Side-chain modifications of phyllobilins may not be essential for chlorophyll degradation in Arabidopsis

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Abstract Disposing efficiently and safely chlorophyll derivatives during senescence requires a coordinated pathway that is well conserved throughout green plants. The PAO/phyllobilin pathway catalyzes the degradation of the chlorophyll during senescence and allows detoxification of the pigment and its subsequent export from the chloroplast. Although most of the chloroplastic reactions involved in chlorophyll degradation are well understood, the diversity of enzymes responsible for downstream modifications of non-phototoxic phyllobilins remains to be explored. More than 40 phyllobilins have been described to date, but only three enzymes catalyzing side-chain reactions have been identified in Arabidopsis thaliana, namely, TIC55, CYP89A9, and MES16. Here, by generating a triple mutant, we evaluate the extent to which these enzymes are influencing the rate and amplitude of chlorophyll degradation at the metabolite as well as its regulation at the transcriptome level. Our data show that major side-chain modifications of phyllobilins do not influence significantly chlorophyll degradation or leaf senescence, letting the physiological relevance of their striking diversity an open question.

KEYWORDS Arabidopsis thaliana, chlorophyll, CYP89A9, MES16, phyllobilins, senescence, TIC55

1 INTRODUCTION

Safe and efficient degradation of the photosynthetic apparatus during leaf senescence, fruit ripening, or maturing seeds is associated with the disposal of chlorophyll, a central but highly photoreactive pigment. Removing chlorophyll from thylakoid membranes and its subsequent detoxification is considered a key step in plant adaptation to land (Schumacher et al., 2021). Most of our understanding of enzymatic degradation of higher plants comes from Arabidopsis thaliana (subsequently Arabidopsis) and is catalyzed via a tightly regulated enzymatic pathway: the pheophorbide a oxygenase (PAO)/phyllobilin pathway (Aubry et al., 2021; Hörtensteiner et al., 2019). This pathway consists of two parts. First, a highly conserved chloroplastic part converts chlorophyll into non-phototoxic linear tetrapyrroles, which is followed by more diversified cytosolic modifications as a second part. The opening of the porphyrin ring, catalyzed by PAO, is the key irreversible step in the process leading to detoxifying chlorophyll (Figure 1). The produced linear tetrapyrroles are then exported to the cytosol, where a whole range of species-specific modifications occur, that could be subsequently stored to the vacuole (Figure 1). This diverse range of chlorophyll catabolites are referred to as “phyllobilins” (Süssenbacher et al., 2014). All phyllobilins described in green plants are ultimately derived from the C5/C6 ring-opening activity of PAO. In Viridiplantae,
Chlorophyll (left) is degraded into a primary fluorescent catabolite (pFCC) (Hörtensteiner et al., 1995; Rodoni et al., 1997). PAO and RCCR are physically interacting, most likely to avoid any accumulation of photoreactive intermediates (Pružinská et al., 2007; Sakuraba et al., 2012). Indeed, defect in any of these steps, like in pao1 or acd2-1 mutants, leads to the accumulation of pheide a or RCC, respectively, and the formation of necrotic lesions (Pružinská et al., 2005). Interestingly, in addition to the observed phototoxicity, light-independent lesions have been observed in pao1 (but not in acd2-1) incubated under constant darkness, suggesting particular toxicity associated with pheide a (Hirashima et al., 2009). Pheide a, shown to be accumulating in the chloroplast (Christ et al., 2013), has been subsequently proposed as part of a signaling pathway associated with jasmonic acid response (Aubry et al., 2020).

