Journal of
Experimental
Botany

www.jxb.oxfordjournals.org

SPECIAL ISSUE REVIEW PAPER

Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated

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Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the predominant protein in photosynthesizing plant parts and the most abundant protein on earth. Amino acids deriving from its net degradation during senescence are transported to sinks (e.g. developing leaves, fruits). Rubisco catabolism is not controlled only by the overall sink demand. An accumulation of carbohydrates may also accelerate senescence and Rubisco degradation under certain conditions. Amino acids produced by proteolysis are rapidly redistributed in plants with proper source–sink relationships. In leaves of wheat plants with reduced sink capacity (e.g. sink removal, phloem interruption by steam girdling at the leaf base), Rubisco is degraded and free amino acids accumulate. They may be washed out in the rain during late senescence. In leaves of depodded soybeans, Rubisco is degraded and amino acids can be reutilized in these leaves for the synthesis of special vacuolar proteins in the paraveinal mesophyll (vegetative storage proteins). Nitrogen deriving from Rubisco degradation in older (senescing) leaves of annual crops is integrated to some extent again in newly synthesized Rubisco in younger leaves or photosynthesizing tissues of fruits. Finally, a high percentage of this nitrogen is accumulated in protein bodies (storage proteins). At the subcellular level, Rubisco can be degraded in intact chloroplasts. Reactive oxygen species may directly cleave the large subunit or modify it to become more susceptible to proteolysis. A metalloendopeptidase may play an important role in Rubisco degradation within intact chloroplasts. Additionally, the involvement of vacuolar endopeptidase(s) in Rubisco catabolism (at least under certain conditions) was postulated by various laboratories.

Key words: Chloroplast, endopeptidase, nitrogen remobilization, phloem transport, proteolysis, Rubisco, stress, vacuole.

Rubisco: the most abundant protein on earth

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is present at very high levels in photosynthesizing cells and must therefore be considered as the most abundant protein in the world (Ellis, 1979). Rubisco is the predominant protein in leaves of C_3 plants and may contribute up to 50% to the soluble leaf proteins (Spreitzer and Salvucci, 2002) and 20–30% of total leaf nitrogen (Evans and Seemann, 1989; Makino 2003; Kumar *et al.*, 2002). In leaves of C_4 plants, Rubisco still contributes ~30% to the soluble proteins (Sugiyama *et al.*, 1984) and 5–9% of total leaf nitrogen (Sage *et al.*, 1987; Makino *et al.*, 2003). Rubisco, phosphoenolpyruvate (PEP) carboxylase, and pyruvate orthophosphate dikinase are the most abundant soluble proteins in leaves of C_4 plants and may contribute together nearly the same percentage to the soluble leaf proteins as Rubisco alone in leaves of C_3 plants (Sugiyama *et al.*, 1984; Sage *et al.*, 1987; Makino *et al.*, 2003). The large subunit of Rubisco is easily detectable on stained gels from C_3 or C_4 plants (Fig. 1). Considering the high percentage of leaf nitrogen bound in Rubisco, it becomes evident that this enzyme is, besides its function in the assimilation of inorganic carbon, highly relevant for the nitrogen budget of a plant. This review is focused on senescence, Rubisco degradation, and the reutilization of Rubisco nitrogen within the plant.

