Tansley review

Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process

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Contents

Key words: abscisic acid (ABA), ascorbate, cysteine protease, glutathione, nodule senescence, reactive oxygen species (ROS), redox signalling, transcript profiling.

Summary

Research on legume nodule development has contributed greatly to our current understanding of plant–microbe interactions. However, the factors that orchestrate root nodule senescence have received relatively little attention. Accumulating evidence suggests that redox signals contribute to the establishment of symbiosis and senescence. Although degenerative in nature, nodule senescence is an active process programmed in development in which reactive oxygen species (ROS), antioxidants, hormones and proteinases have key roles. Nodules have high levels of the redox buffers, ascorbate and glutathione, which are important in the nodulation process and in senescence. These metabolites decline with N-fixation as the nodule ages but the resultant decrease in redox buffering capacity does not necessarily lead to enhanced ROS or oxidative stress. We propose models by which ROS and antioxidants interact with hormones such as abscisic acid in the orchestration of nodule senescence.

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I. Introduction

The symbiotic association between the roots of leguminous plants and soil rhizobia results in the development of specific organs, called nodules, whose primary function is N-fixation. The products of symbiotic N-fixation (amides in temperate legumes and ureides in tropical legumes) are exported from the nodules to the rest of the plant, where they are incorporated into essential macromolecules such as amino acids and proteins that drive plant growth, development and, in the case of agriculture, crop yields. Root nodules not only make a crucial contribution to the N-economy of leguminous crops but also enhance the N-content of the soil, and thus they have a key role in environment-friendly agricultural practices. Legume–rhizobia symbioses are beneficial to both partners. In exchange for exported N-compounds, the symbiotic bacteria (such as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and related genera) are supplied with energy and C skeletons in the form of dicarboxylic acids (Lodwig *et al.*, 2003). The bacteria and plant cells also probably exchange amino acids; for example, the plant supplies the bacteria with glutamate and so enables them firstly to limit ammonium assimilation and secondly to produce aspartate and alanine to be returned to the plant. This would also allow ammonium to be transported out of the bacteria to the plant for assimilation and would favour mutual benefit from the exchange processes (Lodwig *et al.*, 2003).

The establishment of the symbiosis requires extensive recognition and signalling by both partners (Long, 2001). Symbiosis is initiated by release of lipochitooligosaccharide molecules called 'Nod' factors by the bacterium through the expression of *nod* genes in response to plant-derived flavonoid, stachydrine and aldonic acid molecules in the soil (Stougaard, 2000). If penetration occurs via rhizobial attachment to the root hairs, then invasion of the cortical tissue via a structure called the 'infection thread' is accompanied by initiation of meristematic activity in the root cortical and pericycle cells. The Nod factors modify the plant hormone balance in such a way as to stimulate mitosis and to allow development of the symbiosome that houses the bacteria within the plant (Ferguson & Mathesius, 2003). This involves the release of the bacteria into individual cortical cells by endocytosis, a process that results in the enclosure of the bacteria within a plant membrane called the 'peribacteroid' or 'symbiosome' membrane. This membrane effectively isolates the bacteria from the host cell cytoplasm. The peribacteroid membrane thus fulfils essential structural and metabolic roles, separating the bacteria from the plant cell cytoplasm and controlling exchange of metabolites and signals. The constant exchange of information between the symbiotic partners not only allows the plant to regulate bacterial metabolism (for example, the control of dicarboxylate use by modulating amino acid supply; Lodwig *et al.*, 2003) but it also ensures the survival of the bacteria within the potentially hostile environment of the plant. In accepting the rhizobial partner, the plant lowers the inherent endogenous defences that prevent invasion by foreign organisms, so that the bacteria can survive and grow in very close proximity to the plant cell cytoplasm (Colebatch *et al.*, 2004). Moreover, the plant provides a unique microaerobic low-oxygen environment for the bacteria within the symbiosome that controls the expression of the bacterial N-fixation genes as well as cytochromes that work best in these conditions (Long, 2001).

Nodules are classified as 'indeterminate' and 'determinate' according to their mode of development. The biology of both types of nodule has been fully described in a previous Tansley review (Hirsch, 1992). Thus, we will emphasise here only the nodule features that are important in understanding the mode of nodule senescence. In contrast to indeterminate nodules, which have a persistent apical meristem that often yields a cylindrical or branched nodule structure, the determinate nodule has no active meristem and thus has a rather different shape and structure to the indeterminate nodule, as illustrated in Fig. 1. Determinate nodules (Fig. 1a) such as those of soybean are initiated from meristematic cells in the outer cortex, but cell division stops at *c.* 10 d after infection. There is a radial gradient of development, with senescence beginning at the centre and spreading outwards. The bacteria housed in the symbiosomes in both determinate and indeterminate nodules can revert to free-living bacteria as the organs senesce (Müller *et al.*, 2001).

Indeterminate nodules such as those of pea, clover or alfalfa have a very different structure to determinate nodules, being comprised of five easily distinguished zones (Fig. 1b). Zone I is made up of small meristematic cells, which are permanently dividing and do not contain any microsymbionts. Zone II contains the infection zone where bacteria are captured for symbiosome formation. In Zone III the bacteria are housed in the symbiosomes and become competent in atmospheric N-fixation. Zone IV is not present during early nodule differentiation but appears as the nodule develops, becoming progressively larger as the plant ages. Senescence is evident in Zone IV where the symbiotic partnership is lost. Proximal to the senescent zone is a region (Zone V) where the bacteria are essentially free-living and do not show the ultra-structural features of symbiosomes (Timmers *et al.*, 2000).

The following tissue types can be distinguished in both types of nodule from the periphery to the centre: an external nodule cortex, an endoderm, an internal cortex called the nodule parenchyma and the central zone housing bacteria (Van de Wiel *et al.*, 1990). Metabolic exchange between the nodule and the other organs of the plant is ensured by the presence of vascular bundles, localised within the nodule parenchyma that is connected to the root vascular system. Mutual signal exchange begins the nodulation process and we must presume that the two-way communication between bacterial and plant cells extends throughout the nodule lifespan (Stougaard, 2000). The interaction between the plant and the

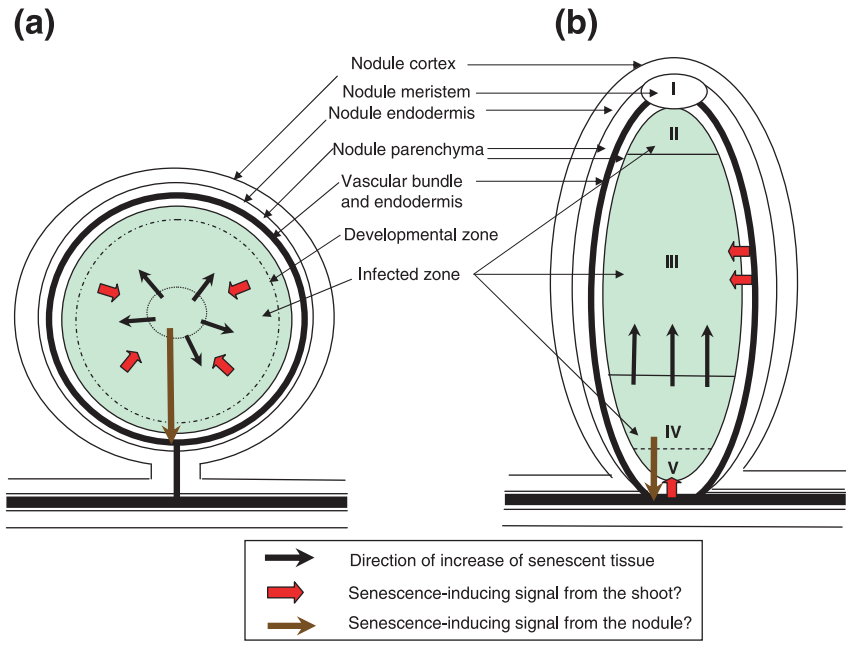

Fig. 1 A diagrammatic representation of the structure of determinate (a) and indeterminate nodules (b).

bacteria is controlled by the plant and is highly specific, with much of the specificity determined by the structure of the Nod factors. While nonnodulating mutants do not respond to bacterial Nod factors (Mitra *et al.*, 2004), the analysis of supernodulation and hypernodulation phenotypes has shown that the degree of nodulation is controlled through metabolite signals (Lodwig *et al.*, 2003) and hormones that control plant development (Ferguson & Mathesius, 2003). In particular, exposure to ethylene and the application of abscisic acid (ABA) inhibits the number of nodules that form (Penmetsa & Cook, 1997; Oldroyd *et al.*, 2001; Ferguson & Mathesius, 2003). In both cases, inhibition appears to result from effects of these hormones on early root responses: ABA prevents the formation of the lateral root system (Signora *et al.*, 2001), whereas ethylene inhibits all the early responses of the plant (Oldroyd *et al.*, 2001). The ratio of ABA to cytokinins is also considered to be important in auto-regulation (Caba *et al.*, 2000; Bano *et al.*, 2002). Thus, it would appear logical to presume that the plant controls nodule longevity in a similar manner to which it controls nodule numbers and the extent of symbiotic root nodule formation through the signalling and crosstalk between metabolite and hormone pathways (Ferguson & Mathesius, 2003; Oldroyd *et al.*, 2001; Lodwig *et al.*, 2003).

