Plant Biology ISSN 1435-8603

REVIEW ARTICLE

The control of chlorophyll catabolism and the status of yellowing as a biomarker of leaf senescence

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Keywords
Gene; hypersensitive response; mutation; plastid; pseudosenescence.

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Guest Editor
K. Krupinska

Received: 5 December 2007; Accepted: 1 February 2008

doi:10.1111/j.1438-8677.2008.00081.x

ABSTRACT

The pathway of chlorophyll catabolism during leaf senescence is known in a fair amount of biochemical and cell biological detail. In the last few years, genes encoding a number of the catabolic enzymes have been characterized, including the key ring-opening activities, phaeophorbide a oxygenase (PaO) and red chlorophyll catabolite reductase (RCCR). Recently, a gene that modulates disassembly of chlorophyll–protein complexes and activation of pigment ring-opening has been isolated by comparative mapping in monocot species, positional cloning exploiting rice genomics resources and functional testing in Arabidopsis. The corresponding gene in pea has been identified as Mendel's I locus (green/yellow cotyledons). Mutations in this and other chlorophyll catabolic genes have significant consequences, both for the course of leaf senescence and senescence-like stress responses, notably hypersensitivity to pathogen challenge. Loss of chlorophyll can occur via routes other than the PaO/RCCR pathway, resulting in changes that superficially resemble senescence. Such 'pseudosenescence' responses tend to be pathological rather than physiological and may differ from senescence in fundamental aspects of biochemistry and regulation.

THE PATHWAY OF CHLOROPHYLL CATABOLISM DURING LEAF SENESCENCE

This paper deals with what happens to chlorophyll during leaf senescence. Loss of green colour is the visible symptom of senescence, the progress of which is usually measured by determining the amount of chlorophyll. This assumes that pigment changes are strongly linked to other parts of the senescence syndrome, notably nutrient recycling. Observations of the coordination of senescence physiology do not always support the assumption (see for example Hidema *et al.* 1991).

During catabolism (Fig. 1), chlorophyll follows a route that begins with its release from pigment–proteolipid complexes within thylakoid membranes. A series of reactions ensues in which stroma and plastid envelope factors bring about the opening of the tetrapyrrole ring. The colourless linear product is exported from the plastid and, in different ways in different plant species, may be conjugated in the cytosol before being transported into the cell vacuole where final chemical modifications may take place (Hörtensteiner 2006; Kräutler & Hörtensteiner 2006; Tanaka & Tanaka 2006).

Chlorophyll and its immediate catabolites are coloured and strongly excited by ambient light. The catabolic pathway is organized so that photodynamic damage by free pigments is limited. Inside the plastid, chlorophylls and their coloured derivatives must be moved around and interconverted within strongly quenching microenvironments. The risk of photodamage is finally removed by opening the tetrapyrrole ring. This is a two-stage reaction, the first of which is catalysed by PaO (phaeophorbide a oxygenase) and adds oxygen across the methine bridge between rings A and B. The product of this reaction is red chlorophyll catabolite (RCC), a photoactive red pigment. Normally, its photodynamic properties are immediately abolished in a channelled reaction catalysed by the enzyme RCC reductase, in which a reduction destroys the residual conjugated bond system to yield a colourless product, primary fluorescent chlorophyll catabolite