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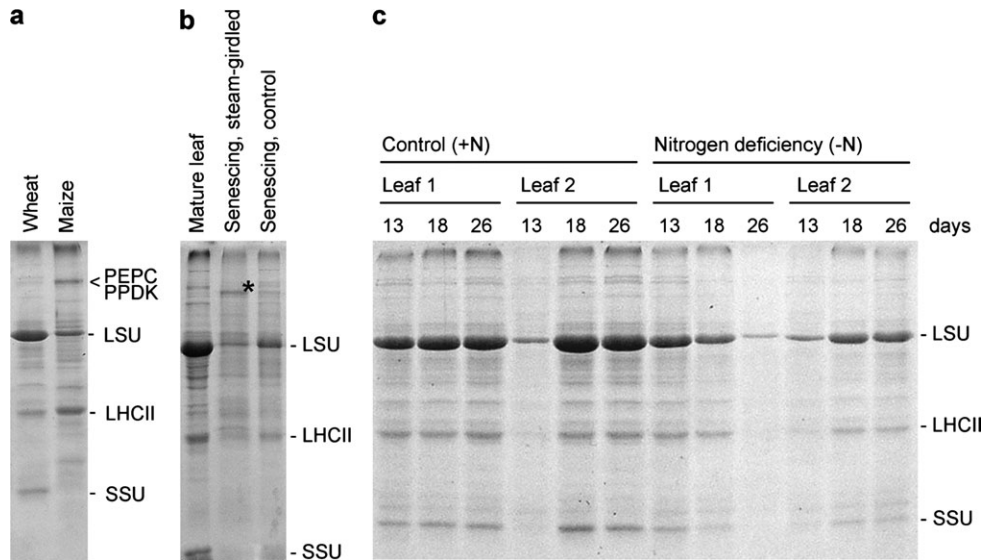
Fig. 1. Rubisco large (LSU) and small (SSU) subunits on SDS–polyacrylamide gels loaded with wheat and maize leaf extracts (a), effect of steam girdling the petiole on the polypeptide pattern in French bean leaves (b), and influence of the availability of inorganic nitrogen in the culture medium on Rubisco levels in wheat leaf extracts (c). (a) The Rubisco LSU is easily detectable in wheat and maize samples (~53 kDa). The strong band in the range 95–110 kDa in the maize sample contains phosphoenolpyruvate carboxylase (PEPC) and pyruvate phosphate dikinase (PPDK). (b) French bean plants were grown on a medium with a low nitrate concentration. The phloem was interrupted in the petiole of the first trifoliate leaf of some plants by steam girdling. The lanes were loaded with an equal percentage (0.2%) of a leaflet. A new band (*) appeared in extracts from steam-girdled leaves, but not or only weakly in extracts from leaves with an intact phloem through the petiole. (c) Wheat seeds were germinated on wet tissue paper in darkness for 2 d and then transferred to a light/dark cycle (14 h light/10 h darkness). The wheat plants grew on deionized water until day 13 and were then transferred to a nutrient medium lacking mineral nitrogen (–N) or to the same medium supplemented with 5 mM NH_4NO_3 (+N). Samples were collected at day 13 (transfer from deionized water to nutrient medium with or without nitrogen), day 18, and day 26 after imbibition. The lanes were loaded with an equal percentage (0.6%) of a leaf, allowing a direct comparison.

Reutilization of Rubisco nitrogen in whole plants

After fulfilling the enzymatic functions, Rubisco is degraded and becomes a major internal nitrogen source. Senescence and Rubisco degradation already occur during the early phases of vegetative growth (Fischer and Feller, 1994). The nitrogen remobilized during Rubisco degradation in the oldest leaves can be translocated to young leaves, and later to even younger leaves or to maturing fruits. A high percentage of the nitrogen present in whole cereal or legume plants is accumulated finally in the protein bodies of mature seeds. Although the degradation of chlorophyll is in general the most obvious senescence symptom, chlorophyll nitrogen remains in the form of colourless chlorophyll catabolites in the vacuole of senescing leaf cells and is not exported to other plant parts (Hörtensteiner and Feller, 2002). Rubisco degradation and chlorophyll catabolism are often well correlated and represent major senescence symptoms, but under certain conditions these two processes may be partially or fully uncoupled (e.g. in stay-green genotypes), as discussed elsewhere (Thomas *et al.*, 2002; Hörtensteiner and Feller, 2002; Hörtensteiner, 2006).

Senescence and protein remobilization in maturing cereals are well organized and controlled on the whole-plant level (Fig. 2). Senescence in wheat progresses from the lowest (oldest) to the uppermost (youngest) leaves, and within the leaves senescence starts at the tip and progresses towards the base and the leaf sheath (Fig. 2a). In corn, senescence also starts at the leaf tip and expands as a V-shaped area towards the leaf base (Fig. 2c). In contrast to wheat, the uppermost (youngest) leaf is not the last one to senesce (Fig. 2b, d). In corn, senescence and protein remobilization start before anthesis in the oldest leaves. During grain maturation, senescence also starts at the top of the plant and the last green leaves are near or slightly above the ear(s) (Feller *et al.*, 1977). From these facts it becomes evident that the age of the leaf is not, or not only, relevant and that the position of major sinks (developing grains) is important in this context.