The second part of the PAO/phyllobilin pathway starts from the export of chloroplastic (epi)-pFCC and ends up in a diverse range of phyllobilins in the cytosol and the vacuole (Christ et al., 2012, 2013; Süssenbacher et al., 2014). To date, at least nine distinct FCC-derived structures have been described in Arabidopsis that are modified on their peripheral side chains at five distinct positions on the molecule (C1, C2, C4, C32, and C84, respectively; Figure 1). A recent survey of over 183 species revealed a much larger diversity, with potentially more than 40 distinct phyllobilin structures to be found in green plants (Schumacher et al., 2021). All these phyllobilins are classified based on their structure into two categories: Type I, nonfluorescent (NCCs), 1-formyl and 19-oxobilins, and Type II, dioxygenbilin-type (DNCCs) (Kräutler, 2014, 2016). Although most of the vacuolar isomerizations of the modified FCC into nonfluorescent catabolites (NCCs) are thought to be nonenzymatic (and associated with the acidic pH of the vacuole), the structural diversity of phyllobilins observed in the cytosol mostly involves dedicated enzymatic process. So far, three enzymes involved in the side-chain modifications have been characterized in Arabidopsis, namely, TIC55 (Hauenstein et al., 2016), MES16 (methyleneesterase family member 16) (Christ et al., 2012), and CYP89a9 (Christ et al., 2013).

**FIGURE 1** Chlorophyll (left) is degraded through the PAO/phyllobilin pathway and produces a diverse range of linear tetrapyroles: the phyllobilins (right). In Arabidopsis, only enzymes modifying side chains of phyllobilins have been identified: TIC55 catalyzing C32 hydroxylation, MES16 catalyzing C82 carboxymethyl group demethylation, and CYP89a9 catalyzing C1 deformation.
hypermodified FCCs (hmFCCs) detected, for example, in ripe banana (Musa sp.) and peace lily (Spathiphyllum wallisii) (Kräutler et al., 2010; Moser et al., 2009). hmFCCs are stabilized due to functionalization of the C12 propionic acid with different groups (Oberhuber et al., 2008).

Some gymnosperms and ferns like Pteridium aquilinum accumulate iso-phyllobilanones, which present a major carbon-skeleton rearrangement (Erhart et al., 2018; Schumacher et al., 2021). Given some phyllobilins are also bioactive compounds with antioxidative and anti-inflammatory compounds (Wang et al., 2021), understanding the molecular basis of their synthesis and accumulation could bring interesting insight outside plant sciences toward potential pharmacological applications.

The physiological relevance of the structural diversity of phyllobilins remains a matter of debate. Interestingly, none of the single mutants depleted in either TIC55, MES16, or CYP89a9 could show any impact on their growth and speed or extent of leaf senescence (Christ et al., 2012, 2013; Hauenstein et al., 2016). Taking advantage of the comprehensive description of these lines in Arabidopsis, we tested here the impact of removing three enzymes responsible for side-chain modifications on leaf senescence. A triple mutant line, cyp89a9-1, mes16-1, and tic55-2 (cmt), was produced, and its phenotype was characterized at the transcriptome and metabolome (phyllobilins) level during dark-induced senescence. Although the triple mutant does accumulate the expected pattern of intermediary phyllobilins and shows a marginal change in its gene expression compared with the WT, the rate of degreening does not appear to be significantly modified in this line. However, we report a change in color of the triple mutant lysate after dark incubation, possibly due to accumulation of a new phyllobilin, but without indication of leaf sensitivity to light. Taken together, our data allow us to confirm that side-chain modifications of chlorophyll catabolites are, at least for the growth conditions we tested, not essential to the process of chlorophyll degradation. We discuss here the implications of these findings and confront some of the recent literature that concluded differently.

