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Research

Accumulation of chlorophyll catabolites photosensitizes the hypersensitive response elicited by *Pseudomonas syringae* in Arabidopsis

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Summary

- The *staygreen* (*SGR*) gene encodes a chloroplast-targeted protein which promotes chlorophyll degradation via disruption of light-harvesting complexes (LHCs).
- Over-expression of *SGR* in Arabidopsis (*SGR-OX*) in a Columbia-0 (Col-0) background caused spontaneous necrotic flecking. To relate this to the hypersensitive response (HR), Col-0, *SGR-OX* and RNAi *SGR (SGRi)* lines were challenged with *Pseudomonas syringae* pv *tomato* (*Pst*) encoding the avirulence gene *avrRpm1*. Increased and decreased *SGR* expression, respectively, accelerated and suppressed the kinetics of HR-cell death. In Col-0, *SGR* transcript increased at 6 h after inoculation (hai) when tissue electrolyte leakage indicated the initiation of cell death.
- Excitation of the chlorophyll catabolite pheophorbide (Pheide) leads to the formation of toxic singlet oxygen ($^1O_2$). Pheide was first detected at 6 hai with *Pst avrRpm1* and was linked to $^1O_2$ generation and correlated with reduced Pheide a oxygenase (PaO) protein concentrations. The maximum quantum efficiency of photosystem II (F_v/F_m), quantum yield of electron transfer at photosystem II (ϕ PSII), and photochemical quenching (qP) decreased at 6 hai in Col-0 but not in *SGRi*. Disruption of photosynthetic electron flow will cause light-dependent H_2O_2 generation at 6 hai.
- We conclude that disruption of LHCs, possibly influenced by SGR, and absence of PaO produce phototoxic chlorophyll catabolites and oxidative stress leading to the HR.

Introduction

Plant resistance to pathogenic challenge can be associated with the elicitation of localized cell death – known as a hypersensitive response (HR) – which is likely to contribute to neutralization of the infection (reviewed by Beers & McDowell, 2001; Lam *et al.*, 2001; Mur *et al.*, 2008). Many signals have been shown in combination to bring about the HR, including H^+/K^+ exchange, calcium fluxes from extracellular and intracellular stores (Lecourieux *et al.*, 2002), nitric oxide (Delledonne *et al.*, 2001) and ceramides (Townley *et al.*, 2005). It remains the case, however, that reactive oxygen species (ROS) are the most intensively investigated initiatory HR signal (Lamb & Dixon, 1997; Bolwell *et al.*, 2002). Light is now emerging as a critical factor in the HR (Zeier *et al.*, 2004) and in this paper we identify the chloroplast-centred events that underlie the light-dependent development of oxidative stress during the HR.

Investigations of ROS have been concerned with largely apoplastic sources. Good evidence supports contributions from polyamine oxidases (Yoda *et al.*, 2006), a pH-dependent extracellular peroxidase (Bolwell *et al.*, 2002) or oxalate oxidase (Lane *et al.*, 1993; Wei *et al.*, 1998). Most work has centred on plant homologues of neutrophil NADPH

oxidases encoded by the *Atrboh* gene family in Arabidopsis, which were shown to be major sources of apoplastic ROS during the HR (Torres *et al.*, 1998, 2002). Intracellular sources of ROS during the HR have come to be seen as important, and the focus is increasingly on organelles as sources of oxidative stress. Ashtamker *et al.* (2007) demonstrated a calcium-mediated increase of ROS in the nuclei of cultured tobacco (*Nicotiana tabacum*) cells treated with a cell elicitor. The redox status of the mitochondrion is essential to cell viability (reviewed by Noctor *et al.*, 2007) and mitochondrial ROS have been implicated in cell death initiated by the elicitors victorin and harpin (Yao *et al.*, 2002; Vidal *et al.*, 2007). Evidence for a chloroplastic source of ROS came from observations that some forms of the HR could be light-dependent (Asai *et al.*, 2000; Zeier *et al.*, 2004; Chandra-Shekara *et al.*, 2006), as is the oxidative burst during a tobacco mosaic virus (TMV)-elicited HR in tobacco (Liu *et al.*, 2007). Recently, expression of a cyanobacterial flavodoxin, which has an antioxidant function, in tobacco was found to suppress chloroplast ROS production and also an HR elicited by *Xanthomonas campestris* pv *vesicatoria* (Zurbriggen *et al.*, 2009). One likely source for such ROS is a disruption of photosynthetic electron transport (PET). Alterations in thylakoid membrane properties suggesting diminished photosynthetic capacity were seen as little as 30 min following inoculation with harpin (Boccara *et al.*, 2007). Examination of chlorophyll (Chl) fluorescence (F_v/F_m) indicated photosystem II (PSII) damage at *c.* 9 h following initiation of a bacterially induced HR in Arabidopsis and 4 h following the elicitation of an HR by TMV in tobacco (Seo *et al.*, 2000; Almeras *et al.*, 2003). Such disruptions in PET will lead to ROS photoproduction as a result of excess excitation energy (EEE), where photon intensity exceeds that required for CO_2 fixation (Karpinski *et al.*, 2003; Szabo *et al.*, 2005; Asada, 2006). Cell death in the spontaneous death (SD) mutant, *lesions simulating disease 1* (*lsd1*), has been linked to a failure in photorespiratory dissipation of EEE coupled to reduced antioxidant capacities (Mateo *et al.*, 2004).

Analyses of other SD mutants suggest another mechanism of photooxidative stress, as several mutants have been linked to a compromised Chl catabolic pathway. The Arabidopsis accelerated cell death mutants *pheophorbide a oxygenase 1* (*pao1*) and *accelerated cell death 1 (acd1-1)* lack functional pheophorbide *a* oxygenase (PaO; Tanaka *et al.*, 2003; Pružinská *et al.*, 2005), the monooxygenase that cleaves the macrocycle of the Chl catabolite pheophorbide *a* (Pheide), resulting in the loss of green colour (Hörtensteiner *et al.*, 1998). The SD phenotype in *acd1* leaves is light-dependent (Greenberg & Ausubel, 1993), as is the similar phenotype in the maize (*Zea mays*) *lethal leaf spot 1 (lls1)* mutant (Gray *et al.*, 2002). The *Lls1* gene is the maize homologue of *Acd1*, and also encodes PaO. Similarly, Spassieva & Hille (2002) produced a light-dependent SD phenotype in tomato (*Solanum lycopersicum*) by viral-induced gene silencing of the tomato *Lls1* homologue. The *acd2* mutant of Arabidopsis is deficient in red Chl catabolite reductase (RCCR), the enzyme that converts the red product of PaO action to a fluorescent but colourless intermediate (Wüthrich *et al.*, 2000), and production of SD lesions in *acd2* plants is light-dependent (Mach *et al.*, 2001). Upon light excitation, Chl can be converted to the triplet state (^{3}P680*), which ultimately transfers its energy to the ground state of molecular oxygen to generate $^1O_2$ (Apel & Hirt, 2004). Release of the pigments from the light-harvesting complexes removes Chl and Chl catabolites from quenching carotenoids and may initiate considerable oxidative damage (Triantaphylides & Havaux, 2009). The Arabidopsis protein fluorescent (FLU) and its barley (*Hordeum vulgare*) orthologue Tigrina have been shown to play vital roles in the regulation of tetrapyrrole biosynthesis (Meskauskiene *et al.*, 2001; Lee *et al.*, 2003; Triantaphylides & Havaux, 2009). Overaccumulation of protochlorophyllide in *flu* and *tigrina* leads to light-induced cell death directly associated with singlet oxygen generation (Triantaphylides & Havaux, 2009). *Flu*-initiated cell death has been linked to two chloroplast-located proteins, EXECUTER 1 and 2, which mediate retrograde chloroplastic signalling to the nucleus (Wagner *et al.*, 2004; Lee *et al.*, 2007). The nuclear-encoded cell death mechanisms initiated via a chloroplast-located $^1O_2$–EXECUTER1/2 pathway remain to be defined, but may involve products of lipid peroxidation (Triantaphylides & Havaux, 2009).

The *staygreen* (*SGR*) mutation was originally identified in *Festuca pratensis* (Thomas & Stoddart, 1975) and was subsequently shown to affect the *Festuca* homologue of Mendel's *I* locus (Armstead *et al.*, 2007). Orthologous genes have been found in rice (*Oryza sativa*), pea (*Pisum sativum*) and Arabidopsis and have been designated *STAY-GREEN* (*SGR*; Jiang *et al.*, 2007; Park *et al.*, 2007; Sato *et al.*, 2007). Mutations within *SGR* result in a 'cosmetic' staygreen phenotype in which photosynthetic competence progressively declines identically to that of wild type during leaf senescence, but without the decline in Chl content that normally parallels it. While the majority of leaf proteins, including Rubisco, decline at the normal rate during senescence in *SGR* plants, Chl-associated plastid proteins are preferentially retained in the mutant, indicating overall failure to dismantle Chl–protein complexes in plants lacking functional SGR. Analysis of *SGR* mutants in pea has demonstrated delayed reduction of maximal PSII fluorescence yield (F_v/F_m) and PSII degradation during senescence, supporting a role for SGR in the disassembly of PSII (Aubry *et al.*, 2008). The SGR protein sequence is highly conserved across all higher plants investigated, but its function is as yet uncertain.

We here show how modification of *SGR* expression can influence an HR elicited by *Pseudomonas syringae* pv *tomato* (*Pst*) *avrRpm1* on Arabidopsis. The modulation of the HR was

at least partially associated with Chl catabolism and particularly the formation of Pheide and hence $^1O_2$. The formation of Pheide was correlated with a reduction in PaO protein. Disruption of PET probably leads to the production of ROS during the HR which could trigger Chl catabolism. Therefore, this adds a photosensitive step in the cell death process.

