Running title: Early disruption of microtubules during leaf senescence

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Environmental Stress and Adaptation
Leaf senescence is accompanied by an early disruption of the microtubule network in Arabidopsis thaliana

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ABSTRACT
The dynamic assembly and disassembly of microtubules is essential for cell function. Although leaf senescence is a well-documented process, the role of the microtubule cytoskeleton during senescence in plants remains unknown. Here we show that both natural leaf senescence and senescence of individually-darkened leaves are accompanied by early degradation of the microtubule network in epidermis and mesophyll cells whereas guard cells which do not senesce, retain their microtubule network. Similarly, entirely-darkened plants which do not senesce, retain their microtubule network. While genes encoding the tubulin subunits and the bundling/stabilizing microtubule-associated proteins (MAPs) MAP65s and MAP70-1 were repressed in both natural senescence and dark-induced senescence, we found strong induction of the gene encoding the microtubule destabilizing protein MAP18. However induction of MAP18 gene expression was also observed in leaves from entirely darkened plants showing that its expression is not sufficient to induce microtubule disassembly and is more likely to be part of a Ca^{2+}-dependant signaling mechanism. Similarly, genes encoding the microtubule severing protein katanin p60 and two of the four putative regulatory katanin p80s were repressed in the dark, but their expression did not correlate with degradation of the microtubule network during leaf senescence. Taken together, these results highlight the earliness of the degradation of the cortical microtubule array during leaf senescence and lead us to propose a model in which suppression of tubulin and MAP genes together with induction of MAP18 play a key role in microtubule disassembly during senescence.
INTRODUCTION

Leaf senescence can be the result of natural aging but can also be initiated in response to various stresses such as ozone, shading or pathogen infections (Woolhouse, 1967; Smart, 1994). In response to these developmental or environmental cues, the leaf will undergo three distinct phases sequentially: i) an initiation period where metabolic changes lead to a decrease in the photosynthetic activity and a transition from source to sink, then ii) a degenerative phase mainly characterized by the disassembly of cellular components and their degradation, and finally iii) a terminal phase where cell integrity is lost prior to cell death and death of the whole organ (Nooden et al., 1997; Yoshida, 2003). As leaf senescence is also induced by abiotic and biotic stresses, understanding its mechanisms is not only important for answering fundamental scientific questions but also as part of a challenge to increase crop yields.

In a recent study with Arabidopsis thaliana (Keech et al., 2007) we showed that regulation of metabolism differed significantly between an individually-darkened leaf attached to a whole plant, and an equivalent leaf from an entirely darkened plant. After 6 days of darkness, photosynthetic capacity was maintained in leaves from a whole darkened plant whereas the capacity for mitochondrial respiration decreased. In contrast, individually darkened leaves showed an accelerated senescence with a rapid decline in photosynthetic capacity while mitochondrial capacity remained high. Additionally we observed drastic ultrastructural alterations including aggregation of both chloroplasts and mitochondria during the accelerated senescence occurring in mesophyll cells of individually-darkened leaves. We consequently hypothesized that the cytoskeleton, and in particular the cortical microtubules (MTs) could undergo modifications contributing to the pronounced rearrangements of the cellular content in the mesophyll cells during leaf senescence.

In plant cells, there are two main cytoskeleton components i) MTs which are heterodimeric polymers of the globular proteins α and β tubulin and ii) microfilaments which are composed of actin monomers (Lloyd and Hussey, 2001; Boutté et al., 2007). Microtubules are assembled in a head to tail fashion to form protofilaments and they are constantly undergoing polymerization and depolymerization at both the plus and the minus end (Hashimoto, 2003). They are mainly involved in cell elongation and cell division (Dixit and Cyr, 2004; Boutté et al., 2007), in the development of the plant cell wall notably by facilitating or guiding cellulose
deposition (Wasteneys, 2004; Lloyd, 2006; Wightman and Turner, 2008; Pesquet et al., 2010) and in organelle positioning (Van Gestel et al., 2002; Wada and Suetsugu, 2004; Romagnoli et al., 2007). Nevertheless, their function is intimately linked to their organizational state, which directly depends on the developmental and/or the environmental conditions that the cells are facing (Dixit and Cyr, 2004). Modifications of these conditions trigger signals which in turn can be transduced through several types of proteins, generally referred to as MT-associated-proteins (MAPs). MAPs, by their stabilizing, bundling or severing activities, can then regulate the MT dynamics and can actively participate in the reorganization of the MT array (Lloyd and Hussey, 2001; Hashimoto and Kato, 2006; Hamada, 2007).

Although the dynamic assembly and disassembly of MTs is essential for cells stability and survival, the state of the MT cytoskeleton during senescence in plants remains largely unknown. Considering this in light of our previous data suggesting a remodeling of cytoskeleton in leaves experiencing dark-induced senescence, we investigated changes in the cortical MT network and potential regulatory mechanisms during both natural and dark-induced leaf senescence in the model plant Arabidopsis thaliana.

