Comparison of Developmental and Stress-Induced Nodule Senescence in *Medicago truncatula*\(^1[C][W][O A]\)

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Mature indeterminate *Medicago truncatula* nodules are zonated with an apical meristem, an infection zone, a fixation zone with nitrogen-fixing bacteroids, and a "developmental" senescence zone that follows nodule growth with a conical front originating in the center of the fixation zone. In nitrogen-fixing cells, senescence is initiated coincidently with the expression of a family of conserved cysteine proteases that might be involved in the degradation of symbiotic structures. Environmental stress, such as prolonged dark treatment, interferes with nodule functioning and triggers a fast and global nodule senescence. Developmental and dark stress-induced senescence have several different structural and expression features, suggesting at least partly divergent underlying molecular mechanisms.

Legume nodules are specialized root organs for biological nitrogen fixation, a process that is carried out by horizontally acquired endosymbiotic rhizobia. This interaction is highly complex and results from interconnected plant developmental programs to allow bacterial infection and organ formation. Nodule formation is triggered by bacterial signal molecules, the lipochitooligosaccharidic nodulation factors, that are thought to be perceived by receptor-like kinase complexes present in compatible host plants (Oldroyd and Downie, 2008).

The understanding of the molecular basis of this interaction has greatly advanced since the study of the model legumes *Medicago truncatula* and *Lotus japonicus*. In both species, rhizobia enter growing root hairs via transcellular infection threads that proceed toward nodule primordia in the cortex, where rhizobia are released into the host cytoplasm through engulfment by the plant plasma membrane. Bacteria differentiate into nitrogen-fixing bacteroids that, together with the peribacteroid membrane (PBM), constitute a new organelle, the symbiosome (for review, see Jones et al., 2007). In the nitrogen fixation zone of the nodule, the cytoplasm of the infected cells contains the oxygen-binding leghemoglobin proteins. The infected cells are interspersed with uninfected cells that are presumed to transport sugars and nitrogenous compounds in connection with the peripheral vascular bundles (Selker, 1988; Gordon et al., 1995; Peiter and Schubert, 2003).

The nodules of *M. truncatula* are indeterminate organs with an apical meristem that functions for many weeks to months. While the meristem activity is in theory unlimited, the normal lifespan of an indeterminate nodule in a temperate climate is restricted to one season or growth cycle of the host (Pate, 1961). In *M. truncatula*, as in most monocarpic legumes, pod filling coincides with the end of the nodule’s lifespan (Lawn and Brun, 1974; Bethlenfalvay and Phillips, 1977).

In the fixation zone, the infected plant cells function for a limited period, after which senescence begins. In this process, cells of both symbiotic partners are degraded. Hence, the nitrogen fixation zone is constantly renewed and the senescence zone increases as the nodule ages. This developmental senescence of infected cells follows two stages (Van de Velde et al., 2006): first, bacteroids are degraded and symbiosomes are resorbed, with the characteristic presence of numerous vesicles in the plant cytoplasm, and afterward, plant cells decay and collapse. A transcriptome analysis revealed that developmental nodule senescence in *M. truncatula* is associated with a carbon sink-to-source transition and with the activation of various genes related to defense and stress.

Here, the question addressed was about which cells in the fixation zone show the first signs of senescence. We carried out a microscopic analysis and, in parallel, identified a family of Cys proteinase (CP) genes that...
turned out to be excellent markers for early stages of nodule senescence. In addition, we compared the process of age-related senescence and stress-induced senescence caused by continuous dark treatment. The lifespan of an indeterminate nodule is not only influenced by nodule age, plant growth phase, and reproduction but can also be triggered or sped up by environmental conditions. Prolonged dark treatment had been shown to drastically induce nodule senescence, with a decrease in nitrogenase activity and in leghemoglobin levels (Romanov et al., 1980; Swaraj et al., 1985, 1986, 2001; Matamoros et al., 1999). Our experiments reveal that developmental and dark-induced senescence differ cellurally and transcriptionally and suggest that the former process is better organized for nutrient remobilization.

