Journal of Experimental Botany, Vol. 64, No. 16, pp. 4967–4980, 2013
doi:10.1093/jxb/ert285 Advance Access publication 8 October, 2013

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

In vivo inhibition of cysteine proteases provides evidence for the involvement of 'senescence-associated vacuoles' in chloroplast protein degradation during dark-induced senescence of tobacco leaves

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Received 14 February 2013; Revised 23 July 2013; Accepted 26 July 2013

Abstract

Breakdown of leaf proteins, particularly chloroplast proteins, is a massive process in senescing leaves. In spite of its importance in internal N recycling, the mechanism(s) and the enzymes involved are largely unknown. Senescence-associated vacuoles (SAVs) are small, acidic vacuoles with high cysteine peptidase activity. Chloroplast-targeted proteins re-localize to SAVs during senescence, suggesting that SAVs might be involved in chloroplast protein degradation. SAVs were undetectable in mature, non-senescent tobacco leaves. Their abundance, visualized either with the acidotropic marker Lysotracker Red or by green fluorescent protein (GFP) fluorescence in a line expressing the senescence-associated cysteine protease SAG12 fused to GFP, increased during senescence induction in darkness, and peaked after 2–4 d, when chloroplast dismantling was most intense. Increased abundance of SAVs correlated with higher levels of *SAG12* mRNA. Activity labelling with a biotinylated derivative of the cysteine protease inhibitor E-64 was used to detect active cysteine proteases. The two apparently most abundant cysteine proteases of senescing leaves, of 40 kDa and 33 kDa were detected in isolated SAVs. Rubisco degradation in isolated SAVs was completely blocked by E-64. Treatment of leaf disks with E-64 *in vivo* substantially reduced degradation of Rubisco and leaf proteins. Overall, these results indicate that SAVs contain most of the cysteine protease activity of senescing cells, and that SAV cysteine proteases are at least partly responsible for the degradation of stromal proteins of the chloroplast.

Key words: Cysteine proteases, proteolysis, Rubisco, *SAG12*, senescence-associated vacuoles, tobacco.

Introduction

Leaf senescence may be viewed as a controlled dismantling process during which most cellular organelles are broken down and substantial amounts of components of the degradation products (e.g. amino acids) are exported to other parts of the plant (Feller, 2004; Mae, 2004; Gregersen *et al.*, 2008; Masclaux-Daubresse *et al.*, 2010). Breakdown of chloroplasts is particularly important, not only because chloroplasts represent the single most important source of remobilizable nutrients in a leaf, but also because their degradation causes a decline in the photosynthetic potential of leaves (Gepstein, 1988; Krupinska, 2007). Therefore, understanding the mechanism and regulation of chloroplast degradation during senescence is crucially important in any attempt to extend the photosynthetic activity of crops or to improve nitrogen use efficiency (Masclaux-Daubresse *et al.*, 2010).

Early ultrastructural studies showed that chloroplasts are dismantled before deteriorative changes can be detected in other organelles (Noodén, 1988). Frequently, the breakdown of

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chloroplast stromal proteins (e.g. Rubisco) precedes the loss of chlorophyll and thylakoid membranes (Krupinska, 2007), but there is a large degree of flexibility, and, therefore, the relative timing of the degradation of various chloroplast components may change depending on species, environmental conditions, senescence-inducing factors, etc. (e.g. Hidema *et al.*, 1991, 1992). The proteolytic mechanism(s) and the protease(s) responsible for degradation of chloroplast proteins, including Rubisco, the most abundant protein in leaves, are largely unknown.

Degradation of chloroplast proteins could be carried out by chloroplast proteases within the plastid itself (Sakamoto, 2006). A number of chloroplast proteases appear to be up-regulated in senescing leaves (for a review, see Roberts *et al.*, 2012). Several members of the *DegP* and *FtsH* families of serine-type proteases have been implicated in the degradation of D1 and other photosystem II (PSII) proteins during repair of photoinhibited PSII centres (Zaltsman *et al.*, 2005; Sun *et al.*, 2007; Kato *et al.*, 2009; Sun *et al.*, 2010). Based on its ability to degrade Rubisco *in vitro* and the delay of Rubisco degradation in antisense lines, it has been suggested that the chloroplastic, DNA-binding aspartic protease CND 41 has an important role in breakdown of Rubisco (Kato *et al.*, 2004). However, since antisense lines display stunted growth and reduced gibberellin levels (Nakano *et al.*, 2003), delayed Rubisco degradation in these antisense lines could merely reflect slower growth and, therefore, an attenuation of the correlative effects that mediate the control of leaf senescence by the growing shoot apex (Noodén, 1988). So far, there is no clear-cut evidence linking chloroplast proteases to degradation of the abundant proteins of the plastid stroma.

Recent evidence suggests that Rubisco, and other important stromal proteins, might be degraded outside the plastid. 'Rubisco vesicular bodies' accumulate in the cytosol of mature tobacco leaves, implying that Rubisco may be transferred outside the plastid (Prins *et al.*, 2008). Spherical bodies containing Rubisco (i.e. 'Rubisco-containing bodies'; RCBs) occur outside the plastids in senescing leaves of wheat and *Arabidopsis* (Chiba *et al.*, 2003; Ishida *et al.*, 2008). RCBs appear to be part of an autophagy-dependent pathway that carries stromal proteins to the central vacuole for degradation (Ishida *et al.*, 2008; Wada *et al.*, 2009). However, a recent study found that the time-course of Rubisco degradation in *Arabidopsis* is very similar in the wild type and autophagy mutants until very late stages of senescence (Lee *et al.*, 2013). 'Senescence-associated vacuoles' (SAVs) are small, acidic vacuoles that develop in chloroplast-containing cells of senescing leaves (Otegui *et al.*, 2005). They are clearly different from the central vacuole (e.g. the central vacuole aquaporin γTIP is absent from SAVs) and contain high levels of peptidase activity (Otegui *et al.*, 2005). The fact that SAVs contain Rubisco and other stromal proteins, and that they appear to be competent for Rubisco degradation *in vitro* (Martínez *et al.*, 2008), suggests that they might be involved in degradation of stromal proteins during senescence. Both RCBs and SAVs contain Rubisco; however, unlike RCBs, SAVs are acidic compartments with high peptidase activity as well as vacuolar markers in their limiting membrane (Otegui *et al.*, 2005).

Transcriptomic and other studies of gene expression consistently show that cysteine proteases represent a large number of the proteases associated with senescence (Hensel *et al.*, 1993; Bhalerao *et al.*, 2003; Buchanan-Wollaston *et al.*, 2003; Andersson *et al.*, 2004; Guo *et al.*, 2004; Gregersen and Holm, 2007; Parrott *et al.*, 2010). The activity of cysteine proteases typically increases during senescence of petals and leaves (e.g. Wagstaff *et al.*, 2002; Martínez *et al.*, 2007), and in ripening fruits (e.g. Neuteboom *et al.*, 2009). Consistent with a role for cysteine proteases in cellular dismantling during senescence, there is one report of reduced protein degradation in senescing leaf segments treated with a cysteine protease inhibitor (Thoenen *et al.*, 2007). Likewise, overexpression of rice cystatin inhibited cystein protease activity and delayed the loss of Rubisco and two Rubisco activase proteins in tobacco (Prins *et al.* 2008). One of the cysteine proteases consistently expressed during senescence is SAG12, which shows a strictly senescence-associated pattern of expression in leaves (Lohman *et al.*, 1994; Noh and Amasino, 1999*a*). This specificity has been exploited to generate transgenic lines where autoregulated production of cytokinins in a senescence-specific manner delays the expression of senescence symptoms by fusing the *SAG12* promoter to the *IPT* gene (Gan and Amasino, 1995). Experiments with a SAG12–green fluorescent protein (GFP) fusion expressed under control of the *SAG12* promoter showed that most of SAG12 localizes to SAVs in *Arabidopsis* (Otegui *et al.*, 2005). Interestingly, probing protease activity *in vivo* with a fluorescent substrate for cysteine proteases shows that most of this activity concentrates in SAVs (Otegui *et al.*, 2005).