RESULTS

Two systems to study leaf senescence

We compared attached leaves submitted to dark-induced senescence with Arabidopsis leaves naturally ageing in order to assess whether the cytological modifications observed in our previous study (Keech et al., 2007) were dark-induced or senescence specific. Individually-darkened leaves (idl), previously described in detail by Weaver and Amasino (2001) and by Keech et al. (2007), offer a more synchronous and a better controlled process of senescence than age induced senescence. Using this method in the present work, we followed the progression of leaf senescence for 6 to 8 days after which the leaves were yellow (Fig 1a). Time zero idl (T0idl) represented leaves from 8 week-old plants with a chlorophyll content of 1.27 ± 0.04 mg/mL/gFW. In parallel, from 11 to 13 week-old plants, we selected leaves at different stages of natural senescence (T0ns, T50ns and T85ns). T0ns represented leaves with a chlorophyll content of 1.85 ± 0.02 mg/mL/gFW which was 30% more than in the T0idl. T50ns and T85ns were the leaves undergoing natural senescence with approximately 50% and more than 85% reduction of
their chlorophyll content, respectively. These 3 stages were consequently representative of the
beginning, the middle and the end of the process of natural senescence in Arabidopsis leaves (Fig
1a). In addition, as we noticed that leaves undergoing natural senescence generally exhibit a
heterogeneous pattern of chlorosis (Supp Fig 1), we proposed that T50ns could thus represent a
combination of the three more distinct and more synchronous stages T2idl, T4idl and T6idl as
suggested in Figure 1a.

To compare the similarities of the developmental stages between our two systems at the protein
level, we ran immunoblot analyses with several relevant biological markers. In line with the
progressive degradation of the chloroplasts during leaf senescence, the large subunit of Rubisco
(Rbc L) and the chloroplastic isoform of glutamine synthetase (GS2) were progressively
degraded during the two time courses of the natural and the dark-induced senescence (Fig 1b).
As expected, the relative content of the cytosolic isoform of glutamine synthetase (GS1)
increased during both natural and dark-induced senescence as part of the nitrogen remobilization
and nutrient recycling pathway in accordance with previous reports (Kawakami and Watanabe,
1988; Masclaux et al., 2000). It is worthwhile to note that the ratio GS1/GS2 was higher in T0ns
leaves than in the T0idl leaves (Fig 1b) reflecting an older stage of development of T0ns when
compared to T0idl (see also qRT-PCR analysis in Figure 5c). Mitochondrial degradation was
tested with antibodies raised against the cytochrome c oxidase subunit 2 (COX-II), subunit of the
complex IV in the electron transport chain. We previously reported that the number of
mitochondria per volume of leaf decreased during dark-induced senescence while their size
increased (Keech et al., 2007). Here we show that the amount of COX-II remained fairly stable
during the 6 days of dark-induced and natural senescence demonstrating that the ratio
mitochondrial proteins to total proteins remained almost unchanged during leaf senescence. This
confirms that the degradation of mitochondria is slower than the degradation of chloroplasts and
strengthens the prominent role of mitochondria during late events of leaf senescence, most likely
to supply energy and the carbon skeleton to the cell for efficient nutrient remobilization. Finally,
two cytoskeleton markers, α-tubulin for the MTs and actin for the actin-filaments, decreased
unexpectedly early during both dark-induced and natural leaf senescence.

In vivo visualization of the microtubule cytoskeleton during leaf senescence
To investigate the consequences of such an early decrease in abundance of α-tubulin during both dark-induced (Fig 2) and natural (Fig 3) leaf senescence, the organization of the MT cytoskeleton was visualized in vivo by confocal laser microscopy using Arabidopsis plants constitutively expressing GFP-β-tubulin 6 (Nakamura et al., 2004). The GFP fusion to the N terminus of the Arabidopsis β-tubulin 6 under the control of the cauliflower mosaic virus 35S promoter was demonstrated to be a reliable reporter to fluorescently label MTs in aerial tissues (Abe and Hashimoto, 2005). In addition, the authors reported the incorporation of labeled β-tubulin subunits to be 20–30% of the endogenous tubulin level. Thus, it is most likely that the in vivo visualization of the polymerized MTs was unbiased despite the construct being driven by a strong promoter. Microtubules were observed in 3 different cell types from the abaxial side of leaf sections. Already 2 days after the induction of dark-induced senescence, a reduction of the MT network was observed in both the epidermis and the adjacent spongy mesophyll cells (Fig 2B,G) and this reduction was accentuated at 4 and 6 days in the two cell types. However, no apparent modification of the MT cytoskeleton was observed in guard cells of stomata (Fig 2K–O). We also noticed a progressive reduction in size and in number of chloroplasts in mesophyll cells (Fig 2 F,G,H,I) which led to their complete degradation after 8 days (Fig 2J). In line with our previous observations (Keech et al., 2007), chloroplasts tended to aggregate after 6 days. Finally, after 8 days, the MT network was completely disrupted with the exception of the stomatal guard cells that retained both their MTs and their chloroplasts (Fig 2E,J,O).

The situation was very similar in leaves undergoing natural senescence. The MT cytoskeleton from the epidermis and the mesophyll cells of T0ns leaves exhibited the same pattern as T0idl. Later, we observed a drastic reduction of the density of the MT cytoskeleton in epidermis and mesophyll cells after 50% degradation of the chlorophyll (T50ns) (Fig 3B,F). After 85% degradation of the chlorophyll (T85ns), the depolymerization of the MTs was apparently complete in these two cell types (Fig 3C,G). We also noticed chloroplasts of a smaller size in mesophyll cells at T50ns (Fig 3F) and their complete degradation at T85ns (Fig 3G). Finally as in dark-induced senescence, guard cells of stomata retained their MT network and their chloroplast until the very end of the leaf’s life (Fig 3H,I,J).