II. The characteristics of legume nodule senescence

Whereas leguminous trees and shrubs have large woody perennial nodules, the nodules of fast-growing annual species are relatively short-lived compared with the parent roots. The N-fixing capacity of the nodules peaks early in the life of the nodule, beginning to decline when the nodule is only 3–5 wk old (as illustrated in Fig. 2). Whereas leaf senescence and cell death related to the development of vascular tissues have been intensively studied (Buchanan-Wollaston *et al.*, 2003), the factors that limit the lifespan of the rhizobia–plant symbiosis have received relatively little attention. Over the last 10 years, more than 30 reviews have been published on the rhizobia–legume interaction. These able texts have considered many key aspects of nodule biology from structure to recognition and signalling, emphasising the interactions occurring at the early stages of the nodulation process. To date, only one

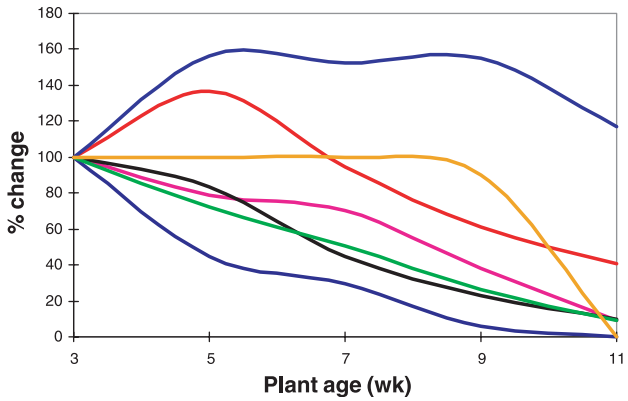

Fig. 2 Developmental changes in parameters associated with the lifespan of legume root nodules. Data is shown for pea nodules harvested at different time-points. The 3-wk value for each parameter was set as 100%. These data are modified from H. Vanacker *et al*. (unpublished). Nitrogenase activity, dark blue; total ascorbate content, black; total glutathione content, green; total protein content, purple; catalase activities, red; relative transcript levels of leghemoglobin (isoform 120–2), orange; glutathione reductase activities, light blue.

review has exclusively concerned the complex and poorly understood process of nodule senescence (Swaraj & Bishnoi, 1996). Moreover, the general dearth of literature on this topic is surprising, given its importance to agriculture. Nodule senescence is genetically controlled, and while legume breeding programmes have yielded varieties with early or delayed senescence, the lack of research may reflect the absence of useful mutants with early or delayed senescence phenotypes with which to tackle the problem. Although a large number of nodulation mutants are now available, no late senescing phenotypes have been described.

It is widely accepted that functional genomics approaches are powerful tools that rapidly accelerate investigations of developmental processes in specialised tissues such as inoculated roots, nodules and mycorrhiza (Gamas *et al.*, 1996; Journet *et al.*, 2002; Mitra *et al.*, 2004). However, there are relatively few reports directed at the application of such technologies to the understanding of the genetics that control nodule senescence (Fedorova *et al.*, 2002). Little database information specific to nodule senescence exists, but The Institute for Genomics Research (TIGR) *Medicago truncatula* Gene Index: Expressed Sequence Tags (ESTs) from senescent nodules supplied by Carrol Vance (MtGI-GVSN) library (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago) is recommended to the reader in this regard. The *Medicago truncatula* expressed sequence tag (EST) database contains over 140 000 sequences, of which < 3000 come from the GVSN library of senescent nodules (Fedorova *et al.*, 2002). Of particular note within the GVSN database are (1) the Cys-cluster proteins and (2) the tentative consensus (TC) sequences similar to the nodule-specific protein nms-22. These sequences suggest firstly that Cys-cluster proteins are induced before N-fixation commences and extend throughout nodule senescence, and secondly that the nms-22-like proteins are specifically expressed during nodule senescence (Fedorova *et al.*, 2002). Transcript profiling has also been used to identify genes that are expressed in nodules and roots from 7-wk-old *Lotus japonicus* plants (Colebatch *et al.*, 2002, 2004), with the aim of identifying genes that are essential for symbiotic N-fixation, in particular the nodulin genes that are expressed only in developing or mature nodules. Nodulins are classed as early or late, based on when the gene transcripts are first detected (Verma *et al.*, 1992). Early nodulin genes are largely involved with nodule development and are activated in roots within hours or days of inoculation with rhizobia. Late nodulin genes are activated around the time that N-fixation commences and include enzymes of C and N metabolism as well as structural components. Of the 850 or so genes that have now been shown to be more highly expressed in nodules compared with roots, about 100 are involved in signalling and related processes, while about one-third of them are involved in metabolism and transport (Colebatch *et al.*, 2004).

The lifespan of an average nodule on a fast-growing herbaceous legume in the absence of stress is *c.* 10–12 wk (maximum) from the point of initiation. Indeterminate nodules contain a senescing zone that starts to form in mature nodules and becomes progressively larger (Fig. 1b). The senescent area spreads as the nodule develops until it extends throughout the nodule. In the case of determinate nodules, senescence begins at the centre of the tissue and then extends progressively reaching the periphery after a few short weeks (Fig. 1a). In addition to developmental controls, nodule senescence can be induced prematurely by dark stress (Gogorcena *et al.*, 1997; Matamoros *et al.*, 1999; Hernandez-Jimenez *et al.*, 2002), defoliation (Vance *et al.*, 1979), treatment with nitrate (De Lorenzo *et al.*, 1990; Swaraj *et al.*, 1993; Escuredo *et al.*, 1996; Matamoros *et al.*, 1999) and drought (Gogorcena *et al.*, 1995; Gonzalez *et al.*, 1998). These observations suggest that shoot and root signals are major factors controlling nodule lifespan. As in leaves, senescence occurs in an age-dependent manner (as illustrated in Fig. 2) and consists of a highly organised process that appears to start with early changes in metabolite contents (after 3–4 wk) and modulation of metabolism. In pea, for example, maximum rates of N-fixation capacity peak early in development (3 wk), whereas loss of leghemoglobin transcripts, protein degradation and organ death occur much later (9–12 wk; H. Vanacker *et al.*, unpublished). Also important is the arrest of the meristematic activity (when a persistent meristem is present). In any assessment of the factors orchestrating the aging processes, it is logical to presume that the number of nodules present at any one time on the roots is dictated at least in part by the activity and sensing of processes in the shoot, because firstly plant N status is sensed in the shoots in such a way as to control the shoot : root ratio (Scheible *et al.*, 1997) and secondly because the net assimilate produced and exported from the shoot determines the extent of nodulation, as demonstrated by the negative impact of shading the aerial plant parts. Even early responses to sugar depletion in leaves are extensive (Thimm *et al.*, 2004), but no data are available yet on how sugar depletion affects gene expression in roots or nodules. Moreover, because the whole plant development programme from seed dormancy to flower senescence is regulated by hormones, it is logical to consider that ABA, auxin, cytokinins, ethylene and gibberellic acid can also affect nodule senescence (Ferguson & Mathesius, 2003). It remains to be seen whether such hormone signalling occurring during nodule senescence involves common elements such as the ORE9 protein that links ABA, ethylene and methyl jasmonate signalling in *Arabidopsis* leaf senescence (Woo *et al.*, 2001).

1. Structural changes

During the senescence process visible changes occur in nodules, for example the colour of the N-fixing tissues of the nodule changes from red (due to the presence of functional leghemoglobin) to green (indicating an alteration of this protein; Swaraj & Bishnoi, 1996). At the cellular level ultra-structural