RESULTS

Initiation of Developmental Senescence in Nitrogen-Fixing Cells

In mature indeterminate nodules, the nitrogen fixation zone is constantly renewed by descendants from the apical meristem. Old nitrogen-fixing cells eventually stop functioning, and a senescence zone is established where nutrient remobilization takes place (Van de Velde et al., 2006). Based on a pink-to-green color change caused by bilirubin accumulation after leghemoglobin dysfunction (Lehtovaara and Perttilä, 1978), the senescence front is typically described as planar (Puppo et al., 2005). Upon toluidine blue staining, infected senescing cells are more purple and healthy cells are more blue (Van de Velde et al., 2006). In longitudinal sections through mature nodules, purple cells were seen occasionally in the center of the fixation zone (Fig. 1, asterisk), where the senescence process might start.

To study the topology of senescence initiation, serial transversal sections were made through nodules at 35 d post inoculation (dpi). In the young fixation zone, no signs of senescence were visible (Fig. 1, A and B). In more proximal sections, an infected senescing cell appeared sometimes in the center (Fig. 1C, arrow) and more frequently in subsequent, more proximal sections, spreading progressively toward the periphery (Fig. 1D), to finally occupy the central tissue completely (Fig. 1E). In sections through the bottom part of the nodule (old senescence zone), mainly irregular dead cells occurred that were empty or invaded by saprophytic bacteria (Fig. 1F). As a consequence, the senescence zone in a mature indeterminate nodule of *M. truncatula* has a conical front that follows nodule growth (Fig. 1G).

CP Genes as Early Markers for Developmental Senescence

Several putative CP tags had been found to be transcriptionally up-regulated during developmental nodule senescence (Van de Velde et al., 2006). To determine the evolutionary relationships of the corresponding genes, a phylogenetic tree of putative CPs was constructed (Fig. 2A) with sequences of *M. truncatula* (Medicago Gene Index at the Dana-Farber Cancer Institute and Mt assembly version 2.0), Arabidopsis (*Arabidopsis thaliana*), poplar (*Populus trichocarpa*), and a few other CPs that had been linked to senescence processes (Kardailsky and Brewin, 1996;
Figure 2. (Legend appears on following page.)
Naito et al., 2000; Fedorova et al., 2002; Alesandrini et al., 2003; Sheokand and Brewin, 2003; Asp et al., 2004).

Tag MtAC160013_1/4_TC93993 was highly related to the pea ( Pisum sativum ) gene PsCyp15a, which had been shown to play a role in nodule development, seed germination, and stress adaptation (Kardaiskly and Brewin, 1996; Vincent et al., 2000; Sheokand et al., 2005). Genes corresponding to three other cDNA-amplified fragment length polymorphism tags clustered with three additional M. truncatula genes. Together, these six M. truncatula genes were designated MtCP1 to MtCP6. The cluster also contained nodule senescence-related CPs of white clover ( Trifolium ripens ; Asp et al., 2004) and of Chinese milk vetch ( Astragalus sinicus ; Naito et al., 2000). A very related cluster contained four poplar genes and one gene of European black alder ( Alnus glutinosa ) but none of Arabidopsis (Fig. 2A, green box).

All members of these two clusters had an N-terminal signal peptide (Fig. 2B) and a putative tetrapeptide vacuolar targeting signal (LQRD; Fig. 2B, gray box; Chrispeels, 1991), while an HDEL or KDEL endoplasmic reticulum retention signal was absent. Interestingly, no LQRD tetrapeptide motif was present in the CP of the senescence-associated gene SAG12 from Arabidopsis, which belongs to a closely related, yet distinctly different, cluster (Fig. 2).

Transcription of MtCP1 to MtCP6 was analyzed by quantitative reverse transcription (qRT)-PCR in young fixing nodules (F) and in nodules with a progressively increased senescence zone (SN1–SN3; Supplemental Fig. S1). During the course of the developmental nodule senescence, the expression patterns with increasing transcript levels were similar for MtCP1 to MtCP6 (Supplemental Fig. S1).