Expression of tubulin-related genes
In order to explain the progressive, tissue-dependent, disruption of the MTs during both dark-induced and natural senescence we investigated the regulation of several molecular players which could be involved in the bundling/stabilization and the destabilization of the MT network. In Arabidopsis, there are 6 genes coding for α-tubulin subunits (Kopczak et al., 1992), 9 for β-tubulin (Snustad et al., 1992) and 2 for γ-tubulin (Liu et al., 1994). From publicly available microarrays (van der Graaff et al., 2006) performed with leaves undergoing natural and dark-induced senescence in similar conditions to ours, we extracted relative expression profiles for all genes encoding tubulin subunits (Supp Table 3) and expressed the data as the Log2 of the ratio between T25%, T50% or T75% and the expression value of the gene at T0 (T0 being a 6 week-old leaf and T25, T50 and T75 being leaves with a loss of 25%, 50% and 75% of their chlorophyll content, respectively). As shown in Fig. 4, the expression pattern for the genes encoding alpha, beta and gamma subunits was fairly similar between natural (Fig 4a) and dark-induced senescence (Fig 4b) and showed a progressive repression for most of the genes. However, only a few of the genes showed a significant down-regulation. We subsequently used quantitative real-time PCR (qRT-PCR) to confirm that expression of TUB 6 (beta-tubulin-6) matches that of van der Graaff et al. (2006) in our systems (Fig 5).

While these results (van der Graaff et al., 2006) show reduced expression of the genes encoding tubulin subunits, we also hypothesized that other regulators could be involved in the disruption of the cortical MT array. To assess this possibility, we examined by qRT-PCR the level of transcripts of several MT regulators: the nine genes representing the MT bundling MAP-65 family (Smertenko et al., 2008), the MT severing p60 subunit of katanin (Burk et al., 2001) and four of its putative p80 subunits containing WD40 repeats (Bouquin et al., 2003; Roll-Mecak and McNally, 2010 and annotations in TAIR9), the MT stabilizing MAP 70-1 (Korolev et al., 2005) and finally the MT destabilizing MAP 18 (Wang et al., 2007). Results were expressed as the log10 of the ratio between the expression of the genes at T50ns and the expression of the genes at T0ns for which the expression value was set to 1 (Fig 5a). Similarly, Fig. 5B represents the log10 of the ratio between the gene expression levels at T2idl, T4idl or T6idl and the expression of the genes at T0idl (for which the value was set to 1). The low abundance of transcripts due to their degradation occurring at T85ns and T8idl did not allow qRT-PCR quantifications.
Often referred as the main molecular marker of leaf senescence, the level of expression of SAG12 (Senescence-Associated-Gene 12, encoding a papain-like cysteine proteinase) was used as a control, and shown to be up-regulated several thousand-fold between T0ns and T50ns and also gradually up-regulated during dark-induced senescence strengthening the similarities between the two systems. Since the GFP-β-TUBULIN 6 construct was used for the visualization of the MTs by confocal laser microscopy (Nakamura et al., 2004), we also examined expression of TUB6 gene. Reinforcing the validity of the arrays of van der Graaff et al. (2006) we found TUB6 to be progressively repressed during natural senescence and dark-induced senescence (Fig 5a,b).

Due to their central role in plant growth and development, MTs undergo constant reorganization during the different phases of cell expansion and actively participate to intracellular transport and signal transduction in response to hormone signals and/or environmental perturbations. As a direct consequence of their multiple functions, MTs are submitted to extremely complex regulation orchestrated by a plethora of proteins notably involved in building, remodeling and interconnecting the MT network (Sedbrook, 2004). Amongst the increasing number of proteins discovered to interact with MTs, we primarily focused on the MAP65 family as numerous studies have reported MAP65 proteins to be MT-bundling-proteins. The Arabidopsis genome contains 9 MAP65 genes sharing 25 to 78% amino acid sequence identity suggesting an important variability in MAP65 activities (Smertenko et al., 2008). In an effort to unravel the localization and the biochemical role of the different members of the MAP65 family in Arabidopsis thaliana, Smertenko et al. (2008) provided strong evidence for divergent dynamic association between the MAP65s and the MTs but also highlighted the difficulties of determining in planta the role of each MAP65 due to their tissue and developmental expression specificity. Nonetheless, most of MAP65 proteins seem to promote MT polymerization at some stage. In the present study, we show the expression of all the nine MAP65 genes to be significantly repressed during natural and dark-induced senescence (Fig 5a,b). In addition, we also report another MAP involved in stabilization of MTs, MAP70-1 (Korolev et al., 2005), to be down regulated during the two processes (Fig 5a,b).