Cysteine proteases and SAVs appear to play a role in chloroplast protein degradation during senescence of leaves. The main objective of this study was to provide pharmacological evidence showing that cysteine-type proteases of SAVs may be, at least partly, involved in Rubisco degradation in senescing leaves.

Materials and methods

Plant material and growing conditions

Plants of tobacco (*Nicotiana tabacum* L. cv. Petit Havana) were grown in 3 litre pots containing soil in a naturally lit greenhouse, at 25/15 ºC day/night temperature in La Plata (Argentina, 34º55´S, 57º54´W), or at 21/18 ºC day/night temperature with a 16 h photoperiod in Halle (Germany, 51º28´N, 11º58´E). A 250–300 mg aliquot of fertilizer (12%N, 5%P, 14%K) was added to pots and plants were grown for 6–8 weeks before leaves were taken for senescence induction. To induce senescence, mature leaves (i.e. leaf number 3–4 counting from the apex) were detached, inmersed in ethephon (2-chloro ethyl phosphonic acid, 100 ppm) for 3 min, placed on moist filter paper in plastic boxes, and induced to senesce in continuous darkness at 20 ºC. In plant tissues, ethephon is converted into ethylene; therefore, ethephon is a convenient source of ethylene to accelerate senescence of leaves or ripening of fruits. In some experiments, ethephon treatment was omitted, and leaves were treated with benzylaminopurine (BAP, 20 ppm), or immersed in water for 3 min to serve as untreated controls.

In vivo treatment with E-64

In some experiments, leaf disks (1 cm diameter) were excised from ethephon-treated leaves and incubated in darkness for 2 d floating on a solution of E-64 (100 µM, dissolved in distilled water).

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Generation of transgenic tobacco plants

Agrobacterium tumefaciens strain LBA4404 was transformed with pPZP221 containing ProSAG12:SAG12–GFP (Otegui *et al.*, 2005), and grown in selection plates containing 25 mg l^{-1} rifampicin and 50 mg l^{-1} spectinomycin. Tobacco (*N. tabacum* cv. Petit Havana) leaf disks were transformed using *Agrobacterium* as described by Wise *et al.* (2006). Transformed disks were selected on a medium containing 25 mg l^{-1} gentamycin, 50 mg l^{-1} carbenicillin, and hormones for callus formation. Transformed calli growing on the side of different leaf pieces were separated and placed in the corresponding media for plant regeneration. Five transgenic plants regenerated from independent calli were examined for SAG12–GFP protein expression. To that end, senescent leaf extracts were subjected to SDS–PAGE followed by western blot. The SAG12–GFP construct was detected with an anti-GFP antibody (Molecular Probes).

Confocal laser scanning and fluorescence microscopy

Leaf pieces or isolated cells were observed under a Zeiss LSM510 META or a LEICA SP5 laser scanning confocal microscope. Excitation/emission settings were 488/505–530 nm for GFP, 543/585–615 nm for Lysotracker Red, and 633/670–730 nm for chlorophyll. Previous studies (Otegui *et al.*, 2005; Martínez *et al.*, 2008) showed that wild-type leaves or cells treated with no fluorescent probe do not emit autofluorescence in the 505–530 nm and 585–615 nm bands. Perfluorodecalin was added to improve image sharpness (Littlejohn *et al.*, 2010).

Isolation of SAVs and chloroplasts

SAVs were isolated as in Martínez *et al.* (2008). Approximately 14 g of tobacco leaves were homogenized on ice in 50 ml of buffer [25 mM HEPES pH 7.5, 0.6 M mannitol, 6.2 mM cysteine, 2 mM EDTA, 1% (w/v) polyvinylpolypyrrolidone (PVPP), 1 mM phenylmethylsulphonyl fluoride (PMSF)]. To stain SAVs with the aciditropic marker Neutral Red, the homogenate was stirred gently for 15 min in the presence of 0.1 mg ml^{-1} Neutral Red, filtered through a nylon mesh, and centrifuged at 3500 *g* for 10 min at 4 °C to pellet chloroplasts and chloroplast membranes. The supernatant was overlayered on top of a discontinuous sucrose gradient [5, 25, 35, 45, and 60% (w/v) sucrose prepared in 25 mM HEPES pH 7.5, 0.6 M mannitol] and centrifuged at 100 000 *g* in a swinging bucket rotor for 1 h at 4 °C in an OptimaMax Ultracentrifuge (Beckman-Coulter, Palo Alto, CA, USA). To remove contaminating soluble proteins, the fraction enriched in SAVs was treated with thermolysin (60 µg ml^{-1} plus 0.5 mM $CaCl_2$) at 4 °C for 30 min. Thermolysin treatment was terminated by adding 0.16 mM EGTA, and SAVs were then re-isolated on a density gradient as described above. In tobacco, this protocol yields a fraction enriched in SAVs, with negligible contamination by chloroplasts, mitochondria, peroxisomes, and the central vacuole, and only traces of endoplasmic reticulum proteins and the Golgi apparatus (Martínez *et al.*, 2008).

To isolate intact chloroplasts, leaves were homogeneized in chilled buffer [50 mM HEPES pH 7.6, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 5 mM ascorbic acid, 0.05% (w/v) bovine serum albumin]. The extract was overlayered on top of a Percoll solution [50 mM HEPES pH 7.6, 330 mM sorbitol, 35% (w/v) Percoll] and centrifuged at 3000 *g* for 5 min. The chloroplast pellet was resuspended in wash buffer (50 mM HEPES pH 7.6, 330 mM sorbitol), thermolysin (60 µg ml^{-1} plus 0.5 mM $CaCl_2$) was added, and the pellet was then incubated at 4 °C for 30 min. Thermolysin treatment was stopped with EGTA as described above; chloroplasts were washed twice with wash buffer and then lysed by resuspension in 50 mM HEPES pH 7.6.

Cell isolation

To isolate cells, leaves were cut into small pieces (~2 mm^2), infiltrated under partial vacuum with a digestion mix [20 mM MES pH 5.5, 0.5 M mannitol, 0.25% (w/v) Macerozima R10], and incubated at 37 °C for 2 h. After filtration through a nylon mesh and centrifugation at 50 *g*, cells were washed twice with buffer (20 mM MES pH 5.5, 0.5 M mannitol). In some experiments, cells were then treated for 2 h with E-64 (100 µM).

Protein analysis, western blot, and autodigestion assays

SDS–PAGE and western blot analysis were carried out as described in Tambussi *et al.* (2000). For autodigestion experiments, SAVs were incubated in darkness during 4 h at 30 °C with/without the addition of protease inhibitors [0.01% (v/v) Protease Inhibitor Cocktail, for plants SIGMA-Aldrich P9599, 50 µM E-64, or 0.1 mM aminoethylbenzene sulphonyl fluoride (AEBSF)]. In autodigestion experiments, the substrate for protease activity was Rubisco naturally localized to SAVs (Martínez *et al.*, 2008); that is, no exogenous substrate was added. Protein content was determined as in Bradford (1976), or by scanning Coomassie-stained SDS–polyacrylamide gels and quantitation with the SigmaGel software.