Materials and Methods

Plant materials and chemicals

Arabidopsis plants of various genotypes were grown at 20 ± 2°C under an 8-h light period and used at *c.* 4 wk following germination. Unless otherwise stated, all mutants were obtained from the Nottingham Arabidopsis Stock Centre (NASC; Nottingham, UK; http://arabidopsis.info/). The *acd1-20* mutant used in this study exhibited an essentially identical phenotype to that of *acd1-1* (Greenberg & Ausubel, 1993); the latter mutant is not available from public stock centres. The derivation of Landsberg erecta (L*er*) transgenic line *PR1-GUS* has been described previously (Clarke *et al.*, 2004) while L*er* transformed with the senescence responsive *SAG12* promoter-*GUS* fusion was derived by Richard Amasino (University of Wisconsin, Madison, WI, USA). For the derivation of the *staygreen* over-expression line (*SGR-OX*), the production of *SGR-OX*, full-length *AtSGR* (Aubry *et al.*, 2008) was cloned into pC-TAPa (Rubio *et al.,* 2005) using Gateway technology (Invitrogen). The 9× myc, 6× His and 2× IgG-binding-domain tags were added at the C-terminus of SGR with the intention to use them for tandem affinity purification experiments. Such experiments will be described elsewhere. As a control, GFP was used, which was tagged with the same domains and was directed to the chloroplast via N-terminal fusion with the chloroplast transit peptide of the small subunit of Rubisco (pNTAP-RBCS-GFP; U. Eckhardt, pers. comm.). Columbia-0 (Col-0) was transformed with the floral dip method and homozygous plants of the T_2 generation were selected.

Plants were illuminated with 55-W (Osram, Sylvania, Munich, Germany) high-frequency lighting tubes (4580 lumen output), supplemented with 2 × 30-W clear tube cooled lighting. Light fluence rates at the top of the plants varied according to the requirements of the experiments.

All chemicals were purchased from Sigma Pharmaceuticals Ltd (Poole, UK).

Pathogen inoculation procedures and estimations of cell death

Arabidopsis plants of various genotypes were inoculated with avirulent *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000 *avrRpm1* or nonpathogenic/non-HR-eliciting *Pst* strain *hrpA* as described previously (Mur *et al.*, 2000) using inocula of 2×10^6 cells ml^{-1}.

Cell death was estimated by electrolyte leakage in 1 cm diameter cores as described in Mur *et al.* (2000).

Visualization of H_2O_2 and singlet oxygen sensor green (SOSG)

After sampling, the excised leaves were immersed in an aqueous solution of 1 mg ml^{-1} 3,3′-diaminobenzidine (DAB), pH 3.8, and incubated at room temperature for 4 h. The leaves were removed from the DAB solution and fixed and cleared in absolute ethanol. The samples were scanned using a flatbed scanner and the intensity of staining was quantified using Image-Quant TL software (GE Healthcare Life Sciences, Little Chalfont, UK). SOSG dye (Invitrogen, Paisley, UK) was used at 10 μM (in deionized water) and infiltrated into areas previously inoculated with *Pst avrRpm1*, *Pst hrpA* or 10 mM $MgCl_2$ at 6 h after inoculation (hai). Infiltrated areas were viewed following excitation with 488-nm light and emission with 520-nm light using an Olympus BH2 microscope (Southend-on-Sea, UK).

Analysis of Chl and its derivatives

Chorophylls and their immediate catabolites were extracted from frozen leaf material using 80% (v/v) acetone and separated by reversed-phase high-performance liquid chromatography (HPLC) as described previously (Roca *et al.*, 2004). Pheide levels were expressed in terms of HLPC trace peak area normalized to leaf fresh weight.

Chl fluorescence parameters

Chl fluorescence from plant leaf areas infiltrated with bacteria was measured in air with a PAM 2000 portable fluorimeter (Waltz, Effeltrich, Germany) as described previously (Kingston-Smith *et al.*, 1997).

β-glucuronidase assays

Histochemical assays of GUS activity involved placing samples into 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (first dissolved in dimethyl formamide at 1 mg ml^{-1}) in 50 mM $NaPO_4$, pH 7.0, and 0.1% Triton X-100. For infiltration, the samples were placed under vacuum twice for 2 min each time and then incubated at 37°C overnight. The Chl was removed by sequential extraction with 30%, 75% and 95% ethanol.

Northern hybridization

RNA was extracted using RNAEasy kits (Qiagen, Crawley, UK) following the manufacturer's instructions. Northern blotting and hybridization were undertaken as described in Draper *et al.* (1988). A probe for *PR1* (At2g19990) was

obtained from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org). *SAG12* (At5g45890) was the gift of Dr Vicky Buchanan Wollaston (Warwick University, Coventry, UK).

Real-time polymerase chain reactions (qPCRs)

cDNA was synthesized from purified RNA by reverse transcription using ImProm II reverse transcriptase (Promega) and oligo dT primers as per the manufacturer's protocol. Similar reactions containing no reverse transcriptase were used to test for genomic contamination. qPCR analysis was carried out using an Applied Biosystem 7500 Real Time PCR System and SYBR green master mix in accordance with the manufacturer's instructions. Initial reactions with actin primers (Act F1 (5′-TCTTGTTCCAGCCCTCG TTT) and Act R1 (5′-TCTCGTGGATTCCAGCAGCT) created using Primer Express software (Applied Biosystems)) were used to standardize template concentration. Relative transcript abundance was determined using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak & Schmittgen 2001) normalized to actin, following qPCR of the cDNA template using gene-specific primers; PaO F1 (5′-TTCAAGGTGGAGTC AAGTGGG), PaO R1 (5′-GTCATCATTCGCACCTT GGA), SGR F1 (5′-GGGAAGCTGTTTCGCCTGA) and SGR R1 (5′-GAGCCTCGGGAAGAGTCTCA) primers were created using Primer Express software. Each cDNA reaction was analysed in triplicate per run, and mean values were obtained from three separate runs.

Salicylic acid (SA) and jasmonic acid (JA) measurements

SA concentrations were determined as described in Clarke *et al.* (2004). JA concentrations were determined as described in Allwood *et al.* (2006).

Western blotting

Western transfer of proteins to nitrocellulose membranes was undertaken as described in Bi *et al.* (1995). PaO antibodies (Aubry *et al.*, 2008) were detected using assays for horseradish peroxidase on the secondary antibody with an ECL chemiluminescence kit (GE Healthcare UK Ltd, Little Chalfont, UK).

Repetition and statistical analysis

Each experiment was performed at least three times, generating similar data to those presented in this paper. The exception was the northern hybridization experiment, which was repeated only once, and gave similar results. All other data were tested for significance by ANOVA using Minitab v 13 (Minitab Ltd, Coventry, UK).

Results

Arabidopsis lines over-expressing SGR exhibit spontaneous necrotic flecking

We previously described an Arabidopsis homologue of Mendel's *I* gene (At4g22920) which, when suppressed by RNA interference in line *SGRi*, resulted in a staygreen phenotype (Armstead *et al.*, 2007). To explore further the roles of *SGR* in plant physiology, over-expression lines (*SGR-OX*) in Arabidopsis were generated (Supporting Information Fig. S1a). These transgenic lines formed discrete necrotic-like flecks when grown under long-day conditions (Fig. 1a) and tended to be smaller than wild-type plants (Fig. S1b,c). Staining with trypan blue established that the flecks in *SGR-OX* lines represent areas of cell death (Fig. 1b). The H_2O_2 stain DAB revealed sites of ROS generation in apparently asymptomatic leaves (Fig. 1c), which were likely to be contributing to the formation of necrotic flecks. Lesions did not occur in control plants expressing chloroplast-directed GFP (data not shown), indicating that *SGR* expression was responsible for the phenotype.

Modulating the expression of *SGR* influences the HR

Given the apparent parallels between flecking and the formation of an HR, *SGR-OX* plants were challenged with avirulent *Pst avrRpm1* bacteria. No apparent macroscopic change in the HR was observed (Fig. 2a), but electrolyte leakage indicated that cell death was accelerated compared with controls (Fig. 2b).

To assess how *SGR* could contribute to the *Pst avrRpm1*-elicited HR in wild-type plants, *SGR* transcript accumulation was determined. Compared with inoculations with the non-HR-eliciting strain *Pst hrpA*, qPCR indicated significant ($P < 0.001$) increases in *SGR* transcript by 6 h after inoculation of Arabidopsis (Fig. 2c).

The HR was investigated in RNAi *SGR* (*SGRi*) and wild-type lines with *Pst avrRpm1* in the light (160 μmol m^{-2} s^{-1}; Fig. 3a). A delay in HR cell death (Fig. 3b) and electrolyte leakage (Fig. 3b) in *SGRi* plants compared with Col-0 was observed. To determine whether these effects were specific to a *Pst avrRpm1*-elicited HR, *SGRi* lines were inoculated with *Pst avrPphB* or *Pst avrRps4*. In both cases, electrolyte leakage suggested that the HR was delayed (Fig. S2). As the kinetics of cell death are significantly influenced by ROS (Levine *et al.*, 1994), Col-0 and *SGRi* were stained with DAB at 6 h after inoculation with *Pst avrRpm1* or a 10 mM $MgCl_2$ infection control. In *SGRi*, DAB staining was reduced but not abolished compared with Col-0 (Fig. 3d). There was a significant but not dramatic increase in *Pst avrRpm1* bacterial numbers in *SGRi* compared with Col-0. This indicates that the delayed formation of an HR does not greatly compromise plant resistance (Fig. 3e).