Interestingly, MAP 18, whose protein has been demonstrated to have a depolymerizing activity on MTs in vitro (Wang et al., 2007) was found to be highly up-regulated during natural senescence: around 14-fold when compared to T0ns, and also during dark-induced senescence.
where its regulation peaked at a $10^4$-fold increase, suggesting a major role for MAP18 during leaf senescence. Strong promoter activity of MAP18 during leaf senescence was confirmed using a promMAP18::GUS (β-Glucuronidase) reporter gene in transgenic plants (Wang et al., 2007). GUS staining was barely visible in either T0idl or T0ns but surprisingly, we saw a pronounced staining in structures likely to be the hydathodes of the leaves. Progressively, the GUS staining appeared concomitantly with the progress of the senescence and had a peak of intensity at T4idl and at T50ns (Fig 6). Although the expression of promMAP18::GUS was still detectable in T6idl and T85ns, the staining was not as strong as suggested by the results of the qRT-PCR analysis (Fig 5 and 6). This discrepancy between the two results may be explained by the fact that the GUS staining reveals only the activity of the promoter of MAP18 whereas qRT-PCR reveals the steady state level of MAP18 mRNA. Indeed, while the promoter activity is essential to produce MAP18 mRNAs, the quantity of steady MAP18 transcripts strongly depends on the MAP18 mRNA stability. From a recently published study on mRNA decay rates in Arabidopsis thaliana (Narsai et al., 2007), we discovered that MAP18 had one of the most stable transcripts in Arabidopsis thaliana with a half life estimated at 46 h. The group of the most stable transcripts comprises transcripts with a half-life of at least 24 h and represents less than 1% of the 13000 mRNAs studied (Narsai et al., 2007). The decrease of the promMAP18 activity in T6idl and the concomitant high level of MAP18 mRNAs during senescence are consistent with a particularly high stability of the MAP18 mRNA in leaves.

Finally, we also investigated the level of expression of katanin subunits. Katanin is a heterodimeric protein that mediates ATP-dependant destabilization of MTs in animals. The small subunit (p60) of katanin has been identified in Arabidopsis and presents a MT-severing activity in vitro whereas a regulatory subunit of 80KDa (p80) is involved in the targeting of katanin (Stoppin-Mellet et al., 2007). In our work, the p60 subunit of katanin and four of its putative p80 partners were differentially regulated between natural and dark-induced senescence. Whereas the genes coding for the characterized p60 and the four putative p80 subunits were all down-regulated during natural senescence, p60 and two of the four putative p80 appeared to be significantly up-regulated during dark-induced senescence. This discrepancy between natural and dark-induced senescence suggests katanin to be somehow regulated by light.

Whole darkened plants as control
In an attempt to dissociate the effects of darkness from the process of cell death occurring in leaves submitted to dark-induced senescence, we examined the expression level of the same set of genes in leaves harvested after 2, 4 and 6 days from entirely-darkened plants. We previously showed (Keech et al., 2007) that after 6 days of darkness, leaves from entirely-darkened plants were in a metabolic ’stand-by’ mode where most of the cellular functions were reduced but still kept functional in order to optimize the recovery of the plant when a source of light becomes available. While individually-darkened leaves are inexorably committed to senesce, this fundamentally different metabolic strategy confers to the entirely darkened plant a crucial survival ability. The qRT-PCR analysis revealed an intermediate transcript profile in the leaves from the entirely-darkened plants. As in individually-darkened leaves, MAP18, p60-At1g80350, p80-4g34280 and p80-5g23430 were progressively up-regulated during the 6 days of darkness. However, individually-darkened leaves and whole darkened plants differed as TUB6 was significantly up-regulated and all genes encoding MAP65 and MAP70-1 were stably expressed (Fig 7a). Interestingly, only a minor reduction of the density of the MT network was observed in leaves from 6 days darkened plants (Fig 7b).

**DISCUSSION**

*Both natural and dark-induced senescence are accompanied with an early, cell specific degradation of the cortical microtubule array*

Although leaf senescence is a thoroughly studied developmental stage, the role of the cytoskeleton remains unknown. Consequently, our objective in this study was to characterize the cortical MT array during leaf senescence and to investigate its potential regulation. Natural senescence is the obvious physiological reference for studies about leaf senescence. However, working with different experimental systems can sometimes be advantageous because the comparison of the results unravels common or specific pathways and also helps to establish more accurately the interconnections between pathways leading to leaf senescence. In the present study, despite T0ns being 4 weeks older than T0idl, no visible sign of senescence was apparent. Indeed, no degradation of chlorophyll was evident (the chlorophyll content was even 30% higher in T0ns than in T0-idl), the immunoblot analysis of Rubisco and of α-tubulin revealed a high amount of protein in the two T0s and finally, the comparison of the MT cytoskeleton between
the two did not show apparent differences. However, for a few genes of interest, the ratios between the expression levels in T0ns and in T0idl demonstrate that the T0ns leaves may have already entered the process of senescence (Fig 5c). For instance, the high expression of \textit{SAG12} clearly indicates that the genetically controlled process of degradation has already begun in T0ns (Fig 5c). In addition, it has been shown that in leaves under normal conditions the chloroplastic isoform (GS2) represents the main fraction of the glutamine synthetase pool, while the cytosolic isoform (GS1) is more abundant during leaf senescence (Kawakami and Watanabe, 1988; Brugiere \textit{et al.}, 2000; Masclaux \textit{et al.}, 2000). The higher protein ratio of GS1/GS2 in T0ns leaves when compared to T0idl indicates that these leaves have already modified their metabolism to enter their nutrient recycling process and in particular the salvage of nitrogen. In addition, as shown by Supp Fig 1, leaves undergoing natural senescence exhibit a more heterogeneous degradation pattern than leaves submitted to a dark-induced senescence. Taken together, these results show the difficulties in determining the starting point of natural senescence without using destructive techniques. For this reason the use of other systems such as dark-induced senescence, which allows a fast, controlled and more synchronous induction of leaf senescence, appears to be a powerful tool to study leaf senescence.

