea mays*) and *Sorghum* (Thomas & Howarth, 2000; Morgan *et al.*, 2002; Valentinuz & Tollenaar, 2004) owe their productivity and stress tolerance, at least in part, to the staygreen trait. The detailed biochemistry, cell biology, physiology, genetics and introgressive gene transfer of the staygreen phenotype from *F. pratensis* have been extensively described (Hauck *et al.*, 1997; Kingston-Smith *et al.*, 1997; Thomas *et al.*, 2002; Moore *et al.*, 2005).

Crops and models: the experimental approach

The development of the initial *Lolium/Festuca* mapping population ($n = 100$) segregating for *F. pratensis*-derived *y* was reported previously (Moore *et al.*, 2005) and this population was used to assign *y* to *Lolium/Festuca* chromosome (C) 5 using anchored comparative mapping markers (Table 1a). Earlier studies (Armstead *et al.*, 2002; Jones *et al.*, 2002; Alm *et al.*, 2003; Sim *et al.*, 2005) had indicated that this region of *Lolium/Festuca* C5 shows a degree of synteny with rice C9, a rice chromosome known to contain a quantitative trait locus (QTL) for staygreen (Cha *et al.*, 2002). Using rice sequences taken from the The Institute for Genome Research (TIGR) rice C9 pseudomolecule (http://www.tigr.org/tdb/e2k1/osa1/), which flanked the position of this QTL as templates for primer design, it was possible to develop comparative mapping markers which demonstrated that *F. pratensis*-derived and rice staygreen phenotypes were determined from syntenically equivalent genomic regions. From an extended mapping family of 1627 individuals, 60 genotypes were identified which showed recombination in a 10-cM interval around *y*. Further implementation of the comparative mapping strategy based on these 60 recombinant genotypes allowed refinement of the relationship between *Lolium/Festuca* C5 and rice C9 to the extent that *F. pratensis*-derived staygreen could be localized to an equivalent region of rice C9 consisting of *c.* 200 kb, which contained 30 rice gene models (Table 1a,b). The annotations of these gene models in the TIGR database indicated that one of these, LOC_Os09g36200, was predicted to be a senescence-inducible chloroplast stay-green protein (Table 1c), although no direct evidence for this function was available in the published literature. To provide further validation for LOC_Os09g36200 as a candidate for *y*, the 30 implicated rice gene models were analysed for the presence of putative chloroplast transit peptides, using ChloroP 1.1 (http://genome.cbs.dtu.dk/services/ChloroP/; Emanuelsson *et al.*, 1999). Additionally, the temporal and organ-specific expression patterns of their most similar Arabidopsis gene models (Table 1d) were obtained through microarray data available in the Genevestigator® Meta-Analyzer database (https://www.genevestigator.ethz.ch/; Zimmerman *et al.*, 2004). Along with seven other gene models from this region, LOC_Os09g36200 was predicted to contain a chloroplast

Table 1 Syntenic relationship between *Lolium/Festuca* C5 and rice C9. (a) Genetic markers mapped in rice (*Oryza sativa*) and *Lolium/Festuca* and their equivalent positions on the TIGR rice 9 pseudomolecule. (b) Diagrammatic representation of the physical position of mapped markers and genes on the TIGR rice 9 pseudomolecule. (c) TIGR gene models and predicted functions for candidate sequences for the *Festuca pratensis* staygreen gene. (d) Most similar Arabidopsis sequence