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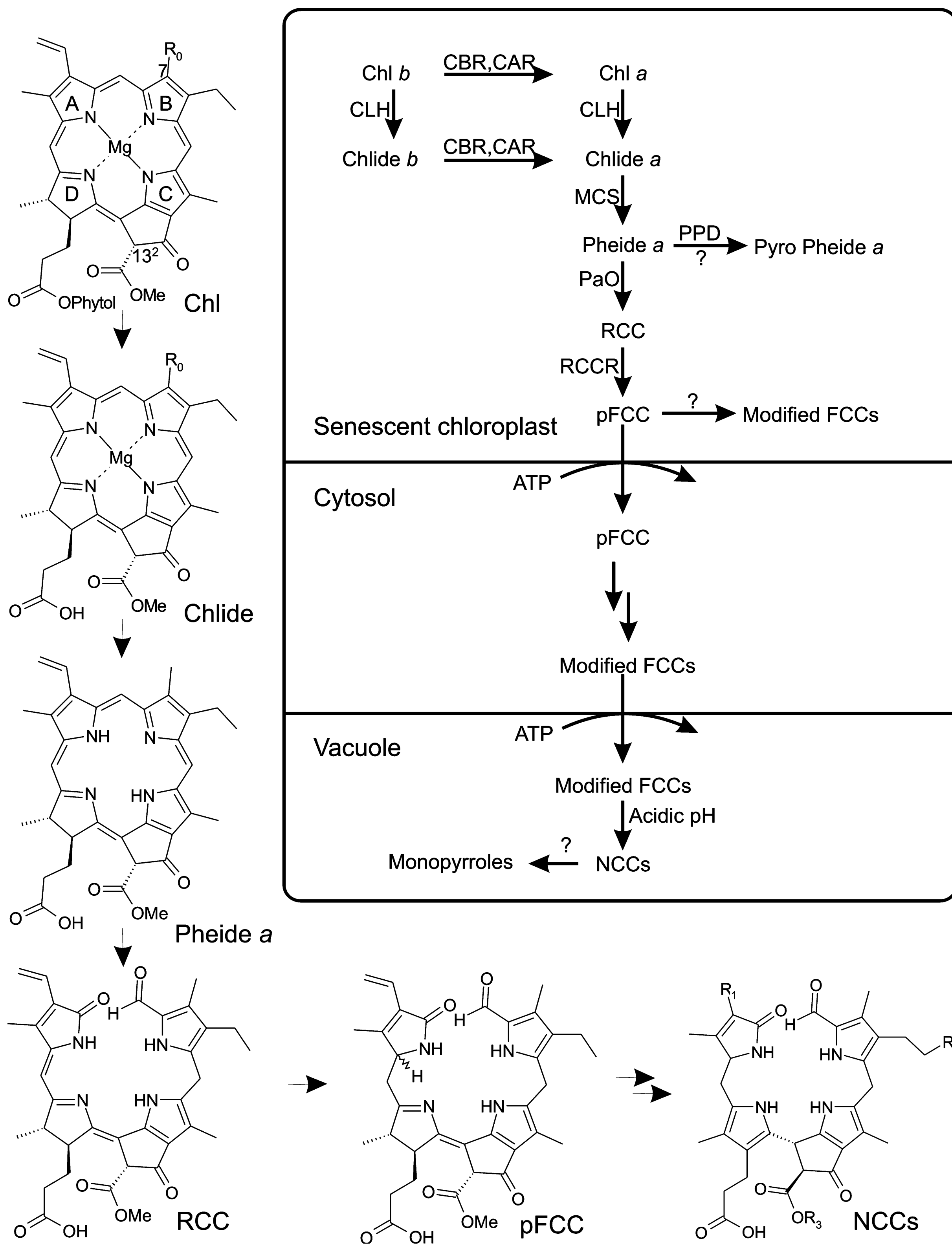

Fig. 1. Chemical structure of chlorophyll and of chlorophyll breakdown products, and topographical model of chlorophyll breakdown. For abbreviations of enzymes, see Table 1. Putative reactions are indicated with a question mark. For abbreviations of catabolites, see the text. Pyrrole rings (A–D) and relevant carbon atoms are labelled in chlorophyll. R0 = CH_3, chlorophyll *a*; R0 = CHO, chlorophyll *b*.

(pFCC). The fate of pFCC is to be conjugated, imported into the vacuole and tautomerized to accumulate there as non-fluorescent chlorophyll catabolites (NCCs) and (possibly) other terminal catabolites. The part of the pathway that takes place outside the plastid closely resembles the biochemistry undergone by xenobiotics and other harmful chemicals (Kreuz *et al.* 1996). This suggests that to dismantle chlorophyll inside the viable cell it is necessary to treat it and its catabolites as dangerous products which must be detoxified.

GENES ENCODING CHLOROPHYLL CATABOLIC ENZYMES

The complete pathway of chlorophyll catabolism (Fig. 1) comprises six more or less characterized enzyme or transporter activities, one factor (MCS, the Mg-removing de-chelating step) that is probably not enzymic (Suzuki & Shioi 2002), one or more species-specific cytosolic conjugating enzymes (Hörtensteiner 2006), the final tautomerization reaction of FCCs to NCCs inside the vacuole (Oberhuber *et al.* 2003) and a number of critical interacting reactions including pigment–protein complex dismantling (Park *et al.* 2007) and the ferredoxin cycle (Hörtensteiner *et al.* 1995). Of these, genes for the four key steps upstream of export from the plastid have been isolated and characterized (Table 1): chlorophyll *b* reductase (Kusaba *et al.* 2007); chlorophyllase (Jakob-Wilk *et al.* 1999; Tsuchiya *et al.* 1999); PaO (Pružinská *et al.* 2003); and red chlorophyll catabolite reductase (RCCR) (Wüthrich *et al.* 2000). An additional reaction leading to catabolites with a pyro configuration (loss of the $C13^2$ methoxycarbonyl group of chlorophyll) has been proposed recently, and a respective esterase been cloned (Suzuki *et al.* 2006). Further reactions can be deduced from NCC structures, and the location of NCCs inside the vacuole implies the requirement of transport systems at the chloroplast envelope and the tonoplast. Table 1 lists the to date (limited) information available on these additional steps.