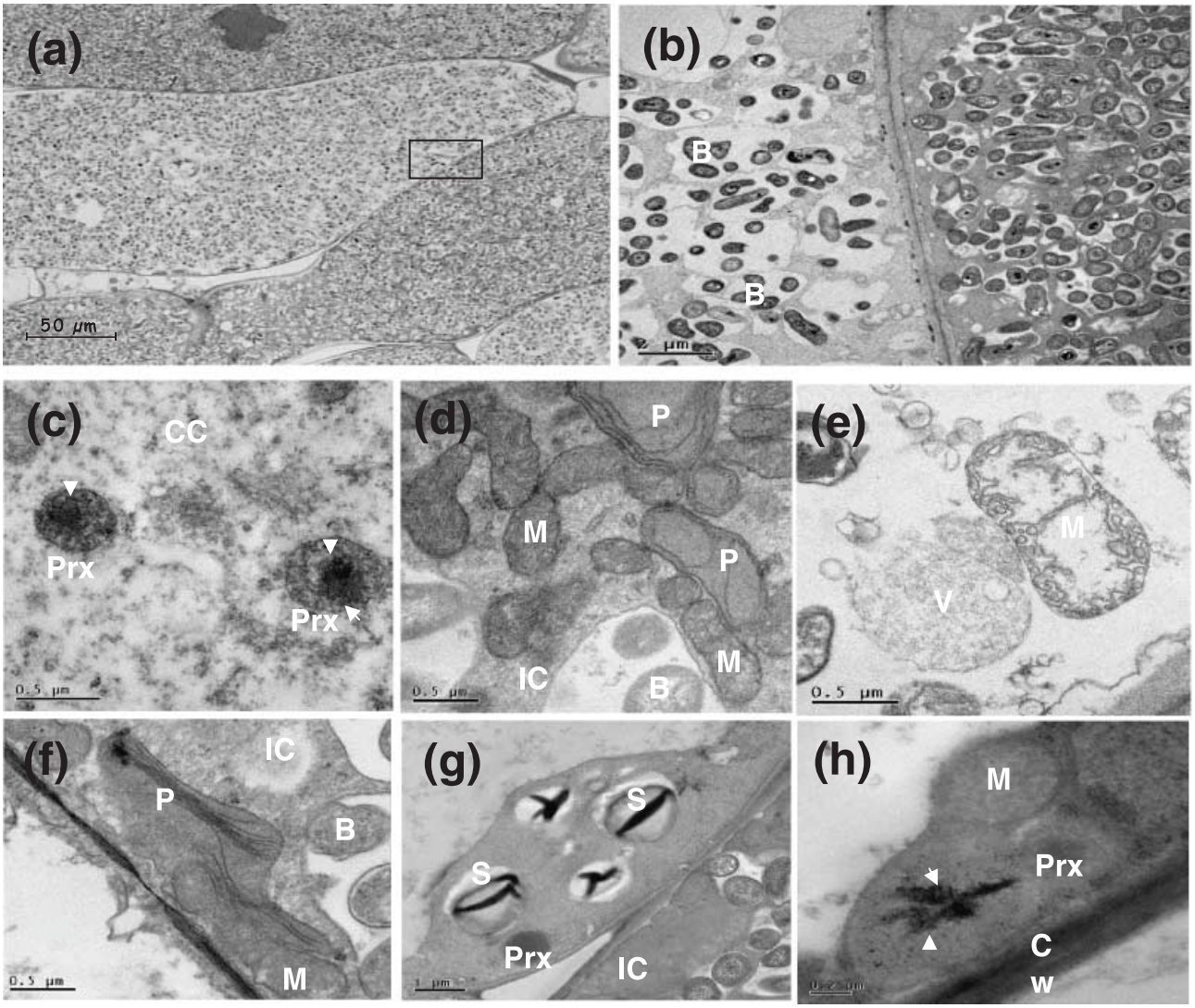

Fig. 3 Changes in determinate nodule structure observed during the natural senescence process. Light microscopy photograph of a transverse section of a 7-wk-old soybean nodule (a) showing infected cells at different stages of senescence. The ultrastructure of the area delineated by the rectangle in (a) is shown in (b). The cytoplasm density is much higher in nonsenescent cells (right) than in senescent cells (left; b). Peroxisomes in the cortex have a central dense matrix (c). Clusters of mitochondria appear in infected senescent cells in 8-wk-old nodules (d), followed by destruction of the internal structure (e). Developmental changes in plastid ultrastructure are also observed in the senescence process (f–h). Plastids become devoid of starch granules (f) relative to those of 6-wk-old nodules, where large starch granules are observed (g). Ferritin (indicated by arrowheads) is induced in the cortical plastids of senescent 8-week-old nodules (h). B, bacteroid; Cw, cell wall; CC, cortical cells; IC, infected-cells; M, mitochondrion; NIC, noninfected-cells; P, plastid; Prx, peroxisomes; S, starch granules; V, vesicle.

changes also take place. In the determinate nodules such as those of soybean, for example, changes in the organisation of the cellular structures are observed (Fig. 3). Even in determinate nodules senescence does not occur homogeneously throughout the nodule; hence ultra-structural differences between senescing and mature adjacent infected cells can be compared (Fig. 3a,b). The cytoplasm of senescent soybean nodule cells becomes progressively less electron-dense and numerous vesicles appear. Moreover, the symbiosomes change in size and shape as the bacteroid deteriorates. Organelle composition and structure also change during senescence (Fig. 3c–h). For example, the number of peroxisomes increases and the mitochondria begin to form complex elongated structures (Fig. 3d,e). There is also a tendency for plastids to accumulate ferritin (Fig. 3h), depending on the state of the nodule tissue (Lucas *et al.*, 1998). Membrane damage, particularly to the symbiosome membrane, appears to occur early in the senescence process. For example, biochemical and cytological evidence from soybean, French bean and alfalfa nodules indicates that the symbiosome membrane may be the first target for degradation in the nodule senescence process (Pladys & Rigaud, 1985; Puppo *et al.*, 1991). However, breakdown of

the symbiosome membrane in lupin was only observed at a very advanced stage of senescence (Hernandez-Jimenez *et al.*, 2002). At any stage, rupture of this membrane is likely to be deleterious to nodule function because regulated metabolite and signal exchange between the partners will be lost.

2. Changes in N-fixation capacity

The loss of measurable N-fixation activity occurs in parallel with a decrease in leghemoglobin during the natural senescence of soybean, French bean and mung bean nodules (Pladys & Rigaud, 1985; Dalton *et al.*, 1986; Lahiri *et al.*, 1993). However, the rapid decrease in N-fixation capacity that occurs following exposure to stress is observed before any observed decrease in leghemoglobin transcripts in pea nodules (H. Vanacker *et al.*, unpublished). Similarly, the rapid decrease in N-fixation capacity that occurs following exposure to stress is observed well before a decline in leghemoglobin (Escuredo *et al.*, 1996; Gogorcena *et al.*, 1997; Matamoros *et al.*, 1999). Moreover, when pea plants were treated with ABA, which is known to induce nodule senescence, N-fixation declined before any observed decrease in leghemoglobin (Gonzalez *et al.*, 2001). ABA has a number of functions in plants but in general it decreases growth rates and enhances cell sustainability, as is perhaps best illustrated by its role in desiccation survival in seed maturation and dormancy. Lateral root development is also inhibited in plants sprayed with ABA, and ABA synthesis and signalling are involved in the pathways by which roots sense and respond to high nitrate levels in the soil (Signora *et al.*, 2001; De Smet *et al.*, 2003). Hence in regard to nodule senescence, ABA has a number of functions: (1) it can induce root nodule senescence (Gonzalez *et al.*, 2001), as it does in leaves (Woo *et al.*, 2001); (2) it is involved in the control of the development of the plant root system in response to soil N; and (3) its synthesis and signalling are integral to sugar- and nitrate-sensing pathways in plants.

3. Changes in proteinase activities

During the final stages of the senescence process, large-scale protein remobilization occurs (Malik *et al.*, 1981). As the aging process progresses, various hydrolytic enzymes are activated. Although no changes in the activities of bacteroid peptide hydrolases have been reported to date (Pfeiffer *et al.*, 1983), a whole spectrum of plant proteases including cysteine proteases (CPs) and aspartic proteases are involved in nodule protein breakdown and remobilisation of resources. Protease genes have now been cloned from a large range of senescent plant tissues (e.g. Smart *et al.*, 1995; Drake *et al.*, 1996; Cercos *et al.*, 1999). Of these, thiol-proteases appear to be the most common proteolytic enzymes induced in senescent plant cells (Beers *et al.*, 2000; Palma *et al.*, 2002). In particular, thiol-type protease activities appear to serve important roles in senescing soybean (Malik *et al.*, 1981), French bean (Pladys *et al.*, 1991) and alfalfa (Pladys & Vance, 1993) nodules. Apoptosis in animals requires the activation of a group of CPs called 'caspases'. While metacaspase genes have now been identified in plants, there are no nodule metacaspases known at present. However, a group of vacuolar CPs, collectively called 'vacuolar processing enzyme', may fulfil a similar role to animal caspases in orchestrating plant programmed cell death (PCD). Expression of TC 28421, encoding a CP, was almost undetectable in Northern blots of N-fixing *M. truncatula* nodules, but five ESTs for this CP were found in the GVSN library (Fedorova *et al.*, 2002). Other CPs may also play a key role in nodule development, with specific forms being induced during senescence. Moreover, the activation of CPs has also been strongly implicated in pea (Kardailsky & Brewin, 1996), Chinese milk vetch (Naito *et al.*, 2000) and soybean (Alesandrini *et al.*, 2003b) nodule senescence. CPs have also been found to be highly up-regulated in senescent *M. truncatula* nodules (Fedorova *et al.*, 2002). The structural and metabolic modifications that occur as a result of this extensive increase in protein degradation activity must inevitably affect the microsymbionts. The bacteroids in *Astralagus sinicus* nodules undergo DNA fragmentation in these circumstances (Kobayashi *et al.*, 2001). However, the bacterial partners are not completely destroyed in these conditions and some bacteria survive the cell death events initiated by the plant to live saprophytically thereafter within the plant cells. The differentiated bacteria in the senescent tissue may then have a higher survival probability than free-living rhizobia in the rhizosphere (Hernandez-Jimenez *et al.*, 2002).

III. Redox homeostasis and programmed cell death during the senescence process

1. General signalling roles of reactive oxygen species

All aerobic organisms, while having the ability to utilise oxygen, have the associated risk of oxidative stress caused by reactive oxygen species (ROS). It is now the generally accepted view that the effects of ROS result from responses to sensing systems involving antioxidant pools, not as a consequence of oxidative damage to proteins or other molecules *per se*. Supporting this is recent evidence that both ROS and antioxidants are powerful signalling molecules (Neill *et al.*, 2002; Foyer *et al.*, 2004), including both the cell cycle and PCD (Lam *et al.*, 2001). Photosynthesis, photorespiration and respiration generate superoxide anions and H_2O_2 at very high flux rates (Foyer & Noctor, 2000; Noctor *et al.*, 2002; Sweetlove & Foyer, 2004). ROS are produced and act as second messengers in plant hormone, e.g. ABA (Pei *et al.*, 2000) and ethylene (Moeder *et al.*, 2002) signalling, innate immune responses (e.g. the hypersensitive response) and in acquired resistance (Neill *et al.*, 2002). Recently it has been demonstrated that in mammalian cells low levels of ROS are synchronously generated with the cell cycle, indicating that

ROS are closely connected with the cell cycle (Takahashi *et al.*, 2004). Moreover, small perturbations in ROS levels prevented normal progression of the cell cycle, indicating that distinct signalling between ROS levels and the cell cycle exists and is biologically significant. Hence, ROS and antioxidants are crucial to plant cell-cycle activities and to the operation of any functional meristem such as that operating in the nodule. In addition to these responses, H_2O_2 also acts as a second messenger in a wide range of hormone-dependent developmental processes. Thus, ROS production is part of the signalling cascades underpinning the development of embryonic axes, lateral root formation, germinating seeds, expanding leaves and growing coleoptiles (Joo *et al.*, 2001; Foreman *et al.*, 2003; Kovalchuk *et al.*, 2003). In particular, ROS, generated by a plasma membrane NADPH oxidase, regulate root hair growth through activation of calcium channels (Foreman *et al.*, 2003). Because initial bacterial penetration can occur via the root hairs, it would be very interesting to know how H_2O_2 functions are regulated in these conditions to allow recognition and resultant root hair growth.