In situ hybridizations of serial sections with a probe corresponding to a conserved region of the MtCP1 to MtCP6 genes revealed no signals in the early (distal) nitrogen fixation zone (Fig. 3, A and B), but expression was detected in infected cells located at the periphery of the conical senescence zone starting in the center of the fixation zone (Fig. 3, A–D). In more proximal sections, signals were absent (Fig. 3, C and D, asterisk). Given the homogeneity of this pattern, a common regulation of the expression of the genes MtCP1 to MtCP6 seems plausible.

The promoter region of MtCP6 was fused to the GUS-encoding gene uidA. Composite plants with transgenic roots expressing the corresponding construct were nodulated and analyzed by GUS staining. In young fixing nodules, no blue staining was observed, but it occurred in the basal part of older nodules (Fig. 3E). Sectioning indicated that the expression pattern was similar to that observed by in situ hybridization. Strong expression was detected in some cells that showed bacteroid degradation and that were interspersed between healthy fixing cells (Fig. 3, F–I). A low background level was seen in older fixing cells (Fig. 3, G–I), whereas decaying cells with degraded bacteroids no longer expressed MtCP6 (Fig. 3I, asterisk).

Features of Dark-Induced Nodule Senescence

Continuous dark treatment interferes with nodule functioning and triggers full nodule senescence (Matamoros et al., 1999; Swaraj et al., 2001; Hernández-Jiménez et al., 2002). Because noduleation of M. truncatula roots is not synchronous, the nodule population on any particular root is heterogeneous. For instance, at 25 dpi, most nodules fix nitrogen, but a few have an incipient senescing zone while a few others just start to differentiate.

Hence, to study the progression of dark-induced senescence, the following scheme was used. Plants were grown and inoculated on vertically stacked agar plates (Supplemental Fig. S2). At 21 dpi, young pink nodules, without visible sign of senescence, were marked with an ink label (see “Materials and Methods”). Subsequently, plants were split in four groups: two groups were kept as controls and treated under the usual regime (16 h of light/8 h of dark) for 3 and 5 d; two groups were placed in continuous darkness for 3 and 5 d. After 3 and 5 d, all marked nodules were inspected for occurrence and size of a green senescence zone.

At 3 d of dark treatment (ddt; n = 35), 29% of the nodules had a senescence zone of 50% or less than the nodule size, 51% were fully senescent, and 20% had no visible senescence zone. In the control roots (n = 30), 76% of the nodules were still functional and 24% had a senescence zone that was 50% or less than the nodule size. At 5 ddt, 89% of the nodules were fully senescent and 11% had no senescence zone. In the control roots, 64% were still functional and 36% had a small senescence zone. In a biological repeat, a similar trend was seen (see “Materials and Methods”).

To analyze dark-induced senescence at the microscopic level, longitudinal sections were stained with toluidine blue (Fig. 4). Three days of continuous darkness caused a drastically reduced fixation zone (Fig. 4, A–D).

Figure 2. Phylogenetic analysis of CPs from several plant species. A, Maximum-likelihood tree of the CPs of Arabidopsis, poplar, and M. truncatula and senescence-related CPs from different plants. The subgroup of genes, indicated in green, is the group to which MtCP1 to MtCP6 belong. The guided alignment is shown in Supplemental Figure S3. B, Alignment of the N-terminal region of CPs belonging to the cluster indicated in green in A. For comparison, the N-terminal part of SAG12, which does not belong to this cluster, is shown. The sequence in gray corresponds to a putative tetrapeptide vacuolar targeting signal (LQRD). For gene nomenclature and keys for retrieving the gene sequences from repositories, see Supplemental Table S3. [See online article for color version of this figure.]
Serial sectioning revealed that the advancing senescence front was planar. After 5 d of continuous darkness, all infected cells stained faintly purple and were senescing (Fig. 4C). High magnification showed intact uninfected cells and infected cells with a completely degraded content, yet still with a rigid cell shape (Fig. 4, D–G). Saprophytic bacteria invaded the senescing cells very rapidly, leading to intensely blue-stained patches (Fig. 4, G–I). These data indicate that dark-induced nodule senescence progresses faster than developmental nodule senescence.

