Leaf necrosis resulting from down-regulation of poplar glycosyltransferase

UGT72A2

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Running title

UGT72A2 and poplar leaf redox status

Abstract

Reactive species causing oxidative stress are unavoidable by-products of various plant metabolic processes, such as photosynthesis, respiration or photorespiration. In leaves, flavonoids scavenge reactive species produced during photosynthesis and protect plant cells against deleterious oxidative damages. Their biosynthesis and accumulation are therefore under tight regulation at the cellular level. Glycosylation has emerged as an essential biochemical reaction in the homeostasis of various specialised metabolites such as flavonoids. This article provides a functional characterization of the *Populus tremula* x *P. alba* (poplar) *UGT72A2* coding for a UDP-glycosyltransferase that is localised in the chloroplasts. Compared to the wild type, transgenic poplar lines with decreased expression of *UGT72A2* are characterised by reduced growth and oxidative damages in leaves, as evidenced by necrosis, higher content of glutathione and lipid peroxidation products as well as diminished soluble peroxidase activity and NADPH to NADP⁺ ratio under standard growing conditions. They furthermore display lower pools of phenolics, anthocyanins and total flavonoids but higher proanthocyanidins content. Promoter analysis revealed the presence of *cis*-elements involved in photomorphogenesis, chloroplast biogenesis and flavonoid biosynthesis. *UGT72A2* is regulated by the poplar MYB119, a transcription factor known to regulate the flavonoid biosynthesis pathway. Phylogenetic analysis and molecular docking suggest that *UGT72A2* could glycosylate flavonoids, however the actual substrate(s) was(ere) not consistently evidenced with *in vitro* assays nor analyses of glycosylated products in leaves of transgenic poplar overexpressing or downregulated for *UGT72A2*. This article provides elements highlighting the importance of flavonoid glycosylation regarding protection against oxidative stress in poplar leaves and raises new questions about the link between this biochemical reaction and regulation of the redox homeostasis system.

Introduction

Cell metabolism is at the origin of the production of reactive species (RS), including reactive oxygen species (ROS) and reactive carbonyl species (RCS). RCS, deriving from ROS, are a group of α , β -unsaturated carbonyl compounds that originate from lipid peroxides, such as malondialdehyde (Mano et al. 2019). While low cellular RS concentrations adjust a panel of parameters related to redox homeostasis (Noctor et al. 2018; Morales & Munné-Bosch, 2019; Mano et al. 2019), RS accumulation results in deleterious molecular and physiological damages typical of oxidative stress. Exogenous factors penalising plant growth or reproduction, collectively referred to as “stress”, often lead to this redox disequilibrium and have typical cellular and molecular signatures (Vaahtera et al. 2014, Farooq et al. 2019). In order to maintain RS abundance at a concentration compatible with suitable cell metabolism under standard conditions, plants have designed a multi-layer mechanism based on enzymatic and non-enzymatic RS scavenging systems fitting the requirements of specific organelles, tissues and/or organs (Noctor et al. 2016).

As a consequence of their metabolism, plant leaves produce large amounts of RS (Khorobrykh et al. 2020) principally in chloroplasts, mitochondria and peroxisomes (Waszczak et al. 2018). Regarding chloroplasts, reaction of O_2 with reduced forms of electron carriers from the photosynthetic electron transfer chain is the main source of ROS of variable chemical reactivity (Khorobrykh et al. 2020). Superoxide anion $O_2^{\bullet-}$ and hydrogen peroxide H_2O_2 are moderately reactive while singlet oxygen 1O_2 and particularly hydroxyl radical $HO^{\bullet}$ are highly damaging for photosystems I and II, membrane lipids, stromal proteins and chloroplast DNA (Waszczak et al. 2018, Khorobrykh et al. 2020). Superoxide dismutases (SODs) convert $O_2^{\bullet-}$ into H_2O_2 , which is then reduced into water through activities of stromal ascorbate peroxidases (sAPX), glutathione peroxidase-like enzymes and peroxiredoxins (Waszczak et al. 2018). Besides, $HO^{\bullet}$ can be scavenged by ascorbate, tocopherols, glutathione and mannitol present in

the chloroplasts (Shen et al. 1997, Edreva 2005). Finally these enzymatic redox systems and specialised metabolites such as flavonoids, carotenoids and tocopherols scavenge $^1\text{O}_2$ in the chloroplast (Waszczak et al. 2018, Gómez et al. 2019).