Here, we show that the degradation of the cortical MT array is an early event and takes place in the epidermis cells and the adjacent layer of mesophyll cells of \textit{Arabidopsis thaliana} leaves undergoing either natural or dark-induced senescence (Fig 2, 3). Only a minor aggregation of the chloroplasts in mesophyll cells from both natural and dark-induced leaf senescence is observed, whereas such aggregation was more apparent in a previous study (Keech \textit{et al.}, 2007). However as infiltrations of calcofluor white were used to visualize the cell wall in our previous study (Keech \textit{et al.}, 2007), this might have accentuated the aggregation of the organelles since they could not be properly anchored to the cytoskeleton due to its destabilization. In contrast, guard cells of stomata conserve their chloroplasts and their MT network, consistent with earlier studies reporting the extended longevity of chloroplasts from guard cells of yellowing leaves from both perennial and annual plants (Zieger and Schwartz, 1982). It has been proposed that guard cells of stomata retain their functionalities to facilitate gas exchanges until the very end of the senescence process (Thimann and Satler, 1979). However, the heterogeneity in the metabolism of the different cell types during leaf senescence raises fundamental questions about cell-specific regulation and potential signaling between the cells undergoing senescence.
Molecular mechanisms of the degradation of the microtubule arrays during leaf senescence

In 2003, Smertenko et al. reported disorganization of the MTs during embryonic Programmed Cell Death (PCD) in the gymnosperm *Picea abies*, and degradation of the MTs was also observed by Zottini et al. (2006) in cytokinin-treated cells of *Medicago truncatula* induced to enter PCD. However, the molecular players and the regulatory mechanisms responsible for this destabilization remain unknown.

We assume that although MT assembly is energy dependant, the lack of ATP/GTP is not likely to be responsible for the disorganization of the MT, as we earlier showed mitochondria to provide sufficient ATP to maintain cellular functions until the end of the process of senescence (Keskitalo et al., 2005; Keech et al., 2007). So, in an attempt to elucidate the molecular mechanisms responsible for the degradation of the MT lattice during leaf senescence, we investigated the regulation of genes coding for tubulin subunits and for several MT-associated proteins in whole leaves. Expression of genes encoding alpha, beta and gamma tubulins is mainly repressed during leaf senescence as shown by published microarray data (van der Graaff et al., 2006). In addition, we show that the MAP65 gene family and MAP70-1 are significantly repressed during both natural and dark-induced senescence but not in leaves from whole plants experiencing prolonged darkness. This consequently suggests that MT polymerization and stabilization mechanisms are specifically down-regulated during leaf senescence.

However, not all MAPs are involved in stabilization of MTs. Recently identified as a new MT-associated-protein (Wang et al. (2007), MAP18 was shown to bind to MTs and to inhibit tubulin polymerization in vitro. MAP18 was reported to be expressed mostly in expanding cells and localized in root, flower, cotyledon, hypocotyl and trichomes but surprisingly not in the root tip or mature leaves (Wang et al. (2007)). Using the GUS reporter gene fused to the promoter of MAP18 we observed a specific staining of structures believed to be hydathodes of mature leaves under normal conditions (T0ns and T0idl). However, both qRT-PCR and GUS staining showed an increased expression of MAP18 during leaf senescence strongly supporting the idea that MAP18 could play a physiological role in the senescence-specific degradation of MTs. However, our analysis showed that MAP18 was also induced in leaves from whole darkened plants whereas only a minor destabilization of the MT network was observed. Furthermore, in a recent study, Kato et al. (2010) investigated the biochemical properties of MAP18 and concluded that this
protein was a plasma membrane associated Ca\textsuperscript{2+} binding protein (consequently renamed PCaP2 by the authors) and was very likely to be involved in the regulation of cellular functions including stress response and cytoskeletal reorganization. In vivo, MAP18/PCaP2 appeared to be associated with the plasma membrane due to its N-myristoylation and strongly interacted with several phosphatidylinositol phosphates in a Ca\textsuperscript{2+}-calmodulin dependant manner (Kato et al., 2010). We consequently conclude that increased expression of MAP18 is not sufficient to destabilize the MT network in vivo but could instead be part of a more complex Ca\textsuperscript{2+}-dependant signaling mechanism potentially leading to the perturbation of the MT cytoskeleton.