Comparison of Developmental and Dark-Induced Senescence by Transmission Electron Microscopy

Transmission electron microscopy revealed marked differences at the cellular level between developmental and dark-induced senescence. The start of the developmental nodule senescence was characterized by numerous vesicles, prominent endoplasmic reticulum, abundant mitochondria and Golgi apparatus, and symbiosome degradation (Fig. 5A). During dark-induced senescence, the bacteroid content condensed, the peribacteroid space increased (Fig. 5B, arrows), and often different symbiosomes fused (Fig. 5B, arrowheads). Strikingly, the PBMs remained intact, even when most of the bacteroid content had disappeared, in strong contrast to the developmental senescence, during which eventually the complete symbiosome had dissolved (Fig. 5, compare C and D).

Vesicle trafficking was not pronounced. After vacuolar collapse, the cytoplasm was completely degraded, the mitochondria disappeared, and the only cellular remains were apparently intact PBMs and remnants of bacteroids (Fig. 5, E and F). Saprophytic bacteria, derived from infection threads, entered senescing cells.
at an early stage and multiplied until they occupied most of the space (Fig. 5, G and H). In conclusion, dark-induced senescence is a rapid process in which the symbiosome content is degraded in the absence of cellular signs for nutrient remobilization and with a fast colonization by saprophytic bacteria.

Comparison of Developmental and Dark-Induced Senescence by Marker Analysis

qRT-PCR was used to compare expression profiles of a number of genes that had previously been shown to be up-regulated during the formation of the senescence zone in developing nodules (Fig. 6; Supplementary Tables S1 and S2). The set included genes coding for proteins of different functional groups, such as signal transduction and transcription factors, hormonal control, protein and non-protein degradation, transport, and defense responses (Fig. 6).

For developmental senescence, expression patterns were compared between young fixing nodules without signs of senescence harvested from roots at 21 dpi (F), nodules with less than 50% senescence zone harvested at 35 dpi (SN1), nodules with equal or more than 50% senescence zone harvested at 50 dpi (SN2), and fully senescent nodules harvested at 61 dpi (SN3).

During dark-induced nodule senescence, expression patterns were analyzed in young fixing nodules (0 ddt) and nodules at 1, 3, and 5 ddt. Samples F and 0 ddt were comparable and used as references for the developmental and dark-induced nodule senescence, respectively.

The qRT-PCR analysis of 58 genes confirmed the up-regulated patterns of the developmental senescence (Fig. 6). Twenty-one genes were also up-regulated upon dark treatment, and their expression levels increased or remained high as the senescence process moved on (Fig. 6, group A). Nine other genes were transiently up-regulated during dark treatment (Fig. 6, group B). Finally, expression of a group of 28 genes was not induced upon dark treatment (Fig. 6, group C).

Also, the expression of MtCP1 to MtCP6 was followed upon dark treatment. The transcript levels were measured in young fixing nodules before (0 ddt) and at 1, 2, 3, 4, and 5 ddt. Except for MtCP4, which was not induced, MtCP1, MtCP2, MtCP3, MtCP5, and MtCP6 were transiently up-regulated (Supplemental Fig. S1). The maximal expression level was at 2 dtt and decreased afterward.

DISCUSSION

Developmental senescence in indeterminate M. truncatula nodules is an organized cell death process characterized by nutrient remobilization and a sink-to-source transition (Van de Velde et al., 2006). To try to
understand the transition from actively functioning infected plant cells to cells destined for decay and degradation, it is important to localize the first signs of senescence. Interestingly, serial transversal sections through mature nodules revealed that the earliest and most distal (apical) signs of degeneration occurred in a few infected cells in the center of the fixation zone. The senescence zone progressively extended toward the nodule periphery in subsequent proximal cell layers, with a conical front as a result.