Activity labelling of cysteine proteases

Cysteine proteases were labelled with DCG-04, a biotinylated derivative of E-64 (Greenbaum *et al.*, 2000). Leaves were ground in chilled distilled water and the homogenate was centrifuged at 13 000 *g* for 15 min. One volume of the supernatant was mixed with 3 vols of 5 µM DCG-04 dissolved in 25 mM Na-acetate buffer pH 5, 10 mM cysteine, and this mixture was incubated at room temperature for 3 h with gentle shaking. Proteins were precipitated with chilled acetone [80% (v/v)], resuspended in Laemmli buffer [25 mM TRIS pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol], boiled for 1 min, and run in a 12% (w/v) acrylamide concentration SDS–polyacrylamide gel. After electrophoresis, proteins were electrotransferred to a nitrocellulose membrane, and labelled proteins were detected with streptavidin conjugated to horseradish peroxidase. To control for specificity, aliquots were treated with E-64 (100 µM dissolved in 25mM Na-acetate buffer pH 5, 10 mM cysteine) 1 h prior to labelling with DCG-04.

qPCR

The expression of the tobacco orthologue of *SAG12* (HQ108340) was analysed, including elongation factor-1α (*EF1-α*, AF120093) and protein phosphatase 2 A, (*PP2A*, X97913.1), which show constant expression in tobacco and do not change during senescence, as reference genes (Schmidt *et al.*, 2010). Primers were designed with the PrimerSelect software (National Center for Biotechnology Information, USA). Supplementary Table S1 available at *JXB* online lists the primers used for PCR. As *NtSAG12* and *NtCP1* (Beyene *et al.*, 2006) show >95% homology it cannot be excluded that *CP1* was also amplified with the primers used. Total RNA was isolated according to Chomczynski and Mackey (1995), and was then treated with recombinant RNase-free, DNase I (Roche-Diagnostics-GmbH). A 1 µg aliquot of total RNA was reverse transcribed with a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) in a volume of 20 µl to generate first-strand cDNA, following the supplier's instructions. PCR was performed in an iCycler (MyiQ™2, Bio-Rad) in a total volume of 15 µl, including 1× Platinium SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.3 µM of each gene-specific primer, and 10 µM fluorescein (Bio-Rad) as the passive reference dye for well-factor calibration. The following PCR program was used: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s 63.4 °C for 25 s, and 60 °C for 30 s, and a melting curve from 55 °C to 95 °C. PCR efficiencies of primers, which were close to 100%, were calculated as described by Pfaffl (2001) using dilutions series (1:4, 1:16, 1:64, 1:256). The expression rate of the gene of interest in senescing leaves relative to the mature leaf controls was calculated as described by Livak and Schmittgen (2001), using the geometric mean of Ct of both reference genes for calculations (Vandesompele *et al.*, 2002).

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Statistical analysis

All experiments were repeated at least three times. Data were subjected to analysis of variance (ANOVA) using the Statistica software, and means were compared by Fisher's LSD test.

Results

Changes in the abundance of SAVs in the course of dark-induced senescence of tobacco leaves

The term 'senescence-associated vacuoles' was chosen to name small vacuoles, clearly different from the large central vacuole, abundant in senescing cells but absent from mature, non-senescent leaves (Otegui *et al.*, 2005; Martínez *et al.*, 2008). However, a detailed description of the changes in abundance of SAVs in the course of senescence is lacking. To follow the changes in abundance of SAVs, mature tobacco leaves were detached, treated with ethephon, and incubated in darkness for up to 5–6 d to induce senescence. SAVs were detected in wild-type leaves by staining with the acidotropic dye Lysotracker Red, which is routinely used to label lytic compartments (e.g. Munafó and Colombo, 2001), and by GFP fluorescence in a tobacco line expressing a fusion of the senescence-associated protease SAG12 to GFP (Otegui *et al.*, 2005). Figure 1A shows that in the wild-type genotype, chlorophyll content declined slowly for the first 3 d of dark incubation, and thereafter the rate of chlorophyll loss increased and leaves lost ~50% of their chlorophyll between days 3 and 6. Rubisco and overall leaf protein levels remained relatively unchanged during the first day of dark incubation, but there was a substantial drop in their levels between days 1 and 2 (Fig. 1B, C). After day 2, protein levels continued to decrease, but at a slower rate. In mature, non-senescing leaves taken before ethephon treatment and dark incubation, no SAVs were detected (Fig. 2A). Numerous SAVs were detected with Lysotracker Red after 2 d of dark incubation (Fig. 2B). The number of SAVs remained high after 4 d (Fig.2C), and declined thereafter (Fig. 2D). A quantitation of the number of SAVs per cell, in a 1 µm thick section (Fig. 2E), shows a steep increase between days 0 and 3, and then the number of SAVs levelled off, to drop substantially after day 4. When the average remaining protein content (expressed as a percentage of day 0) is plotted against the average number of SAVs per cell, an inverse, close linear relationship (r^2=0.956) was obtained (Fig. 2F).

The link between SAVs and the rate of chloroplast degradation was also probed with hormonal manipulations that modulate the rate of senescence. The changes in the abundance of SAVs were consistent with their involvement in chloroplast degradation. Compared with control (non-treated) leaves, ethephon accelerated the rate of chlorophyll loss. In contrast, incubation with BAP, a synthetic cytokinin, reduced chlorophyll degradation; in fact, chlorophyll levels in leaves treated with BAP for 3 d were not significantly

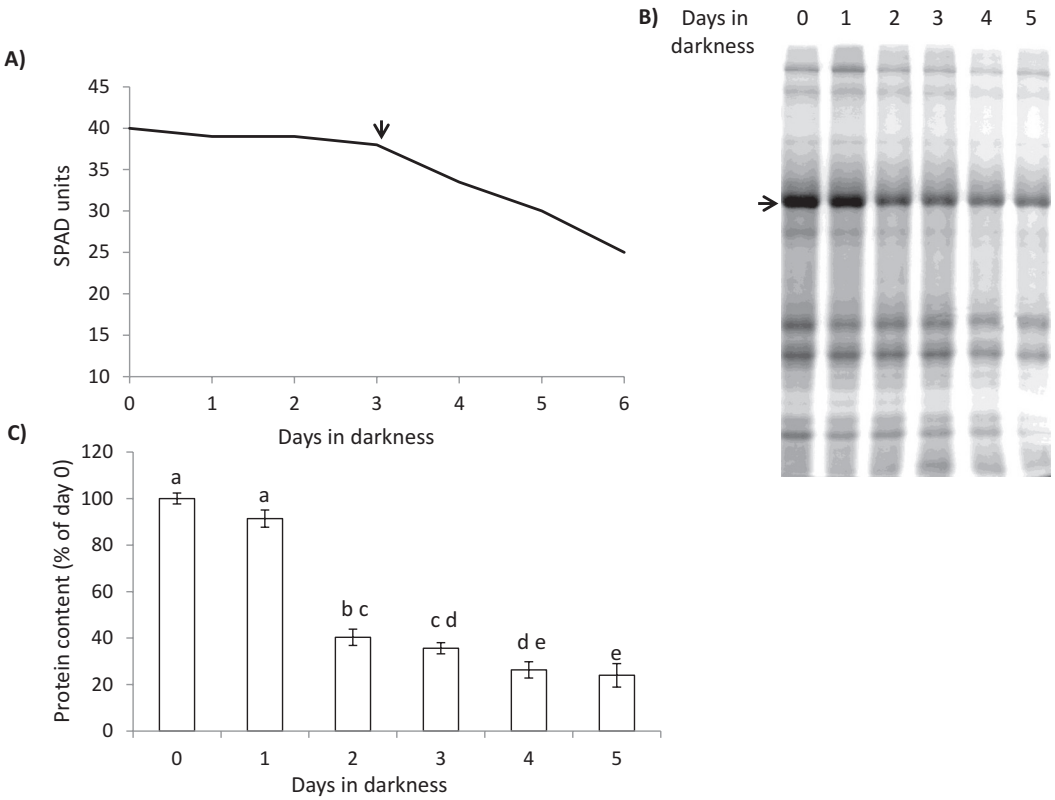

Fig. 1. Changes in chlorophyll (A) and protein (B, C) content of wild-type tobacco leaves induced to senesce in darkness for several days. (A) Chlorophyll was measured with a SPAD (Soil Plant Analysis Development) 502 Chlorophyll Meter and expressed as SPAD units. (B) A representative Coomassie blue-stained gel of leaf proteins. (C) A quantitation of these changes. Arrows show the start of rapid chlorophyll loss (A) or the position of the large subunit of Rubisco (B).