A further redistribution of nitrogen occurs within the wheat ear. Glumes (containing glume, lemma, and palaea) contain Rubisco and are photosynthetically active after ear emergence (Wirth *et al.*, 1977; Feller, 1978). Glumes also senesce in a later phase of maturation and Rubisco nitrogen becomes available for export again (Fig. 2e). Furthermore, the maturing wheat grains contain a photosynthetically active tissue, the cross cells (Fig. 2g). This peripheral layer in the caryopses also senesces at the end of the maturation period (Fig. 2f). The cross cells and the glumes may reassimilate CO_2 released by respiration in the inner parts of the grain (Wirth *et al.*, 1977; Watson and Duffus, 1991; Gebbing and Schnyder, 2001; Tambussi *et al.*, 2007). The CO_2 assimilation within the ear may be
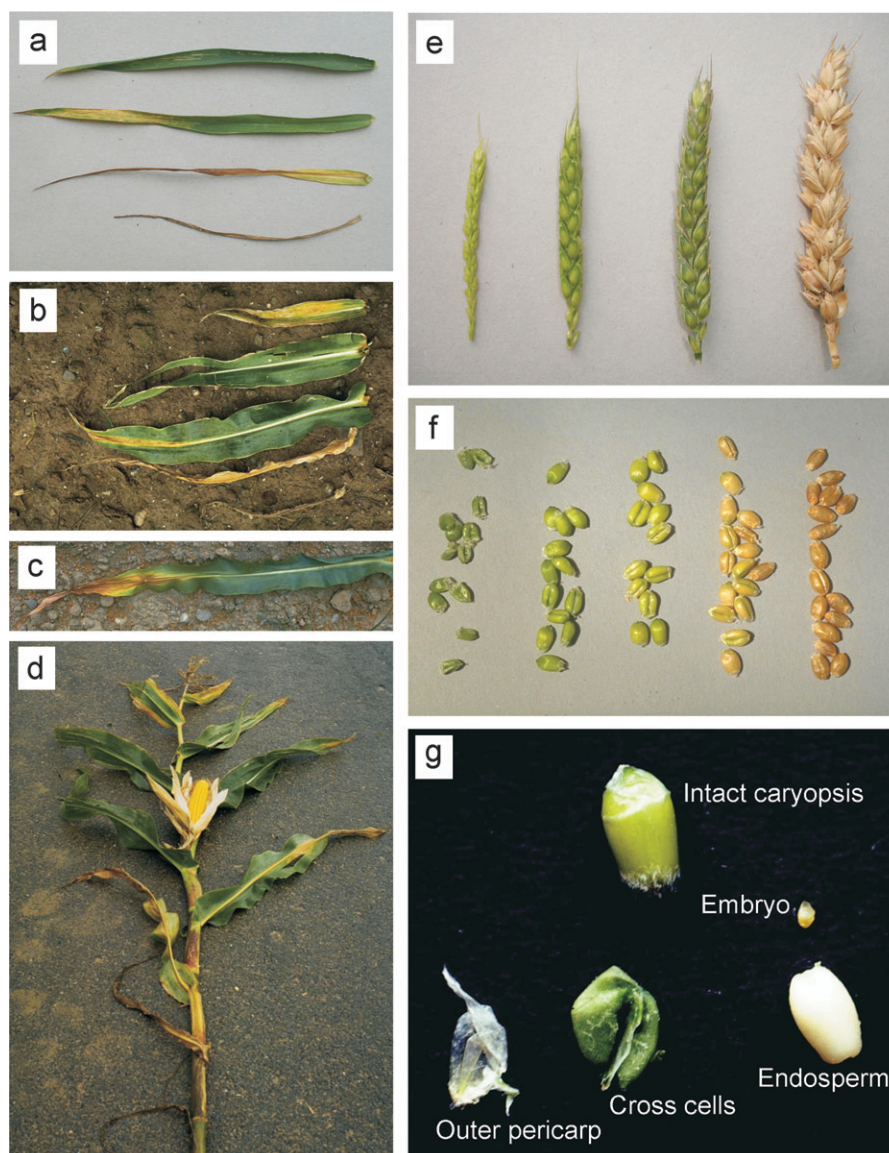
Fig. 2. Loss of photosynthetic capacity in maize and wheat. A picture of the four uppermost leaves of wheat taken shortly after anthesis is shown in (a) (arranged according to their position on the plant). Leaves 1, 4, 7, 10, and 13 from the top of corn (early grain filling period) are shown in (b). The progress of senescence within a leaf and in the whole corn plant is shown in (c) and (d), respectively. Wheat ears 1 week before ear emergence, at ear emergence, at anthesis, and at maturity are shown from left to right in (e). Wheat grains collected at weekly intervals from anthesis (left) to maturity (right) are documented in (f). A grain was dissected 2 weeks after anthesis to illustrate the photosynthetically active (green) cross cells (g).

especially relevant under drought stress (Martinez *et al.*, 2003; Tambussi *et al.*, 2005). In legumes and other dicotyledonous plants, a redistribution of nitrogen from the carpels and from the seed coat to the storage tissues in the seeds can also be observed (Feller, 1990, and references therein). While Rubisco is the predominant protein in green (photosynthesizing) plant parts, storage proteins in the protein bodies are the most abundant proteins in the storage tissues.