(a)	Genetic distance of markers on *L. perenne* C5 and rice C9			Genetic distance from* or no. recombinants with† *y* on rice C9 or *Lm/Fp* C5			(b)	(c)		(d) AGI proteins (TAIR)[b]	
Marker	*Lp* C5	riceA C9	riceB C9	riceC *y**	*Lm/Fp yA**	*Lm/Fp yB†*	TIGR C9 gene model	TIGR gene model	TIGR putative annotation	Arabidopsis gene model	*P*
								Os09g36060	Endoglucanase 1 precursor	AT4G39010	2.4e-161
E51174S	3.1	–	–	–	49.7	–	–	Os09g36070	Cytochrome P450	AT3G26310	4.4e-70
C1176.2	8.2	–	35.2	–	–	–	Os09g17830	Os09g36080	Cytochrome P450	AT1G13080	1.0e-72
BCD1087	8.2	–	38.5	–	–	–	Os09g17730	Os09g36090	Plus-3 domain	AT3G51120	3.8e-148
RZ206	9.2	–	38.5	–	–	–	Os09g19730	Os09g36100[c]	Expressed protein	AT3G51100	1.2e-24
R1751	15.2	49.3	–	–	–	–	Os09g24200	Os09g36110	Subtilisin N-terminal region	AT4G10550	6.1e-170
PSR574	12	62.4	–	–	–	–	Os09g27870	Os09g36120[c]	Expressed protein	AT5G17280	4.8e-12
CDO412	19	–	61.9	–	10.7	–	Os09g28019	Os09g36130[c]	Expressed protein	AT2G21385	2.5e-102
RM160	–	–	–	9.7	–	–	–	Os09g36140	RNA recognition motif 2 family protein	AT3G26120	2.5e-24
RG570	–		80.3	7.2	–	–	Os09g33780	Os09g36150	Retrotransposon protein	AT2G38950	0.060
S2655	–	77.7	–	–	3.4	–	Os09g33810	Os09g36160	Expressed protein	AT5G66350	2.1e-40
C1263	–	78.5	–	4.2	–	–	Os09g34960	Os09g36170	Hypothetical protein	AT1G17980	0.26
RG662	–	–	–	1.8	–	–	–	Os09g36180[c]	Glycosyl transferase family 8 protein	AT3G61130	1.1e-221
R3330	–	79.1	–	–	–	4	Os09g35710	Os09g36190[c]	Glycosyltransferase QUASIMODO1	AT3G61130	8.9e-249
9g35920	–	–	–	–	–	2	Os09g35920	Os09g36200[c]	Senescence-inducible chloroplast stay-green protein	AT4G22920	1.5e-73
9g36030	–	–	–	–	–	4	Os09g36030	Os09g36210	Harpin-induced protein 1	AT4G01410	6.6e-15
9g36060	–	–	–	–	–	1	Os09g36060	Os09g36220	Two-component response regulator-like PRR95	AT5G24470	2.9e-79
9g36200	–	–	–	–	–	0	Os09g36200	Os09g36230[c]	Hypothetical protein	AT5G46100	5.0e-33
9g36270	–	–	–	–	–	0	Os09g36270	Os09g36240	Deoxyribodipyrimidine photolyase	AT5G46100	2.7e-104
9g36320	–	–	–	–	–	0	Os09g36320	Os09g36250	ODORANT1 protein	AT4G22680	3.1e-65
9g36350	–	–	–	–	–	2	Os09g36350	Os09g36260	Hypothetical protein	AT3G57900	0.16
C985[a]	–	82.1	–	2.1	–	–	Os09g36420	Os09g36270	Pantothenate kinase 2	AT4G32180	6.8e-297
R2710	65	–	–	–	3.4	–	Os09g38030	Os09g36280	Glycosyl hydrolases family 17	AT3G57270	3.4e-50
T4	–	90.1	–	10.8	–	–	–	Os09g36290	Serine/threonine protein phosphatase	AT5G63140	5.7e-119
RM189	–	–	–	16.3	–	–	Os09g38370	Os09g36300	Lon protease homologue 1	AT5G47040	0.
RZ404	78	94.2	–	–	–	–	Os09g38580	Os09g36310	Hypothetical protein	AT5G28560	0.21
								Os09g36320[c]	Serine/threonine-protein kinase NAK	AT5G47070	4.5e-103
								Os09g36330	Hypothetical protein	**NONE**	–
								Os09g36340	HVA22-like protein i	AT5G42560	1.5e-38
								Os09g36350	Glycosyl hydrolase family 9	AT1G75680	5.0e-168



riceA, RGP 2003 high-density rice genetic map; riceB, Cornell RFLP 2001 rice genetic map (data obtained from Gramene database; http://www.gramene.org/); riceC, Cha *et al*. (2002) *sgr* (*y*) population.
Lm/Fp yA, original *Lolium/Festuca* introgression *y* screening population (*n* = 100); *Lm/Fp yB*, recombinant genotypes (*n* = 60) identified in expanded *Lolium/Festuca* introgression *y* screening population (*n* = 1627).
[a]Alternative position to that in Gramene database consistent with relative genetic map position. Identified by BLAST search of GenBank accession number D22707 against rice pseudomolecules database at http://www.tigr.org/tdb/e2kl/osal/.
[b]Most significant alignment present in Genevestigator® chipset database as determined by WU-BLAST2 searches at The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/wublast/index2.jsp). AGI, Arabidopsis Genome Initiative.
[c]Chloroplast transit peptide predicted by ChloroP 1.1.