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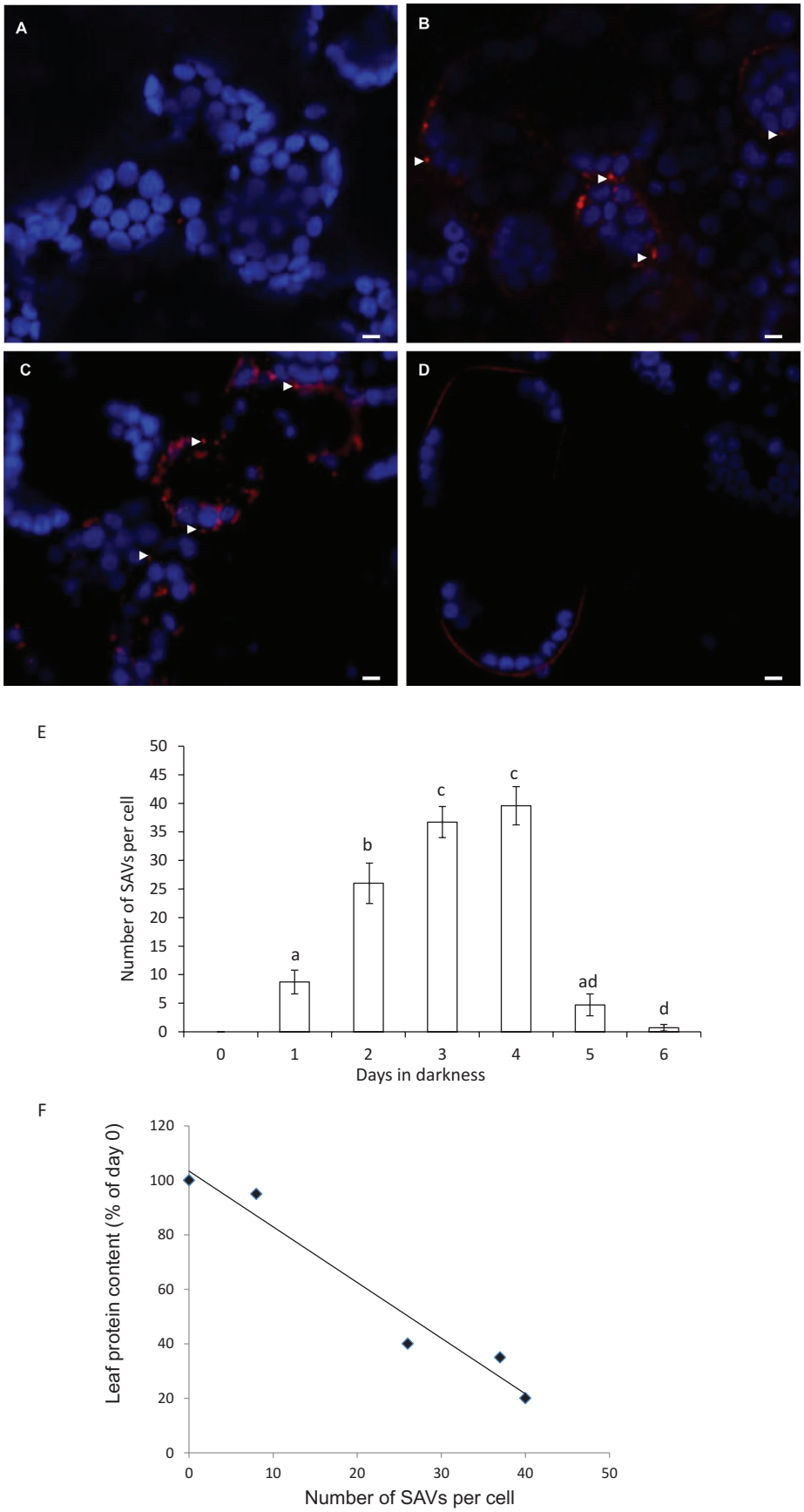

Fig. 2. Confocal images of senescing cells of tobacco leaves. Wild-type leaves were detached, treated with ethephon, and induced to senesce in darkness. Images were taken after 0 (A), 2 (B), 4 (C), and 6 (D) d of incubation. Autofluorescence of chlorophyll is pseudocoloured blue, whereas Lysotracker Red emission is pseudocoloured red. White arrows show a few examples of SAVs in each

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different ($P < 0.05$) from values recorded on day 0 (Fig. 3A). Consistent with this, SAVs were more abundant (~2-fold) in leaves treated with ethephon compared with untreated leaves, and scarce under BAP treatment (Fig. 3B). In some cells, SAVs were completely undetectable after 3 d of dark incubation in BAP-treated leaves. Figure 3C–E shows SAVs in representative sections of leaves incubated for 3 d in darkness and pre-treated with BAP or ethephon.

Previously, Otegui *et al.* (2005) localized a fusion of SAG12 and GFP to SAVs of *Arabidopsis thaliana*. Likewise, SAG12–GFP and Lysotracker Red co-localized in SAVs of tobacco leaves (Supplementary Fig. S1 at *JXB* online). To follow the appearance of SAVs tobacco SAG12–GFP leaves were detached, pre-treated with ethephon, and incubated in darkness as described. SAVs (i.e. vesicular structures containing SAG12–GFP) were completely undetectable in mature leaves (day 0, Fig. 4A) but increased in number on days 2 and 3 (Fig. 4B, C). At 3 d of incubation, after the period of intense protein degradation between days 1 and 2, and when about half of Rubisco was already lost, some SAG12–GFP signal was also detected in plastids.

Expression of SAG12 parallels the appearance of SAVs

Development of SAVs may involve increased transcription of genes coding for their components. To analyse changes in gene expression, mRNA was isolated from the wild-type leaves used for confocal microscopy observations (Fig.2). SAG12 is a cysteine-type protease highly up-regulated during senescence, at both the transcript and protein levels (Lohman *et al.*, 1994; Grbic, 2003; Gombert *et al.*, 2006). Interestingly, SAG12 localizes to SAVs of *Arabidopsis* (Otegui *et al.*, 2005) and tobacco (Fig. 4B, C; Supplementary Fig. S1 at *JXB* online). The expression of *SAG12* mRNA was analysed by qPCR of samples taken from wild-type leaves at different stages during dark-induced senescence. It has to be mentioned that *NtSAG12* shares high homology with another cysteine protease of tobacco, *NtCP1*, and it cannot be excluded that *NtCP1* was also amplified with the primers used. The amino acid sequences of NtSAG12 and NtCP1 differ in only 13 amino acids (Supplementary Fig. S2), which is less than the difference between the two *SAG12* gene products of *Brassica napus* (Noh and Amasino, 1999*b*). Moreover, *NtSAG12* and *NtCP1* cluster with *Arabidopsis* and *Brassica napus SAG12* genes in a phylogenetic tree of cysteine proteases (Supplementary Fig. S3). Therefore, it is surmised that *NtSAG12* and *NtCP1* represent homologous genes in the tobacco genome, possibly with the same cellular localization. Figure 5 shows that transcript levels for *SAG12* increased several fold during the first days of dark incubation, concomitantly with the appearance of SAVs. *SAG12* mRNA levels declined slightly between days 2 and 4 of dark incubation. Overall, increased expression of *SAG12* appeared to correlate with the appearance of SAVs in senescing cells, although it must be noted that *SAG12* expression peaked on day 2, whereas the number of SAVs continued to increase between days 2 and 4 (Fig. 2E).