The nitrogen fluxes into and out of various parts of wheat and corn plants are schematically shown in Fig. 3 (Feller *et al.*, 1977; Feller and Erismann, 1978; Mae, 2004). Leaf senescence is not restricted to the maturation phase, since the oldest leaves already senesce during the early phases of vegetative growth (Fischer and Feller, 1994). Nitrogen may enter expanding leaves via the phloem or via the xylem (Feller and Fischer, 1994). After the sink-to-source transition, the flux of nitrogen into the leaves is restricted to the xylem. Reduced nitrogen may be exported throughout the life span of a leaf via the phloem, but this export becomes most important during the rapid degradation of chloroplast proteins after the onset of senescence (Feller, 1990; Crafts-Brandner *et al.*, 1996, 1998). In mature leaves, amino acids entering a leaf in the transpiration stream may be transferred to the phloem and exported again without being taken up into mesophyll
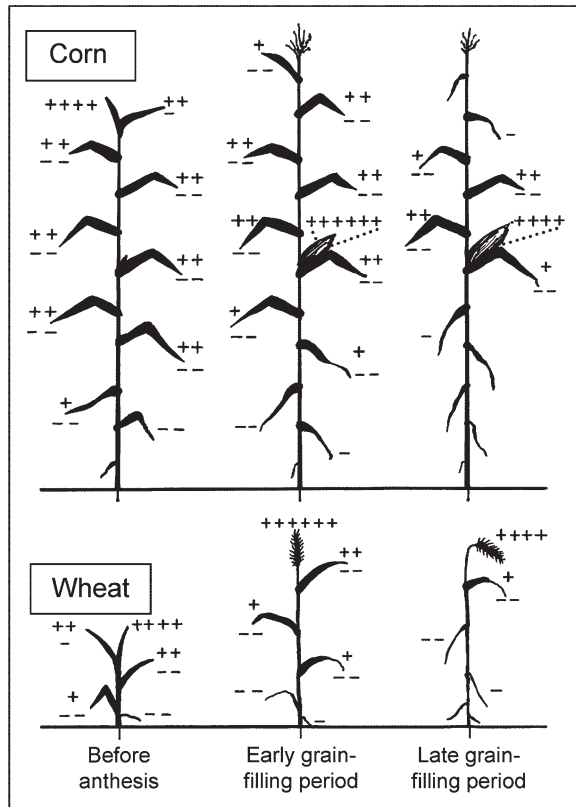
Fig. 3. Schematic overview of nitrogen transport into and out of maize and wheat leaves. Minor (+) and major (++++++) fluxes into a plant part are indicated as well as minor (–) and major (– –) nitrogen fluxes out of the leaves for three developmental stages.

cells (Feller and Fischer, 1994, and references therein). Nitrate on the other hand is not mobile in the phloem, and the nitrogen of this compound may be exported after assimilation in the mesophyll cells. Rubisco is synthesized at a high rate during leaf expansion, is usually turned over very slowly in mature leaves, and its net degradation starts shortly after full leaf expansion, when Rubisco becomes a major nitrogen source for other plant parts (Mae, 2004).

Rubisco degradation and redistribution of Rubisco nitrogen under altered source–sink relationships

Amino acids deriving from Rubisco catabolism are in general exported via the phloem, avoiding an accumulation of free amino acids in the senescing plant parts (Fröhlich and Feller, 1992). However, altered source–sink relationships affect senescence and protein catabolism in leaves on one hand and the export of the amino acids deriving from proteolysis on the other hand (Feller and Fischer, 1994). After panicle or ear removal, assimilates may be allocated to alternative sinks (e.g. tillers), avoiding accumulation in the source leaves (Feller and Fischer, 1994, and references therein; Nakano *et al.*, 1995). When

the export of assimilates from cereal leaves is interrupted, the degradation of Rubisco is accelerated and the content of free amino acids reaches high levels (Fröhlich and Feller, 1992; Feller and Fischer, 1994; Parrott *et al.*, 2005). Under field conditions, accumulated free amino acids may be washed out from the leaves in the rain during a late senescence phase (Debrunner and Feller, 1995; Debrunner *et al.*, 1999). In contrast to potassium and other inorganic ions, the leakage of amino acids is usually not relevant in plants with proper source–sink relationships. From these results, it becomes evident that the net degradation of Rubisco is not only controlled by the demand for amino acids in growing plant parts. Loss of photosynthetic capacity, net degradation of Rubisco and PEP carboxylase, and senescence are accelerated in maize after ear removal (Crafts-Brandner and Poneleit, 1987). The fact that the response to ear removal in cereals depends on the species and even on the genotype supports the assumption that the control of Rubisco catabolism is complex (Crafts-Brandner and Poneleit, 1987; Nakano *et al.*, 1995).