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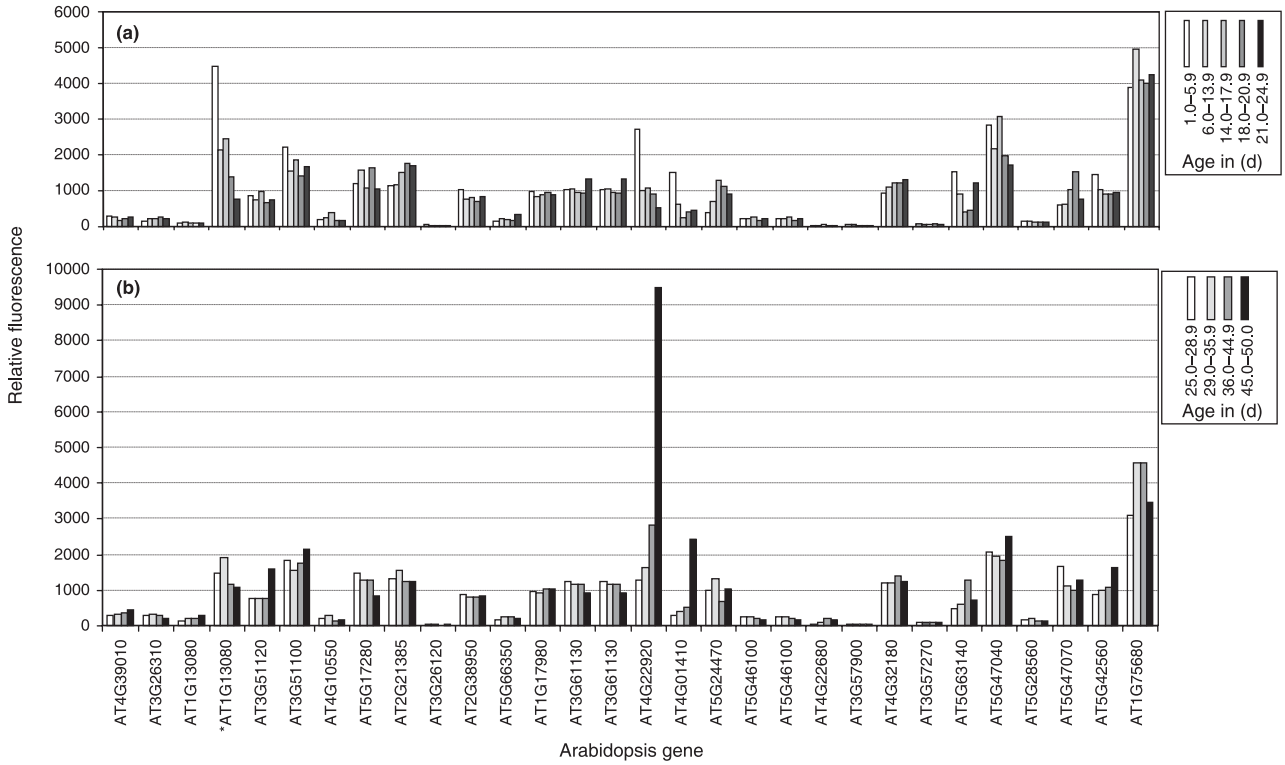

Fig. 1 Relative fluorescence values from microarray analysis of selected Arabidopsis genes; each Arabidopsis gene is an orthologue of a TIGR rice (*Oryza sativa*) gene model predicted to be nonrecombinant with *Festuca pratensis*-derived *y* on the basis of synteny (Table 1). Results are expressed according to Arabidopsis whole-plant growth stage: (a) 1–24.9 d and (b) 25–50 d (see https://www.genevestigator.ethz.ch/ for growth stage definitions). Values were obtained from the Genevestigator® Meta-Analyzer database. *Data from two different probe sets.