2 RESULTS
2.1 A new pattern of phyllobilin accumulation observed in the triple mutant tic55 mes16 cyp89a9

Arabidopsis cyp89a9-1 mes16-1 tic55-2 (cmt triple mutant) was produced by crossing subsequently cyp89a9-1 into mes16-1 double mutant (Christ et al., 2013) and tic55-2 (Hauenstein et al., 2016). Homozygosity was tested by PCR with gene and T-DNA-specific primers (Table S1). None of the single/double or triple mutant lines cmt show any significant phenotypic differences to the WT (Figure 2a) and no statistically significant difference (t-test, p < 0.05) in the rate and speed of chlorophyll degradation (Figure 2b). Measurement of the electrolyte leakage provide a proxy for the potential accumulation of phototoxic compound during dark-induced senescence. For example, pao1 and acd2-1 electrolyte leakage increase significantly quicker compare to WT (Figure 2c). This is due to the accumulation of pheophorbide a and RCC, respectively. No difference in the dynamics of electrolyte leakage could be observed for the triple mutant cmt (Figure 2c), suggesting that no light-independent cell death is triggered, despite the absence of these side-chain modifications of phyllobilins. Accumulation of phyllobilins during dark incubation was profiled by UPLC-MS/MS in the triple mutant leaves. Interestingly, all modified phyllobilins detected in WT, like the most
abundant DNCC_618 (Christ et al., 2016), were absent. Instead, a mixture of hydroxylated NCCs and/or demethylated DNCCs was detected. The most important peak corresponds to unmodified pFCC (m/z 629.2967 [M + H]^+) and its nonfluorescent isomer pNCC (Figure 3a). These MS/MS fragmentation patterns matched previously described pFCC/pNCC patterns (Figure S1) (Hauenstein et al., 2016). Interestingly, although the triple mutant did not show any difference in the rate of chlorophyll degradation, the leaf lysate appears yellow/orange after 7 days in the dark (Figure 3b,c). Due to the similarity to the acd2-1 phenotype that accumulates RCC (and thereby induces light-dependent phototoxicity), the leaf lysate was monitored via liquid chromatography–tandem mass spectrometry (LC-MS/MS). A compound that presents a similar mass compared with RCC (m/z 627.28 [M^+H]^+), but neither the retention time nor the MS/MS fragmentation pattern (Figure S2) matched an RCC. Indeed, RCC has two mass units less compared with a pFCC, due to the oxidation of double bond in C16. This double bond makes it unlikely the loss of ring D of RCC during fragmentation. The compound observed in the triple mutant cmt accumulates at a relatively low concentration and has a very similar mass compared with RCC but does show loss of ring D (Figure S2). Further work is necessary to elucidate the exact structure of this compound.

None of the phyllobilins accumulating in the triple mutant cmt, the new 627.28 mass, DNCC_628, and FCC_628 seem to have an impact on light-independent cell death (Figure 1c). It remained to be shown whether these compounds would affect the light-dependent cell death (indicative of actual phototoxicity). Ion leakage was measured again in these lines after re-exposure to light (Figure S3). Consistent with the growth phenotype observed, the triple mutant cmt does not show any significant difference to WT in terms of electrolyte leakage after re-exposure to light. We, therefore, conclude that despite an accumulation of unusual patterns of phyllobilins in absence of three side-chain modifying enzymes, the phototoxic chlorophyll is degraded safely and efficiently in the triple mutant cmt.

### 2.2 The marginal impact of the triple mutation tic55 mes16 cyp89a9 on leaf transcriptome

Using a genome-wide transcriptome profiling (RNAseq), we monitored the extent of variation in gene expression during dark-induced senescence in detached leaves of single mutant lines cyp89a9-1, mes16-1, tic55-2 and the triple mutant cmt (Figure 4a,b). The transcriptome of detached leaves incubated in the dark has been described previously (Aubry et al., 2020; van der Graaff et al., 2006). Our data correlate with the literature when comparing WT leaves between 0 and 3 days in the dark: No particular perturbation of the genes underlying the process of leaf senescence could be observed (Figure 4a,b and Table S2). The analysis of gene ontologies shows that genes involved in photosynthesis, Calvin cycle, and tetrapyrrole synthesis were underrepresented during dark-induced senescence, whereas genes involved in protein degradation and ABA metabolism were overrepresented (Figure 4e). The global signature of response to dark incubation for the single or triple mutant lines did not differ substantially from WT (Figure 4e). Interestingly, when comparing transcriptional signature across senescing samples (after 3 days in the dark), the triple mutant cmt shows the highest number of differentially expressed genes (2825 genes, log2FC <> 2, padj ≤ .05) when compared with WT (Figure 4c,d). Most of the differences observed were caused by the absence of Tic55 (673 and 902 genes commonly differentially expressed compared with WT in tic55-2 and cmt, respectively) (Figure 4c,d). In cmt, an additional set of 902 genes (452 up- and 455 downregulated compared with WT, respectively) are being differentially expressed only in the cmt line after dark-induced senescence. Although no particular ontology term could be enriched significantly

![Figure 3](image-url)