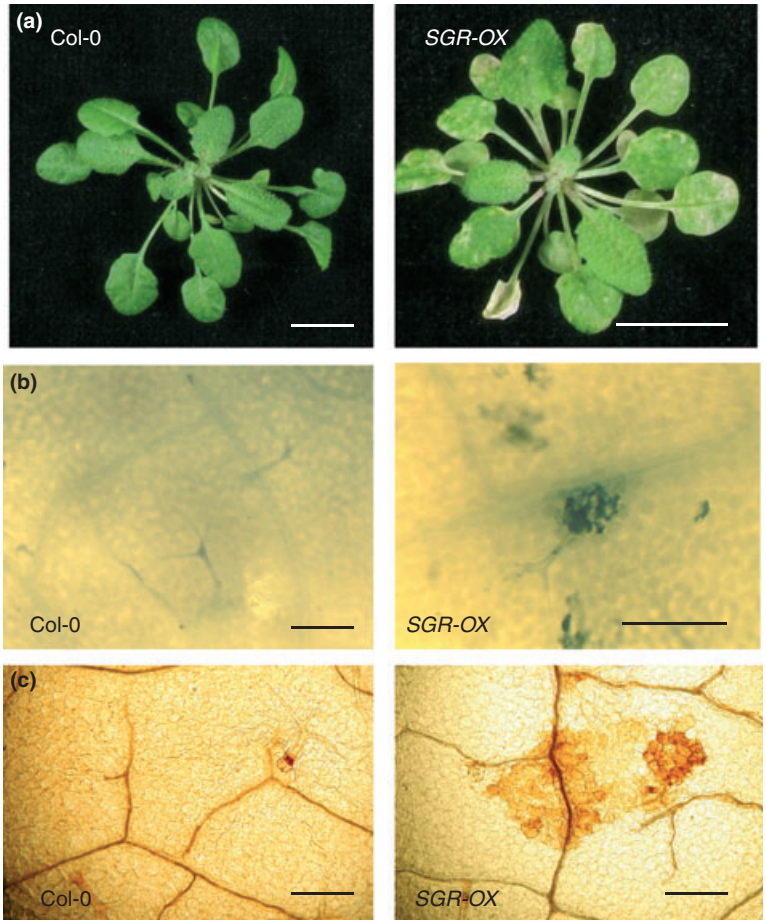

Fig. 1 Necrotic flecking in *staygreen*-over-expressing (*SGR-OX*) lines. (a) Long-day-grown *Arabidopsis thaliana* Columbia (Col-0) (wild type (WT)) and transgenic *SGR-OX* lines, grown at 160 μmol m^{-2} s^{-1}, at 4 wk following germination. Note the contained necrotic flecks within leaves on *SGR-OX* plants. Bars, 1 cm. The phenotypes of two other *SGR-OX* transgenic lines are given in Supporting Information Fig. S1. (b) Trypan-blue and (c) 3,3-diaminobenzidine (DAB) staining of asymptomatic areas of Col-0 and *SGR-OX* leaves (bars, 1 mm).

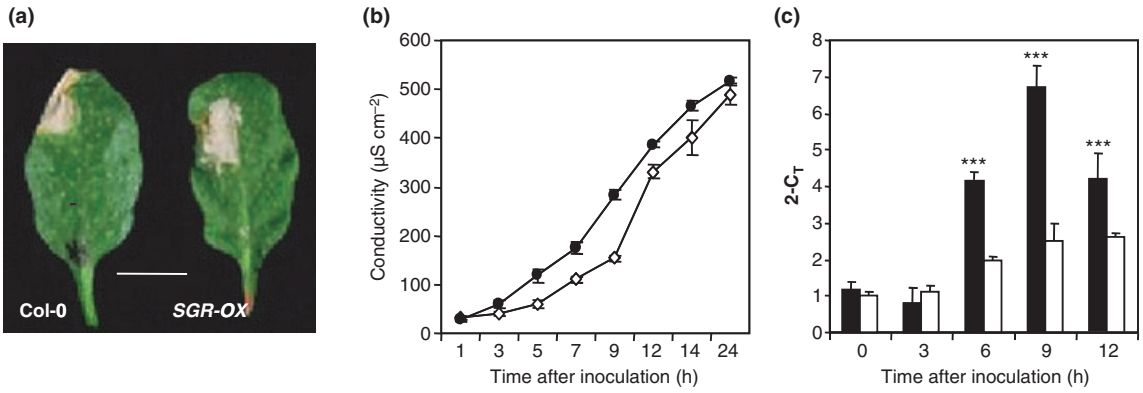

Fig. 2 The *Pseudomonas syringae* pv *tomato* (*Pst*) *avrRpm1*-elicited hypersensitive response (HR) is augmented in *staygreen*-over-expressing (*SGR-OX*) lines. (a) The HR in *Arabidopsis thaliana* Columbia (Col-0) and *SGR-OX* lines at 48 h after inoculation (hai) with *Pst avrRpm1*; plants were maintained at 160 μmol m^{-2} s^{-1} (bar, 1 cm). (b) Electrolyte leakage in Col-0 (wild type (WT), diamonds) and *SGR-OX* (circles) plants following inoculation with *Pst avrRpm1*. Results represent the mean of six samples per treatment (± SE). All differences after 3 hai were significant ($P < 0.001$). Significant differences ($P < 0.001$) between plant responses to *Pst avrRpm1* and *Pst hrp A* are indicated by ***. (c) Quantitative PCR assessments of *SGR* mRNA accumulation in Col-0 following inoculation with *Pst avrRpm1* (closed columns) or the non pathogenic and non HR-eliciting mutant *Pst hrpA* (open columns) mutant.

Chl catabolism occurs during the HR

One feature of *SGRi* lines is a greatly reduced accumulation of phytotoxic Chl catabolites (Armstead *et al.*, 2007). We hypothesized that a build-up of catabolites could contribute to oxidative events. To test this hypothesis, the kinetics of HR cell death were investigated in *acd1-20* and *acd2-2* mutants, where breakdown of Chl catabolites would be perturbed. *acd1-20* exhibited augmented electrolyte leakage at 6 hai, while *acd2-2* did not significantly differ from

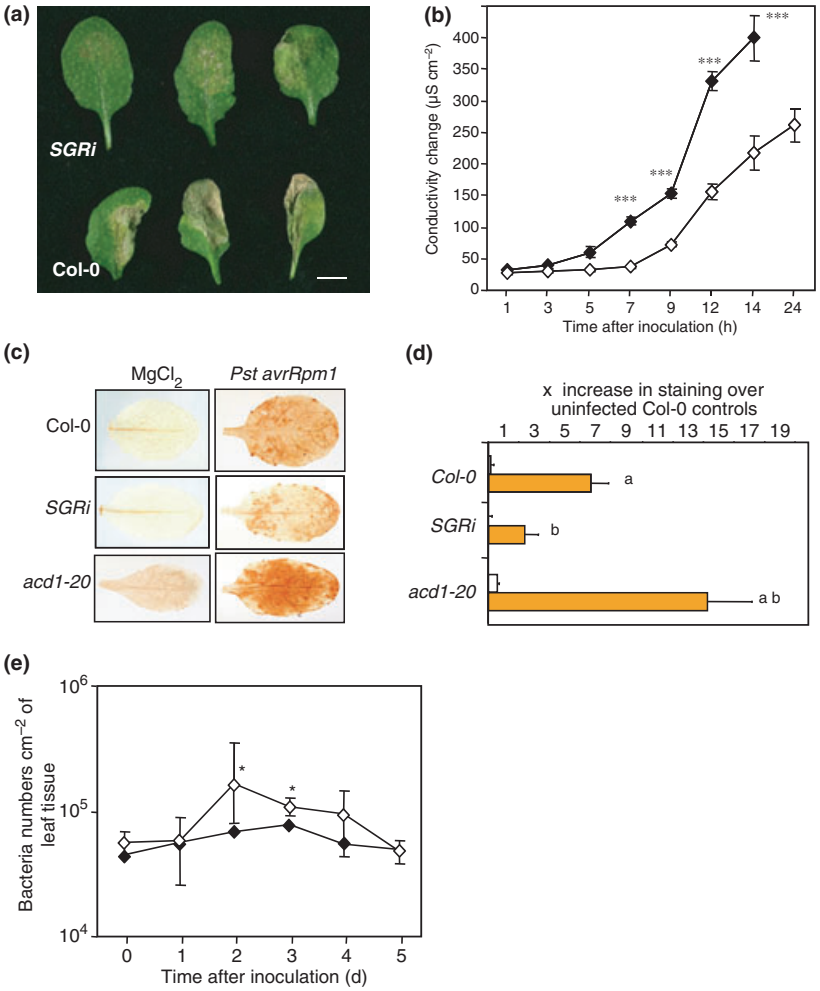

Fig. 3 Hypersensitive response (HR) type cell death in an At4g22920 silenced *staygreen* line. (a) The HR phenotype in Columbia (Col-0) (wild type (WT)) and RNAi *staygreen* (*SGRi*) at 48 h after inoculation (hai) in the light (164 μmol m^{-2} s^{-1}) with the HR-eliciting *Pseudomonas syringae* pv *tomato* (*Pst*) *avrRpm1*. Three examples of inoculated leaves for *SGRi* and Col-0 are provided. Note the minor necrotic flecks and chlorosis within inoculated areas in the *SGRi* lines. (Bar, 1 cm.) (b) Electrolyte leakage in Col-0 (WT, closed diamonds) and *SGRi* (open diamonds) plants following inoculation with *Pst avrRpm1*. Results represent the mean of six samples per treatment (± SE). Significant differences ($P < 0.001$) between responses of plant genotypes are indicated by ***. (c) H_2O_2 accumulation in Col-0, *SGRi* and *accelerated cell death 1 (acd1-20)* at 6 h after inoculation with *Pst avrRpm1* or 10 mM $MgCl_2$ when plants were maintained in the light (164 μmol m^{-2} s^{-1}). H_2O_2 accumulation was visualized by staining with 3,3-diaminobenzidine (DAB). (d) The results of four replicates of inoculations with either 10 mM $MgCl_2$ (white bars) or *Pst avrRpm1* (orange bars) were quantified and are represented graphically. Different letters ('a' and 'b') indicate significant differences ($P < 0.01$) between genotypes. (e) Bacterial numbers in Col-0 (closed diamonds) and *SGR1i* (open diamonds) after inoculation with *Pst avrRpm1*. *Statistically significant difference ($P < 0.05$).

wild-type plants in this respect (Fig. 4a). These data imply that Chl catabolites were rapidly generated during the HR. To verify this, Chl and its photolabile catabolites were extracted from dark-incubated Col-0, *acd1-20* and *acd2-2* following inoculations with *Pst avrRpm1* and analysed by HPLC. The dark incubation was essential, otherwise the Chl catabolites would immediately photobleach in the light and would therefore be undetectable. On the basis of retention times and absorption spectra, the major compounds present were identified as Chl*a*, Chl*b* and Pheide *a* (Fig. S3 shows a typical trace for Col-0, in which these three compounds are all represented). No accumulation of Pheide was detected in any genotype either before inoculation with *Pst avrRpm1* or at any time-point in tissue inoculated with *Pst hrpA* (data not shown). Following inoculation with *Pst avrRpm1,* Pheide was first detected in *acd1-20* at 6 hai (Fig. 4b) and in wild-type plants at 12 hai. Given the results obtained with *acd1-20,* it may be presumed that Pheide is produced earlier than 12 hai in wild-type plants, but is further metabolized by PaO. Interestingly, no Pheide was observed in *acd2-2* before 24 hai. When Pheide accumulation was assessed in *SGRi* up to 3 d following inoculation of *SGRi* and Col-0 with *Pst avrRpm1*, none was detected (Table 1). Thus, the altered kinetics of the HR in *SGRi*, Col-0 and *acd1-20* correlated with differing Pheide accumulation.