Cysteine protease activity in isolated SAVs

Previously it was found that SAVs are heavily stained by a fluorescent probe for cysteine proteases and that they seem to concentrate most of the cysteine protease activity of senescing cells (Otegui *et al.*, 2005). Furthermore, isolated SAVs can degrade Rubisco *in vitro* (Martínez *et al.*, 2008). To determine the mechanistic class of proteases active in Rubisco degradation *in vitro*, SAVs were isolated and allowed to digest Rubisco for 4 h. Here it is worth mentioning that the substrate for these assays was Rubisco naturally present in SAVs (Martínez *et al.*, 2008). Consistent with changes in SAV abundance estimated from confocal microscopy observations, the amounts of SAVs recovered with the isolation protocol used here increased during the first 2 d of dark incubation (Supplementary Fig. S4 at *JXB* online). Figure 6A shows the results of an experiment where isolated SAVs were incubated and allowed to degrade Rubisco contained within them. Isolated SAVs degraded ~25% of their Rubisco in 4 h of incubation, and this was completely blocked by a cocktail of protease inhibitors or E-64, a diagnostic inhibitor for cysteine proteases (Barrett *et al.*, 1982), whereas AEBSF, a water-soluble inhibitor of serine proteases (van der Hoorn and Jones, 2004), was ineffective.

Activity labelling of cysteine proteases located to SAVs

An activity tagging approach (Greembaum *et al.*, 2000; van der Hoorn *et al.*, 2004; Martínez *et al.*, 2007) was used to detect cysteine proteases in senescing leaves and isolated SAVs. Three active bands of ~40, 33, and 21 kDa were detected in the crude supernatant of senescing leaves (i.e. leaves incubated in darkness for 2 d; Fig. 6B). The 21 kDa band was the least active/least abundant, and it was clearly absent from SAVs. The two most abundant bands in the leaf homogenate, of 40 kDa and 33 kDa, were also detected in SAVs. In contrast, the same approach did not detect active cysteine proteases in chloroplasts isolated from senescing leaves (Fig. 6C). This suggests that a large part of the active cysteine proteases of senescing cells localize to SAVs, with no detectable, or at least much lower levels of, cysteine proteases in senescing plastids, which is consistent with previous *in vivo* confocal microscopy observations (Otegui *et al.*, 2005). Cysteine proteases in SAVs are active, as

image. Bars represent 5 μm. In (E), a quantitation of the number of SAVs per cell, in a 1 μm thick section, is shown. Values are the average and standard deviation of at least nine independent observations (1 μm thick section per cell) each day. Different letters indicate statistically significant differences at $P < 0.05$. No SAVs were detected on day 0; therefore, this sampling date was not included in the statistical analysis. (F) The relationship between the average number of SAVs per cell (in a 1 μm thick optical section) and remaining leaf protein content (as a percentage of day 0) in leaves pre-treated with ethephon and incubated in darkness for 0–4 d (i.e. the period of most intense protein degradation). The coefficient of determination (r^2)=0.956.

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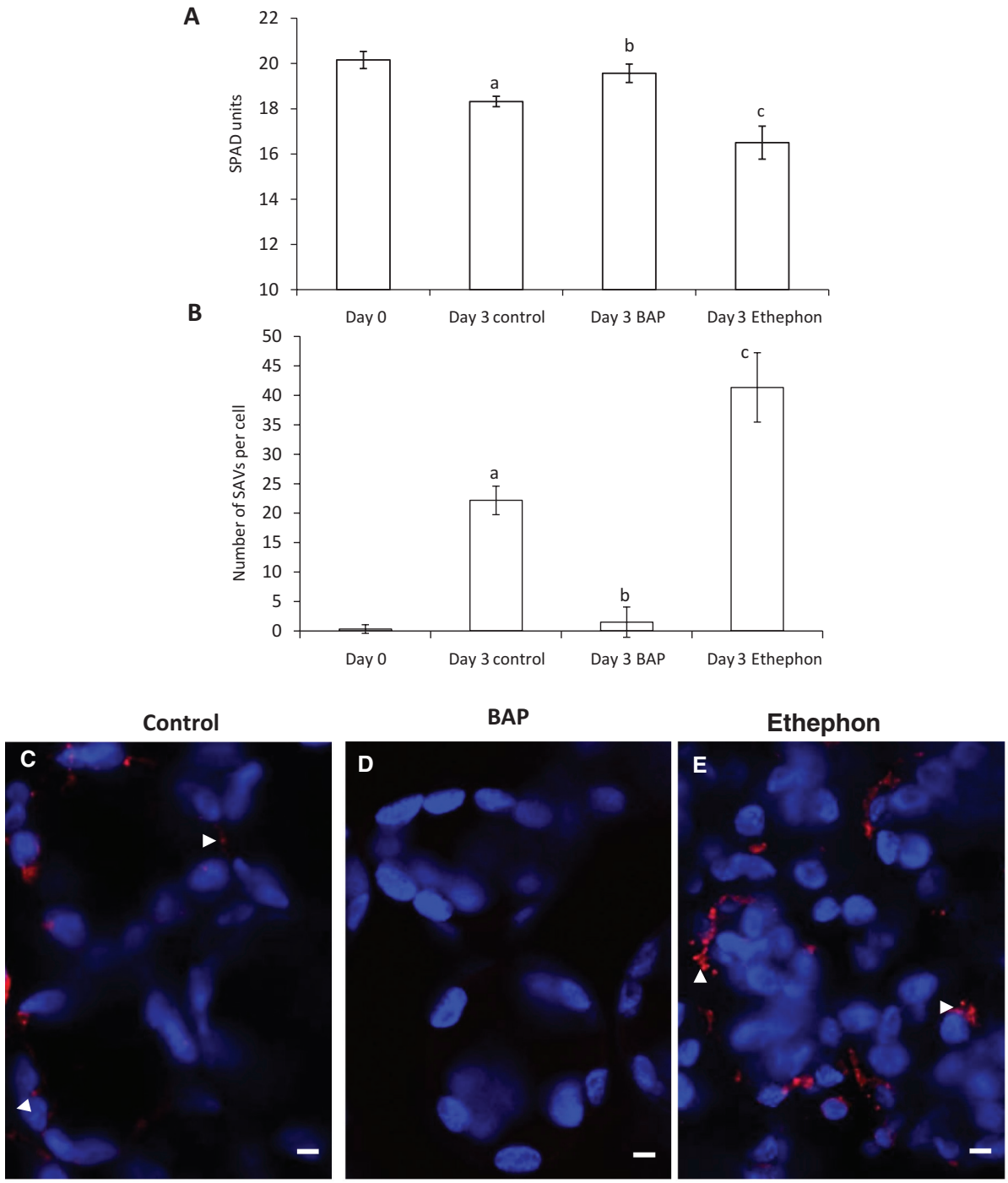

Fig. 3. Effects of ethephon and benzylaminopurine (BAP; a synthetic cytokinin) on the relative abundance of SAVs. (A) Chlorophyll content (SPAD values) in wild-type leaves measured on day 0 or after 3 d of incubation in darkness (untreated control, or treated with BAP or ethephon). (B) The number of SAVs per cell (in a 1 μm thick section) in leaves senescing under the same treatments. Bars show the standard error, and different letters indicate significant differences between hormonal treatments at $P < 0.05$. (C–E) Representative confocal microscope observations of leaf cells treated with BAP (D) or ethephon (E), or untreated controls (C). Chloroplasts are pseudocoloured blue and SAVs stained with Lysotracker Red are pseudocoloured red. Bars represent 5 μm.

shown by three different, complementary approaches: (i) cleavage of a fluorescent probe (Otegui *et al.*, 2005); (ii) labelling by an activity tag (Fig. 6B, C); and (iii) the results of autodigestion experiments (Fig. 6A). Thus, it seems that most of the cysteine protease activity of senescing cells localizes to SAVs, where they may participate in degradation of chloroplast proteins.