A GENE MODULATING DISASSEMBLY OF CHLOROPHYLL–PROTEIN COMPLEXES

Chlorophyll catabolism is activated during senescence, but most if not all of the enzyme machinery is present in the cell at a basal level before senescence begins (Wüthrich *et al.* 2000; Mach *et al.* 2001; Pružinská *et al.* 2005; Park *et al.* 2007). Figure 2 shows an example of PaO abundance in green and senescent Lolium leaves. The enzyme largely localizes to the membrane fraction and its abundance is highly increased during senescence. It may be that the pathway is responsible for pigment turnover during plastid assembly and at the steady state, though the evidence is not conclusive. It is clear, however, that chlorophyll catabolism increases from a low baseline on induction of senescence as a consequence of the removal of one or more bottlenecks. Chief amongst these is the need to take apart the pigment–proteolipid complexes of the thylakoid membrane and expose their

Table 1. Enzymes of chlorophyll catabolism and the corresponding genes and mutants.

name	abbreviation	gene code in Arabidopsis	mutants in Arabidopsis	other mutants	references
steps with cloned genes					
chlorophyll *b* reductase	CBR	NYC1: At4g13250 NOL1: At5g04900	–	rice: nyc1	(Kusaba *et al.* 2007)
chlorophyllase	CLH	CLH1: At1g19670 CLH2: At5g43860	–	–	(Tsuchiya *et al.* 1999)
phaeophorbide a oxygenase	PaO	At3g44880	acd1; pao1	maize: lls1	(Greenberg & Ausubel 1993; Gray *et al.* 1997; Pružinská *et al.* 2003)
RCC reductase	RCCR	At4g37000	acd2	–	(Greenberg *et al.* 1994; Mach *et al.* 2001; Pružinská *et al.* 2007)
pheophorbidase	PPD	At4g16690	–	–	(Suzuki *et al.* 2006)
stay-green	SGR/SID	SGR1: At4g22920 SGR2: At4g11910	nye1	rice: sgr Festuca/Lolium: y Pea: JI2775 (i)	(Armstead *et al.* 2006, 2007; Jiang *et al.* 2007; Park *et al.* 2007; Ren *et al.* 2007; Sato *et al.* 2007)
ABC transporter (Tonoplast)		AtMRP2: At2g34660 AtMRP3: At3g13080			(Lu *et al.* 1998; Tommasini *et al.* 1998)

name	abbreviation	identification	localization	references
biochemically identified steps				
hydroxychlorophyll a reductase	CAR	enzyme activity	plastid	(Scheumann *et al.* 1998, 1999)
catabolite exporter		activity (ATP-dependent)	plastid envelope	(Matile *et al.* 1992)
malonyltransferase		enzyme activity	cytosol	(Hörtensteiner 1998)

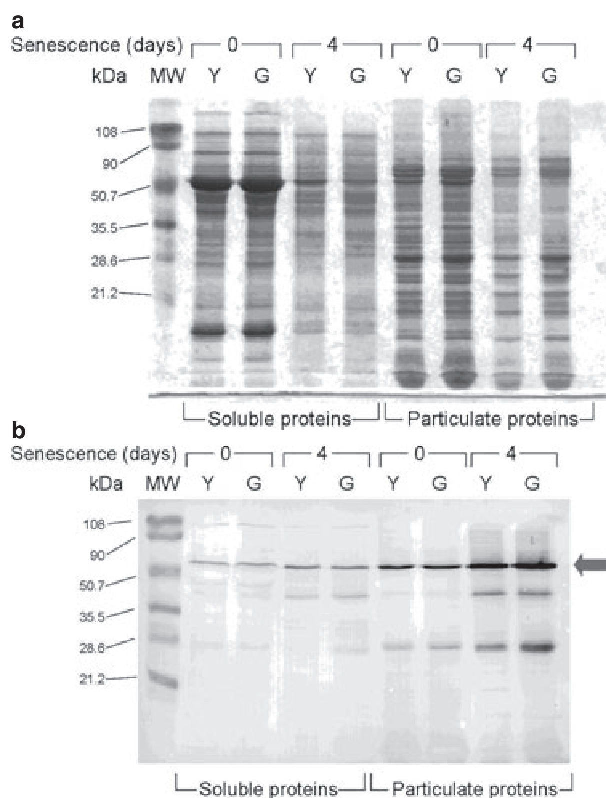

Fig. 2. Immunoblots of PaO in soluble and particulate protein fractions from leaf tissue of wild-type (Y) and stay-green introgression (G) lines of Lolium temulentum genotype Ceres. Senescence was induced in leaf segments and proteins were analysed by electrophoresis as described by Roca *et al.* (2004). Immunoblotting was carried out according to Thomas *et al.* (1999) and PaO visualized using the antibody and detection method of Pružinská *et al.* (2005). (a) Polyacrylamide gel stained with Coomassie Brilliant Blue. (b) Immunodetection of PaO antigens on nitrocellulose blot corresponding to separation in (a). PaO is the band at 56 kDa (arrowed); other bands are degradation products and/or Rieske protein antigens cross-reacting with the polyclonal antiserum.

components to their respective catabolic systems (Thomas 1997).