To correlate these observations with $^1O_2$ generation, *in planta* visualization was carried out using the sensitive and specific SOSG dye (Flors & Nonell, 2006). $^1O_2$ generation was detected at 6 hai with *Pst avrRpm1*, that is, before plant cell collapse and when Pheide concentrations would be increasing during the HR. SOSG staining indicated that $^1O_2$ concentrations in Col-0 were increased in *acd1-20* and *SGR-OX* but reduced in *SGRi* (Fig. 4c). No SOSG staining was observed in uninoculated control plants of any genotype (data not shown).

Levels of PaO protein were determined in wild-type plants following whole-leaf inoculation with *Pst avrRpm1*, and also with *Pst hrpA* and 10 mM $MgCl_2$ as controls (Fig. 4d). The control inoculations caused a transient increase in amounts of PaO between 3 and 12 hai, probably as a consequence of the wounding associated with the inoculation procedure (Yang *et al.*, 2004). With *Pst avrRpm1*, this increase was not observed and, indeed, by 12 hai PaO

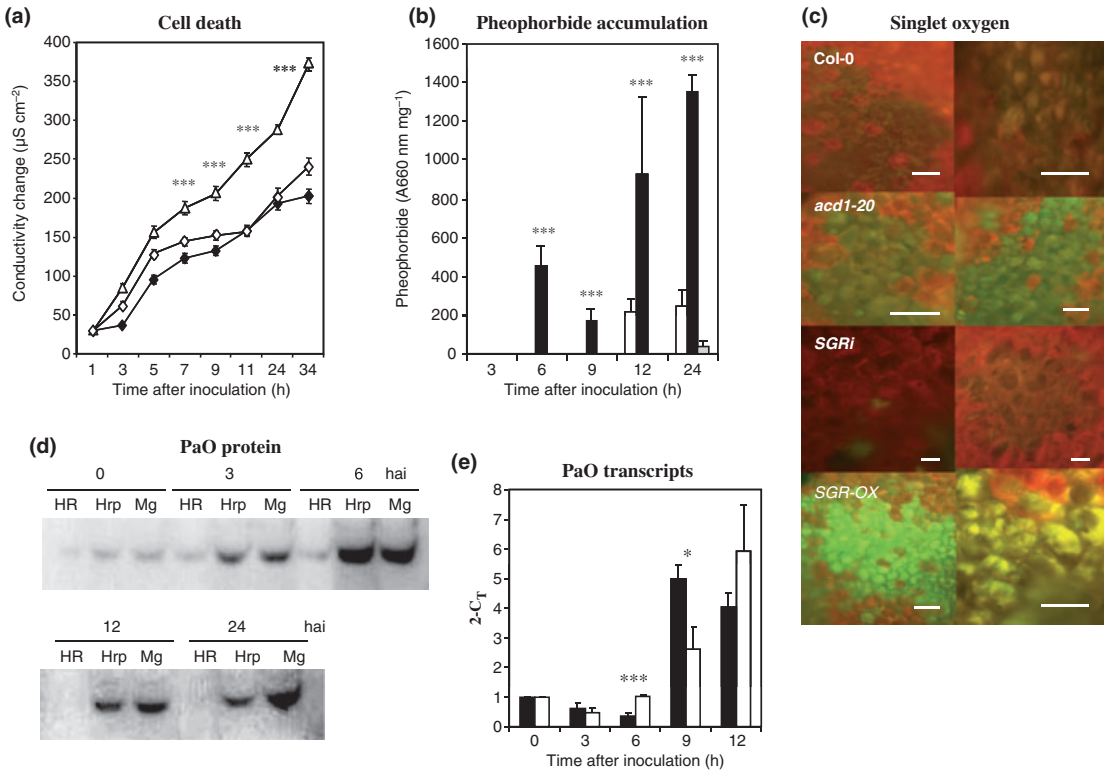

Fig. 4 Pheide *a* oxygenase (PaO) influences the development of Hypersensitive Response (HR)-associated cell death. (a) Electrolyte leakage in Columbia (Col-0) (open diamonds), *accelerated cell death 1* (*acd1-20*) (triangles) and *accelerated cell death 2* (*acd2-2*) (closed diamonds) in Arabidopsis in the light (164 μmol m^{-2} s^{-1}) following inoculation with the HR-eliciting strain *Pseudomonas syringae* pv *tomato* (*Pst*) *avrRpm1* . Results represent the mean of six samples per treatment (± SE). Significant differences ($P < 0.001$) between responses of plant genotypes are indicated by ***. (b) Pheide *a* accumulation in Col-0 (open squares), *acd1-20* (black squares) and *acd2-2* (grey squares) following inoculation with *Pst avrRpm1* and incubation in the dark. Pheide peak areas in the high-performance liquid chromatography (HPLC) are normalized with respect to fresh weight. Each point represents the average of three samples (± SE). (c) Singlet oxygen sensor green (SOSG) detection of singlet oxygen generation in Col-0, *acd1-20*, RNAi *staygreen* (*SGRi*) and *staygreen*-over-expressing (*SGR-OX*) at 6 h after inoculation with *Pst avrRpm1*. Typical replicate images are shown for each genotype. (Bars, 100 μm.) (d) Concentrations of PaO with *Pst avrRpm1* (hypersensitive response (HR)), the non-disease forming and HR-eliciting strain *Pst hrpA* mutant (Hrp) and 10 mM $MgCl_2$ (Mg) at various time-points (hours after inoculation (hai)) as detected using PaO monoclonal. (e) Quantitative PCR assessments of PaO mRNA accumulation in Col-0 following inoculation with *Pst avrRpm1* (HR, closed squares) or the *Pst hrpA* mutant (Hrp, open squares). **, $P < 0.01$; ***, $P < 0.001$.

Table 1 Pheophorbide accumulation in *Arabidopsis thaliana* Columbia (Col-0) and RNAi *staygreen* (*SGRi*) following inoculation with the Hypersensitive Response eliciting *Pseudomonas syringae* pv *tomato* DC3000 *avrRpm1*

	Day 0	Day 1	Day 2	Day 3
Wild type	nd	140 (70)	520 (67)	570 (53)
SGRi	nd	nd	nd	nd

Pheide levels represent high-performance liquid chromatography (HPLC) peak areas normalized with respect to mg fresh weight. Each value represents the average ratio of three samples (± SE). nd, not detected.

protein was not detected, which agreed with the timescale of Pheide appearance in wild-type plants (Fig. 4a). Parallel assays for Rubisco large subunit (LSU) protein using monoclonal antibody demonstrated equally loading (data not shown) and also that PaO protein loss preceded the general proteolysis known to occur during the HR (Mur *et al.*, 2008). Conversely, increased PaO transcript levels following inoculations of both *Pst avrRpm1* and *Pst hrpA* after 9 hai (Fig. 4e) suggested that reduced PaO protein concentrations seen with the *Pst avrRpm1* inoculations arose from a control mechanism operating at the post-transcriptional and/or post-translational level.

Cell death associated with the *Pst avrRpm1*-elicited HR are influenced by light

One prediction of accumulation of Chl catabolites during the HR is that they will contribute to a light-mediated effect. *Arabidopsis thaliana* Col-0 was inoculated with *Pst avrRpm1* and incubated under different light fluence rates, leading to distinctive phenotypes (Fig. 5a). As light fluence rates were progressively reduced from *c.* 160 μmol m^{-2} s^{-1}, the inoculated areas became correspondingly less necrotic, appearing increasingly green, while the surrounding areas

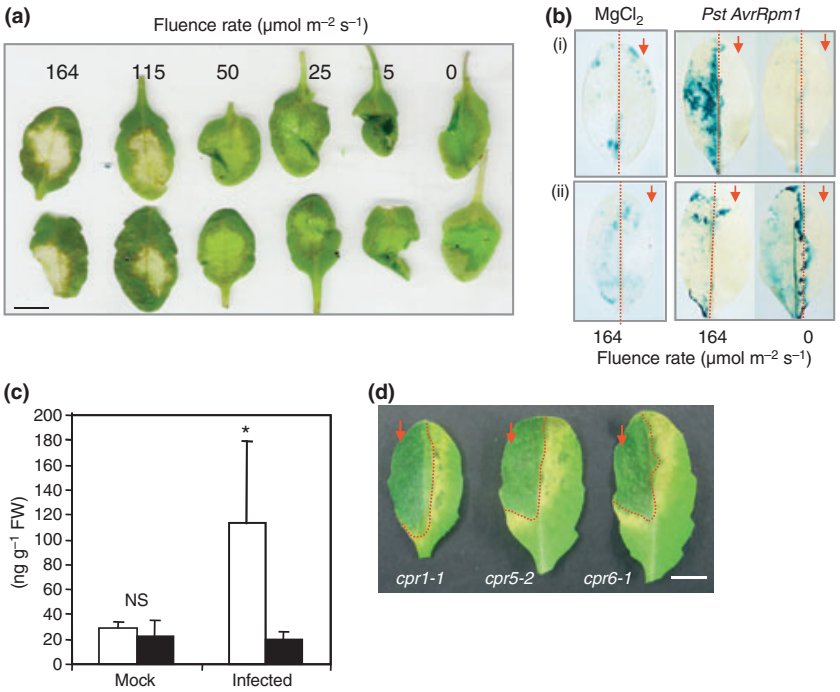

Fig. 5 Light-dependent effects on the hypersensitive response (HR) elicited by *Pseudomonas syringae* pv *tomato* (*Pst*). (a) Phenotypes following inoculation with the HR eliciting *Pst* DC3000 *avrRpm1* at 48 h after inoculation (hai) under the light fluence rates indicated. The leaves remained attached until photography. Two examples of HR at each fluence rate are given. (b) Histochemical GUS assay using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) following half leaf inoculation with 10 mM $MgCl_2$ or *Pst avrRpm1* and incubation under light (164 μmol m^{-2} s^{-1}) or dark conditions in (i) *Pathogenesis-Related 1 (PR1) PR1-GUS* and (ii) *Senescence associated gene 12 (SAG) SAG12-GUS* transgenic lines. Inoculated half leaves are arrowed. (c) The accumulation of salicylic acid (SA) around HR lesions at 48 h following challenge with *Pst avrRpmI* and incubation under 164 μmol m^{-2} s^{-1} (open squares) or in darkness (closed squares). A significant difference (*, $P = 0.05$) between light- and dark-incubated samples is indicated. (d) HR lesions in Arabidopsis *constitutive pathogenesis related* mutants *cpr1-1*, *cpr5-2* and *cpr6-1* mutants at 48 h following incubation in the dark. Inoculated areas are surrounded by a red dotted line and arrowed. (Bar, 1 cm.)

became more chlorotic. Measurements of electrolyte leakage indicated that diminishing light fluence rates also reduced cell death (Fig. S4).