We also questioned whether katanin could be involved in the degradation of MTs. In a recent study, Stoppin-Mellet et al. (2006) examined in Arabidopsis thaliana the overexpression of the p60 subunit (also called AtKSS or AtKTN1) fused to the reporter mRFP (monomeric Red Fluorescent Protein). Under the control of an ethanol-inducible promoter, overexpression of katanin p60 subunit triggered MTs to organize into numerous and thick bundles, which ultimately depolymerized. In the present work, our findings showed that all katanin subunit genes (i.e. the p60 and putative p80s genes) were all repressed during natural senescence whereas the genes encoding the p60 and 2 of the putative p80s were significantly up regulated in individually-darkened leaves (Fig 5a,b). Despite this up-regulation during dark-induced senescence, the formation of short and thick bundles was not apparent. Consequently, katanin does not seem to be required for the depolymerization of MTs during leaf senescence. However, the discrepancy between the data from natural senescence and from dark-induced senescence suggests that the severing activity of katanin could be involved in the rearrangement of MT in a light-dependant manner. In fact, this idea is further supported by the two following points. First, qRT-PCR performed on leaves from entirely darkened plants revealed the same p60 and p80 transcripts to be significantly up-regulated during 6 days of darkness (Fig 7a) although only a minor degradation of the cortical MT array was noticed (Fig 7b). Second, when we examined the ChIP-chip data set from Lee et al. (2007), we observed that AtKSS (p60-At1g80350) was among the in vivo binding targets of the transcription factor HY5 (LONG HYPOCOTYL5). The bZIP protein HY5 is an extensively studied transcription factor involved in promoting photomorphogenesis. Mutations in HY5 cause a defect in the inhibition of hypocotyl elongation in all light conditions, suggesting that HY5 acts downstream of PHYA, PHYB, cryptochromes, and UV-B (Lee et al., 2007). Taken together, these results strongly suggest that the severing
activity of katanin on the remodeling of the cortical MT array can be light dependant since darkness in one hand enhances katanin expression and in another hand represses the expression of HY5. However, much work remains to elucidate the regulation of the cortical MTs in response to developmental and environmental stimuli.

To conclude, in view of these new findings, we propose a tentative model (Fig 8) for the early degradation of the cortical MT lattice during both natural and dark-induced senescence. Katanin p60 and p80 subunits may not be required during natural senescence; they could however contribute to the reorganization and the severing of the MTs during prolonged darkness including dark-induced senescence, potentially via the repression of the transcription factor HY5. In contrast, the co-repression of the tubulin subunits and the bundling/stabilizing MAP65s / MAP70-1 could contribute to the destabilization of the MT network during leaf senescence; the minor disruption of the MTs observed in leaves from entirely darkened plants suggesting that MAP18 expression alone is not sufficient for the disruption of the MTs. All together, this work highlights a novel trait of leaf senescence by demonstrating the surprising earliness of the degradation of the cortical MT array. This also leads to further questions about how vesicle shuttling, organelle mobility and re-organization of metabolism are achieved in cells having a drastically reduced MT network.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0), Col-0 transformed with GFP-β-tubulin 6 (Nakamura et al., 2004; Abe and Hashimoto, 2005) were grown in a controlled environment growth chamber with a short-day photoperiod (8 h light/16 h dark), an irradiance of 250 µmol quanta m⁻² s⁻¹, a relative humidity of 75% and a temperature cycle of 22ºC day / 17ºC night.

Induction of senescence

Leaves undergoing natural senescence were collected from rosettes (leaves 6 to 11) from plants aged 11 to 13 weeks. The three stages were labeled T0ns, T50ns and T85ns. T0ns represented leaves with a Chlorophyll content of 2.2 ± 0.4 mg/mL/gFW. T50ns and T85 were respectively
the leaves undergoing natural senescence with respectively 50% and more than 85% degradation of their chlorophyll content.

-Dark-induced senescence was carried out as described by Keech et al. (2007). Briefly, with Arabidopsis plants aged 8 weeks, rosettes leaves from 6 to 11 were covered by mittens for 2, 4, 6 and 8 days while the rest of the plant remained in the light. This refers to individually darkened leaves (idl). In parallel, whole plants were placed in darkness for 2, 4 and 6 days.

**Protein extraction and Immunoblot analysis**

For each treatment, two leaf discs (Ø 15mm) were ground in 500 µL of extraction buffer (50 mM Tris-HCl pH 7.5, 5 mM EGTA, 5 mM MgCl2, 100 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton-X-100, 0.5 % (w/v) SDS, 2 mM DTT). After centrifugation (4°C, 15 min, 20000g), supernatants were collected and protein amount was quantified with the DC protein assay kit from Bio-Rad®. Immunoblot analysis was done on nitrocellulose and primary antibodies were used to the following concentration: anti-Rbc L (from Agrisera®, Vännäs, Sweden) 1 / 5000, anti-CoxII (from Agrisera®) 1 / 1000, anti-GS1 and anti-GS2 (Sakakibara et al., 1992) 1 / 1000, anti-Tubα (from Sigma®, T5168) 1 / 2000, Anti-Actin (from Sigma®, A0480) 1/1000. Western blots were visualised by ECL (GE healthcare®, Rydalmere, Australia).

**Confocal laser imaging**

*Microtubule network visualization* - Imaging was performed with a SP2 inverted-microscope (Leica, Mannheim, Germany) under an x63 water immersion objective (numerical aperture 1.20). MT cytoskeleton was visualized in the epidermis and the adjacent layer of spongy mesophyll cells from the abaxial side of the leaf sections. Green Fluorescent Protein was excited using 488nm Ag laser and emitted light was collected in the 503-562nm window. Chloroplasts revealed by chlorophyll fluorescence were excited using a 633nm HeNe laser and emitted light was collected in the 662-721 nm window. Spectral measurements were performed to confirm emission spectra. Three-dimensional images of leaves were performed by sequential scans. For each time point, at least 4 biological replicates were processed. Images were mounted with the Image J software (version1.38x).
Transcript quantification by quantitative RT-PCR