In leaves of depodded soybean plants, Rubisco is finally also degraded and a special set of proteins is synthesized (Wittenbach, 1982; Franceschi *et al.*, 1983; Crafts-Brandner and Egli, 1987). These newly synthesized polypeptides represent vegetative storage proteins and are located in the vacuole of the paraveinal mesophyll (Franceschi and Giaquinta, 1983; Klauer *et al.*, 1996). The 94 kDa vegetative storage protein in soybean has lipoxygenase activity (Tranbarger *et al.*, 1991). The paraveinal mesophyll may act in intact plants as a buffer between the mesophyll (production of amino acids) and the phloem (export of amino acids from the leaf), as suggested by Franceschi and Giaquinta (1983). In addition to these predominant vegetative storage proteins, several other proteins accumulate in soybean leaves after fruit removal (Crafts-Brandner and Salvucci, 1994; Salvucci and Crafts-Brandner, 1995, and references therein). A Rubisco complex protein represents an interesting polypeptide in this context (Crafts-Brandner and Salvucci, 1994). An insoluble complex is formed *in vitro* when this polypeptide (30 kDa subunits) and Rubisco are incubated in a 1:1 ratio by weight, while the complex is soluble when one of the components is present in a several-fold excess. Furthermore, it has been demonstrated that this Rubisco complex protein is located in the cytosol and not in the plastids (Crafts-Brander and Salvucci, 1994). The function of this polypeptide in intact cells is so far unknown. After pod removal, a 28 kDa pod storage protein accumulates in newly formed pods of French bean plants, indicating that special proteins are also produced in young fruits after the removal of the major sinks (Zhong *et al.*, 1997). It must be borne in mind that in depodded legume plants, leaf senescence and net Rubisco degradation may be delayed as compared with intact control

plants (Nakano *et al*., 2000). The findings mentioned above indicate that in leaves of depodded soybean and other legume plants, amino acids deriving from Rubisco degradation may be incorporated into newly synthesized special proteins. New proteins may accumulate in various organs, in various cell types, and in various subcellular compartments.

The production of new polypeptides is also initiated in French bean leaves after interrupting the phloem in the petiole by steam girdling (Fig. 1b). This finding supports the hypothesis that the limited export from leaves of plants with a reduced sink capacity (e.g. after depodding) plays a key role in this context.

Levels of Rubisco and Rubisco fragments under environmental stress

The catabolism of Rubisco depends on a series of endogenous and environmental factors (Nooden *et al*., 1997; Demirevska-Kepova and Feller, 2004; Marín-Navarro and Moreno, 2006). Only some examples for abiotic and biotic stress effects can be summarized here.

Nitrogen limitation causes an accelerated senescence in the oldest leaves of young bean (Crafts-Brandner *et al*., 1996) and wheat (Crafts-Brandner *et al*., 1998) plants. Rubisco is lost earlier in leaves of nitrogen-starved plants than in those of control plants. Transcript levels of nuclear-encoded stromal enzymes as well as their protein levels decline more rapidly under nitrogen starvation than in control plants. The effect of inorganic nitrogen in the culture medium on Rubisco levels is illustrated in Fig. 1c. After germination of wheat, nitrogen is available from the hydrolysis of storage proteins in the endosperm, allowing the proper development of the first leaf. If inorganic nitrogen is present in the nutrient medium, the level of Rubisco in the first leaf is maintained over many days and the level in the second (expanding) leaf increases to a high level. Under nitrogen deficiency, the Rubisco level declines in the first leaf and increases moderately in the second leaf. This pattern illustrates the reutilization of amino acids deriving from Rubisco degradation in the first leaf and the acceleration of leaf senescence under nitrogen deficiency. Rubisco fragments are usually not detectable on immunoblots with samples of extracts from senescing leaves of control or nitrogen-starved plants, indicating that the further hydrolysis of the fragments occurs rapidly. The amino acids produced by the degradation of Rubisco and other chloroplast proteins can be reutilized in other plant parts after transport via the phloem, allowing the growth of sink organs even under strong nitrogen limitation (Feller and Fischer, 1994).

Light is important for the net degradation of Rubisco (Feller and Fischer, 1994). Dark-induced senescence is frequently used as a model system, but under these conditions energy becomes limiting (low carbohydrate level) and protein catabolism may partially differ from that in illuminated leaves (initially high carbohydrate level). In wheat leaves, senescence and the net degradation of stromal proteins including Rubisco are accelerated by a depletion as well as by an accumulation of carbohydrates (Herrmann and Feller, 1998; Parrott *et al*., 2005: Thoenen *et al*., 2007).

Extreme climatic conditions (e.g. heat, drought, and waterlogging) are important stress factors for plants (Herrmann and Feller, 1998; Demirevska-Kepova and Feller, 2004). Elevated temperature can accelerate protein degradation in leaf segments (Demirevska-Kepova *et al*., 2005). It is interesting to note that a Rubisco fragment slightly smaller than the intact large subunit accumulates in wheat leaf segments incubated in the light in the absence of external CO_2 (Herrmann and Feller, 1998; Thoenen *et al*., 2007).