In addition to the roles of ROS in the progression of the cell cycle and the activation of calcium channels, they are also important in growth and movement responses triggered by hormones such as auxin and ABA, as well as in pathogen resistance and cell death responses (Neill *et al.*, 2002). This dual action in growth and defence is possible because plants have a robust tolerance to ROS. The endogenous H_2O_2 contents of plant cells are far higher than those found in animals and bacteria. Indeed, plant cells happily support H_2O_2 levels that would kill animal cells. This tolerance is linked to the presence of an extensive antioxidant system, in which ascorbic acid and glutathione fulfil crucial roles (Noctor & Foyer, 1998). When there is an imbalance between ROS formation and their scavenging by the antioxidant defence, ROS signalling leads to orchestration of defence strategies but cells are faced with the possibility of oxidative stress, where the chances of oxidative damage are greatly increased. While the expression of relatively few genes involved in secondary metabolism is up-regulated in nodules, mRNAs encoding proteins involved in ROS and lipid peroxide scavenging such as glutathione peroxidase are much higher in nodules compared with the parent roots (Colebatch *et al.*, 2002, 2004). It is interesting to note that in other tissues such as barley aleurone cells, where developmental regulation of PCD is observed, the plant hormones ABA and gibberellic acid appear to control antioxidant gene expression and sensitivity to H_2O_2 (Fath *et al.*, 2001). Application of ABA greatly decreases sensitivity to H_2O_2-mediated PCD while application of gibberellic acid represses antioxidant gene expression, causing the cells to become highly susceptible to oxidative damage (Fath *et al.*, 2001). It would be interesting to determine the effects of hormones such as ABA and gibberellic acid on H_2O_2 sensitivity and stress-induced PCD in nodules.

The plant presents a very hostile environment for invading microbes, even potentially beneficial partners. The plasma membrane is not a passive interface with the external world, but contains many receptors that identify potential threats and elicit appropriate action. Perception of physical or chemical changes in the environment provokes a transient short oxidative burst that can be followed by a prolonged burst. Plasma membrane receptors such as MLO, which is part of the innate immune response network in plants, control this response. The MLO protein dampens the cell-wall-restricted H_2O_2 burst at points of attempted fungal penetration of the epidermal cell wall, and in subtending mesophyll cells. It suppresses the second prolonged oxidative burst and PCD (Piffanelli *et al.*, 2002). The prolonged oxidative burst occurs around forming papilla and in the mesophyll cells surrounding attacked cells undergoing cell death (Hückelhoven *et al.*, 1999; Vanacker *et al.*, 2000; Piffanelli *et al.*, 2002). It also appears to be important in the orchestration of systemic acquired resistance (SAR), a process that involves salicylic acid (SA) and the concerted expression of pathogenesis related (PR) genes via thiol–disulphide interactions (Mou *et al.*, 2003). Homologues of receptor-like kinases and other proteins involved in plant microbe interactions are induced in nodules compared to the parent *L. japonicus* roots (Colebatch *et al.*, 2004). In particular, two homologues of plant MLO proteins were higher in nodules than roots (Colebatch *et al.*, 2004). It is suggested that MLO induction helps to prevent the triggering of plant defence responses against the rhizobia and thus averts the potential threat of microbe-induced HR and cell death (Colebatch *et al.*, 2004). Exposure to Nod factors is important in suppression of the ROS-generating system that sustains the prolonged oxidative burst associated with plant defence responses (Shaw & Long, 2003). It is logical to suggest that this suppression of the innate plant immune system within the nodule is reversed as the nodules senesce.

2. Roles for mitochondria and PCD in senescence processes

It is now clear that longevity is controlled genetically. In animal species from *Caenorhabditis elegans* to man, calorie restriction in the diet prolongs life (Weindruch, 1996), but it is uncertain whether this relationship can be extrapolated to plants. According to the free-radical theory of aging, ROS produced by respiration contribute to the aging of all organisms (Harman, 1956). There is an inverse relationship between the rate of mitochondrial ROS production and the maximum lifespan of mammalian species. It is not surprising therefore that ROS accumulation has also been implicated in plant senescence. For example, ROS generation by microsomes (Thompson *et al.*, 1987) and peroxisomes (Pastori & del Rio, 1997; Palma *et al.*, 2002) has been implicated in leaf senescence. However, although there is a

clear distinction between PCD and aging in animals, cell aging decreasing the susceptibility of cells to PCD, the two processes can be integrated in plant development. ROS accumulation in plants clearly leads to PCD as it does in animals. The inability of transgenic tobacco cells to induce the alternative oxidase, an enzyme considered to minimise superoxide production in the mitochondrial electron transport chain leads to PCD (Vanlerberghe *et al.*, 2002). Moreover, oxidative stress induces PCD in *Arabidopsis* cell cultures; in this case, the mitochondria produce increased amounts of H_2O_2 and release cytochrome c (Tiwari *et al.*, 2002). Such observations of mitochondrial dysfunction following exposure to stress have led to the suggestion that these organelles act as stress targets and/or sensors (Lam *et al.*, 2001). More recently, it has become clear that mitochondrial electron transport and energy production have important roles in determining basal resistance levels in plants. For example, the cytoplasmic male sterile II (CMSII) mutant of *Nicotiana sylvestris* has a lesion in the mitochondrial *NAD7* gene and this results in the loss of complex I function. Thus, the mutant lacks a major cellular NADH sink (Dutilleul *et al.*, 2003). Nevertheless, CMSII plants have high rates of rotenone-insensitive respiratory electron transport, and by virtue of many modifications in gene expression that are induced as a result of altered cellular redox poise, they show greatly increased tolerance to abiotic (ozone) and biotic (tobacco mosaic virus) stresses (Dutilleul *et al.*, 2003). Mitochondrial electron transport rates also control poly (ADP ribose) polymerase (PARP). Recent results have shown that down-regulation of the PARP provides substantial protection against a range of abiotic stresses in both monocot and broad leaf crops (De Block *et al.*, 2004), allowing plants to continue growing under conditions where controls not only stop growing but exhibit extreme stress responses including necrosis. PARP is closely linked with cell-cycle control in mammals. This raises the possibility that PARP may be a key player linking stress responses to growth and cell division at the cellular level. Although little is known about nodule mitochondria, it is clear from structural studies, such as that shown in Fig. 3, that mitochondrial form changes as the nodule ages, current evidence suggesting that mitochondria may fuse and undergo functional changes as part of the senescence process. Because plant mitochondria are an important source of regulatory redox signals in plant cells, it will be intriguing to discover how mitochondrial redox signals are modulated in the symbiotic partnership and whether PARP has a role in nodule sustainability.

Leaf senescence is characterised by extensive loss of protein and chlorophyll, decreased expression of genes related to photosynthesis and protein synthesis, and increased expression of senescence-associated genes (SAGs; Nam, 1997). No SAGs have been described to date in relation to nodule senescence, but nodule senescence is demonstrably a genetic process. Plant senescence programmes sometimes involve PCD, which is an essential cell suicide programme that eliminates targeted cells during development and disease to maintain physiology and homeostasis. PCD requires new gene expression and protein synthesis. H_2O_2 accumulation, enhanced expression of a CP gene and internucleosomal DNA fragmentation have been observed at the periphery of the central infected zone of 5-wk-old nodules (Alesandrini *et al.*, 2003b). This zone is considered to be an area where development is occurring in determinate nodules (Jørgensen *et al.*, 1999). Interestingly, the peripheral cells show enhanced ascorbate peroxidase, dehydroascorbate reductase and monodehydroascorbate reductase activities as well as higher ascorbate than the infected zone (Dalton *et al.*, 1998). Internucleosomal DNA fragmentation was observed close to the vascular bundles in 5-wk-old nodules, suggesting perhaps that this process is controlled by a signal originating from the plant (Alesandrini *et al.*, 2003b).

In animal and plant systems, mitochondria play a crucial role in the orchestration of PCD (Sweetlove & Foyer, 2004). Because of the high energy demand of the nitrogenase, mitochondria are abundant in the cortex and infected region of nodules. Mitochondria are the site of ascorbate synthesis in plants (Millar *et al.*, 2003) and they contain all the enzymes of the ascorbate–glutathione cycle that are important in ROS detoxification (Chew *et al.*, 2003). While there is no evidence to date that the legume nodule can synthesise ascorbate, the enzymes of the ascorbate-glutathione cycle are very high in nodule mitochondria (Iturbe-Ormaetxe *et al.*, 2001). Because these organelles are very sensitive to oxidative stress, any decline in the mitochondrial antioxidant capacity might act as a trigger for PCD. However, only one report to date has shown any evidence that PCD occurs during nodule senescence (Alesandrini *et al.*, 2003b). In older determinate nodules (7–8 wk), the zone with high CP expression and PCD and H_2O_2 accumulation progressively enlarges toward the centre of the organ (Fig. 1a). It is interesting to note that these results correspond well with the pattern of senescence in soybean leaves (Solomon *et al.*, 1999), where oxidative stress activates a specific subset of CPs, some of them instrumental in the PCD of soybean cells.