A mutant with a lesion in dismantling chlorophyll–protein complexes was originally identified in Festuca pratensis (Thomas 1987). The mutant gene, sid, was subsequently introgressed into a number of Lolium species (Thomas *et al.* 1999; Armstead *et al.* 2006). All individuals homozygous for the mutant gene retain green colour during leaf senescence. Much of the senescence syndrome proceeds normally in these genotypes – for example, decrease in photosynthetic capacity, loss of stroma enzymes, including rubisco degradation, and plastid RNA. But the chlorophylls and their associated membrane proteins are comparatively stable and recognizable thylakoid structures persist into late senescence, though stacking is disrupted and pigment–protein complexes become randomized throughout the membrane (Hilditch *et al.* 1989). The corresponding wild-type protein, SID, is a factor that leads to unpacking of the complexes of the photosynthetic membrane, thereby facilitating catabolism of chlorophylls, pigment-binding proteins and lipids (Thomas *et al.* 2002). It has been suggested that the factor is part of a multicomponent machine that systematically dismantles thylakoids in senescing plastids (Armstead *et al.* 2006). The orthologous SGR protein of rice has been shown to physically interact with LHCII (Park *et al.* 2007). Another observation of SGR/SID-deficient plants is reduced PaO activity (in some instances combined with the accumulation of phaeophorbide a), which suggests regulation of PaO through SGR/SID (Thomas *et al.* 1996; Roca *et al.* 2004; Ren *et al.* 2007). It is significant that although measurable PaO activity is low in senescing leaves of Lolium homozygous for the sid mutation, immunoblotting detects an essentially normal pattern of increased PaO protein (Fig. 2).

The gene has been isolated by a novel version of positional cloning called introgression landing (Fig. 3). To get from a locus on a genetic map to a DNA sequence is far from routine, especially in an organism with relatively few genomics resources. But the occurrence of the stay-green mutation in Festuca also has some important advantages for gene isolation. Intergeneric gene transfer from Festuca to Lolium is relatively simple because of interfertility and near-homologous levels of recombination between species (King *et al.* 2007a). Substituting a Festuca gene for the homoeologous Lolium gene is the sexual equivalent of introducing an alien transgene into a new genetic background. Furthermore, the high degree of species-specific polymorphisms in coding and non-coding DNA means that a chromosome segment introgressed from Festuca can be quickly located and mapped in Lolium using the appropriate markers. Lolium–Festuca mapping also benefits from a high degree of synteny with the genome of the model monocot, rice (King *et al.* 2002, 2007b).

By bringing together the introgression tools developed for Lolium–Festuca with the genomics and bioinformatics resources of rice, Armstead *et al.* (2006) succeeded in identifying a candidate locus in a stay-green Lolium introgression mapping family that corresponded to a sequence in a syntenous region of the rice genome that, in turn, related to a stay-green locus in that species (Fig. 3). When the equivalent gene in Arabidopsis was knocked out by RNAi, the resulting plants had the same stay-green phenotype as the mutant Festuca and the derived Lolium introgressions (Armstead *et al.* 2007; Fig. 3 panel 5). The molecular basis of the lesion in Festuca was found to reside in a four base-pair insertion in the first exon, possibly representing the footprint of a departed mobile element. The abundance of transcripts of both wild-type and mutant SID increases markedly in senescence, but presumably the frame-shift leads to aborted translation or a nonsense protein.