Biotic stress (e.g. infection of plants by micro-organisms) may induce or accelerate senescence. Victorin, a fungal toxin, induces senescence and Rubisco fragmentation in oats (Navarre and Wolpert, 1999). The first 14 amino acids from the N-terminus of the large subunit of Rubisco have been cleaved in oat leaf segments exposed to victorin for 4 h in darkness, while no such fragmentation could be observed in controls incubated under the same conditions in the absence of victorin. This cleavage of Rubisco is sensitive to E-64 (an inhibitor of sulphydryl endopeptidases). Navarre and Wolpert (1999) reported that victorin causes multiple effects in plant cells and might induce a signal cascade. The sequence of events leading to cell death after such fungal infections is not yet clear.

Fate of Rubisco at the subcellular level

During the past two decades, impressive numbers of genes encoding different types of plant peptide hydrolases or proteins with similarities to peptide hydrolases have been described by various groups (Schaller and Ryan, 1995; Shanklin *et al*., 1995; Buchanan-Wollaston, 1997; Bhalerao *et al*., 2003; Buchanan-Wollaston *et al*., 2003). The peptide hydrolases belong to different classes and are located in defined subcellular compartments. The functions of many of these peptide hydrolases have not yet been identified. The expression of genes encoding peptide hydrolases alters during senescence. The abundance of some mRNAs for peptide hydrolases strongly increases during this final phase of leaf development (Bhalerao *et al*., 2003; Buchanan-Wollaston *et al*., 2003). The question of whether and how these peptide hydrolases might be involved in the catabolism of Rubisco and other plastidial proteins is not yet satisfactorily answered. It is not only enzymes located inside the plastids that may

contribute to the degradation of Rubisco, as suggested by various laboratories (Yoshida and Minamikawa, 1996; Guiamét *et al.*, 1999; Thoenen *et al.*, 2007).

Enzymatically active Rubisco is located in the stroma. Caution is recommended when using isolated chloroplasts as an experimental system, since contaminating endopeptidases of vacuolar origin may act on stromal proteins after chloroplast rupture (Miyadai *et al.*, 1990). Treatment of the isolated chloroplasts with thermolysin (Miyadai *et al.*, 1990) or the reisolation of intact chloroplasts after incubation of the samples (Mitsuhashi *et al.*, 1992; Roulin and Feller, 1997, 1998*a*) may be suitable to avoid such artefacts. The predominant stromal protein Rubisco can be degraded in intact chloroplasts (Mitsuhashi *et al.*, 1992; Desimone *et al.*, 1996, 1998; Roulin and Feller, 1997, 1998*a*; Ishida *et al.*, 1998; Zhang *et al.*, 2007). Several exo- and endopeptidases are located in plastids (Adam and Clarke, 2002). A metalloendopeptidase is able to degrade the large subunit of Rubisco (Bushnell *et al.*, 1993; Roulin and Feller, 1998*a*). Chelators (e.g. EDTA), which are often standard constituents of chloroplast isolation and incubation media, can inhibit the degradation of Rubisco in darkness. This inhibition is reversible by the removal of EDTA and the addition of zinc in the micromolar range and of magnesium in the millimolar range, strongly supporting the hypothesis that a metalloendopeptidase is involved (Roulin and Feller, 1998*a*). The N-terminus was still present in the Rubisco large subunit fragments produced during the incubation of isolated chloroplasts, indicating that the first cut(s) occurred near the C-terminus (Roulin and Feller, 1998*a*; Ishida *et al.*, 1999). A 44 kDa fragment of the large subunit of Rubisco missing the N-terminus was detected after the incubation of chloroplast lysates in darkness (Kokubun *et al.*, 2002). This finding and the observations mentioned above suggest that different mechanisms for the degradation of Rubisco exist in plastids. Carbamylation and substrate binding of Rubisco may still be functional after limited proteolysis, although the catalytic activity may be lost (Gutteridge *et al.*, 1986).

The degradation of Rubisco in intact chloroplasts can be achieved, initiated, or accelerated by reactive oxygen species (Desimone *et al.*, 1996; Ishida *et al.*, 1997; Stieger and Feller, 1997; Roulin and Feller, 1998*b*). It has been demonstrated that Fe^{2+} ions catalyse a site-specific cleavage of the large subunit of Rubisco near the catalytic centre (Luo *et al.*, 2002). The *in vivo* fragmentation of the large subunit of Rubisco has been reported recently for cucumber leaf discs incubated in the light under chilling conditions (Nakano *et al.*, 2006).