PR1 gene expression, which was modified in *SGR* modulated lines, has been reported to be light-dependent (Zeier *et al.*, 2004). We have tested this using northern hybridization (Ougham *et al.*, 2008), in conjunction with assays of *PR1-GUS* transgenic plants (Fig. 5bi). Both approaches indicated that *PR1* expression at the periphery of the HR zone was influenced by light fluence rates. To assess whether the chlorosis observed around the HR at low fluence rates could represent a senescence-like symptom, we investigated expression of the senescence marker gene *SAG12* (Pontier *et al.*, 1999). Both *SAG12* northern hybridization (Ougham *et al.*, 2008) and *SAG12-GUS* promoter-fusion activity showed increasing up-regulation with lowering light fluence rates (Fig. 5bii), supporting the view that a form of senescence is initiated at low light fluence rates.

PR1 is a marker for SA, and a compromised HR in the dark has been associated with reduced accumulation of this stress hormone (Zeier *et al.*, 2004). We also observed that SA concentrations were significantly ($P < 0.05$) reduced in dark incubated HR (Fig. 5c). To investigate whether this reduced concentration of SA was the source of the altered HR in the dark, Arabidopsis *constitutive pathogenesis related* mutants *cpr1-1, cpr5-2* and *cpr6-1* (Fig. 5d), each of which exhibits elevated concentrations of SA, were inoculated with *Pst avrRpm1*. In these mutants the dark-incubation HR was not restored to that seen in the light. This indicated that the dark HR phenotype did not arise as a consequence of reduced concentrations of SA.

Photosynthetic light-harvesting efficiencies are perturbed during the HR leading to ROS production

Although we have focused on the accumulation of Chl catabolites and $^1O_2$ generation, SGR is also likely to disrupt PET, leading to the light-dependent production of O_2^- and H_2O_2. To establish the role of PET, the intrinsic efficiency of electron transport through photosystem II (F_v/F_m) was examined in Col-0 following inoculation with *Pst avrRpm1* and *Pst hrpA* (Fig. 6a). F_v/F_m was compromised by 5.5 hai in *Pst avrRpm1*- but not in *Pst hrpA*-inoculated leaves in the light. This decrease in F_v/F_m was coincident with increased electrolyte leakage and was associated with a decrease in the quantum yield of electron transfer at photosystem II (ϕ

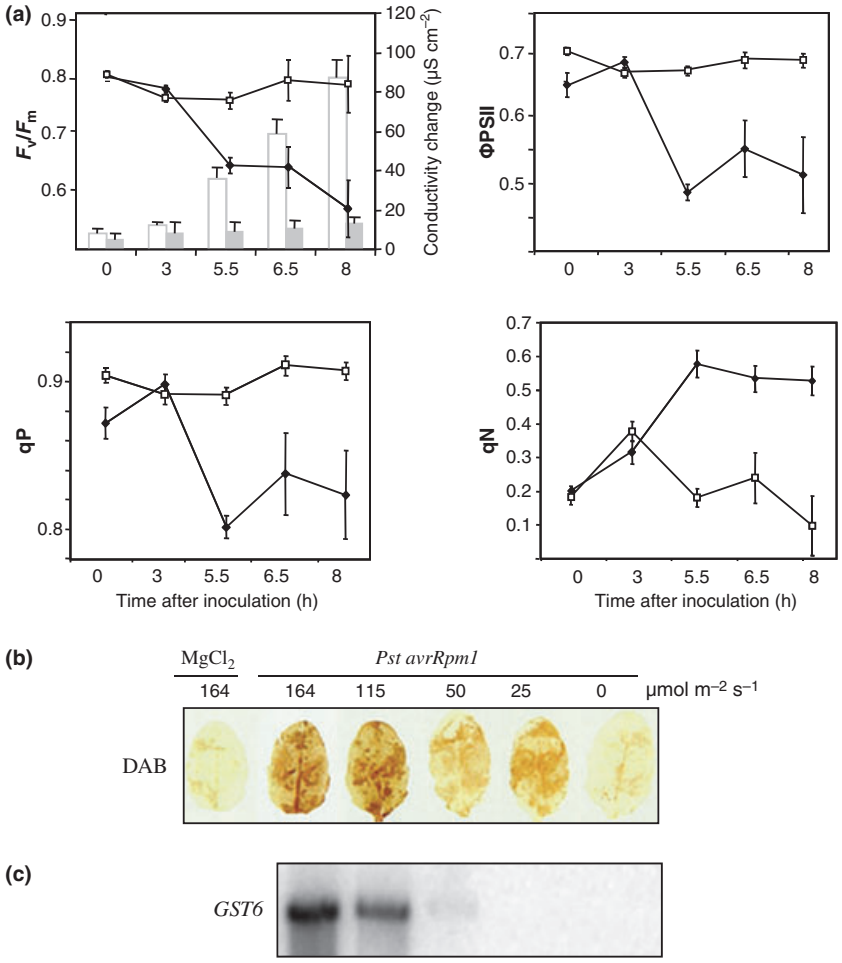

Fig. 6 Influence of light on oxidative effects and chlorophyll (Chl) fluorescence changes in Arabidopsis during a hypersensitive response (HR). (a) Chl fluorescence parameters in Arabidopsis plants following inoculation with HR-eliciting *Pseudomonas syringae* pv *tomato* (*Pst*) *avrRpm1* (diamonds) and a non-HR-eliciting mutant variant *Pst hrpA* (squares) when incubated in the light (164 μmol m^{-2} s^{-1}). Data for optimum photosynthetic quantum yield (F_v/F_m), quantum yield of electron transfer at photosystem II (ϕPSII), photochemical quenching (qP) and nonphotochemical quenching (qN) are presented. Results represent the mean of six biological replicates (± SE). F_v/F_m is related to levels of electrolyte leakage (conductivity changes) into the solution bathing leaves inoculated with either *Pst avrRpm1* (white bar) or *Pst hrpA* (grey bar). Results represent the mean of six samples per treatment (± SE). (b) H_2O_2 accumulation as indicated by staining with 3,3-diaminobenzidine (DAB) and (c) *Glutathionine S transferase 6 (GST-6)* expression at 6 h after inoculation (hai) with *Pst avrRpm1* when plants were incubated at the different fluence rates indicated.

PSII) and photochemical quenching (qP), indicating that infection resulted in a decrease in the number of available reaction centres, limiting flux through PET. As consumption of electrons in photochemistry declined, nonphotochemical quenching (qN) increased. Significantly, the impact of a *Pst avrRpm1*-induced HR on F_v/F_m, ϕPSII, qP and qN was reduced in *SGRi* (Fig. S5).

To correlate PET disruption in Col-0 with ROS generation, the extent of DAB staining was examined under different fluence rates at 6 h after inoculation with *Pst avrRpm1*, before there was any visible sign of leaf collapse. More intense staining was seen with higher fluence rates, while in the dark very little staining was observed (Fig. 6b). Expression of the H_2O_2-responsive marker gene *glutathione S-transferase (GST-6)*, determined at 6 h after inoculation with *Pst avrRpm1*, also proved to be light-dependent (Fig. 6c).

Discussion

The SGR protein plays a key role in the initiation of Chl degradation. Mutations in *SGR* genes have been associated with a cosmetic, Chl-retaining phenotype during senescence in a *Lolium/Festuca* introgression (Armstead *et al.*, 2006), rice (Jiang *et al.*, 2007; Park *et al.*, 2007), pea (Sato *et al.*, 2007), tomato and pepper (*Capsicum annuum*) (Barry *et al.*, 2008). The SGR mode of action remains to be clearly resolved but the protein is known to be chloroplast targeted and to bind to the light-harvesting complex (LHC) *in vivo* (Park *et al.*, 2007). In a pea *SGR* mutant line, senescence-associated proteolysis of the LHCs, reduction of F_v/F_m and the appearance of Chl catabolites were delayed (Aubry *et al.*, 2008). The weight of evidence suggests that SGR is required for LHC disassembly with concomitant effects on photosynthetic efficiency and Chl catabolism.