3. ROS accumulation in symbiosis and senescence

The nodule is a special organ housing interactive bacteria where ROS play a key part in establishing and maintaining the symbiosis between the plant and the rhizobia (Santos *et al.*, 2000; D'Haeze *et al.*, 2003; Shaw & Long, 2003). As previously mentioned, ROS are important in the cell-cycle turnover and in functional meristems. Thus it is not surprising that ROS are readily detectable in the meristematic and N-fixing zones of nodules, as illustrated for pea nodules in Fig. 4. Detectable levels of superoxide (Fig. 4a,b) and H_2O_2 (Fig. 4c,d) decline as the symbiosis ends and the nodule ages (H. Vanacker *et al.*, unpublished). In contrast, H_2O_2 and lipid hydroperoxides increase during senescence of soybean nodules (Evans *et al.*, 1999). Similarly, there is

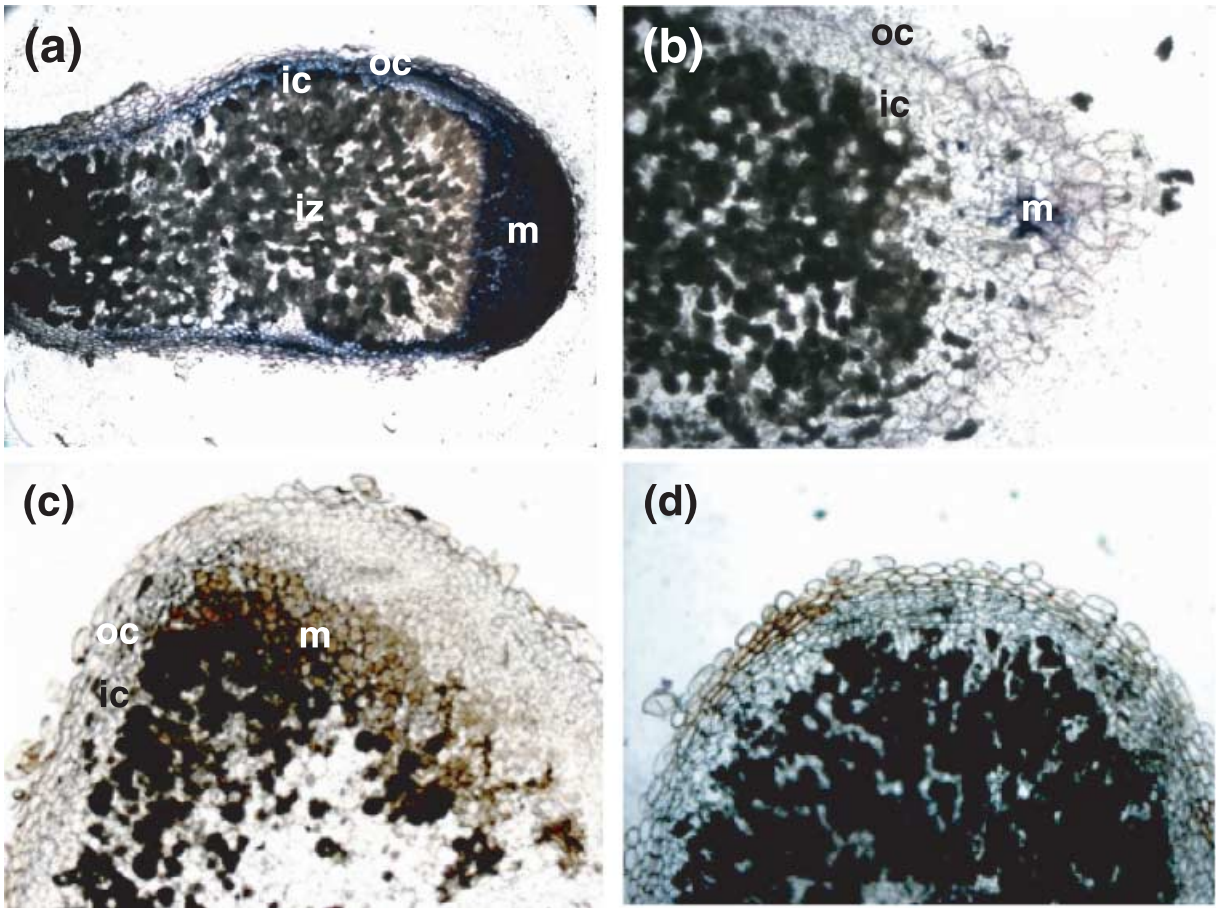

Fig. 4 *In situ* localization of H_2O_2 and superoxide anions in pea root nodules. during development. Sections (50–70 μM) of attached pea root nodules that had been vacuum-infiltrated with either nitroblue tetrazolium (NBT) for the detection of superoxide anions or diaminobenzidine (DAB) for the detection of H_2O_2 were observed under the light microscope. (a) Young (4-wk-old) and (b) senescent (11-wk-old) pea root nodules showing regions of blue staining where superoxide accumulates in the presence of NBT. (c) Young (5-wk-old) and (b) senescent (11-wk-old) pea root nodules showing brown staining in regions where H_2O_2 accumulates in the presence of DAB. ic, Inner cortex; oc, outer cortex; iz, infection zone; m, meristem.

evidence of senescence- and ROS-linked enhanced lipid peroxidation and degeneration of membrane integrity in leaf (del Rio *et al.*, 1998) and nodule senescence (Becana & Klucas, 1992; Mathieu *et al.*, 1998). During the senescence of both organs, there is a period of fatty acid degradation which generates ROS at relatively high rates. Moreover, the application of exogenous H_2O_2 (Desikan *et al.*, 2000) or enhanced metabolic H_2O_2 accumulation caused by catalase depletion (Vandenabeele *et al.*, 2003) induces the expression of a large number of genes linked to PCD and senescence. An increase in tissue H_2O_2 content in parallel with a decrease in antioxidant enzyme activity occurs during flower senescence (Panavas & Rubinstein, 1998). Different models have been proposed to define the threshold after which senescence becomes an inevitable consequence of exposure to stress. For example, the 'decay' model suggests that there is a 'point of no return', when a breakdown in the endogenous defence processes that prevent uncontrolled oxidation leads to senescence (Leshem, 1988; Swaraj & Bishnoi, 1996). It is now time perhaps to re-evaluate such models in the light of new information and concepts concerning the processes of aging, senescence and PCD and their interrelationships.

There are a number of processes that contribute to the high ROS levels found in functional nodules. Firstly, ROS formation by the plasmamembrane-bound enzyme systems is important in the establishment of the plant–rhizobia symbiosis (Santos *et al.*, 2000; D'Haeze *et al.*, 2003). Secondly, the highly reducing conditions required for N-fixation in nodules inevitably lead to ROS formation as many electron transfer components including ferredoxin, uricase and hydrogenase are susceptible to auto-oxidation resulting in superoxide formation (Dalton *et al.*, 1991). Oxyleghemoglobin can also produce superoxide radicals, which disproportionate to H_2O_2 (Puppo *et al.*, 1991). The reaction of leghemoglobin with H_2O_2 can then generate very oxidizing species such as ferryl haem proteins and protein radicals (Davies & Puppo, 1992; Moreau *et al.*, 1996). Peroxides, protein carbonyls and modified DNA base concentrations increase with age in certain nodules (Evans

et al., 1999) and also in stress-induced nodule senescence (Escuredo *et al.*, 1996; Gogorcena *et al.*, 1997). Free iron is a powerful catalyst in hydroxyl radical production and the level of free iron may also increase significantly during the ageing process (Becana & Klucas, 1992; Mathieu *et al.*, 1998; Hernandez-Jimenez *et al.*, 2002). Enhanced membrane lipid peroxidation has been reported during the natural senescence process in lupin nodules (Hernandez-Jimenez *et al.*, 2002). These results indicate a shift in the redox homeostasis of the nodule from initiation through development to senescence. ROS generation is important at the outset of nodule development, declining with nodule age. Some antioxidant enzymes such as iron-superoxide dismutase increase in senescent nodules at a time of active leghemoglobin degradation (Moran *et al.*, 2003), but others remain relatively constant through nodule development (H. Vanacker *et al.*, unpublished).

4. The nodule redox buffers: ascorbate and glutathione

Ascorbate and glutathione are the major redox buffers of the plant cell (Noctor & Foyer, 1998). The relationship between ROS and these antioxidants in legume nodules with regard to protection against oxidative damage has been discussed previously by Becana *et al.* (2000) and Matamoros *et al.* (2003) and therefore will not be covered in detail here. However, it is important to consider other functions of ascorbate and glutathione in plants in addition to their roles as antioxidants. Nodule ascorbate and glutathione levels begin to decrease early in development, declining in a similar manner to nitrogenase activity (Fig. 2), but the activities of most enzymes of the ascorbate-glutathione cycle such as ascorbate peroxidase activity are not significantly altered during nodule senescence (Dalton *et al.*, 1986).