The sensitivity of enzyme proteins to proteolysis depends on the actual conditions in the relevant compartment (Feller, 2004). The pH value as well as solute concentrations may affect protein catabolism. Rubisco is protected against proteolytic degradation under catalytic conditions (Houtz and Mulligan, 1991) or in the presence of an inhibitor (reaction intermediate analogue) binding to the active site in the large subunit (Mulligan *et al.*, 1988; Khan *et al.*, 1999). Such interactions may allow a fine-tuning of the degradation. Rubisco can be present in an active (carbamylated) or an inactive form (Spreitzer and Salvucci, 2002). CND41, a DNA-binding aspartic protease in plastids, can degrade Rubisco and may play a role in senescence and Rubisco nitrogen remobilization (Kato *et al.*, 2004). These authors have shown that active (carbamylated) Rubisco is less sensitive to this protease than the inactive form. Already moderately elevated temperatures negatively affect the activation of Rubisco by Rubisco activase (causing a lower percentage of carbamylated molecules) and may, as a consequence, also alter the susceptibility of Rubisco to attack by certain proteases (Feller *et al.*, 1998).

Although often no Rubisco fragments are detected in naturally senescing leaves, typical breakdown products may accumulate in leaves or leaf segments under certain conditions (Hildbrand *et al.*, 1994; Nakano *et al.*, 2006; Thoenen *et al.*, 2007). Under hypoxia or anoxia, a strong band becomes visible on gels (SDS–PAGE) below the large subunit of Rubisco (Hildbrand *et al.*, 1994). [^{35}S]Methionine present in the incubation medium is not incorporated into this band, suggesting that the polypeptide is not synthesized *de novo*. On the other hand, this polypeptide cross-reacts with antibodies raised against the large subunit of Rubisco, indicating that this band is a breakdown product of Rubisco. From more recent experiments with antibodies raised against the C- and N-terminus of the large subunit of Rubisco (Fig. 4), it became evident that under these conditions the N-terminus is removed and the C-terminus is still present. Two fragments of ~50 kDa and 40 kDa become visible on blots with antibodies directed against the C-terminus. The larger (but not the smaller) fragment also reacts with antibodies against amino acids 9–28 from the N-terminus, while both fragments are not detected with antibodies raised against the first 25 amino acids from the N-terminus. These results document that both fragments lack the N-terminus. These fragments are similar to those reported by Yoshida and Minamikawa (1996). Under hypoxia, the small subunit of Rubisco is well maintained, although a portion of most large subunit molecules is removed (Hildbrand *et al.*, 1994). The removal of an N-terminal portion has also been detected in wheat leaf segments incubated under conditions causing a low carbohydrate level (Thoenen *et al.*, 2007). The formation of such fragments is inhibited by E-64 (an inhibitor of cysteine endopeptidases). Furthermore, it could be shown that this cleavage occurs when the large subunits are still integrated together with the small subunits in the Rubisco holoenzyme (Thoenen *et al.*, 2007). This type of fragmentation was not observed in isolated pea chloroplasts. The
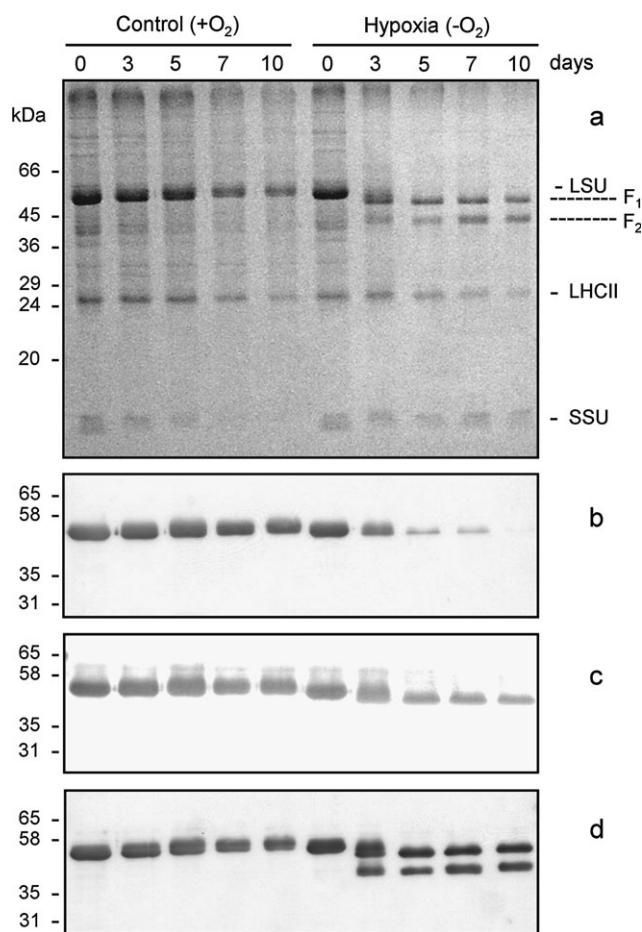
Fig. 4. Fragmentation of the large subunit of Rubisco in bean leaves incubated under hypoxia in darkness. Detached bean leaves were incubated in darkness for 0, 3, 5, 7, and 10 d either floating on deionized water ($+O_2$) or submerged in deionized water ($-O_2$). Extracts were analysed by SDS–PAGE with an equal percentage (0.2%) of a leaf loaded per lane. The stained gel with the fragments F_1 and F_2 is shown in (a). Specific antibodies raised in rabbits against a synthetic version of the first 25 amino acids from the N-terminus of the large subunit from spinach Rubisco (b), against a synthetic version of amino acids 9–28 from the large subunit of wheat Rubisco (c), or against a synthetic version of a sequence near the C-terminus (amino acids 425–440) of the large subunit of Rubisco from wheat (d) were used to develop the immunoblots.