There have been many reports demonstrating that light/shading influences either resistance or the progression of disease (reviewed by Roberts & Paul, 2006). Light can influence plant cell death, for example in Arabidopsis protoplast cultures treated with the toxin fuminosin B1 (Asai *et al.*, 2000) or in whole plants inoculated with either the avirulent *P. s.* pv *maculicola* (Zeier *et al.*, 2004) or turnip crinkle virus (TCV; Chandra-Shekara *et al.*, 2006). Reduced resistance to pathogens in the dark has been noted under experimental conditions (Zeier *et al.*, 2004) and,

given the importance of the HR to resistance, may explain why certain pathogens infect at night. Indeed, light can suppress phytopathogenic fungal conidial germination or germ tube growth (Beyer *et al.*, 2004). The present study shows that over-expression of *SGR* is sufficient to elicit cell death in the absence of infection (Fig. 1a), while suppression of *SGR* may delay the *Pst avrRpm1*-elicited HR (Fig. 3a). In the light of these observations, we suggest that SGR has a function in triggering certain forms of HR and that under these conditions Chl catabolites accumulate and play a role in the cell death process, thereby contributing to the often-reported light sensitivity of certain types of HR. It should be noted that, as in a recent study where suppression of chloroplast-generated ROS reduced an HR (Zurbriggen *et al.*, 2009), our results suggest that the light-mediated step in the HR contributes little to plant resistance (Fig. 3e).

Correct metabolic processing of the Chl macrocycle is essential for plant homeostasis. Tetrapyrrole pigments are photosensitizers, transferring energy from a short-lived singlet excited state to molecular oxygen, thereby generating $^1O_2$. Within the LHCs, $^1O_2$ is quenched by carotenoids (Triantaphylides & Havaux, 2009). Unsurprisingly, several mutants exhibiting SD have been mapped to genes involved in Chl metabolism; for example, the *flu* mutation which leads to protochlorophyllide accumulation (Meskauskiene *et al.*, 2001). Lesions in *acd1* and *acd2* correspond to enzymes involved in the catabolism of Pheide *a* and RCC, respectively (Fig. 7; Hörtensteiner, 2006). Our observations also imply a role for Chl catabolites in the *Pst avrRpm1*-elicited HR. Pheide *a* could be detected in *acd1-20* as early as 6 hai (Fig. 4b), indicating an early, rapid initiation of Chl catabolism during the HR. Crucially, where Pheide concentrations were either increased or decreased in the *acd1-20* mutant or *SGRi* line, respectively, there was a corresponding modification in the rate of HR-associated cell death. Further, examination of PaO/ACD1 protein concentrations during the HR showed that these decreased after 6 hai, following which increases in Pheide were observed. This suggests that the HR has features in common with that of the *acd1-20* mutant, where the accumulation of Pheide in the absence of PaO contributes directly to spontaneous cell death. We observed that $^1O_2$ generation could be modulated by manipulating *SGR* transcript levels (Fig. 4c), thereby linking *SGR* effects on the HR to $^1O_2$ generation. Further, although defence-associated phenalenone phytoalexins can generate $^1O_2$ (Flors & Nonell, 2006), the increases seen with *acd1-20* would suggest that Chl catabolites, perhaps especially Pheide, are the source of $^1O_2$. Based on this, it may be hypothesized that Chl catabolites could be triggering the HR; but we consider this to be unlikely, for the following reasons. Pheide accumulation occurred after 6 hai, some time after other HR initiatory events, for

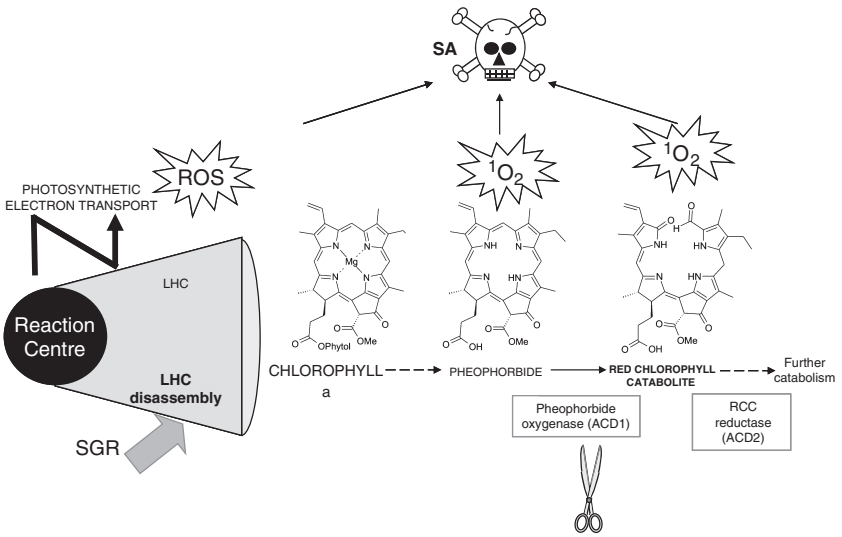

Fig. 7 Model for the contribution of chlorophyll (Chl) catabolites to a hypersensitive response (HR). Dismantling of the photosystem reaction centres and light-harvesting complex (LHC) disrupts photosynthetic electron transport leading to reactive oxygen species (ROS) production. This triggering event is proposed to be influenced by a staygreen (SGR) or SGR-like protein. The Chl porphyrin moiety is removed from the LHCs and is first dechelated to form pheophytin and then dephytylated by pheophytinase to form chlorophyllase (Schelbert *et al.*, 2009). The central magnesium (Mg) is removed by Mg dechelatase to form pheophorbide (Pheide) *a*, which is localized to the stroma. Pheide is cleaved by Pheide *a* oxygenase (PaO, encoded by *ACCELERATED CELL DEATH 1 (ACD1) ACD1* in Arabidopsis) to form red Chl catabolite (RCC). RCC is further processed by RCC reductase (encoded by *ACD2* in Arabidopsis) to form colourless Chl catabolites. Porphyrin and tetrapyrrole excitation by light giving rise to ROS is indicated. This pathway is reviewed by Hörtensteiner (2006). The accumulation of Pheide from LHCs is exacerbated as a result of a reduction in PaO concentrations (indicated by scissors on the figure). Photoactivation of Pheide, causing photobleaching, makes a significant light-dependent contribution to ROS production during the HR. The light-dependent ROS production results in the observed impact of light on HR cell death and *Pathogenesis-related 1 (PR1)*, as salicylic acid (SA) production is modulated by ROS.

example nitric oxide (NO) generation and the initiation of an oxidative burst, are known to occur (Mur *et al.*, 2008). It is more probable that Chl catabolite accumulation is associated with cellular collapse which is first indicated by electrolyte leakage, which also increases at *c.* 6 hai (Fig. 2b). Indirect evidence that supports this later role for Chl catabolites and $^1O_2$ is our observation that there was no major disruption in the HR when the EXECUTER mutants *exe1* and *exe2* were inoculated (Fig. S6).

Interference with PET leading to ROS production has been suggested to initiate tetrapyrrole release and thence lead to $^1O_2$ formation (Triantaphylides & Havaux, 2009; Zurbriggen *et al.*, 2009). Thus, SGR-triggered Chl catabolism could be initiated by disruption of PET. The modulation of photosynthesis following pathogenic challenge has been noted by many workers (e.g. Chou *et al.*, 2000; Berger *et al.*, 2004). Bonfig *et al.* (2006) examined photosynthetic parameters following inoculation of Arabidopsis with *Pst avrRpm1*, with virulent *Pst* or with 10 mM $MgCl_2$ and showed that F_v/F_m decreased 3 h after inoculation with *Pst avrRpm1* but not the virulent *Pst.* In our experiment, reduced F_v/F_m was observed between 3 (Fig. S5) and 5 (Fig. 5a) hai, the latter being coincident with the first detection of Pheide in *acd1-20* (Fig. 4b). Interestingly, the reduction of F_v/F_m was not observed in *SGRi* lines (Fig. S5) which also failed to exhibit Pheide accumulation (Table 1). The behaviour of *SGRi* plants is consistent with a triggering role for PET disruption. In Col-0 the decrease in F_v/F_m was coupled with declining PSII efficiency (ϕ PSII), and plastoquinone pools (qP) becoming progressively reduced. The resulting over-reduction of the electron transport components would contribute to death through electron donation to molecular oxygen to produce reactive oxygen and H_2O_2, as visualized by DAB staining (Fig. 6b).

The timing of light-associated ROS generation coincided with the persistent rise in ROS that forms part of the HR-associated oxidative burst (Lamb & Dixon, 1997; Mur *et al.,* 2005), perhaps suggesting that this could be of chloroplastic origin. The source of this oxidative burst has been most often linked to an NAPDH oxidase (Torres *et al.*, 1998, 2002) and Zeier *et al.* (2004) also suggested that ROS generation was light-independent. However, in Zeier *et al.* (2004), oxidative events were assessed at 24 hai rather than 6 hai as in this study, which is coincident with the oxidative burst (Mur *et al.,* 2005). In this context, it is relevant that Zeier *et al.* (2004) demonstrated light-dependent expression of the ROS-responsive *gst1* gene at 4 hai. However, PET perturbation is not a feature of all forms of cell death. Garmier *et al.* (2007) investigated cell death in *Nicotiana sylvestris* leaves infiltrated with harpin, which forms ion-conducting pores in lipid bilayers and is likely to be a virulence factor involved in mobilizing nutrients from the host (Lee *et al.*, 2001). Unlike the *Pst avrRpm1*-elicited HR, harpin-induced cell death was only marginally affected by light intensities, and programmed cell death-linked nuclear fragmentation was observed in both the light and the dark. As in our study, analyses of Chl parameters indicated considerable PSII damage but DAB staining was only trivially affected in dark-adapted leaves. It may be relevant that dark-maintained harpin-infiltrated leaves were simply delayed in dehydration, but did not remain green as we observed with *Pst avrRpm1* (Fig. 5a).