Infected cells degenerate before uninfected cells, which makes sense because the latter are thought to function in nutrient transport to the vascular tissues (Selker, 1988; Gordon et al., 1995; Peiter and Schubert, 2003). The conical organization of the senescence front presumably promotes optimal remobilization of breakdown products, because the last cells to die would be located near the vascular bundles at the periphery. Thus, a simple model to explain the initiation of senescence would be based on cellular age and topology. A central position would correspond to the greatest distance from the peripheral vasculature and the nodule surroundings (such as medium and atmosphere), hinting at a role for concentration gradients of oxygen or of molecules derived from vascular bundles.

The architecture of senescence initiation was confirmed by the expression pattern of a small family of conserved genes (MtCP1–MtCP6) that turned out to be excellent early markers for nodule senescence, in accordance with earlier reports of a putative role of CPs in legume nodulation, both in determinate and indeterminate nodules (Kardaisky and Brewin, 1996; Alessandri et al., 2003; Sheokand and Brewin, 2003; Asp et al., 2004; Sheokand et al., 2005). Transcripts corresponding to MtCP1 to MtCP6 were detected in senescing, infected cells with numerous vesicles located near the symbiosomes. An N-terminal signal peptide presumably directs the proteins MtCP1 to MtCP6 toward secretion pathways, and a putative vacuolar targeting signal suggests vacuole-like structures as the destination. The PBMs have been reported previously to have tonoplast characteristics (Vincent and Brewin, 2000). Recently, cell biological studies have shown that symbiosomes obtain a vacuolar identity at the onset of senescence (Limpens et al., 2009). Hence, the symbiosomes might be very likely targets. The proteins MtCP1 to MtCP6 might be specifically involved in bacteroid degradation for nutrient remobilization. Recent experiments with knockdown of the related CP gene of the Chinese milk vetch AsNODF32 support such a role (Li et al., 2008).

Interestingly, in a phylogenetic tree of CP proteins, a cluster of four poplar genes and one black alder gene was the most closely related to the M. truncatula gene family. All these genes contained the putative vacuolar targeting signal of the actinomycete Frankia species, and its CP transcripts are enhanced in nodules (Goetting-Minesky and Mullin, 1994). These particular CPs might thus be involved in senescence phenomena in Frankia nodules and in the degradation of mycorrhizal arbuscules. The latter endosymbiotic structures function for approximately 7 d, after which they are resolved. Several features of developmental nodule senescence are reminiscent of leaf senescence. In leaves of

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**Figure 5.** Transmission electron micrographs of dark-induced and developmental nodule senescence. A, Enlargement of an infected cell at an early stage of developmental nodule senescence. Arrows and arrowhead mark prominent endoplasmic reticulum and a mitochondrion, respectively. B, Enlargement of an infected cell from a 3-ddt nodule at an early stage of dark-induced nodule senescence. Arrows and arrowheads indicate the enlarged symbiosome space and symbiosome fusions, respectively. C, Infected cell during developmental nodule senescence, in which all symbiosomes are degraded. D, Infected cell from a 3-ddt nodule during dark-induced nodule senescence. The bacteroids are degraded, while the PBMs are still visible. E, Infected cell from a 3-ddt nodule during dark-induced nodule senescence, in which the vacuole is collapsed but the PBMs are intact. F, Enlargement of an infected cell at the same stage as in E. Arrow indicates an intact PBM. G, Enlargement of infected cells from a 5-ddt nodule during dark-induced nodule senescence, when bacteria are liberated from the infection threads. H, Infected cells from a 5-ddt nodule at the last stage of dark-induced nodule senescence and completely filled with saprophytic bacteria. Bars = 2 μm (A, B, F, and G) and 10 μm (C–E and H).
Arabidopsis, senescence-associated vesicles have been described in chloroplast-containing cells. These vesicles contain the SAG12 protein, a CP that might be involved in chloroplast degradation (Guo et al., 2004; Otegui et al., 2005). SAG12 is not part of the MtCP-containing cluster and has not the putative vacuole-targeting motif. Also, CP genes involved in *M. truncatula* leaf senescence (MtAC183777_49.4 and MtACJ85307; De Michele et al., 2009) did not cluster there. These observations again support the hypothesis that MtCP1 to MtCP6 are specific for degradation of endosymbiotic structures.