While it is tempting to categorise ascorbate primarily as a low molecular weight antioxidant, considerable evidence now points to the central importance of this metabolite in plant biology, with roles in hormone synthesis, gene expression, cell division and growth. For example, ascorbate is involved in dioxygenase reactions, such as those required for the synthesis of ABA, gibberellic acid and ethylene (Arrigoni & De Tullio, 2002). Ascorbate is required both for the activity of 9-cis-epoxycarotenoid dioxygenase (NCED), an enzyme catalysing the formation of xanthoxin, the precursor of ABA, and for the expression of the *NCED* gene, such that *NCED* transcripts are increased when ascorbate is low and decreased when ascorbate is high (Pastori *et al.*, 2003). Ascorbate is also involved in the regulation of quiescence, mitosis and cell growth (Potters *et al.*, 2000, 2004). Such effects might have important implications for nodule function, particularly if the decline in nodule ascorbate that is observed rather early in the nodule stimulates localised ABA synthesis. It is important to note that when soybean roots were supplied with exogenous ascorbate, striking increases in nodule nitrogenase activity were observed (Bashor & Dalton, 1999). Moreover, while Bashor and Dalton (1999) focused their studies regarding the effects of enhanced ascorbate on protection against oxidative damage showing that lipid peroxides were decreased, these authors noted that ascorbate feeding increased the average number of nodules per plant. Earlier studies had also reported that exogenous ascorbate led to increased nodule numbers as well as earlier nodule initiation and delayed senescence, with resultant large increases in plant yields (Swaraj & Garg, 1970). Furthermore, the inclusion of ascorbate and ascorbate peroxidase in a model system containing *Bradyrhizobium japonicum* bacteroids and leghemoglobin resulted in large increases in nitrogenase activity and enhanced oxygenation of haem proteins (Ross *et al.*, 1999). Perhaps it is also interesting to note that ascorbate regulates apoptosis in human cells (Vissers *et al.*, 2001). In this case, ascorbate-mediated (but not thiol-mediated) protection of an oxidant-sensitive step in the initiation phase allowed apoptosis to proceed under sustained oxidation.

The redox coupling between the ascorbate and glutathione pools linked to ascorbate peroxidation is a necessary part of a H_2O_2 detoxification pathway. The relative redox potentials of the ascorbate and glutathione couples strongly favour net electron flow from glutathione to dehydroascorbate (DHA, Foyer & Noctor, 2000) and this reaction can occur at significant rates even in the absence of dehydroascorbate reductase (DHAR), particularly at alkaline pH values (Winkler, 1992; Polle, 2001). However, whereas glutathione will always reduce DHA, the degree of coupling between the ascorbate and glutathione redox couples varies greatly between different cellular compartments. The flexibility of coupling between these antioxidant pools is crucial to differential signalling by ascorbate and glutathione (Noctor *et al.*, 2002; Foyer *et al.*, 2004). However, it is important to note that recent evidence has been provided for the presence of a glutathione-independent pathway of ascorbate regeneration from DHA in tobacco BY-2 cell cultures (Potters *et al.*, 2004), and similar pathways may occur in other cell types. Like ascorbate, glutathione is a multifunctional compound with functions that extend beyond the antioxidative system (May *et al.*, 1998; Noctor & Foyer, 1998). Decreases in total glutathione have also been linked to nodule senescence (Dalton *et al.*, 1993; Evans *et al.*, 1999). Thus, the observed changes in ascorbate and glutathione content during nodule development may not only be important in limiting oxidative stress but they may also be important in controlling signal transduction in nodules, as they are in leaves (Kiddle *et al.*, 2003; Pastori *et al.*, 2003).

IV. Antioxidants and programmed cell death during the senescence process

The evidence discussed here strongly implicates ascorbate and glutathione in nodule senescence. Both hypotheses are based on the following observations. (1) The plant supplies the nodule with essential metabolites such as ascorbate. Cysteine, glycine and other amino acids are required for the synthesis of

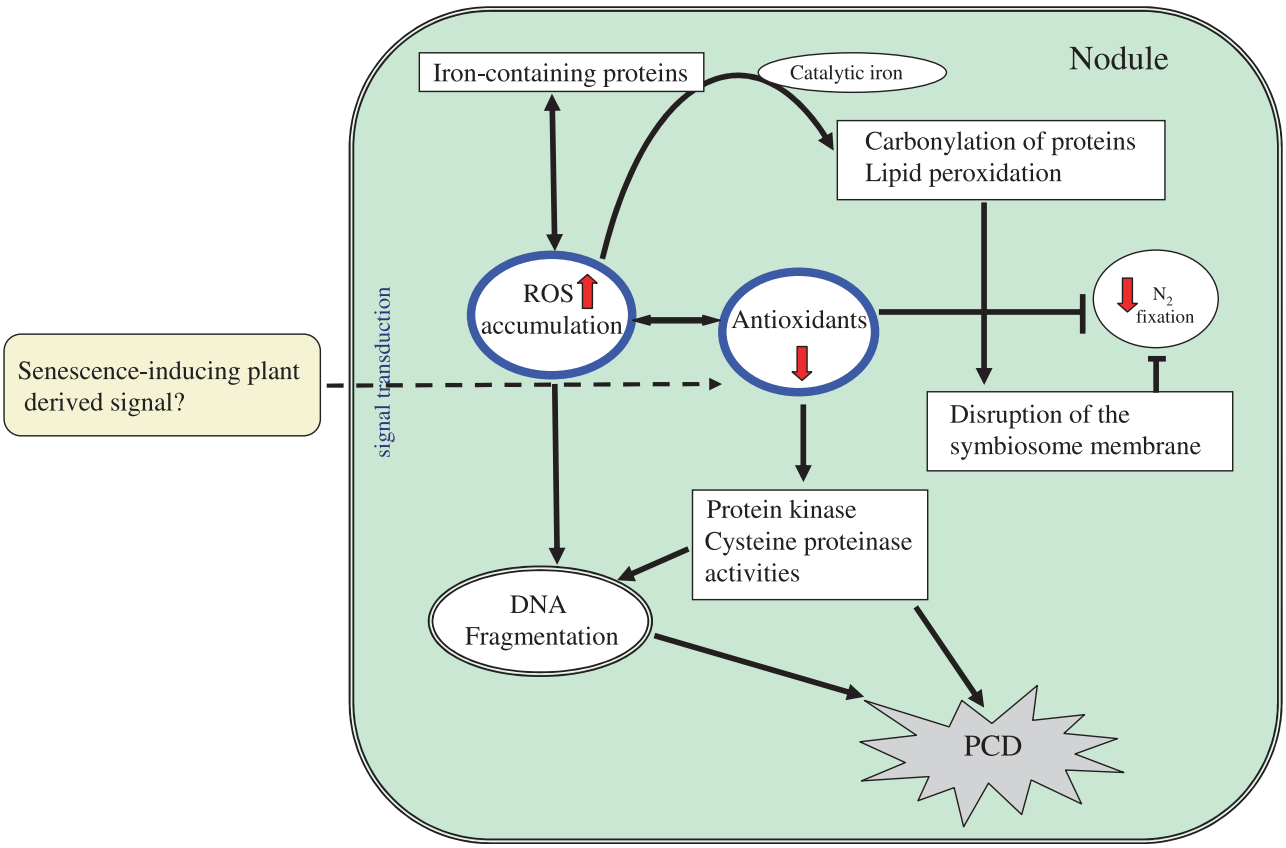

Fig. 5 Possible roles of oxidative processes in stress-induced nodule senescence. A signal from the plant orchestrates processes involving a decline in soluble low molecular weight antioxidants. This leads to an increase in reactive oxygen species and consequently oxidative stress, cysteine protease activation and programmed cell death. The direction of the broad arrow indicates increase and decline, respectively. ROS, reactive oxygen species; PCD, programmed cell death.

glutathione and glutathione homologues in the nodules. There are no data on the production of these amino acids by the bacteria but they are certainly synthesised in the leaves. (2) The capacity of leaves to produce ascorbate declines with leaf age (Foyer, 2004). (3) Nodule ascorbate and glutathione contents decline in parallel with N-fixation as nodules age (H. Vanacker *et al.*, unpublished). (4) Exogenous ascorbate supply stimulates nodulation and enhances N-fixation (Swaraj & Garg, 1970; Bashor & Dalton, 1999). (5) Ascorbate and glutathione are required for the operation of the cell cycle (Potters *et al.*, 2004). Based on the evidence of these five observations, it is possible to suggest two hypotheses regarding the roles of ROS, ascorbate and glutathione in legume nodule senescence. In the simplest scenario, as illustrated in Fig. 5, the progressive loss of ascorbate and glutathione could lead to a progressive increase in oxidative stress, triggering nodule senescence. Studies on ROS accumulation and oxidative damage suggest that this series of events could also be triggered in nodulated plants exposed to abiotic stress. With the initiation of senescence peroxisomes are replaced by glyoxysomes and catalase plays an essential function in the removal of H_2O_2 generated by β-oxidation of fatty acids. In the second hypothesis, illustrated in Fig. 6, a progressive decline in nodule ascorbate and in nodule glutathione during the aging process does not lead to a progressive increase in oxidative stress because it is accompanied by a similar decrease in ROS, and the redox balance of the nodule is maintained. In this hypothesis, a high level of ROS and antioxidant signalling is commensurate with meristem activity and symbiosis, being part of the repertoire of communication between the bacteria and host cells. We suggest that the decline in tissue ascorbate is sensed, and this together with high N-metabolite availability relative to C-metabolite levels induces signalling that leads to increased ABA synthesis in the plant, which is transported to the nodule. The decline in nodule ROS and ascorbate with age slows mitosis and meristematic activity in the nodule contributing to the aging process. The increase in nodule ABA activates key proteinases and the 26S proteasome that are intrinsic to the senescence process. The levels of ABA in pea and soybean nodules are higher than those of the parent roots (Charbonneau & Newcomb, 1985). Nodule ABA may have a role in the induction of enzymes that increase the sink capacity and thus this hormone may support the early development of