**Figure 3** (a) Extracted ion chromatogram of wild-type phyllobilins (top panel) shows the accumulation of diverse phyllobilins. On the contrary, cmt mutant (bottom panel) accumulates unmodified and nonfluorescent chlorophyll catabolites. MS/MS fragmentation pattern and chemical structure of these two phyllobilins (F_628 and N_628) are shown in Figure S1. (b) Rosettes of 6-week-old short-day grown Arabidopsis and detached leaves that were dark incubated for senescence induction. (c) Methanol extracts of the 7-dDD wild-type (Col-0) and triple mutant cyp mes tic leaf.
in this subset, the molecular basis for these variations remains unknown.

The transcriptome analysis also allowed us to monitor the pattern of expression of all genes involved in the PAO/phyllobilin pathways (Figure 5). As expected, expression of PAO, MES16, and CYP89a9 was significantly increasing during dark-induced senescence, whereas TIC55, RCCR, and CLD1 were decreasing. These results are consistent with previous reports (Aubry et al., 2020). Noteworthy, the remaining expression that could be observed, particularly in cyp89a9-1 lines is not corresponding to a functional transcript (transcription of a portion of the first exon could be detected in cyp89a9-1). No major differences in the PAO, RCCR, or CLD1 expression level could be observed due to the absence of CYP89a9, MES16, and TIC55 (Figure 5). These results do not seem to indicate a particular link, at the transcriptional level, between the chloroplastic and the cytosolic subsections of the PAO/phyllobilin pathway.
DISCUSSION

3.1 | A central regulatory function of TIC55 in leaf senescence is unlikely

Unraveling TIC55 function was a particularly circumvoluted path in the recent history of chloroplast research. It has been first identified as a member of the TIC complex of protein import to the envelope of the chloroplast (Küchler et al., 2002). Although a role for TIC55 in redox regulation of protein import has been suggested earlier, no significant change in chloroplastic import could be measured in tic55 mutants (Boij et al., 2009). More recently, though its localization to the inner envelope of chloroplast has been confirmed, TIC55 has been identified as being involved in hydroxy-pFCC, one of the major phyllobilin in Arabidopsis (Hauenstein et al., 2016). No detectable phenotype could be observed, neither during normal growth, nor dark-induced senescence of detached leaves, suggesting that hydroxy-pFCC conversion is not a necessary step to efficient degradation of chlorophyll.

Recent reports claimed the possible role of TIC55 in the regulation of chlorophyll degradation (Chou et al., 2018; Hsu et al., 2022). While characterizing some senescence-related transcription factors binding to the promoter of TIC55, namely, NAC003 and MYB108, the actual dynamics underlying such a potential regulation remain unclear. Indeed, the TIC55 expression profile during dark-induced senescence decreases unlike other modifying enzymes like MES16 and CYP89a9 (Figure 5). This peculiar pattern of TIC55 expression profile during dark-induced senescence has been reported several times independently (Aubry et al., 2020; van der Graaff et al., 2006). TIC55 is therefore very unlikely to be a positive regulator of the PAO/phyllobilin pathway. In addition, the absence of TIC55 in the tic55-2 mutant does not change the speed of overall chlorophyll degradation, nor does it when MES16 and CYP89a9 activities are absent (Hauenstein et al., 2016; Figure 2). The side-chain modification catalyzed by TIC55 has little consequences on the speed of degradation. A slight increase in substrate affinity of MES16 for hydroxy-pFCC has been reported, but again without consequence on the rate of degradation. Genes that were shown to be differentially expressed in Chou et al. (2018)
between WT and tic55-2 after dark-induced senescence of detached leaves could not be identified as differentially expressed in our current dataset. For example, none of the four NAC (NAC003, NAC010, NAC042, and NAC075) found to be downregulated in tic55-2 compared with WT could be confirmed in our assay (Table S3). Therefore, in the current state of knowledge, TIC55 is unlikely to be a key control point neither for the PAO/phyllobilin pathway of chlorophyll degradation nor for leaf senescence. Unless a new function or new substrate could be reported (particularly more natural/changing growing conditions must be tested), TIC55 is most likely at the “bottom line” of the chlorophyll detoxifying pathway and does not influence its speed, nor leaf senescence.