When developmental and dark-induced nodule senescence are compared at the microscopic and gene expression levels, a number of features differ. Dark stress, caused by keeping plants under continuous darkness, provoked a fast-progressing senescence. Bacteroid degradation was not accompanied by any pronounced vesicle mobilization in the host cytoplasm. PBMs remained intact and fused and preserved

**Figure 6.** Comparison of developmental and dark-induced nodule senescence by qRT-PCR analysis of developmental nodule senescence marker genes. The expression profiles of 58 genes that were up-regulated during developmental nodule senescence were analyzed during the dark-induced nodule senescence and divided into three groups. Group A corresponds to genes that are up-regulated during both types of senescence; group B contains genes that are gradually up-regulated during developmental nodule senescence and transiently induced during dark-induced nodule senescence; group C consists of genes that are uniquely induced after developmental nodule senescence. For each group, a representative expression profile is shown. The genes to which the expression profiles belong are indicated under the graphs. Expression profiles are shown during developmental nodule senescence (left graphs) and dark-induced nodule senescence (right graphs). Note the difference in scale of each individual graph. For developmental nodule senescence, expression patterns are compared between 21-dpi-fixing nodules without signs of senescence (F), nodules with less than 50% senescence zone (SN1), nodules with equal or more than 50% senescence zone (SN2), and fully senescent nodules (SN3). For dark-induced nodule senescence, expression patterns were compared between young fixing nodules (0 ddt) and nodules at 1, 3, and 5 ddt.
a round shape, while during developmental nodule senescence, the symbiosome content mixed with the cytoplasm after collapse of the central vacuole.

Thus, membrane dismantling and fatty acid degradation are features of developmental senescence, in contrast to dark-induced nodule senescence, which is more reminiscent of a general stress response with rapid death of both the microsymbiont and the host cell as a consequence. Similar features have been described in aberrant nodules of Fix− mutants or transgenic plants or induced by lipopolysaccharide-defective bacteria (Perotto et al., 1994; Tsyganov et al., 1998; Campbell et al., 2002; Suganuma et al., 2003; Wan et al., 2007; Den Herder et al., 2008).

At the transcriptional level, of the 58 selected up-regulated genes during developmental nodule senescence, 50% were not up-regulated during dark-induced nodule senescence, which included regulatory and transport functions, membrane and protein degradation, and stress resistance as well as a syntaxin and two phosphatidyl-4-phosphate-5-kinases suggestive of specific vesicle transport (Roth, 2004). Several other genes were only transiently up-regulated in dark-induced nodule senescence. Genes seemingly not involved in dark-induced nodule senescence encode many functions related to proteasome-mediated protein degradation and several CPs, hinting at a less organized degradation process in dark-induced than in developmental nodule senescence. Comparable results have been described for leaf senescence in Arabidopsis: 34% of the genes that were up-regulated during developmental senescence had no altered expression during dark-induced nodule senescence (Buchanan-Wollaston et al., 2005).

These observations will be useful to further unravel the molecular basis of senescence processes in plants, in general, and in nodules, in particular. To understand the initiation of developmental nodule senescence, future experiments should deal with the cell-specific transformations at the gene expression level. An approach to identify target cells might be by means of molecular markers, and the CP genes are suitable for cells at the onset of senescence. Laser capture microdissection of MtCP-expressing cells will undoubtedly reveal many interesting insights into how nodule senescence is triggered and proceeds.

MATERIALS AND METHODS

Plant Material, Bacterial Strains, and Growth Conditions

For developmental nodule senescence, growth of Medicago truncatula Jemalong J5, Sinorhizobium meliloti 1021, and plant inoculation were as described (Mergaert et al., 2003). Plants were grown in sterilized perlite as substrate and watered with sterilized nutrient solution I (SOLi; Blondon, 1964). For each nodulation stage, nodules from 30 plants were harvested. Senescent nodules at 35 and 61 dpi were isolated with a MZFLIII stereomicroscope (Leica). Root material was obtained from noninoculated, nitrogen-defective bacteria (Perotto et al., 1994; Tsyganov et al., 1998; Campbell et al., 2002; Suganuma et al., 2003; Wan et al., 2007; Den Herder et al., 2008).