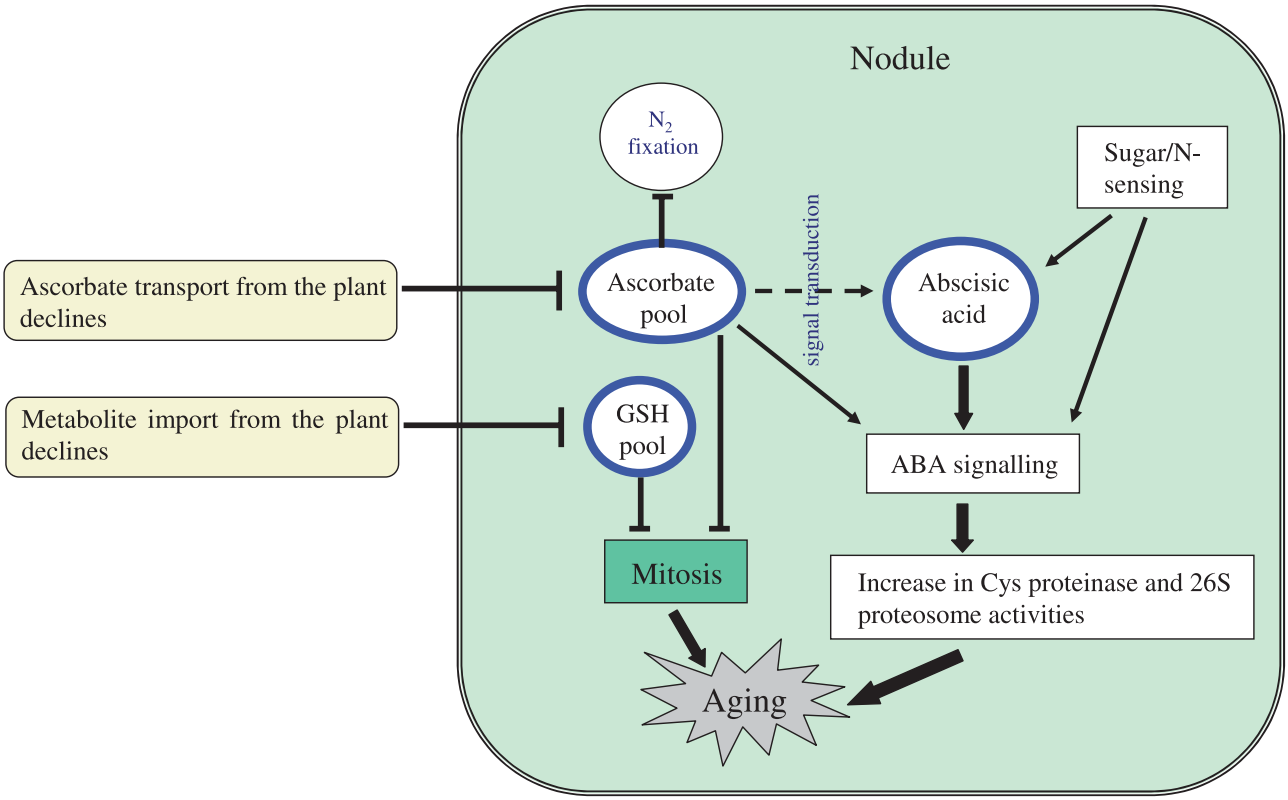

Fig. 6 A model for ascorbate–hormone interactions in the orchestration of nodule senescence. This hypothesis is based on the evidence that the plant supplies the nodule with acorbate and nodule ascorbate contents decline in parallel with N-fixation as nodules age as it does in leaves (Foyer, 2004; H. Vanacker *et al*., unpublished). We suggest that, the decline in tissue ascorbate is sensed by the plant and this, together with a high N : C ratio, induces signalling that leads to increased abscisic acid (ABA) synthesis in the plant, which is transported to the nodule. The decline in nodule ROS and ascorbate with age will also slow mitosis contributing to the aging process in indererminate nodules where there is a persistent meristem but not in determinate nodules where meristematic activity ceases early in development. The increase in nodule ABA activates key proteinases and the 26S proteasome that are intrinsic to the senescence process. GSH, glutathione.

individual nodules (Ferguson & Mathesius, 2003). However, increasing rates of ABA synthesis during senescence may cause nodule death. Pea nodule ABA levels are high during the first 2 wk, then reach a plateau, increasing again in the later stages of development (Charbonneau & Newcomb, 1985).

In either scenario (Fig. 5 vs Fig. 6), events might be triggered by a loss of plasmodesmata-mediated communication because the establishment of a symplasmic field is associated with increased plasmodesmata density in *Medicago* nodules (Complainville *et al*., 2003). It is possible, for example, that nodules cannot synthesise ascorbate; the last enzyme of the ascorbate biosynthetic pathway, galactono-lactone dehydrogenase, could not be detected in pea nodules (H. Vanacker *et al*., unpublished). Thus ascorbate has to be imported from the parent plant through the vascular system, which is established early in nodule development to facilitate export of N-containing compounds from the nodule and delivery of sucrose and other metabolites to the nodule tissues. Long-distance ascorbate transport through the phloem to sink tissues has been described in *Medicago* (Franceschi & Tarlyn, 2002). Thus, import of ascorbate from the parent plant might be a key feature of the plant–symbiosome relationship. While GDP-mannose pyrophosphorylase transcripts were much higher in *L. japonicus* nodules than in the parent roots (Colebatch *et al*., 2002), this enzyme occurs early in the pathway before ascorbate synthesis branches from cell wall metabolism. Thus the high level of GDP-mannose pyrophosphorylase mRNA found in nodules may reflect the structural requirements of nodule formation rather than ascorbate synthesis.

Nodule senescence might be viewed as a delayed response of the plant to the presence of a foreign organism, the rhizobia. It is possible that there are slow recognition signals that identify the bacterium as a potential pathogen. Alternatively, this recognition may be an early event, but the natural pathogen defence responses of the plant are suppressed during the productive stage as other signals that identify a potential symbiotic partner override the defence response (Mellor, 1989). The potential for a symbiosis–pathogenesis transition exists with regard to rhizobia. The bacteria could be controlled by the plant in a number of ways. For example, the rhizobia–legume symbiosis could represent a controlled incompatible reaction (Rolfe & Gresshoff, 1988) because the two situations

are variations on a common theme (Baron & Zambrysky, 1995). A recently isolated molecular marker of soybean nodule senescence exhibits significant homology with an EST from *M. truncatula* corresponding to a gene expressed upon elicitor induction in cell cultures (Alesandrini *et al.*, 2003a). This would again suggest that nodule senescence is a delayed response on the part of the plant to rhizobium as a potential pathogen. However, caution must be exercised in such considerations as this gene also exhibits high homology with multiple ESTs from different cDNA libraries being part of a gene family that encodes proteins involved in general remobilisation events (Alesandrini *et al.*, 2003a). Indeed, CPs can also be considered to be essential enzymes in re-mobilisation processes in plants that are induced during natural or stress-induced senescence as well as in the utilisation of reserves during the germination (Drake *et al.*, 1996).

V. Perspectives for manipulating nodule senescence

The breakdown of the rhizobia–legume symbiosis is not only of intrinsic scientific interest but it is also of environmental and agronomic importance. The lifespan of an effective, N-fixing symbiosis between legumes and rhizobia is affected by main three factors: (1) the genotype of the host plant; (2) the genotype of the rhizobia microsymbiont; and (3) the growth environment (Vessey, 1992). The initiation and lifespan of the nodule is also regulated by environmental cues such as nitrate availability and stress. High temperatures, acid soil conditions, root pathogen infection and drought lead to an early nodule senescence and rapidly destroy the symbiosis.

The N-content of the host plant plays a part in regulating nodulation. If the soil is well-fertilised and plants are N-replete they will not become nodulated. Similarly, if ammonium or nitrate is applied to nodulated plants the rhizobia will not fix N. It has been assumed therefore that an important signal terminating nodule activity is generated when the plant achieves the required developmental state and N-saturation for pod filling. At this stage no further nodules are initiated and active nodules cease fixation and senesce. However, the plant can still utilise further N, since late N application during pod filling in the field of white lupins leads to greater seed yields with higher seed protein contents (Merbach & Schilling, 1980). Establishing the precise sequence of events is crucial to improving nodule sustainability. In particular, it is not clear whether N-fixation ceases because the plant development cycle is nearing completion or because an optimal or critical plant N-content is reached. N-sensing mechanisms control the development of the plant root system and also that of the shoot, and presumably this is true for seed production also. In the field, the rapid decline in the N-fixation capacity occurs at the onset of pod filling in soybean, white lupin, pea and common bean (Lawn & Brun, 1974; Bethlenfalvay & Phillips, 1977; Merbach, 1984). The mechanistic basis for this relationship remains unexplored. It has been suggested that competition for resources between pods and nodules leads to senescence of the latter, but so far no direct interdependence between C use efficiency and N-fixation capacity has been demonstrated (Schulze *et al.*, 2000). Moreover, the effect of symbiotic plasmids on overall C costs is relatively small, different rhizobial strain/host combinations allow flexibility in nodulation (for example, fewer but larger nodules or faster N-fixation rates), but the end result in terms of total plant N-content is remarkably similar (Skot *et al.*, 1986).