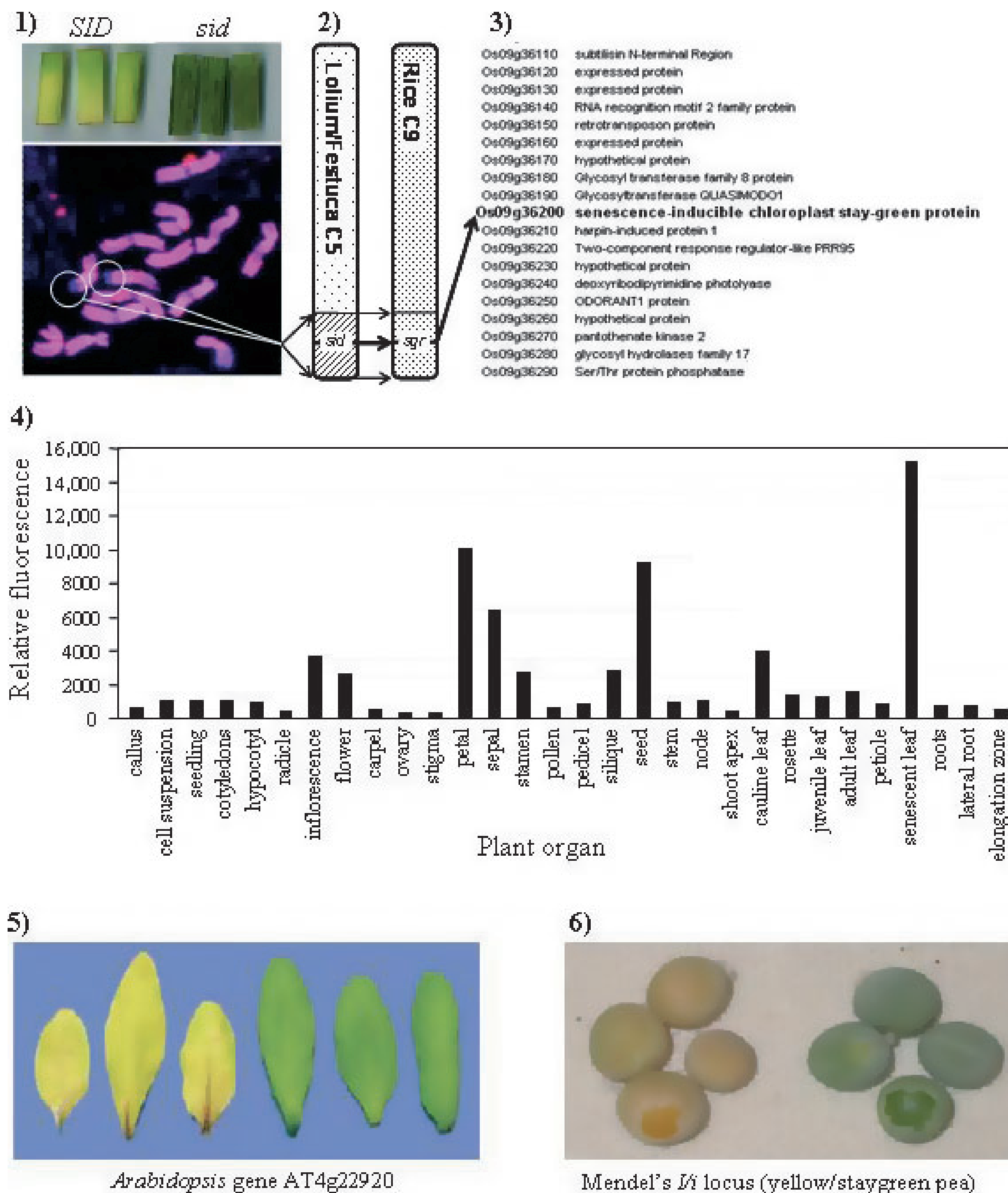

Fig. 3. Identification of a candidate gene for the recessive staygreen mutant sid by introgression landing and comparative fine mapping. (1) Homologous chromosome (C) segments expressing sid were introgressed from Festuca pratensis (dark blue, circled in white) into the Lolium background (pink). (2) Genetic mapping showed that sid was located on Lolium/Festuca C5, a region known to be syntenous with rice C9; fine mapping of common markers showed that sid on Lolium/Festuca C5 and sgr on rice C9 were located in equivalent regions. (3) Predicted functions of gene models in this region of the rice genome identified the candidate gene Os09g36200. Genotype/phenotype relatioship was confirmed by: (4) The Arabidopsis thaliana orthologue of Os09g36200 (At4g22920) is up-regulated during plant leaf senescence; (5) Gene silencing of At4g22920 (right) reproduced the staygreen phenotye relative to wild type (left). (6) The orthologue of At4g22920 and Os9g36200 in pea mapped with Mendel's staygreen cotyledon locus (i).