transit peptide (Table 1c). In terms of the expression profiles, the relative fluorescence of At4g22920 (the most similar Arabidopsis protein to LOC_Os09g36200) was clearly up-regulated in days 45–50, coinciding with maximal senescence in the Arabidopsis life-cycle (Fig. 1b). At4g01410 (the most similar Arabidopsis protein to LOC_Os09g36210) also showed a similar expression pattern, although of smaller relative magnitude (Fig. 1a). None of the remainder of the candidate genes indicated obvious specific induction in days 45–50. Examination of the tissue-specific pattern of expression of At4g01410 and At4g22920 indicated that the former was most strongly expressed in the seed and showed no specific association with the senescent leaf (Fig. 2a). The latter was also strongly expressed in the seed as well as in the petals and sepals. Notably, however, it was associated most strongly with the senescent leaf (Fig. 2b). Subsequent northern analysis of this gene in *Lolium temulentum* and staygreen *F. pratensis* confirmed senescence-associated expression in *Lolium*/*Festuca* leaves. Therefore, on the basis of genetic association, comparative genomics, putative gene function and expression profile, a recessive mutation in the *F. pratensis*-derived homologue of LOC_Os09g36200 was considered to be a strong candidate for *y*.

In order to develop an allele-specific molecular marker for staygreen, the genomic sequence of the *L. perenne* homologue of LOC_Os09g36200 was obtained by polymerase chain reaction (PCR) screening of an *L. perenne* bacterial artificial chromosome (BAC) library (Farrar *et al.*, 2006) and this was used to develop primers for the amplification of regions of *y*. Allele-specific primers are often most efficiently developed by amplifying intronic sequences; however, in this case a 4-bp ATAT insertion was identified in the second predicted exon of the candidate gene (Fig. 3). This allowed the development of both a size-specific molecular marker suitable for high-throughput screening and a cleaved amplified polymorphic sequence (CAPS) marker (the ATAT insertion removed an existing *Bst*F5I restriction enzyme site present in the wild-type sequence) suitable for analysis on agarose gels (Fig. 3a). To date, assays of these markers on experimental mapping populations and application in existing breeding lines have identified no recombination between *y* and the molecular markers.

Beyond the development of a molecular marker, the presence of a 4-bp insertion in an exon represents a translational frameshift, and comparison of the rice and *Lolium* candidate genes with homologous sequences from other species indicates that this 4-bp insertion is peculiar to staygreen lines containing the *F. pratensis*-derived *y* locus