The mechanism whereby plants sense levels of available nutrients and adjust their development accordingly involves complex regulatory circuits that control gene expression and nutrient dependent cell metabolism. Genes involved in C and N pathways are cross-regulated by both C and N metabolites including sugars, nitrate and amino acids (Coruzzi & Bush, 2001; Forde, 2002). Thus genes associated with N-assimilation are up-regulated when C is abundant and down-regulated when C skeletons are limiting or when organic N is abundant (Coruzzi & Zhou, 2001). There is also considerable crosstalk between the sugar and N-signalling pathways and those of ethylene and ABA, two hormones that are strongly implicated in the nodule senescence process. Genes involved in ABA synthesis and signalling and also in ethylene perception are critical for sugar and nitrate signalling (Arenas-Huertero *et al.*, 2000; Signora *et al.*, 2001). Moreover, there is strong genetic evidence in support of an integration of sugar and ethylene signalling pathways (Leon & Sheen, 2003). For example, the etr1 and ein2 Arabidopsis mutants show slower growth and development in the presence of sugar (Price *et al.*, 2003) and sugars reduce the stability of the EIN3 and EIL1 proteins that are critical components of ethylene signalling (Yanagisawa *et al.*, 2003). At the later stages of nodule development, low ascorbate availability coupled with high N-metabolite availability would favour stimulation of ABA synthesis and signalling. Moreover, a low sugar supply from the plant might favour an increase in ethylene. Because ethylene decreases the sensitivity of seedlings to ABA (Beaudoin *et al.*, 2000; Gazzarrini & McCourt, 2001), we suggest that the two hormones might work together in nodules to orchestrate senescence, ethylene triggering remobilisation processes and ABA ensuring that the defences of the nodule are strong to avoid disease and attack by nematodes, etc. at this vulnerable stage of plant development.

A key aim of legume (pea, soybean) improvement programmes is delayed nodule senescence together with amelioration of sustainability under field conditions. It is possible to envisage a number of mechanisms where intervention either on the side of the plant or on the side of the rhizobial partner may yield useful results. These are as follows.

1. Manipulation of root : shoot signal transduction

The control of root formation in response to soil nitrate is orchestrated through signals originating in the shoot (Stitt

et al., 2002). Therefore, it has been tempting to suggest that changes in nodule N-fixation are similarly perceived by the shoot and similar signals are then deployed to modify nodule numbers and development (Schubert, 1995). Additionally, the timing of nodule senescence involves a dialogue between shoot and root signals. If signals originating in the plant determine the lifetime of the nodule then it should be possible to modify the signal transduction process and to manipulate the source–sink relationship. However, the nature of senescence-inducing signals and receptors remain to be elucidated.

Intercultivar differences in pod filling and storage of fixed N are observed with the same rhizobial strain. If signals from the nodule determine leaf senescence, then stay-green phenotypes with delayed leaf senescence might have better sustainability and hence improved yield characteristics (Schulze *et al.*, 1998). Alternatively, sustaining leghemoglobin, phosphoenolpyruruvate carboxylase activity and ureide degrading capacity for longer would have a beneficial effect (Nath *et al.*, 2002).

2. Inhibition of nodule senescence and amelioration of stress tolerance

Oxidative stress, antioxidant decline and activation of CPs are intrinsic features of the senescence process. Hence the expression of CP inhibitors (cystatins) or antioxidant genes under the control of a nodule-specific promoter might delay nodule senescence. The occurrence and putative role of cystatins in legumes nodules has only been described for early stages in the nodulation process of *Sesbiana rostrata* (Lievens *et al.* 2004), but oryzacystatin I expression in transformed tobacco showed enhanced chilling tolerance (Van der Vyver *et al.*, 2003). Similarly, manipulation of nodule hormone contents and production is an attractive target because the ratio of ABA to cytokinins is crucial to nodule suppression phenomena. In particular, the role of ABA as a negative and positive regulator of nodule numbers and sustainability, respectively, requires further examination. In addition, the postulated inverse relationship between ascorbate and ABA indicates that manipulation of nodule ascorbate contents might be a valuable tool in regulating nodule ABA levels as well as limiting oxidative stress. This may also lead to increased nodule sustainability in stress situations.

The senescence process is often initiated by stress conditions such as extremes of temperature, drought and pathogen attack; exposure to heavy metals or acids causes rejection of the microsymbiont. Hence a general increase in plant stress tolerance of the plant would favour better nodule sustainability. In this regard it is interesting that arbuscular mycorrhizal symbiosis can alleviate drought-induced nodule senescence in soybean plants (Ruiz-Lozano *et al.*, 2001).

It might be argued that a prolonged life of the nodules could also lead to a decline in pod or seed yield because of the high-energy demand of nitrogenase. However, studies with male-sterile soybean plants that set only 15% of the pods found on fertile controls, showed similar rates of decline in N-fixation during pod filling (Imsande & Ralston, 1982; Riggle *et al.*, 1982). Such observations indicate that the supply of carbohydrate does not make a major contribution to the controls that determine the lifetime of the nodule. All experiments to date suggest that a prolonged N supply to the plant would have a positive effect on yield.

3. Exploitation of key genes controlling symbiosis.

A global analysis of gene and protein expression in *Sinorhizobium meliloti* as well as cDNA arrays derived from N-fixing nodules of *M. truncatula* and *L. japonicus* indicated important genes participating in the symbiosis (Colebatch *et al.*, 2002, 2004; Fedorova *et al.*, 2002; Ampe *et al.*, 2003; Djordjevic *et al.*, 2003). These are putative targets for future manipulation to modify nodule sustainability. The identification of transcription factors involved in N and C metabolism might also be important tools for yield improvement.

4. Increased competitiveness and improvement of rhizobia sustainability

The development of more efficient N-fixing rhizobial strains with a high competitiveness compared with indigenous rhizobial populations might lead to higher N-fixation rates or improved resistance to oxygen, and hence leads to better yields. When the same pea cultivar was infected with three different rhizobial genotypes, significant differences in the capacity for N-fixation were measured (Santalla *et al.*, 2001). However, as noted above, the end result in terms of plant N gain may be similar. It is possible to obtain rhizobial strains with enhanced stress resistance (Zahran, 2001), for example by specific DNA amplification (Castillo *et al.*, 1999).

VI. Conclusions and Perspectives

It has long been known that abiotic stress accounts for the loss of 30–70% of global agricultural yield. The development of crop varieties displaying resistance to environmental stresses such as heat, cold and drought is thus essential to meet the combined challenges of a growing world population, an increasingly unpredictable global climate and competition for use of agricultural land. The above evidence suggests that the symbiotic balance existing in legume nodules is rather fragile as they are susceptible to early senescence, particularly when the plant is faced with stress. Stress resistance is indeed a major trait target of commercial and public sector breeding programmes, but relatively little of this effort is directed at delayed nodule senescence. Moreover, progress is significantly hampered by the lack of knowledge of the biochemical and molecular processes linking the perception of stress within the plant to the causative effects of senescence within the

symbiotic organs. Exposure to stress results in changes in levels of antioxidants, particularly in glutathione and ascorbate. These antioxidants affect nodule numbers and stability, and they decline with nodule age. In other plant organs, altered levels of these compounds and the ratio of their reduced to oxidised forms act as a signal to trigger specific cellular responses (Noctor & Foyer, 1998; Pastori *et al.*, 2003). One of these effects is specific blocking of the cell cycle causing the cessation of cell division. Clear evidence now links levels of reduced glutathione and ascorbate to specific cell-cycle effects on the G1 phase of the cell cycle (Potters *et al.*, 2004). Because overall plant growth is intimately related with meristem cell division rates (Cockcroft *et al.*, 2000), and a major consequence of stress is the cessation of plant growth, we may postulate that these processes are linked and that cell-cycle arrest in the nodule meristem in response to abiotic stress contributes to growth arrest. Recent evidence shows that nodules will not form on roots if glutathione synthesis is blocked by addition of l-buthionine-SR-sulfoximine, an inhibitor of glutathione synthesis, suggesting that like the root meristem, the nodule meristem is unable to develop in the absence of glutathione (Frendo *et al.*, 2004).

Nodule senescence can be triggered by a signal or signals from the shoot that appear to arise early if the plant is stressed. There is increasing evidence to suggest that at least in the case of the rhizobia–legume symbiosis, N_2 fixation is under the tight metabolic control by the host plant (Schubert, 1995; Schulze, 2003). Deprivation of ascorbate during stress may contribute to nodule aging both by inhibition of mitosis and by induction of ABA synthesis. However, control must work via a dialogue of signals between root and shoot rather than a one-way control. The nature of the plant signal that terminates symbiosis and induces nodule senescence remains unknown; however, the rapidly increasing amount of genome information becoming available for *M. truncatula*, together with the identification of mutations such as SUNN (Varma Penmetsa *et al.*, 2003) which are involved in the control of nodulation, will yield much greater insights into signals and receptors involved in nodule senescence.

Nodule senescence has a number of striking similarities to the processes underpinning leaf senescence. Both are developmentally orchestrated aging processes that can be triggered by stress. Both involve the engagement of the plant hormones ABA and ethylene (Ferguson & Mathesius, 2003), increases in peroxisome number, loss of ascorbate and the activation of lipoxygenases, CPs and the ubiquitin-proteasome system. There is perhaps a functional significance to this similarity. Leaf senescence is rather distinct because it is essentially reversible, according to the needs of the plant. The aging and senescence of individual nodules may also be a reversible process such that it can be delayed or reversed, if for example other nodules are destroyed by insects. Finally, it remains crucial to identify the signals originating in within the plant that determine the initiation of nodule senescence. Only then will it be possible to modify the signal transduction process leading to more prolonged nodule sustainability and thus more stress-tolerant legume N-fixation and crop yields.

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