It has been suggested that light dependence during the HR is attributable to reduced SA concentrations (Asai *et al.*, 2000; Zeier *et al.*, 2004). For example, in the *P. s.* pv *maculicola*-elicited HR, SA concentrations and expression of SA-marker genes were increased in the light (Zeier *et al.*, 2004); features shared with the *Pst avrRpm1*-elicited HR (Fig. 5). Phytochrome A and B also influence cell death, at least in part, through SA-mediated effects (Genoud *et al.,* 2002). This could reflect the chloroplastic location of the SA biosynthetic gene isochorismate synthase (Wildermuth *et al.*, 2001). However, as *avrRpm1*-elicited cell death was not restored in Arabidopsis mutants where SA concentrations are increased (Fig. 5d), our data suggest that light-dependent SA biosynthesis does not explain the light-modulated character of certain types of HR. Similarly, Chandra-Shekara *et al.* (2006) could only partially restore resistance to TCV in the dark when SA was applied. A lack of SA has been shown to alter but not abolish the HR even in the light, through a compromised potentiation mechanism (Mur *et al.*, 1996, 2000). It may be that some workers are similarly observing a reduced potentiating effect when the HR is incubated in the dark. The early stages of jasmonate biosynthesis are also chloroplast-located (Weber, 2002) and Zeier *et al.* (2004) suggested that concentrations of jasmonate were increased in a dark-incubated HR. However, in our experiments, jasmonate concentrations within the HR were actually reduced in the dark (Fig. S7), which was in accord with the light-dependent expression of the jasmonate-marker gene *thionin2.1 (thi2.1)* reported by Zeier *et al.* (2004). However, as jasmonates are elevated in the Arabidopsis mutant *cpr5* (Boch *et al.,* 1998) and this did not display a normal HR in the dark (Fig. 5d), we suggest that the reduced concentrations of jasmonate are not a major cause of light effects on the HR.

Taking all of our data together, we suggest a sequence of chloroplast-centred events occurring in the elaboration of an HR (Fig. 7). During an initial phase (up to 6 hai), well-established triggering events take place: for example, Ca^{2+} influxes and NO, are likely to initiate SGR-mediated LHC disassembly resulting in PET disruption and light-dependent H_2O_2 generation. LHC disassembly also liberates Chl, leading to the HR-associated production of Pheide, mediated by the loss of PaO enzyme; Pheide then becomes a significant source of singlet oxygen. In the light, the olive-green Pheide pigmentation is lost from inoculated areas through photobleaching. This model is currently being tested in our laboratories.

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References

Allwood JW, Ellis DI, Heald JK, Goodacre R, Mur LAJ. 2006. Metabolomic approaches reveal that phosphatidic and phosphatidyl glycerol phospholipids are major discriminatory non-polar metabolites in responses by *Brachypodium distachyon* to challenge by *Magnaporthe grisea*. *Plant Journal* **46**: 351–368.

Almeras E, Stolz S, Vollenweider S, Reymond P, Mene-Saffrane L, Farmer EE. 2003. Reactive electrophile species activate defense gene expression in Arabidopsis. *Plant Journal* **34**: 202–216.

Apel K, Hirt H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, **55**: 373–399.

Armstead I, Donnison I, Aubry S, Harper J, Hörtensteiner S, James C, Mani J, Moffet M, Ougham H, Roberts L *et al.* 2007. Cross-species identification of Mendel's *I* locus. *Science* **315**: 73.

Armstead I, Donnison I, Aubry S, Harper J, Hörtensteiner S, James C, Mani J, Moffet M, Ougham H, Roberts L *et al.* 2006. From crop to model to crop: identifying the genetic basis of the staygreen mutation in the *Lolium/ Festuca* forage and amenity grasses. *New Phytologist* **172**: 592–597.

Asada K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* **141**: 391–396.

Asai T, Stone JM, Heard JE, Kovtun Y, Yorgey P, Sheen J, Ausubel FM. 2000. Fumonisin B1-induced cell death in Arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. *Plant Cell* **12**: 1823–1835.

Ashtamker C, Kiss V, Sagi M, Davydov O, Fluhr R. 2007. Diverse subcellular locations of cryptogein-induced reactive oxygen species production in tobacco bright yellow-2 cells. *Plant Physiology* **143**: 1817–1826.

Aubry S, Mani J, Hörtensteiner S. 2008. Stay-green protein, defective in Mendel's green cotyledon mutant, acts independent and upstream of pheophorbide a oxygenase in the chlorophyll catabolic pathway. *Plant Molecular Biology* **67**: 243–256.

Barry CS, McQuinn RP, Chung MY, Besuden A, Giovannoni JJ. 2008. Amino acid substitutions in homologs of the STAY-GREEN protein are responsible for the green-flesh and chlorophyll retainer mutations of tomato and pepper. *Plant Physiology*, **147**: 179–187.

Beers EP, McDowell JM. 2001. Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. *Current Opinion in Plant Biology* **4**: 561–567.

Berger S, Papadopoulos M, Schreiber U, Kaiser W, Roitsch T. 2004. Complex regulation of gene expression, photosynthesis and sugar levels by pathogen infection in tomato. *Physiologia Plantarum* **122**: 419–428.

Beyer M, Roding S, Ludewig A, Verreet JA. 2004. Germination and survival of *Fusarium graminearum* macroconidia as affected by environmental factors. *Journal of Phytopathology* **152**: 92–97.

Bi YM, Kenton P, Mur L, Darby R, Draper J. 1995. Hydrogen-peroxide does not function downstream of salicylic-acid in the induction of PR protein expression. *Plant Journal* **8**: 235–245.

Boccara M, Schwartz W, Guiot E, Vidal G, De Paepe R, Dubois A, Boccara AC. 2007. Early chloroplastic alterations analysed by optical coherence tomography during a harpin-induced hypersensitive response. *Plant Journal* **50**: 338–346.

Boch J, Verbsky ML, Robertson TL, Larkin JC, Kunkel BN. 1998. Analysis of resistance gene-mediated defense responses in *Arabidopsis thaliana* plants carrying a mutation in CPR5. *Molecular Plant-Microbe Interactions 1* **11**: 1196–1206.

Bolwell GP, Bindschedler LV, Blee KA, Butt VS, Davies DR, Gardner SL, Gerrish C, Minibayeva F. 2002. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of Experimental Botany* **53**: 1367–1376.

Bonfig K, Schreiber U, Gabler A, Roitsch T, Berger S. 2006. Infection with virulent and avirulent *P. syringae* strains differentially affects photosynthesis and sink metabolism in Arabidopsis leaves. *Planta* **225**: 1–12.

Chandra-Shekara AC, Gupte M, Navarre D, Raina S, Raina R, Klessig D, Kachroo P. 2006. Light-dependent hypersensitive response and resistance signaling against Turnip Crinkle Virus in Arabidopsis. *Plant Journal* **45**: 320–334.

Clarke SM, Mur LAJ, Wood JE, Scott IM. 2004. Salicylic acid dependent signaling promotes basal thermotolerance but is not essential for acquired thermotolerance in *Arabidopsis thaliana*. *Plant Journal* **38**: 432–447.

Chou HM, Bundock N, Rolfe SA, Scholes JD. 2000. Infection of Arabidopsis thaliana leaves with *Albugo candida* (white blister rust) causes a reprogramming of host metabolism. *Molecular Plant Pathology*, **1**: 99–113.

Delledonne M, Zeier J, Marocco A, Lamb C. 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceedings of the National Academy of Sciences, USA* **98**: 13454–13459.

Draper J, Scott R, Armitage P, Walden R. 1988. *Plant genetic transformation and gene expression. A laboratory manual.* Oxford, UK: Blackwell Scientific Publications.

Flors C, Nonell S. 2006. Light and singlet oxygen in plant defense against pathogens: phototoxic phenalenone phytoalexins. *Accounts of Chemical Research* **39**: 293–300.

Garmier M, Priault P, Vidal G, Driscoll S, Djebbar R, Boccara M, Mathieu C, Foyer CH, De Paepe R. 2007. Light and oxygen are not required for harpin-induced cell death. *Journal of Biological Chemistry* **282**: 37556–37566.

Gray J, Janick-Buckner D, Buckner B, Close PS, Johal GS. 2002. Light-dependent death of maize *lls1* cells is mediated by mature chloroplasts. *Plant Physiology* **130**: 1894–1907.

Genoud T, Buchala AJ, Chua NH, Metraux JP. 2002. Phytochrome signalling modulates the SA-perceptive pathway in Arabidopsis. *Plant Journal* **31**: 87–95.

Greenberg JT, Ausubel FM. 1993. Arabidopsis mutants compromised for the control of cellular-damage during pathogenesis and aging. *Plant Journal* **4**: 327–341.

Hörtensteiner S. 2006. Chlorophyll degradation during senescence. *Annual Review of Plant Biology* **57**: 55–77.

Hörtensteiner S, Wüthrich KL, Matile P, Ongania KH, Kräutler B. 1998. The key step in chlorophyll breakdown in higher plants – Cleavage of pheophorbide alpha macrocycle by a monooxygenase. *Journal of Biological Chemistry* **273**: 15335–15339.

Jiang HW, Li MR, Liang NB, Yan HB, Wei YL, Xu X, Liu JF, Xu Z, Chen F, Wu GJ. 2007. Molecular cloning and function analysis of the stay green gene in rice. *Plant Journal* **52**: 197–209.

Karpinski S, Gabrys H, Mateo A, Karpinska B, Mullineaux PM. 2003. Light perception in plant disease defence signalling. *Current Opinion in Plant Biology* **6**: 390–396.

Kingston-Smith AH, Thomas H, Foyer CH. 1997. Chlorophyll a fluorescence, enzyme and antioxidant analyses provide evidence for the operation of alternative electron sinks during leaf senescence in a stay-green mutant of *Festuca pratensis*. *Plant, Cell & Environment* **20**: 1323–1337.

Lam E, Kato N, Lawton M. 2001. Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411**: 848–853.

Lamb C, Dixon RA. 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 251–275.

Lane BG, Dunwell JM, Ray JA, Schmitt MR, Cuming AC. 1993. Germin, a protein marker of early plant development, is an oxalate oxidase. *Journal of Biological Chemistry* **268**: 12239–12242.

Lecourieux D, Mazars C, Pauly N, Ranjeva R, Pugin A. 2002. Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell* **14**: 2627–2641.

Lee J, Klessig DF, Nurnberger T. 2001. A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. *Plant Cell* **13**: 1079–1093.

Lee KP, Kim C, Landgraf F, Apel K. 2007. EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **104**: 10270–10275.

Lee KP, Kim C, Lee DW, Apel K. 2003. TIGRINA d, required for regulating the biosynthesis of tetrapyrroles in barley, is an ortholog of the FLU gene of *Arabidopsis thaliana*. *FEBS Letters* **553**: 119–124.