### 3.2 Could phyllobilins be considered xenobiotics by the cell?

The diversity of phyllobilin across the Viridiplantae reported so far at least 12 distinct modifications on eight positions on the porphyrin (Schumacher et al., 2021). With only three enzymes known to date responsible for the side-chain modification of phyllobilins, more work is needed to identify other enzymatic processes responsible for this chemodiversity. Removing “only” three side-chain modification enzymes might not be enough to change the phyllobilin pool to observe a significant change in chlorophyll degradation in optimal conditions. In Arabidopsis, screening for transferases responsible for the accumulation of malonylated or glucosylated phyllobilins is at reach (Hörtensteiner et al., 2019). Taken collectively, side-chain modification is a common process dealing with xenobiotics and allowing a shift in polarity that facilitate their import to the vacuole (Kreuz et al., 1996). Therefore, it may not be surprising that both CYP89a9 and MES16 belong to large gene families, cytochrome P450 and a/b-hydrolase, respectively. Extension of gene families (by gene duplication) has been correlated during evolution to secondary metabolite diversification (Flagel & Wendel, 2009). It is therefore intriguing to observe that phyllobilins outside the chloroplast are considered somehow xenobiotics by the cell and to be disposed swiftly into the vacuoles.

### 3.3 The physiological relevance of phyllobilin’s side-chain modification remains unclear

Considering the lack of significant phenotype when three of the major side-chain modification were impaired, the obvious question concerning the physiological relevance of accumulating a diversified but species-specific set of phyllobilins remains open. Meanwhile, care should be taken when interpreting the absence of phenotype in the triple mutant, as plants were grown in constant light and temperature conditions and senescence induced “artificially” using detached leaves incubated in the dark. Indeed, some slightly significant differences between leaves from WT and cmt ion leakage at 5 and 7 DDD could be observed (Figure 1c). Multiple possible functions for phyllobilins accumulation (above their primary role as non-phototoxic intermediates) have been reported (for an extensive review, see Kräutler, 2016), for example, as an antioxidant in ripened fruits (Müller et al., 2007), as well as contributing to the color of some senescent leaves or ripened fruits (viz., yellow YCCs and pinked colored PICC) (Li & Kräutler, 2019; Moser et al., 2008). The two subections (chloroplastic and cytosolic) of the PAO/phyllobilin pathway appear to have evolved independently: up to the pFCC export from the chloroplast, tight evolutionary constraints channeled the pathway to an efficient and likely enzymatic complex, a met abolon-like organization (Sakuraba et al., 2012), followed by a less specific detoxication that allows a whole range of promiscuous enzymatic activities to modify side chains of the resulting phyllobilins and subsequent vacuolar disposal. This cytosolic subsection of the pathway could be actually thought as a totally independent module that is not exclusively dedicated to chlorophyll or its derivatives, but to virtually any other xenobiotics. Although this work could rule out any particular central function associated with the accumulation and modification of phyllobilins, exploring the recently accessible genomic space in other (photosynthetic) non-model species will surely help identify more enzymes involved in side-chain modifications of phyllobilins.

### 4 MATERIAL AND METHODS

#### 4.1 Plant material, growth conditions, and leaf senescence induction

Arabidopsis (A. thaliana) Col-0 WT was used together with the T-DNA lines tic55-2 (Hauenstein et al., 2016), mes16-1 (Christ et al., 2013), and cyp89a9-1 (Christ et al., 2012) as well as pao1 and acd2-1 lines used as controls (Aubry et al., 2020; Pruzinska et al., 2007). For ph notyping, plants were grown in short-day conditions (8-h light/16-h dark, 23°C, 65% humidity, 100 µmol photons m⁻² s⁻¹) for 8 weeks, whereas for while plants for crossing, seed production and DNA isolation plants were grown in a greenhouse (16-h light/8-h dark, 22°C, 65% humidity, 100–200 µmol photons m⁻² s⁻¹). mes16-1 cyp89a9-1 tic55-2, a triple mutant, was generated by crossing and confirmed by PCR using gene-specific primers (Data S1). Leaf senescence was induced using incubation in complete darkness, at least four leaves, size no. 8 for each triplicate, harvested and frozen in liquid nitrogen after up to 8 days of incubation, on water-soaked filter paper at 23°C.