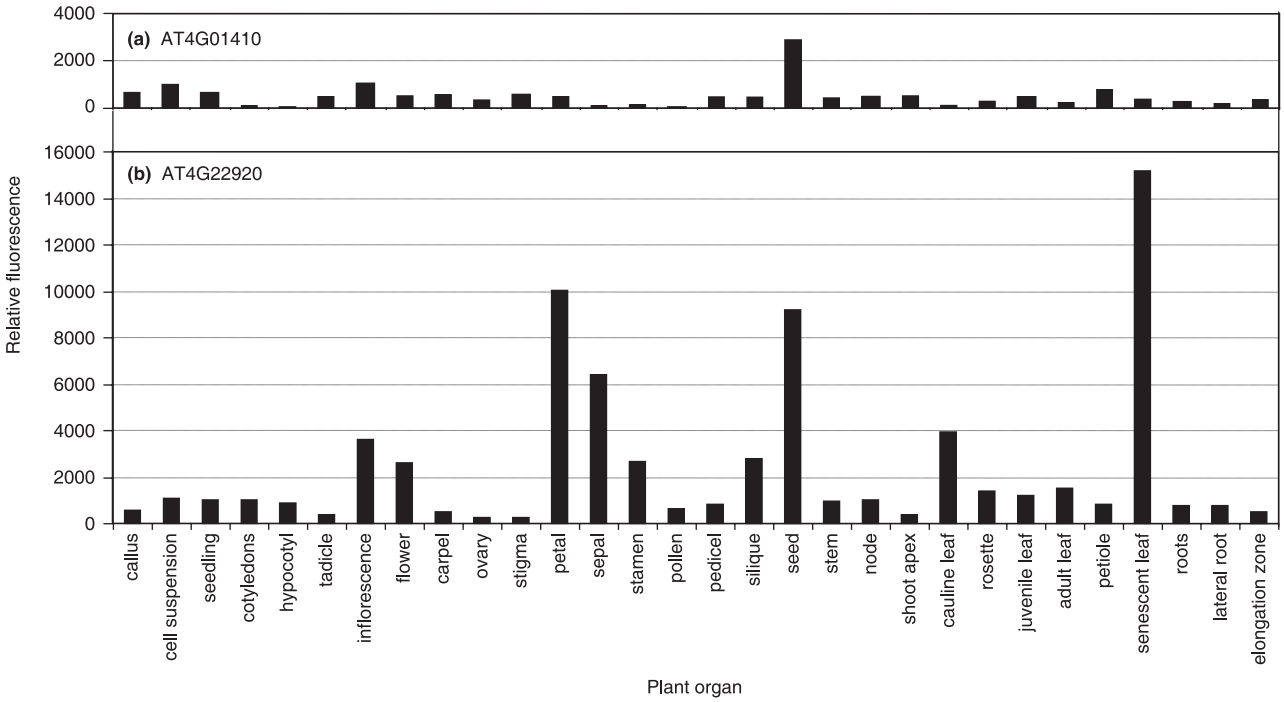

Fig. 2 Relative fluorescence values from microarray analysis of Arabidopsis genes (a) AT4g01040 and (b) AT4G22920, orthologues of TIGR rice (*Oryza sativa*) gene models LOC_Os09g36210 and LOC_Os09g36200, respectively. Results are expressed according to plant organ specificity. Values were obtained from the Genevestigator® Meta-Analyzer database.

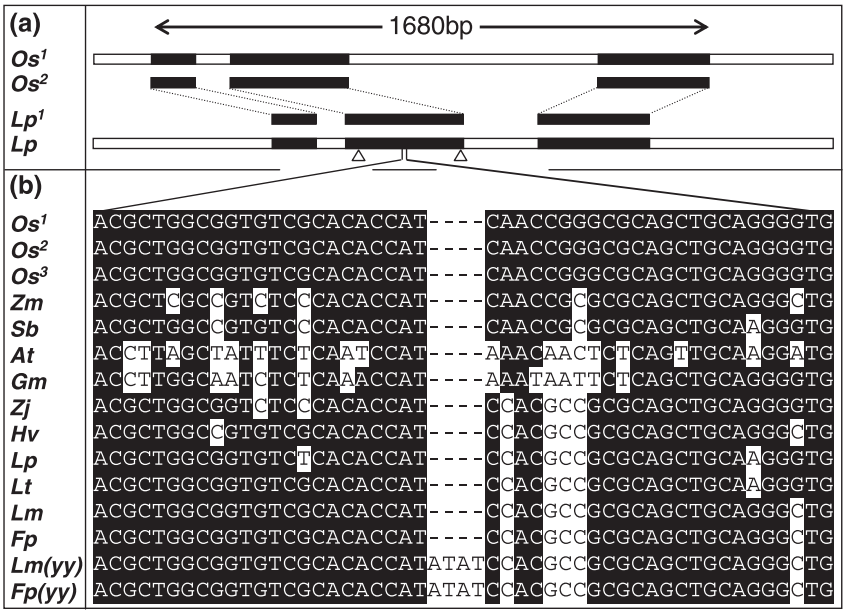

Fig. 3 (a) Diagrammatic representation of the physical distribution of exons in rice (*Oryza sativa*) LOC_Os09g36200 and its homologue from *Lolium perenne*: black horizontal bars, exons; white horizontal bars, noncoding genomic sequence; open triangles, priming sites for marker used in the detection of the ATAT insertion. Dotted lines connect equivalent exons. (b) Partial sequence alignment of exon 2 from LOC_Os09g36200 and its homologues from a number of plant species, illustrating the ATAT insertion in *Lolium/Festuca yy* genotypes. *Os*[1], LOC_Os09g36200 gene sequence; *Os*[2], LOC_Os09g36200 CDS; *Os*[3], *O. sativa* (AY850134); *Lp*, *L. perenne* bacterial artificial chromosome (BAC) sequence; *Lp*[1], *L. perenne* BAC sequence predicted coding sequences (CDS); *Hv*, *Hordeum vulgare* (AY850135); *Zj*, *Zoysia japonica* (AY850154); *Zm*, *Zea mays* (AY850138); *Sb*, *Sorghum bicolor* (AY850140); *Gm*, *Glycine max* (AY850141); *At*, *Arabidopsis thaliana* (AY850161); *Lt*, *Lm* and *Fp*, *Lolium temulentum*, Lolium multiflorum and *Festuca pratensis* genomic polymerase chain reaction (PCR) products covering the second predicted exon; (*yy*), mutant genotype expressing staygreen. GenBank accession numbers are indicated in parentheses.