RNA isolation and cDNA synthesis

Ten leaves at similar stage of senescence – i.e. T0ns and T50ns for natural senescence, and T0, 2d, 4d and 6d for idl and whole darkened-plants (see above for description) – were dissected from five different plants, pooled, frozen in liquid nitrogen and ground into powder. Two sets of samples were prepared from two independent biological replicates. Total RNA was extracted using the hot phenol protocol described in Gutierrez et al. (2006). The resulting RNA preparations were treated with DNaseI using a DNase I free Kit (Ambion) and cDNA was synthesized by reverse-transcribing 10 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The reaction was stopped by incubation at 70°C for 10 min, the reaction mixture was then treated with RNaseH (Invitrogen) according to the manufacturer’s instructions and diluted by adding 700 μL of dH2O. All cDNA samples were tested by PCR using specific primers flanking an intron sequence to confirm the absence of genomic DNA contamination.

Quantitative RT-PCR

Transcript levels were assessed by quantitative RT-PCR, in assays with triplicate reaction mixtures (final volume, 20 μL) containing 5 μL of cDNA, 0.5 μM of both forward and reverse primers and 1x FastStart SYBR Green Master mix (Roche), using an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad), and CT values for each sample were acquired using iCycler iQ™ software 3.0 (Bio-Rad). The following standard protocol was applied for each amplification: 10 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 15 s at 60°C, and 15 s at 72°C. The sequences of primers used for all target genes are presented in Supp Table 1.

Reference genes were selected and validated as follows. Ten genes (see Supp Table 2 for primer sequences) were chosen for their putative stability of expression according to Czechowski et al. (2005) and Gutierrez et al. (2008). Their expression in our experimental material (i.e. leaves at different stages of natural or artificial senescence grown under our experimental conditions) was then assessed, and they were ranked according to their stability of expression using the geNorm software (Vandesompele et al., 2002). APT1 or TIP41 were the most stably expressed genes among the 10 tested (Supp Fig 2), and thus were used to normalize the quantitative RT-PCR data. The normalized expression patterns obtained using both reference genes were similar, so
only the data normalized with APT1 are shown in Figs 4 and 5, and in supplemental data. Relative standard curves describing the PCR efficiencies for each primer pair were generated for each amplicon according to Larionov et al. (2005), and the resulting values were used to calculate expression using the formula $E_T = \frac{(CT_{T0} - CT_X)}{E_R (CT_{T0} - CT_X)}$, where (T) is the target gene and (R) the reference gene, CT is the crossing threshold value (i.e. the number of PCR cycles required for the accumulated fluorescence signal to cross a threshold above the background), (X) is related to cDNA from senescing leaves (i.e. T2idl, T4idl, T6idl and T50ns) and (T0) from the related T0 control (i.e. T0ns and T0idl). A melting curve analysis was added to each PCR program and the size of PCR products was assessed by electrophoresis in agarose gels to check that the fluorescence signal was derived from the single intended amplicon.

**Map18 Promoter GUS analysis**

Stable transformants for MAP18 promoter:GUS (in Columbia 0 background) were kindly obtained from Professor Ming Yuan Gus staining was performed according to Wang et al. (2007).

**SUPPLEMENTAL MATERIAL**

Supplemental Figure 1 S1. Example of heterogeneity in the degradation of the microtubule network during natural leaf senescence.

Supplemental Figure 2 S2. Validation of the reference genes (GeNorm) for the qRT-PCR analysis.

Supplemental Table 1 S3. Sequences of primers used for target genes.

Supplemental Table 2 S4. Sequences of primers used for reference genes.

Supplemental Table 3 S5. Relative expression values for natural and dark-induced senescence time courses.

**AKNOWLEDGEMENTS**

The authors would like to thank for their kind gift Dr. Hitoshi Sakakibara for of GS1 and GS2 antibodies, Dr. Akashi Hashimoto for the transgenic Arabidopsis lines GFP-Beta-tub6 and Prof.
Ming Yuan for the promMAP18::GUS line. Dr Andreas Sjödin is acknowledged for his comments at the early stages of this work.

REFERENCES


LEGENDS
**Figure 1** Representative time course of a mature rosette leaf of *Arabidopsis thaliana* plant (Col0) undergoing either dark-induced senescence or natural senescence. For dark-induced senescence, leaves were individually darkened (idl) for 2 to 8 days. For natural senescence (ns), T50ns and T85ns represent 50% and at least 85% degradation of their chlorophyll content, respectively, in comparison with T0ns. Scale bars = 1cm (a). Immuno-blot analysis of total extracts from natural senescence (ns) and from individually-darkened leaves (idl). Rubisco large subunit (Rbc L), glutamine synthetase cytosolic (GS1) and chloroplastic (GS2) isoforms, cytochrome c oxidase subunit II (COX), alpha tubulin (Tub-α) and F-actin (actin). In each lane, 10µg protein was loaded (b).