finding reported by Zhang *et al.* (2006) that a 50 kDa fragment of Rubisco is produced in crude leaf extracts but not in chloroplast lysates is consistent with the hypothesis that extraplastidial enzymes degrade Rubisco in a manner different from the intraplastidial peptide hydrolases. It appears likely that the observed extraplastidial cleavage is caused by a vacuolar cysteine endopeptidase, while in isolated plastids other types of peptide hydrolases (e.g. metalloendopeptidases) or a direct fragmentation by reactive oxygen species are involved in the degradation of Rubisco.

The evidence for the involvement of vacuolar peptide hydrolases in Rubisco degradation raises the question of how the plastidial substrate protein (Rubisco) may become accessible to the vacuolar enzyme(s) separated by several membranes from the stroma (Yoshida and Minamikawa, 1996; Thoenen *et al.*, 2007). It has been proposed that chloroplasts (Minamikawa *et al.*, 2001) or spherical bodies deriving from chloroplasts and containing stromal proteins but not thylakoid components (Chiba *et al.*, 2003) reach the vacuole for the final degradation of the chloroplast constituents. Guiamét *et al.* (1999) reported that, in leaves of wild-type French bean, globules with lipophilic contents are released from the chloroplasts into the cytosol (and perhaps later into the vacuole) during senescence, while these globules are absent from leaf cells in a stay-green genotype. Such lipophilic globules are the counterpart to the hydrophilic spherical bodies and may be involved in the degradation of pigments or membrane proteins rather than of stromal enzymes. The ricinosomes, organelles containing high levels of sulphydryl endopeptidase precursor, must be considered as important compartments involved in the increase of sulphydryl endopeptidase activity and of the rapid net protein degradation during senescence (Gietl and Schmid, 2001). The question as to what extent Rubisco is degraded intraplastidially and extraplastidially is still open and represents a challenge for future research.

Conclusions

Rubisco is degraded in leaves of intact plants during natural senescence as well as under various stress conditions. Rubisco nitrogen can be exported from senescing organs in the form of amino acids or peptides via the phloem, allowing the supply of growing plant parts. However, the net degradation of Rubisco is not always well correlated with the export of nitrogen from the leaves, since free amino acids may accumulate to high levels or may be incorporated in the same organ into newly synthesized proteins in other cells or other subcellular compartments. The question of how the net degradation of Rubisco is controlled remains open. There is not necessarily a switch allowing the initiation of Rubisco degradation. The opposite situation appears possible: the degradation of Rubisco might be the default pathway in the case of a metabolic disorder and might be initiated by a series of internal and external factors influencing leaf metabolism and interactions between organs. If so, then the question would be: which conditions must be fulfilled to maintain the Rubisco level prior to senescence?

Several laboratories presented good evidence that there is not one well-defined pathway for Rubisco degradation with well-defined proteolytic events. Depending on the actual conditions, reactive oxygen species may directly cleave the polypeptide chain, or plastidial or vacuolar enzymes may act on this stromal protein with or without prior modification. The concentrations of solutes

interacting with Rubisco may affect its susceptibility to proteolytic attack and allow a fine-tuning of the net degradation rate. From the results available today, it appears likely that under photoinhibitory conditions and when carbohydrates are available in excess the degradation inside the plastids plays a major role, and that under energy deficiency (low carbohydrate levels) vacuolar endopeptidases may come into play earlier. Caution is recommended when generalizing mechanisms of Rubisco catabolism.

The following questions remain to be addressed in future experiments. (i) How is the remobilization of Rubisco nitrogen controlled *in vivo*? (ii) Which enzymes are involved in which phase of its catabolism? (iii) Which steps are located in which subcellular compartments? (iv) Is the native Rubisco protein degraded or are modifications necessary prior to proteolysis?

Acknowledgements

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