Levine A, Tenhaken R, Dixon R, Lamb C. 1994. H_2O_2 from the Oxidative Burst Orchestrates the Plant Hypersensitive Disease Resistance Response. *Cell* **79**: 583–593.

Liu YD, Ren DT, Pike S, Pallardy S, Gassmann W, Zhang SQ. 2007. Chloroplast-generated reactive oxygen species are involved in hypersensitive response-like cell death mediated by a mitogen-activated protein kinase cascade. *Plant Journal* **51**: 941–954.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **25**: 402–408.

Mach JM, Castillo AR, Hoogstraten R, Greenberg JT. 2001. The Arabidopsis-accelerated cell death gene ACD2 encodes red chlorophyll catabolite reductase and suppresses the spread of disease symptoms. *Proceedings of the National Academy of Sciences, USA* **98**: 771–776.

Mateo A, Muhlenbock P, Rusterucci C, Chang CCC, Miszalski Z, Karpinska B, Parker JE, Mullineaux PM, Karpinski S. 2004. *Lesion simulating disease 1* – is required for acclimation to conditions that promote excess excitation energy. *Plant Physiology* **136**: 2818–2830.

Meskauskiene R, Nater M, Goslings D, Kessler F, den Camp RO, Apel K. 2001. *FLU*: a negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **98**: 12826–12831.

Mur LAJ, Brown IR, Darby RM, Bestwick CS, Bi YM, Mansfield JW, Draper J. 2000. A loss of resistance to avirulent bacterial pathogens in tobacco is associated with the attenuation of a salicylic acid-potentiated oxidative burst. *Plant Journal* **23**: 609–621.

Mur LAJ, Kenton P, Draper J. 2005. *In planta* measurements of oxidative bursts elicited by avirulent and virulent bacterial pathogens suggests that H_2O_2 is insufficient to elicit cell death in tobacco. *Plant Cell and Environment* **28**: 548–561.

Mur LAJ, Naylor G, Warner SAJ, Sugars JM, White RF, Draper J. 1996. Salicylic acid potentiates defence gene expression in tissue exhibiting acquired resistance to pathogen attack. *Plant Journal* **9**: 559–571.

Mur LK, Kenton P, Lloyd AJ, Ougham H, Prats E. 2008. The hypersensitive response; the centenary is upon us but how much do we know? *Journal of Experimental Botany* **59**: 501–520.

Noctor G, De Paepe R, Foyer CH. 2007. Mitochondrial redox biology and homeostasis in plants. *Trends in Plant Science* **12**: 125–134.

Ougham H, Hortensteiner S, Armstead I, Donnison I, King I, Thomas H, Mur L. 2008. The control of chlorophyll catabolism and the status of yellowing as a biomarker of leaf senescence. *Plant Biology* **10**: 4–14.

Park SY, Yu JW, Park JS, Li J, Yoo SC, Lee NY, Lee SK, Jeong SW, Seo HS, Koh HJ *et al.* 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. *Plant Cell* **19**: 1649–1664.

Pontier D, Gan SS, Amasino RM, Roby D, Lam E. 1999. Markers for hypersensitive response and senescence show distinct patterns of expression. *Plant Molecular Biology* **39**: 1243–1255.

Pruzinska A, Tanner G, Aubry S, Anders I, Moser S, Muller T, Ongania KH, Krautler B, Youn JY, Liljegren SJ, Hörtensteiner S. 2005. Chlorophyll breakdown in senescent Arabidopsis leaves. Characterization of chlorophyll catabolites and of chlorophyll catabolic enzymes involved in the degreening reaction. *Plant Physiology* **139**: 52–63.

Roberts MR, Paul ND. 2006. Seduced by the dark side: integrating molecular and ecological perspectives on the influence of light on plant defence against pests and pathogens. *New Phytologist* **170**: 677–699.

Roca M, James C, Pruzinska A, Hortensteiner S, Thomas H, Ougham H. 2004. Analysis of the chlorophyll catabolism pathway in leaves of an introgression senescence mutant of *Lolium temulentum*. *Phytochemistry* **65**: 1231–1238.

Rubio V, Shen YP, Saijo Y, Liu YL, Gusmaroli G, Dinesh-Kumar SP, Deng XW. 2005. An alternative tandem affinity purification strategy applied to Arabidopsis protein complex isolation. *Plant Journal* **41**: 767–778.

Sato Y, Morita R, Nishimura M, Yamaguchi H, Kusaba M. 2007. Mendel's green cotyledon gene encodes a positive regulator of the chlorophyll-degrading pathway. *Proceedings of the National Academy of Sciences, USA* **104**: 14169–14174.

Schelbert S, Aubry S, Burla B, Agne B, Kessler F, Krupinska K, Hortensteiner S. 2009. (Pheophytin Pheophorbide Hydrolase (Pheophytinase) is Involved in Chlorophyll Breakdown during Leaf Senescence in Arabidopsis. *Plant Cell* **21**: 767–785.

Seo S, Okamoto M, Iwai T, Iwano M, Fukui K, Isogai A, Nakajima N, Ohashi Y. 2000. Reduced levels of chloroplast FtsH protein in tobacco mosaic virus-infected tobacco leaves accelerate the hypersensitive reaction. *Plant Cell* **12**: 917–932.

Spassieva S, Hille J. 2002. A lesion mimic phenotype in tomato obtained by isolating and silencing an Lls1 homologue. *Plant Science* **162**: 543–549.

Szabo I, Bergantino E, Giacometti GM. 2005. Light and oxygenic photosynthesis: energy dissipation as a protection mechanism against photo-oxidation. *EMBO Reports* **6**: 629–634.

Tanaka R, Hirashima M, Satoh S, Tanaka A. 2003. The Arabidopsis-accelerated cell death gene ACD1 is involved in oxygenation of pheophorbide a: Inhibition of the pheophorbide a oxygenase activity

does not lead to the "Stay-Green" phenotype in Arabidopsis. *Plant and Cell Physiology* **44**: 1266–1274.

Thomas H, Stoddart JL. 1975. Separation of chlorophyll degradation from other senescence processes in leaves of a mutant genotype of meadow fescue (*Festuca pratensis* L). *Plant Physiology* **56**: 438–441.

Torres MA, Dangl JL, Jones JDG. 2002. Arabidopsis gp91(phox) homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proceedings of the National Academy of Sciences, USA* **99**: 517–522.

Torres MA, Onouchi H, Hamada S, Machida C, Hammond-Kosack KE, Jones JDG. 1998. Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (*gp91(phox)*). *Plant Journal* **14**: 365–370.

Townley HE, McDonald K, Jenkins GI, Knight MR, Leaver CJ. 2005. Ceramides induce programmed cell death in Arabidopsis cells in a calcium-dependent manner. *Biological Chemistry* **386**: 161–166.

Triantaphylides C, Havaux M. 2009. Singlet oxygen in plants: production, detoxification and signaling. *Trends in Plant Science* **14**: 219–228.

Vidal G, Ribas-Carbo M, Garmier M, Dubertret G, Rasmusson AG, Mathieu C, Foyer CH, De Paepe R. 2007. Lack of respiratory chain complex I impairs alternative oxidase engagement and modulates redox signaling during elicitor-induced cell death in tobacco. *Plant Cell* **19**: 640–655.

Wagner D, Przybyla D, Camp ROD, Kim C, Landgraf F, Lee KP, Wursch M, Laloi C, Nater M, Hideg E *et al.* 2004. The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. *Science* **306**: 1183–1185.

Weber H. 2002. Fatty acid-derived signals in plants. *Trends in Plant Science* **7**: 217–224.

Wei YD, Zhang ZG, Andersen CH, Schmelzer E, Gregersen PL, Collinge DB, Smedegaard-Petersen V, Thordal-Christensen H. 1998. An epidermis/papilla-specific oxalate oxidase-like protein in the defence response of barley attacked by the powdery mildew fungus. *Plant Molecular Biology* **36**: 101–112.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**: 562–565.

Wuthrich KL, Bovet L, Hunziker PE, Donnison IS, Hortensteiner S. 2000. Molecular cloning, functional expression and characterisation of RCC reductase involved in chlorophyll catabolism. *Plant Journal* **21**: 189–198.

Yao N, Tada Y, Sakamoto M, Nakayashiki H, Park P, Tosa Y, Mayama S. 2002. Mitochondrial oxidative burst involved in apoptotic response in oats. *Plant Journal* **30**: 567–579.

Yang ML, Wardzala E, Johal GS, Gray J. 2004. The wound-inducible Lls1 gene from maize is an orthologue of the Arabidopsis Acd1 gene, and the LLS1 protein is present in non-photosynthetic tissues. *Plant Molecular Biology* **54**: 175–191.

Yoda H, Hiroi Y, Sano H. 2006. Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. *Plant Physiology* **142**: 193–206.

Zeier J, Pink B, Mueller MJ, Berger S. 2004. Light conditions influence specific defence responses in incompatible plant-pathogen interactions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. *Planta* **219**: 673–683.

Zurbriggen MD, Carrillo N, Tognetti VB, Melzer M, Peisker M, Hause B, Hajirezaei M-R. 2009. Chloroplast-generated reactive oxygen species play a major role in localized cell death during the non-host interaction between tobacco and *Xanthomonas campestris* pv. *vesicatoria*. *Plant Journal* **60**: 962–973.

Supporting Information

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

Fig. S1 Spontaneous death in two *staygreen*-over-expressing (*SGR-OX*) transgenic lines.

Fig. S2 *Staygreen* (*SGR*)-dependent effects following pathogen challenge.

Fig. S3 Chlorophyll and chlorophyll catabolites detected during a hypersensitive response in Arabidopsis.

Fig. S4 Light-dependent hypersensitive response (HR) type cell death in Arabidopsis.

Fig. S5 Chlorophyll fluorescence changes in Columbia-0 (Col-0), RNAi *staygreen* (*SGRi*) and *staygreen*-over-expressing (*SGR-OX*) Arabidopsis during a hypersensitive response (HR).

Fig. S6 The formation of a hypersensitive response (HR) in Columbia (Col-0) and in the *executer1* and *executer2* mutants.

Fig. S7 Light-dependent jasmonic acid biosynthesis.

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