In vivo inhibition of cysteine proteases reduces chloroplast protein degradation

To probe the role of cysteine proteases in the degradation of chloroplast proteins during senescence, leaf disks were detached, treated with ethephon, and incubated in darkness

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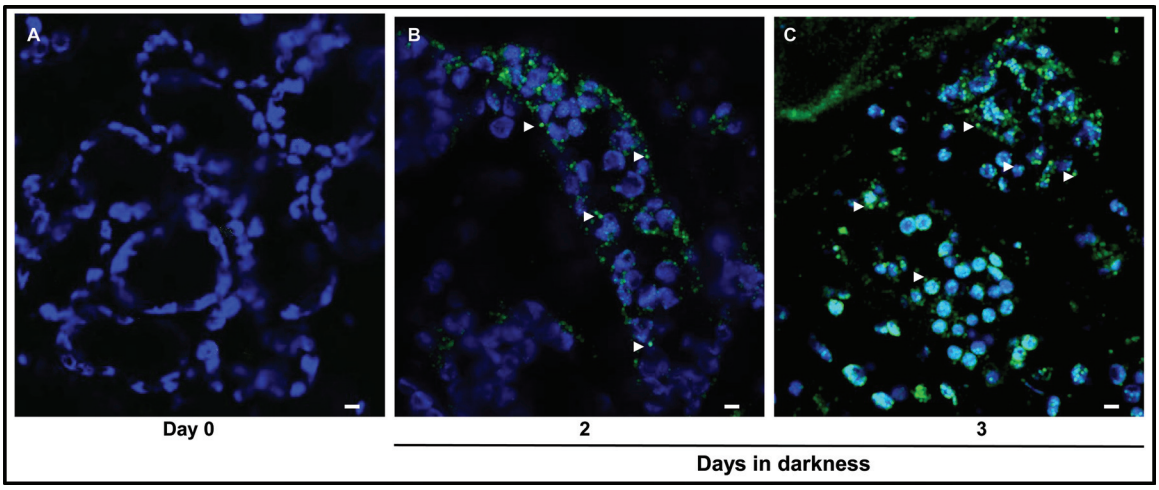

Fig. 4. Confocal microscope images of senescing leaf cells of a tobacco line expressing the senescence-associated cysteine protease SAG12 fused to GFP (SAG12–GFP). SAG12–GFP leaves were induced to senesce by ethephon treatment and dark incubation. Images were taken after 0 (A), 2 (B), and 3 (C) d. The GFP signal is pseudocoloured green, whereas chlorophyll fluorescence is shown in blue. White arrows show a few examples of SAVs in each image. Bars represent 5 μm.

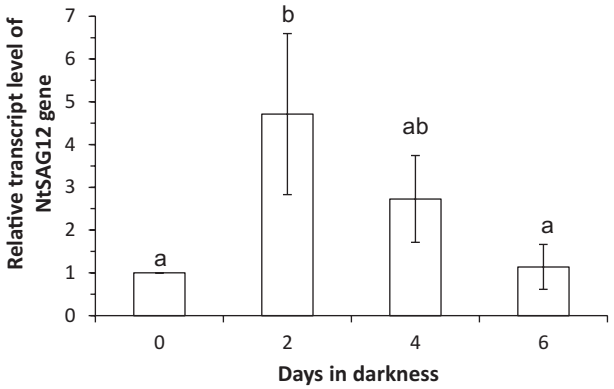

Fig. 5. Changes in the expression of the senescence-associated cysteine protease gene *SAG12* during senescence induced by incubation of wild-type tobacco leaves in darkness. RNA was extracted from leaves at different time points (0, 2, 4, and 6 d of dark incubation). The mRNA level of *SAG12* is shown normalized to the geometric mean of expression of elongation factor-1α (*EF1α*) and protein phosphatase 2 A, (*PP2A*) as reference genes (Vandesompele *et al.*, 2002). The transcript level of senescing leaves relative to the mature controls was calculated as described by Livak and Schmittgen (2001) and the mature control leaf is set as 1. Vertical bars represent ± the standard deviation. Different letters indicate significant differences at $P < 0.05$.

floating on a solution of E-64, as in Thoenen *et al.* (2007). Figure 7 shows that in control cells (not treated with E-64) most of the protease activity detected by R6502, a fluorescent probe for cysteine protease activity (www.probes.com) (Fig. 7C), localizes to SAVs stained with Lysotracker Red (Fig. 7B), which is consistent with previous observations with *Arabidopsis* protoplasts (Otegui *et al.*, 2005). E-64 effectively reduced the cysteine protease activity of SAVs. Senescing cells incubated with E-64 for 2 h showed a substantial decrease of labelling by R-6502, (compare Fig. 7G and C). This implies that E-64 can permeate through cell membranes and reach SAVs *in vivo*. Levels of the large subunit of Rubisco decreased substantially during 2 d of incubation in control disks floating on water (Fig. 7I). Treatment of leaf disks with E-64 during 2 d of incubation in darkness reduced protein degradation, and these leaf disks retained higher amounts of the large and small subunits of Rubisco than untreated controls. This implies that cysteine proteases in SAVs are involved in degradation of the stromal proteins of the chloroplast.

Discussion

The presence and number of SAVs correlate with intensity of chloroplast degradation

The number of SAVs clearly increases in soybean, *Arabidopsis*, and tobacco leaves senescing naturally attached to the plant, or in excised tobacco leaves senescing in darkness (Otegui *et al.*, 2005; Martínez *et al.*, 2008). Overall, these observations indicated that SAVs are senescence specific, possibly independently of the specific treatment inducing or modulating senescence. Although the signalling pathways and some regulatory events involved in senescence differ between leaves attached to the plant, or detached and incubated in darkness, the underlying chloroplast breakdown mechanism may be the same. For example, many stay green mutants delay chloroplast breakdown both in leaves attached to the plant and in detached leaves incubated in darkness (e.g. Guiamet *et al.*, 1991; Guiamet and Gianibelli 1994; Oh *et al.*, 1997; Thomas *et al.*, 2002). Likewise, many cysteine proteases are up-regulated during senescence in both scenarios (e.g. Buchanan-Wollaston *et al.*, 2005; Martínez *et al.*, 2007), and in response to ethylene (Drake *et al.*, 1996; Grbic, 2003). In this study, a more detailed time-course analysis of the appearance of SAVs was carried out during dark-induced senescence of detached leaves. Both in wild-type tobacco, where SAVs