Microscopic Analysis

Light and electron microscopy were done as described previously (D’Haeze et al., 1998). For light microscopy, nodules were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer, washed, dehydrated, and embedded in Technovit 7100 (Kulzer Histo-Technik) according to the manufacturer’s instructions. Next, 2-μm sections were cut on a microtome (Reichert-Jung) and mounted on Vectabond-coated slides (Sigma-Aldrich) that were stained with toluidine blue (Van de Velde et al., 2003). Photographs were taken with a Diaplan microscope equipped with bright-field optics (Leitz).

For electron microscopy, nodules were cut longitudinally and fixed in 2.5% formaldehyde/3% glutaraldehyde in 0.1 M cacodylate buffer, washed, dehydrated, and embedded in LR White hard grade (London Resin). Serial sections were collected on collodion-coated copper grids, stained with 2% uranyl acetate for 12 min, and examined with a transmission electron microscope (Elmicon 101; Siemens).

qRT-PCR Analysis

Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. These RNA preparations were DNase treated and purified through NH4Ac (5 μL) precipitation. Quality and quantification were controlled with the NanoDrop Spectrophotometer (Isogen). RNA (2 μg) was used for cDNA synthesis with the SuperScript Reverse Transcriptase Kit (Invitrogen), subsequently diluted 50 times, and stored at −20°C until further use.

qRT-PCR analysis was carried out with the LightCycler 480 (Roche Diagnostics) and SYBR Green for detection in a total reaction volume of 5 μL. Reactions were done in triplicate on a 384-multwell plate to allow determination of means and s.d. Cycle threshold values were obtained with the accompanying software, and data were analyzed with qBASE software version 1.3.3 (Hellemans et al., 2007). The relative expression was calculated by normalization against the constitutively expressed 40S ribosomal S8 protein (TC100533; www.tigr.org) and the translation elongation factor 1-α (TC106485; www.tigr.org). Three independent experiments were done with two technical repeats, each providing similar results. Representative runs are shown. Primers used are presented in Supplemental Table S1.

Phylogenetic Analysis of CPs

A data set of Arabidopsis (Arabidopsis thaliana), poplar (Populus trichocarpa), and M. truncatula CPs as well as of M. truncatula EST transcript assemblies and

Scientific Nalgene and Nunc) were opened at one side, and draining holes were made at the opposite side. M. truncatula Jemalong J5 seedlings were placed on the opened edge of the plate between a layer of sterile Whatman paper (35mM) and the dish cover. A layer of foam rubber filled the rest of the plate, allowing some pressure on the root system (for details, see Supplemental Fig. S2). The seedlings were inoculated with a culture of S. meliloti 2011 at an optical density at 600 nm = 0.05, until the Whatman paper got completely wet. Plates were placed vertically. The temperature and light regimes were the same as those for the developmental nodule senescence experiments. Plants were watered with sterilized SOLi medium. After 21 dpi, all young fixing nodules (completely pink) were marked. While half of the plates were kept at the 16-h-light/8-h-dark regime, the other half were brought to darkness. After 3 and 5 d, the marked nodules were analyzed for the presence and size of a green senescence zone.