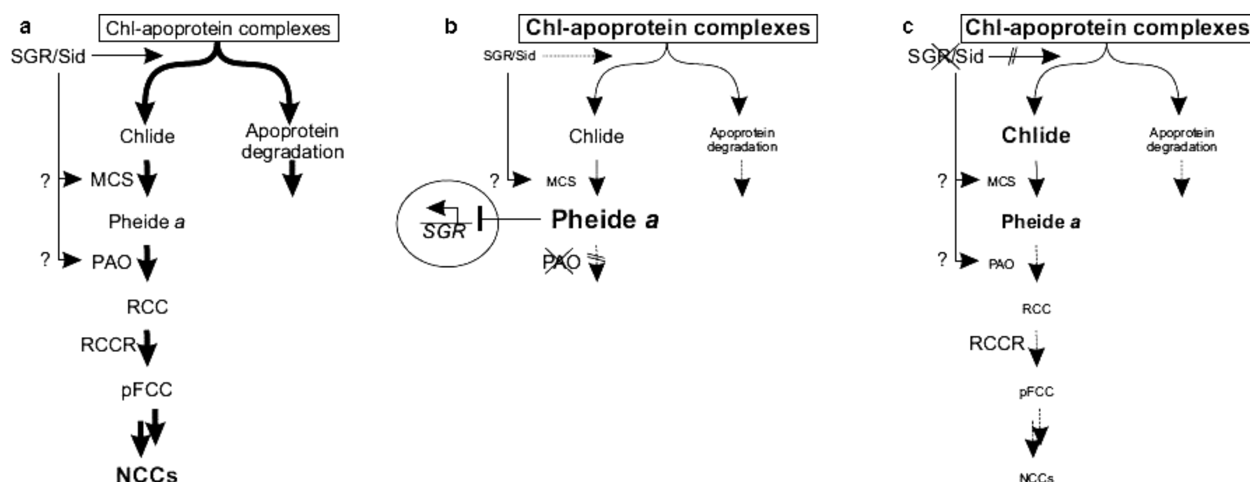

Fig. 4 Model for the regulation of chlorophyll breakdown based on available published data. (a) Wild type; (b) in pao1 mutants; (c) in sgr/sid mutants. Thickness of arrows shows relative flux of metabolites, and font size represents relative changes in catabolite amounts or enzyme activities. The circle in panel (b) represents the nucleus and indicates SGR gene expression. For further explanation, see the text.

The gene is highly conserved between species. As well as cereals and Festuca–Lolium, it has been identified in legumes. In a further study, Armstead *et al.* (2007) showed that the gene co-locates with Mendel's I locus (determining green or yellow cotyledons) on the pea genetic map (Fig. 3, panel 6). The green mutation in pea is associated with low levels of transcription during senescence. Stay-green variants closely resembling Mendel's pea phenotype are particularly common across legume species. Hauck *et al.* (1997) found that the green mutation in Festuca compromizes the plant's nitrogen economy, by immobilizing thylakoid proteins in complexes that would normally be recycled. We speculate that nitrogen fixation in legumes might to some degree compensate for the inefficiencies of nitrogen salvage in stay-green variants.

MORE MUTATIONS IN SENESCENCE GENES

Although a few fast-senescing mutants have been described, most attention has been given to stay-green variants (Thomas & Smart 1993; Thomas & Howarth 2000) with disrupted senescence, of which there are two broad classes. In the functional stay-greens the senescence syndrome remains integrated but starts late, or proceeds slowly, or both. Because of the importance of extended photosynthesis and green area duration for grain production, plant breeding has introduced and exploited extreme genetic variation for the delayed senescence trait and many of the advanced varieties of crops such as maize and sorghum are functional stay-greens.

The second class of senescence mutant is the cosmetic stay-greens. Here, senescence starts and proceeds essentially on cue, but yellowing is disrupted because of a blockage in the chlorophyll catabolism pathway. The mutant gene sid in stay-green Festuca–Lolium, and Mendel's I/i in pea, are examples. As mentioned above, the phenotype of sgr/sid mutants and biochemical analysis of SGR (Park *et al.* 2007) indicates a likely function in dismantling chlorophyll–protein complexes, but a regulatory role in MCS and PaO activity cannot at present be excluded (Fig. 4a).

Normally the integrity of the chlorophyll catabolism pathway ensures that the pigments are safely disposed of, releasing thylakoid proteins for hydrolysis and salvage of amino N. But disruption can and does happen, resulting in a range of symptoms and consequences for the physiology of the organ and the plant. Thus, the loss of PaO or RCCR in acd1/pao1 and acd2 mutants, respectively, results in the formation of a lesion mimic phenotype, because of the accumulation of respective photodynamic chlorophyll breakdown intermediates. Particularly in PaO mutants, this effect has drastic consequences not only for leaf development, but also impairs flower development and seed formation (Gray *et al.* 2002; Pružinská *et al.* 2005).

PaO has an important function in the regulation of chlorophyll breakdown, as demonstrated by its high specificity of expression in senescence, which, in turn, suggests a key role for phaeophorbide a. In pao1, SGR expression is not upregulated during senescence, indicating that phaeophorbide a might act as a retrograde signal, ultimately preventing further chlorophyll breakdown, i.e. minimizing the risk of surplus accumulation of photodynamic intermediates (Fig. 4b). A major difference between pao1 and sgr/sid mutants is the absence of a cell death phenotype in the latter. In sgr mutants, the accumulation of intermediates might not reach a threshold required for cell death induction and, indeed, general concentrations of phaeophorbide a are lower compared to pao1 (Fig. 4c). Alternatively, the early catabolites may be shielded from photodynamism through binding with carrier proteins.

Such chlorophyll–pigment carriers have been postulated (Matile *et al.* 1999) but have not been identified in molecular terms yet. There is also a possible role here for plastoglobuli, lipid-rich bodies containing antioxidants such as carotenoid derivatives (Tevini & Steinmüller 1985; Brehelin *et al.* 2007).