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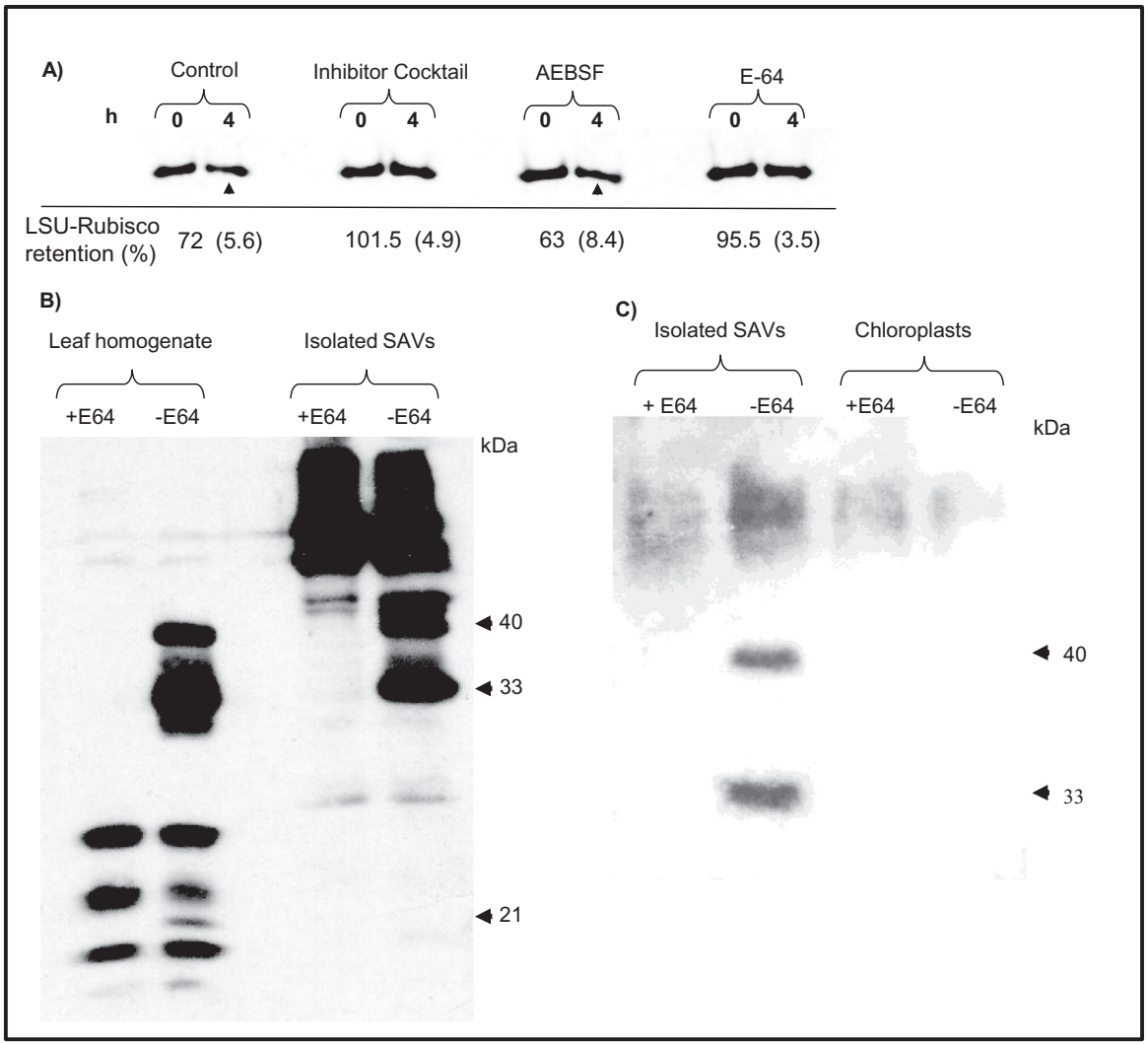

Fig. 6. (A) Autodigestion of Rubisco large subunit by isolated SAVs. This procedure took advantage of the fact that SAVs normally contain Rubisco (Martínez *et al.*, 2008). Thus, SAVs were isolated and incubated at 30 ºC for 4 h to follow degradation of Rubisco contained within them. After ultracentrifugation, the SAV fraction was aliquoted into equal volumes and allowed to autodigest for 4 h. Arrowheads show decreased Rubisco contents after 4 h of incubation. Films developed from western blots were scanned and quantitated using SIGMAGEL software, and the amount of Rubisco remaining after autodigestion is expressed as a percentage of the initial (0 h) Rubisco content (average of three independent experiments; values in parentheses represent the standard deviation). (B) Detection of cysteine proteases in the leaf homogenate and isolated SAVs of senescing tobacco leaves (2 d of incubation in darkness). Proteins binding DCG-04, a biotinylated derivative of the specific cysteine protease inhibitor E-64, were detected with streptavidin conjugated to horseradish peroxidase and a chemiluminescence detection system. To differentiate specific binding of cysteine proteases to biotinylated E-64 (DCG-04) from unspecific protein binding to streptavidin or biotin, a parallel set of samples (leaf homogenate and isolated SAVs) was treated with E-64 (+E-64) before labelling with DCG-04. Arrows show cysteine proteases (i.e. bands showing complete inhibition of labelling by E-64). An 8 (SAVs) and 25 (leaf homogenate) μg aliquot of protein was loaded per lane. (C) Detection of cysteine proteases in chloroplasts and isolated SAVs from leaves incubated in darkness for 2 d. A 5 μg aliquot of protein was loaded per lane. Apparent molecular masses are indicated on the right of B and C.

were detected by their labelling with the acidotropic vacuolar marker Lysotracker Red, and in a transgenic tobacco line harbouring the SAG12–GFP fusion which localizes to SAVs (Otegui *et al.*, 2005), SAVs were undetectable in mature, non-senescing leaves but their number increased significantly after 1–2 d of senescence induction, when chloroplast protein degradation was progressing very rapidly. In addition to this temporal association between SAVs and chloroplast degradation, manipulations with senescence-accelerating (ethylene) and senescing-retarding (BAP) hormones reinforced the link between chloroplast breakdown and appearance of SAVs (i.e. more SAVs in leaves treated with ethylene; fewer SAVs with cytokinin). Thus, SAVs are a cellular structure specific to senescing leaf cells, and, because of the link between SAV abundance and rates of chloroplast protein loss, it may be surmised that SAVs fulfil an important function during chloroplast dismantling.

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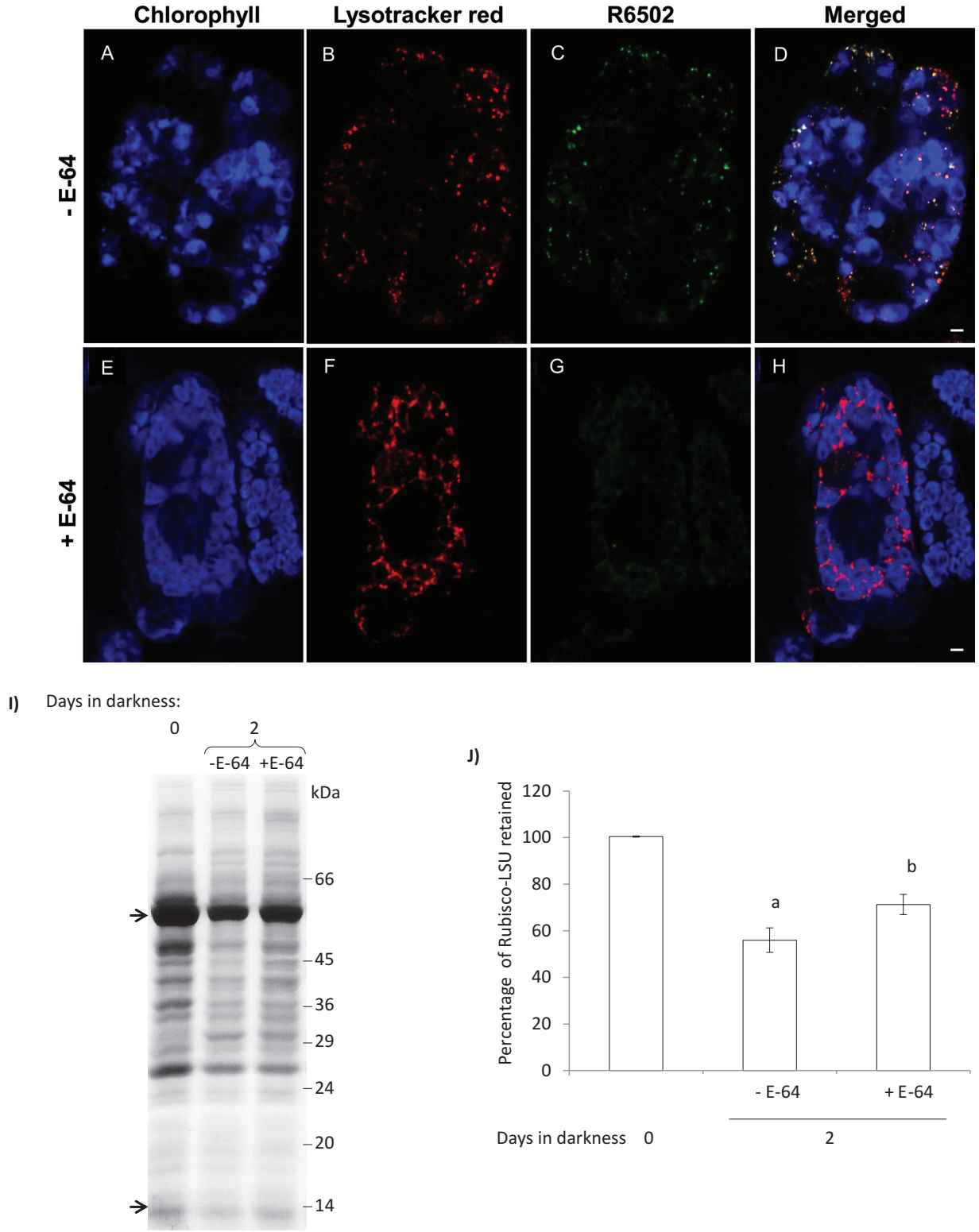