**Figure 2** *in folio* imaging of the progressive disruption of microtubules in epidermis (A-E), spongy mesophyll (F-J) and guard cells of stomata (K-O) during dark-induced senescence. Scale bars A-J = 50µm, K-O = 10µm

**Figure 3** *in folio* imaging of the progressive disruption of microtubules in epidermis (A-C), spongy mesophyll (E-G) and guard cells of stomata (H-J) during natural senescence. The late longevity of stomatal guard cells is evidenced by the conserved microtubule lattice (H-J). Scale bars A-J = 50µm, K-O = 10µm

**Figure 4** Gene expression analysis of alpha, beta and gamma tubulins during natural senescence (a) and dark-induced senescence (b). Data were primarily extracted from publicly available microarrays (van der Graaff et al. 2006). The normalized expression values were converted into the Log2 of the ratio between the samples (25%, 50% and 75% degradation of chlorophyll, respectively) and control (leaves from 6 week-old plants). * significant difference (p<0.05) in comparison to respective T0 determined by Student’s-t-test. α1 (At1g64740), α2 (At1g50010), α3 (At5g19770), α4 (At1g04820), α5 (At5g19780), α6 (At4g14960), β1 (At1g75780), β2 (At5g62690), β3 (At5g62700), β4 (At5g44340), β5 (At1g20010), β6 (At5g12250), β7 (At2g29550), β8 (At5g23860), β9 At4g20890, γ1 (At3g61650), γ2 (At5g05620).

**Figure 5** qRT-PCR analysis of microtubule related genes during natural senescence (a) and during dark-induced senescence (b). SAG12 was also included as a molecular marker of leaf senescence.
senescence. Results are expressed on a log_{10} scale and represent the ratio between T50ns and T0ns in (a) and between T2-idl, T4-idl and T6-idl, respectively, and T0-idl in (b). The ratio between the level of expression of the genes at T0ns and at T0idl indicates that senescence has already begun in T0ns (c). Tub6 (At5g12250), MAP18 (At5g44610), MAP65-1 (At5g55230), MAP65-2 (At4g26760), MAP65-3 (At5g51600), MAP65-4 (At3g60840), MAP65-5 (At2g38720), MAP65-6 (At2g01910), MAP65-7 (At1g14690), MAP65-8 (At1g27920), MAP65-9 (At5g62250), MAP70-1 (At1g68060), SAG12 (At5g45890), p60 (At1g80350), p80 (At5g23430), p80 (At1g03110), p80 (At4g34280), p80 (At4g04940).

**Figure 6** GUS expression analysis of *promMAP18* during dark-induced senescence and natural senescence. Experiments were performed with 6 independent biological replicates.

**Figure 7** qRT-PCR analysis of microtubule related genes in leaves from entirely darkened plants. Results are expressed on a log_{10} scale and represent the ratio between 2 days, 4 days and 6 days, respectively, and T0 (a). *in folio* imaging of the microtubule array during 0, 2, 4 and 6 days in darkness (b). Scale bars = 20μm. All locus identifiers are available in the legend of figure 5.

**Figure 8** Proposed model for the destabilization of the cortical microtubule array in epidermis and mesophyll cells during leaf senescence.
Figure 1

Representative time course of a mature rosette leaf of *Arabidopsis thaliana* plant (Col0) undergoing either dark-induced senescence or natural senescence. For dark-induced senescence, leaves were individually darkened (idl) for 2 to 8 days. For natural senescence (ns), T50ns and T85ns represent 50% and at least 85% degradation of their chlorophyll content, respectively, in comparison with T0ns. Scale bars = 1 cm (a). Immuno-blot analysis of total extracts from natural senescence (ns) and from individually-darkened leaves (idl). Rubisco large subunit (Rbc L), glutamine synthetase cytosolic (GS1) and chloroplastic (GS2) isoforms, cytochrome c oxidase subunit II (COX), alpha tubulin (Tub-α) and F-actin (actin). In each lane, 10µg protein was loaded (b).
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Figure 5

qRT-PCR analysis of microtubule related genes during natural senescence (a) and during dark-induced senescence (b). SAG12 was also included as a molecular marker of leaf senescence. Results are expressed on a log_{10} scale and represent the ratio between T50ns and T0ns in (a) and between T2-idl, T4-idl and T6-idl, respectively, and T0-idl in (b). The ratio between the level of expression of the genes at T0ns and at T0idl indicates that senescence has already begun in T0ns (c). Tub6 (At5g12250), MAP18 (At5g44610), MAP65-1 (At5g55230), MAP65-2 (At4g26760), MAP65-3 (At5g51600), MAP65-4 (At3g60840), MAP65-5 (At2g38720), MAP65-6 (At2g01910), MAP65-7 (At1g14690), MAP65-8 (At1g27920), MAP65-9 (At5g62250), MAP70-1 (At1g68060), SAG12 (At5g45890), p60 (At1g80350), p80 (At4g34280), p80 (At4g34280), p80 (At4g34280).
**Figure 6**

Individually-Darkened Leaves

Natural Senescence

*Figure 6* GUS expression analysis of *promMAP18* during dark-induced senescence and natural senescence. Experiments were performed with 6 independent biological replicates.
Figure 7 qRT-PCR analysis of microtubule related genes in leaves from entirely darkened plants. Results are expressed on a log<sub>10</sub> scale and represent the ratio between 2 days, 4 days and 6 days, respectively, and T0 (a). *in folio* imaging of the microtubule array during 0, 2, 4 and 6 days in darkness (b). Scale bars = 20μm. All locus identifiers are available in the legend of figure 5.
Figure 8

Proposed model for the destabilization of the cortical microtubule array in epidermis and mesophyll cells during leaf senescence.