In contrast to sgr/sid and pao1 mutants, mutations in RCCR/ACD2 (e.g. acd2) do not prevent chlorophyll breakdown from proceeding more or less normally. In addition, the finding of mitochondrial RCCR localization, combined with cell death in acd2 caused by a mitochondrial oxidative burst (Yao *et al.* 2004), raised questions about whether RCCR truly has a role in the chlorophyll breakdown pathway. It was also observed that ACD2 could shield root protoplasts that lack chlorophyll from cell death induced by light or by protoporphyrin 1X, showing that chlorophyll catabolism is not obligatory for ACD2 anti-cell death function under all circumstances (Yao & Greenberg 2006). However, by complementation of acd2 with RCCRs exhibiting a defined stereospecific activity, it has been demonstrated unequivocally that RCCR participates in chlorophyll catabolism *in vivo*.

THE RELATIONSHIP BETWEEN SENESCENCE AND HYPERSENSITIVITY TO PATHOGEN CHALLENGE

The hypersensitive response (HR) refers to an area of cell death that forms at the point of attempted pathogen ingress and which correlates with the exhibition of resistance (Mur *et al.* 2007). A distinctive feature of both macro and micro HR lesions is a sharp delineation between the dead cells and surrounding living tissue. HR shares many of the generalized biological features of programmed cell death (PCD), including roles for some apoptotic markers and for the redox state of the mitochondrion. A distinctive characteristic of this kind of PCD in green tissues, however, is the contribution of the plastid and the significance of light. The development of cell death in HR is compromized at low light fluence rates, whilst the periphery of the leaf not in direct contact with the infected area appears chlorotic (Fig. 5A). Such chlorosis does not occur at high-fluence rates, where the uninfected surrounding areas remain green. Experiments with the HR–associated defence gene marker PR1 (Fig. 5B) and the senescence marker SAG12 (Fig. 5C) at different levels of illumination revealed a striking reciprocal relationship between the expression of HR on the one hand and senescence on the other. Chlorophyll catabolism was determined to be an early event in the elaboration of the HR. Significantly, in SGR knockout lines of Arabidopsis no chlorophyll catabolism was observed during the HR, and cell death was delayed. Therefore, photosensitive HR cell death and the associated gene expression are likely to be influenced by the generation of photoreactive chlorophyll catabolites (L.J. Mur and H.J. Ougham, unpublished results.).

Like SGR knockouts in Arabidopsis, the Festuca stay-green mutation does not result in a photosensitive phenotype, in contrast to PaO and RCCR knockouts (acd1 and acd2). It follows that chlorophyll and its immediate catabolites must remain in a cellular environment that quenches or dissipates incoming light energy until they arrive at the point of ring-opening in the metabolic sequence, whereupon propagating oxidative damage and cell death kick in.

The realization that knockout or mutation of some components required for chlorophyll catabolism result in photosensitivity whereas disabling other components has no such consequence has implications for the classification of stay-greens. Thomas & Howarth (2000) described five general classes of senescence variant, one of which is the cosmetic group with lesions in the pigment breakdown pathway. It is now clear that this category of

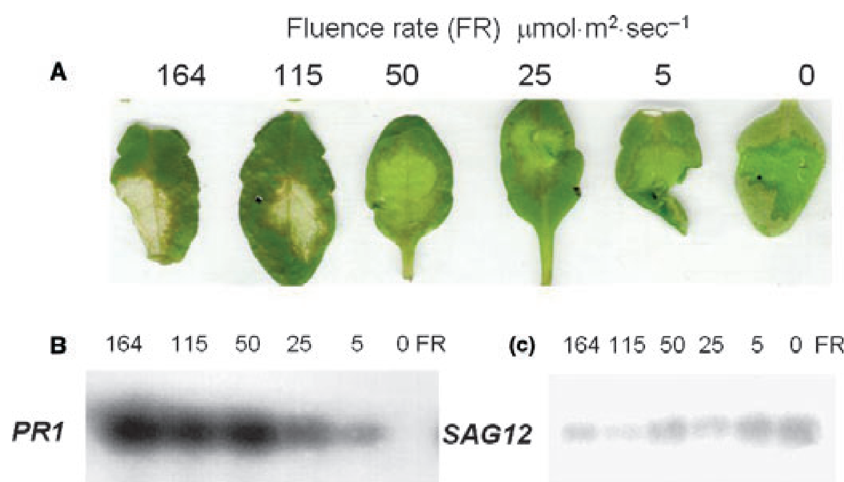

Fig. 5. Light dependence of a Hypersensitive Response. (A) HR phenotypes at 24 h following inoculation with Pseudomonas syringae pv. tomato DC3000 (Pst) avrRpm1 under the light fluence rates indicated. Expression of (B) PR1 and (C) SAG12 in response to a Pst avrRpm1 elicited HR under different light fluence rates as indicated by northern blotting and hybridization.

stay-green should be further divided into the light-sensitive and light-insensitive types, exemplified by acd1/acd2 and sid, respectively.