(Fig. 3b). The mechanism by which this mutation was produced is unknown, but short insertion sequences of this type have been shown to arise as footprints following mobilization of a transposable element (Pooma *et al.*, 2002), although active transposition has not been characterized directly in *Lolium*/*Festuca*. Approximately 1250 bp of the *F. pratensis y* genomic sequence has been isolated so far, spanning the complete first and second exons and, up to the ATAT insertion, the peptide predicted from this is identical to the wild-type *L. perenne* protein. Use of the complete *L. perenne* gene, with and without the ATAT insertion, as a model for the comparison of mutant and wild-type proteins shows that the insertion radically changes the amino acid sequence of the mutant protein from position 100 and gives a final protein consisting of 232 residues, as compared to 279 in the wild type. The region of the protein derived from the second exon is highly conserved across a number of different species (Fig. 3b), indicating that one or more active sites may be affected by the frameshift mutation; this could give rise to a functional knockout mutation, as seen for *y*. Work is under way, using immunochemical approaches, to understand and localize the effect of mutation in *Y* on protein expression in both crop and model species

Staygreen and chlorophyll catabolism

It is established that *Y* is a key gene involved in chlorophyll catabolism, and it is interesting to speculate upon its function. The biochemical lesion represented by *y* in staygreen *F. pratensis* has been shown to be located in the pathway of chlorophyll catabolism at the step where the chlorin macrocycle is opened by oxygenolytic cleavage (Vicentini *et al.*, 1995). Measured *in vitro*, activity of the enzyme responsible (phaeophorbide a oxygenase, PaO) is abnormally low in staygreen lines. There is, however, good genetic and biochemical evidence to show that PaO is expressed, although inactive, in staygreen *Festuca* and *Lolium* (Vicentini *et al.*, 1995; Roca *et al.*, 2004): it is therefore likely that the product of the *Y* gene modulates the activity of PaO. Chlorophyll degradation is organizationally complicated, as the pigment substrates for the catabolic enzymes are complexed with thylakoid membrane proteolipids (Thomas, 1987). Disassembly of these complexes must take place in a regulated fashion if the photodynamic tendencies of chlorophyll catabolites are to be kept in check (Thomas, 1987; Eckhardt *et al.*, 2004; Hörtensteiner, 2004). Recently, a protease (FtsH6) has been described that specifically breaks down chlorophyll-binding proteins in senescence (Zelisko *et al.*, 2005). We propose that disassembly and degradation of the pigment-proteolipids of thylakoids is mediated by a complex of enzymes, including PaO (which in turn interacts with a second enzyme, red chlorophyll catabolite reductase (RCCR) reductase (Hörtensteiner *et al.*, 2000; Eckhardt *et al.*, 2004)), *Y* (Hörtensteiner, 2006) and FtsH6 (Zelisko *et al.*, 2005) – and possibly other activities too, such as acyl hydrolase (He & Gan, 2002). A search for a multienzyme complex that functions as a machine for dismantling chloroplast membranes is justifiable. Identification of *Y* and other recently reported genes as the putative components of such a machine is a significant step towards an understanding of the mechanism and control of this central event in senescence.

Conclusion

This report has described how classical genetics and detailed biochemical analysis paved the way for the identification of a strong candidate for a key gene in the chlorophyll catabolic pathway using the tools of modern comparative genomics and transcriptomics. It has also shown how, by identifying this candidate and determining its protein product, it has now become possible to formulate and test new hypotheses concerning the biochemical regulation of chlorophyll catabolism. The practical application of this work has also been significant, as it has led to the development of an allele-specific marker which, to date, has shown no recombination with *y* and which is currently being employed in breeding programmes to transfer this recessive phenotype.

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