#### 4.2 Chlorophyll quantification

Chlorophyll was extracted from in liquid nitrogen-homogenized tissue during 2 h at –20°C in 10% (v/v) 0.2 M Tris–HCl, pH 8.0, in acetone, precooled to –20°C (5 ml g⁻¹ fresh weight). After twice centrifugation (4 min, 16,000 g, 4°C), supernatants were analyzed by spectrophotometry according to (Strain et al., 1971) using the following
equations. Chl \(a (\mu g/ml) = 11.63 \times A665 - 2.39 \times A649; Chl \(b (\mu g/ml) = 2.11 \times A649 - 5.18 \times A665; Chl \(a + b (\mu g/ml) = 6.45 \times A665 - 17.71 \times A649.

4.3 | Ion leakage quantification

For determining cell death in the lines during senescence, leaf discs (0.4 cm in diameter) were punched with a cork borer under green safe light, avoiding the mid vein. At least 25 leaf discs per time point and genotype were placed in a multiwell plate ion conductivity meter (Reid & Associates; 1.5 ml of water and two discs per well), and relative ion leakage (displayed as microsiemens) was determined in the dark.

4.4 | Chlorophyll catabolite profiling

Metabolite profiling was performed by LC-MS/MS according to a published protocol (Christ et al., 2016). Briefly, plant material was collected in 2-ml microfuge tubes containing 500 µl of 1.25- to 1.65-mm glass beads. The tissue was ground in liquid nitrogen using an MM300 Mixer Mill (Retsch) at 30 Hz for 5 min. Chlorophyll catabolites were extracted with five volumes (w/v) of ice-cold 80% methanol, 20% water, and 0.1% formic acid (v/v/v) containing 1 µg/ml ampicillin as internal standard, sonicated for 2 min, and twice centrifuged at 16,000 g for 2 min. Extracts were analyzed by LC-MS/MS Thermo Fisher Scientific UltiMate 3000 RSLC system, coupled to a Compact Q-TOF mass spectrometer with electron spray ionization (Bruker Daltonics) as described (Christ et al., 2016). The C-18 column was developed with a gradient (flow rate of 0.3 ml min\(^{-1}\)) of solvent B acetonitrile with 0.1% (v/v) formic acid in solvent A water with 0.1% (v/v) formic acid as follows (all v/v): 30% for 0.5 min, 30%–70% in 7.5 min, 70%–100% in 0.1 min, and 100% for 3.9 min. Phyllobilins were identified and quantified using an in-house-built spectrum library (Schumacher et al., 2021).

4.5 | RNA isolation and sequencing

RNA was isolated using an RNAeasy minikit (Qiagen). Quality was assessed using Bioanalyzer RNA nanochip (Agilent). Three replicate samples for each condition were multiplexed randomly on two lanes (12 samples per lane) of HiSeq 2500 (Illumina). Analysis of the RNA-seq reads was performed following standard protocol (Aubry et al., 2020). Single-end 100-bp reads were subjected to adapter trimming and removal of low-quality bases in leading, trailing, and sliding window (4 bp) mode with the tool Trimmomatic v0.35 (Bolger et al., 2014). Reads shorter than 40 bp after trimming were discarded. The remaining reads were aligned to the protein-coding transcripts from the ENSEMBL release of the TAIR10 Arabidopsis transcriptome using the software BowTie v1.0.1 (Langmead et al., 2009). The expression of genes and transcripts was quantified using the software RSEM v1.2.11 taking into account strand-specific information (Li & Dewey, 2011). Differential expression was estimated using DESeq2 (Bioconductor) by estimating the posterior probability of genes to be differentially expressed across all conditions (Love et al., 2014). GO enrichment was performed using a corrected Benjamini–Hochberg enrichment score implemented in the program Pageman. The raw sequencing data from RNA-seq are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-11614.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

MH, SH, and SA designed the research. MH performed the research. SA analyzed the data and wrote the paper.

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