PSEUDOSENESCENCE

So far as is known, the PaO-RCCR pathway is the physiological route for chlorophyll removal wherever green tissues undergo senescence. Because chlorophyll is a pigment, it is intrinsically photolabile. Instability in the light may be enhanced by non-biological factors, such as oxidation and the pathological enzymic reactions associated with oxidative damage. By definition, a tissue from which chlorophyll is disappearing should only be classified as undergoing true senescence if we know that the pigment has been processed by the physiological pathway. Loss by non-physiological bleaching, although superficially resembling senescence, is fundamentally different and essentially pathological. We may call behaviour of this kind pseudosenescence (Cots *et al.* 2002; Ougham *et al.* 2007). In practice, it is often difficult to decide whether the de-greening response of a leaf to a particular treatment represents true or pseudo-senescence.

One suggestive criterion is the differential response to light and darkness. In general, a treatment that stimulates de-greening in light but which has no, or a delaying, effect in darkness is likely to be working through a pseudosenescence route. Examples of such factors are carbohydrates, chelators, intermediates of tetrapyrrole biosynthesis and extreme temperatures (Holden 1972; van Hasselt & Strikwerda 1976; Thimann *et al.* 1977; Satler & Thimann 1983; Thimann 1985; Thomas & Matile 1988). The light-dependent bleaching seen in the HR response, and enhanced by mutations that cause buildup of photodynamic chlorophyll catabolites, is an example of pseudosenescence.

Use of a non-photosensitive stay-green line of the Festuca type is another way of discriminating between modes of de-greening. Because such genotypes are constitutively incapable of breaking down chlorophyll by the physiological route, a de-greening response must mean that the treatment in question works by stimulating pseudosenescence. We have observed in stay-green Lolium introgression populations that there is marked heritable variation for sensitivity of pseudosenescence to low-temperature stress (A. Gay, D. Thorogood, E. Smith, A. Kingston-Smith, C. James, H. Thomas, H.J. Ougham, unpublished results), which strongly indicates that physiological and pathological de-greening responses are genetically distinct.

CONCLUSIONS

The removal of greenness is more than simply the visible symptom, and most easily measured index, of leaf senescence. Chlorophyll catabolism is a pace-setting activity for the senescence syndrome as a whole, regulating the release of a significant proportion of salvageable protein nitrogen and the duration and integrity of photosynthesis (Thomas 1997). Mutations show unequivocally that chlorophyll has a defined job in senescence. In this regard it is much more robust than other indices such as SAG12 transcript, which is widely used as a molecular biomarker of senescence but for which knockouts have failed to establish a necessary function (Otegui *et al.* 2005).

Mutations in chlorophyll catabolism are generally expressed whether senescence is observed in whole plants or in the artificial but experimentally more convenient circumstances of surgical modification, detachment or tissue excision (Matile *et al.* 1999). Furthermore, genes of chlorophyll breakdown are reliably up-regulated, independent of the way physiological degreening is induced (Buchanan-Wollaston *et al.* 2005; Van der Graaff *et al.* 2006). This tells us that the physiological pathway of yellowing is a robust and consistent component of the senescence syndrome and justifies its study in isolated organs and tissues. It may also say something about the source–sink interactions between senescing foliage and the rest of the plant through which nutrient recycling is transacted (Hörtensteiner & Feller 2002). Genetic or chemical perturbation of chlorophyll degradation simultaneously compromises the remobilization of the proteins with which pigments are associated *in vivo* (Thomas 1997; Gay *et al.* 2008). In this respect, the behaviour of chlorophyll suggests one answer to the long-standing question as to whether nutrient recycling is driven by sucking (the demands of the rest of the plant) or blowing (the pressure of salvaged nutrients built up in senescing leaves). It seems clear that the portion of (particularly nitrogen) remobilization that is directly coordinated with pigment catabolism is more or less indifferent to sink demand, a conclusion with implications for defining genetic targets in crop breeding aimed at improved harvest index and resource allocation.

Because of the photodynamic properties of chlorophyll and its immediate catabolites, removal of the pigments is a delicate business and susceptible to disruption, with pathological consequences (Matile *et al.* 1999; Ougham *et al.* 2005). This means that the plastid plays a critical part in light-dependent HR and related PCD processes in green tissues, a role mediated by the pathway of chlorophyll catabolism (Hörtensteiner 2004). Pathological de-greening has only a superficial resemblance to true senescence (we suggest the term pseudosenescence) and employs fundamentally different genetic and biochemical components. Paradoxically, it seems that one of the jobs of senescence as a coherent and integrated syndrome is to maintain viability in tissues that otherwise would rapidly divert into the pseudosenescence route that leads to cell death.

ACKNOWLEDGEMENTS

We thank Caron James for expert technical assistance.

CONFLICTS OF INTEREST

The Institute of Grassland and Environmental Research is sponsored by the UK Biotechnology and Biological

Sciences Research Council. SH is supported by the Swiss National Science Foundation and the Swiss National Center of Competence in Research on Plant Survival. HT is grateful to the Leverhulme Trust for the award of an Emeritus Fellowship.

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