Fig. 7. *In vivo* inhibition of cysteine protease activity with the specific inhibitor E-64. Cells were isolated from senescing (3 d) tobacco leaves, and treated for 2 h with E-64 (100 μM, E–H), or left in buffer as controls (A–D). Cells were later stained with R6502, a probe for protease activity that becomes brightly fluorescent upon cleavage (C and G), and Lysotracker Red, an acidotropic marker for acid organelles (B and F). A and E show chlorophyll autofluorescence, whereas D and H show a merged image of chlorophyll, Lysotracker Red, and R6502. Co-localization of Lysotracker Red and R6502 is pseudocoloured white. Bars represent 5 μm. (I) Mature wild-type leaves were pre-treated with ethephon, and disks were cut with a cork borer and incubated for 2 d in darkness floating either on water or on 100 μM E-64. Arrows show the large and small subunits of Rubisco. The position of molecular mass markers is shown on the right. (J) A quantitation of the changes in Rubisco large subunit, expressed as a percentage of the initial (day 0) Rubisco content, after 2 d of incubation in darkness. Different letters indicate significant differences at $P < 0.05$ between treatments (i.e. minus or plus E-64).

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Up-regulation of SAG12 coincides with increased abundance of SAVs

The appeareance of SAVs might require up-regulation of genes coding for SAV components. SAG12, a cysteine protease located in SAVs (Otegui *et al.*, 2005), is probably unique in that it is a strictly senescence-associated gene, with no, or only barely detectable, expression in mature non-senescing leaves (Lohman *et al.*, 1994; Grbic, 2002, 2003; Gombert *et al.*, 2006). *SAG12* mRNA levels increased significantly after 2 d of dark incubation, in parallel with accumulation of SAVs. However, the number of SAVs continued to increase after the peak in *SAG12* gene expression. Thus, increased expression of genes coding for components of SAVs (e.g. *SAG12*) may be necessary for SAV formation and/or activity. Even though it cannot be ruled out that qPCR may have also detected the expression of the extremely highly homologous *NtCP1*, this gene is so similar to *NtSAG12* that it is very likely that the NtCP1 protease also localizes to SAVs.

Cysteine proteases in SAVs may be involved in chloroplast protein degradation

Earlier biochemical work showed that the activity of cysteine proteases increases in senescing leaves (e.g. Beyene *et al.*, 2006; Martinez *et al.*, 2007), and these observations have been substantiated more recently by transcriptomic studies consistently showing increased transcript levels for cysteine proteases (Bhalerao *et al.*, 2003; Buchanan-Wollaston *et al.*, 2003; Andersson *et al.*, 2004; Guo *et al.*, 2004; Gregersen and Holm, 2007; Parrott *et al.*, 2010). In this work, an activity tag was used to detect cysteine proteases in the crude extract and in SAVs isolated from senescing leaves. The most abundant cysteine proteases in the whole leaf extract (i.e. the proteases of 40 kDa and 33 kDa) appear to be SAV proteases. The SAG12 protein has an apparent molecular mass of 38 kDa (Grbic, 2003), which raises the possibility that the upper protease band in isolated SAVs represents SAG12, or contains SAG12. The enrichment of cysteine proteases in SAVs is consistent with the localization of SAG12, and with observations by confocal microscopy of senescing leaf cells treated with the cysteine protease probe R6502, where most of the activity resides in SAVs (Otegui *et al.*, 2005; Fig. 7). Previously, it was shown that some proteins of the chloroplast stroma relocate to SAVs in the course of leaf senescence (Martínez *et al.*, 2008). SAV proteases can break down the chloroplast proteins contained therein, and most of the proteolytic activity of SAVs against Rubisco is abolished by the cysteine protease diagnostic inhibitor E-64 (Fig. 6A).

The presence of cysteine proteases in SAVs prompted testing of the effects of a cysteine protease inhibitor on degradation of these proteins *in vivo*. Incubation of leaf disks with E-64 substantially reduced the degradation of Rubisco during 2 d of incubation, which is consistent with inhibition of protein degradation by E-64 during senescence of excised wheat leaves (Thoenen *et al.*, 2007), and with the observation that E-64 completely abolishes *in vitro* protease activity of senescing tobacco leaves (Beyene *et al.*, 2006). Likewise, leaf N loss is delayed in maize knockout lines for *See2β*, whose product is a legumain-type cysteine protease (Donnison *et al.*, 2007). *In vivo* treatment with E-64 impairs the protease activity detected in SAVs by confocal microscopy (Fig. 7C). This suggests that cysteine proteases in SAVs break down Rubisco *in vivo* in senescing leaf cells. In contrast, the possible involvement of chloroplast cysteine proteases, at least during the initial stages of senescence, does not seem very likely. First, cysteine proteases targeted to the plastid are not common. For example, of 32 papain-type cysteine proteases of *Arabidopsis*, only two (At2g27420 and At3g49340) are predicted to be targeted to the plastid (Beers *et al.*, 2004). A third cysteine protease, At3g58710, has been detected in non-senescing chloroplasts by a proteomic approach (Bayer *et al.*, 2011). However, none of these chloroplast cysteine proteases seems to be up-regulated during senescence (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Moreover, no cysteine proteases were detected by activity tagging of chloroplasts isolated from senescing tobacco leaves (Fig. 6C). However, the detection of SAG12–GFP signal in plastids after 3 d of incubation in darkness, when around half of Rubisco was degraded, merits further studies to examine whether cysteine proteases in plastids might contribute to protein degradation at later stages of senescence.

In this study it is shown that SAVs increase in abundance in parallel with chloroplast protein degradation, and that this increase may be linked to up-regulation of genes coding for SAV components. Most of the cysteine proteases of senescing leaves appear to reside in SAVs, and treatment with a specific inhibitor of cysteine proteases blocks *in vivo* degradation of proteins in leaf disks incubated with the inhibitor. Taken together, a simple explanation for these observations is that cysteine proteases present in SAVs are, at least to some extent, responsible for the degradation of stromal proteins of the chloroplast. Further manipulation of SAV proteolytic activity by genetic or pharmacological means will help to establish firmly the role of SAVs in protein degradation in senescing leaves, and to estimate the proportion of leaf proteins degraded in SAVs.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Co-localization of SAG12-GFP and Lysotracker red signals in SAVs of tobacco leaves pre-treated with ethephon and incubated in darkness for 2 days.

Figure S2. Comparison of the amino acid sequences of NtSAG12, NtCP1, BnSAG12-1, BnSAG12-2, AtSAG12, and AlSAG12.

Figure S3. Phylogenetic tree for cysteine proteases with the highest similarity to AtSAG12.

Figure S4. Isolation of SAVs from tobacco leaves at different times after senescence induction.

Table S1. List of forward (F) and reverse (R) primers used for qPCR.

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Acknowledgements

This work was funded by FONCYT (Argentina) through PICT 0784, and by DAAD–MINCYT collaboration agreement (PROALAR 50117073). CAC holds a doctoral fellowship from CONICET, MLC and DEM are researchers of CONICET, and JJG is a researcher at CICBA (Argentina).

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