In addition to the results shown, one biological repeat was done. At 3 ddt (n = 52), 16% of the nodules had a senescence zone that was 50% or less of the nodule size, 38% were fully senescent, and 46% of the nodules were still completely functional. After 3 d, 92% of the nodules on control roots (n = 80) not subjected to dark treatment were functional, 63% had a small senescence zone, and 1.5% were fully senescent. At 5 ddt, 97% of the nodules were fully senescent, a residual 1.5% were functional, and 1.5% had only a small senescence zone. In contrast, on the control roots after 5 d, 87% of the nodules were still functional, 10.5% had a senescence zone that occupied less than 50% of the nodule size, and 2.5% were fully senescent. For qRT-PCR experiments, previously marked nodules (fully fixed) were harvested at 0, 1, 3, and 5 ddt, followed by RNA extraction.
several senescence-associated CPs from other plant species was constructed with the BLAST analysis (Alschul et al., 1997). The corresponding protein sequences were aligned with MUSCLE (Edgar, 2004), and the multiple alignment was manually controlled to delineate the gene family and to remove incomplete sequences. The alignment was edited with BioEdit (Hall, 1999) and resulted in the conserved residues of the CPs. A maximum-likelihood tree was constructed with PHYML (Gouyin and Gascuel, 2003) with the amino acid substitution model (Jones et al., 1992) and one category of substitution rates. To assess the inferred relationships, 1,000 data sets were generated. Midpoint rooting and collapsing of the nodes with a bootstrap value smaller than 50 were done for most of the older nodes in the tree. Consequently, no conclusions could be drawn on the relationships between the different subgroups supported by bootstrap values lower than 50 in this phylogenetic tree, whereas the nodes within most of these subgroups are considerably better supported.

In Situ Hybridization of CPs

Ten-micrometer sections of paraffin-embedded nodules at 21, 35, and 61 dpi were hybridized as described (Gooiymachtig et al., 1997). The sequence 5'-ATCTAAGCTCCACATCCGGTCACAGTGAGTTCGAGGAAAGAAGGAGCAGTGCACCGTGAGAACCAAGGTGATGGTGATGGTGGCCATGCATGTCGTTTACGAGCAACACCGGAAAAGATGACGGTGAAGAACCAAGGTCAGTGTGGATGTTGTTGAGCAAGTGACACCTGTGAAGAACCAAGGTCAGTGTGGATGTTGTTGGGCA-5' was digested with BamHI to yield the template for 32P-labeled antisense probe production with T7 RNA polymerase (Invitrogen; Sambrook et al., 1989).

CP Promoter-GUS Fusion

The promoter from MtCP6MtAC174294_27.3/TC100437 (1,732 bp) was amplified with the specific primers PROMTC85913FWD (5'-CAGTGACACCTGTGAAGAACCAAGGTCAGTGTGGATGTTGTTGGGCA-5') and ATTB1REVPROMTC85913 (5'-GGGGACTGCTTTTTTGTACAAACTT-3') to introduce the corresponding attB recombination sites. The promoter region was recombined into pDONR P4-P1 (Invitrogen). The Multisite Gateway Three-Fragment Vector Construct kit (Invitrogen) was used to fuse the promoter region with the GUS gene (from pDONR207-GUS) and the T3SS terminator (from pENTR-R2-T35S-L3) in the pKm43GW-rolD vector that carries the GFP for selection of cotransformed plants. The promoter region was amplified with the specific primers PROMTC85913REV (5'-AAGAAGTGCTGTGCTT-3') and ATTRIBREVPRMTCT85913 (5'-GGGGACTGCTTTTTTGTACAAACTT-3') to introduce the corresponding attB recombination sites. The promoter region was recombined into pDONR P4-P1 (Invitrogen). The Multisite Gateway Three-Fragment Vector Construct kit (Invitrogen) was used to fuse the promoter region with the GUS gene (from pDONR207-GUS) and the T3SS terminator (from pENTR-R2-T35S-L3) in the pKm43GW-rolD vector that carries the GFP for selection of cotransformed plants.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression profiles of MiCP1 to MiCP6 during developmental and dark-induced senescence.

Supplemental Figure S2. Plate system to study the dark-induced senescence.

Supplemental Figure S3. Guided alignment of the phylogenetic tree.

Supplemental Table S1. Overview of primers used for qRT-PCR analysis.

Supplemental Table S2. Expression analysis of 58 gene markers during developmental and dark-induced nodule senescence.

Supplemental Table S3. Keys for retrieving gene sequences from repositories.

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