Glycosylation regulates the biosynthesis and the subcellular location of several antioxidants such as flavonoids and coumarins (Taguchi et al. 2000, Li et al. 2017). This reaction is catalysed by enzymes from the UDP-glycosyltransferase (UGT) super-family. These enzymes catalyse the glycosylation of a large panel of molecules (Tiwari et al. 2016), impacting on the stoichiometry of upstream and downstream biochemical pathways (Taguchi et al. 2000, Agati et al. 2012, Li et al. 2018). Metabolites that are glycosylated by UGTs generally fit specific cellular and molecular requirements, such as phytohormones to regulate development notably through modulation of gene expression (Goda et al. 2008) or phenylpropanoids (such as flavonoids, coumarins, among others classes) to protect leaf tissues from oxidative damages (Agati et al. 2012). By conjugation of an acceptor to a sugar moiety, UGTs may modify the metabolic flux of the corresponding pathway by releasing substrate-induced feedback inhibition, but also the physicochemical properties of the substrate (such as water solubility or volatility), its biochemical activity (such as reductive power) and its subcellular location (De Bruyn et al. 2015, Behr et al. 2020). UGTs glycosylating flavonoids have been identified in several food crops, such as soybean (*Glycine max*) (Yin et al., 2017b), tea (*Camellia sinensis*) (He, Zhao, et al. 2018) and rice (*Oryza sativa*) (Dong et al. 2020), as well as in the model plant species *Arabidopsis thaliana* (Jones et al. 2003, Yonekura-Sakakibara et al. 2012, Li et al. 2017).

The high number of *UGT* genes makes the comprehension of their roles in planta challenging. For instance 281 protein-coding transcripts have been reported in poplar (Kumar et al. 2019). Some functional and biochemical studies targeting UGTs were previously conducted in poplar, highlighting a role in xenobiotic detoxification (Su et al. 2012), in the

response to fungal pathogen (Veljanovski and Constabel 2013) and more recently in monolignol glucosylation (Speeckaert et al. 2020). More widely, a number of plant UGTs partake in redox homeostasis under various (a)biotic stresses, such as cold temperature, exposure to oxidising agents, salt or high osmotic condition and bacterial infection (reviewed in Behr et al. 2020). Because of their antioxidant power, flavonoids are broadly documented molecules in that context. For instance, the increased glycosylation of quercetin and kaempferol in *Arabidopsis* lines overexpressing *UDP-glucose:flavonoid glucosyltransferase 2 (UGT2)* improves tolerance to salt and osmotic stresses, as well as exposure to H₂O₂ (Li et al. 2018). Similar results were obtained in tea, with silencing *UGT78A14* glycosylating kaempferol and quercetin leading to lower tolerance to cold stress (Zhao et al. 2019). By contrast, there are currently no functional studies performed on forest tree species to investigate the function of UGTs dedicated to specialised metabolites and their involvement in redox status imbalance.

This article highlights the role of the *Populus tremula* x *P. alba* UGT72A2 (orthologous to Potri.007G030500) in leaf redox buffering. *UGT72A2* expression is activated by a MYB transcription factor regulating the expression of flavonoid biosynthesis genes. Transgenic poplar lines with different degrees of *UGT72A2* silencing display various phenotypical traits typical of oxidative damages, suggesting a major partaking role of this UGT in redox homeostasis.

Materials and Methods

Plant material and production of *UGT72A2* silenced lines through RNAi

Hybrid poplar *Populus tremula* x *P. alba* (clone INRA 717-1B4) was used in this study. Silenced *UGT72A2* poplars were produced through targeting a 275 bp region of the coding sequence (between c.70 and c.344 from start codon) with the RNAi method (Figure S1). The

sequence of interest was PCR-amplified (the sequence of all primers used in this study is provided in Table S1) and inserted in the destination vector pK7GWIWG2D(II) (Karimi et al. 2002) using the Gateway technology. Full length cDNA sequence of *UGT72A2* was cloned into pK7WG2D to produce overexpressing lines as described previously (Speeckaert et al. 2020). Poplar transformation and selection (kanamycin) were performed as described in Leple et al. 1992. Transgenic lines were propagated in vitro and transferred to soil in phytotron (photoperiod 16 h : 8 h, light : dark, 22-24 °C, humidity 30-60%, light intensity *ca.* 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Leaf sampling

For comparing WT and silenced lines, leaves were sampled at different positions, according to the phenotype observed in the silenced lines. In these trees, green leaves correspond to expanded (source) leaves located just above the first yellowing leaf (usually the 14th-15th leaf from the apex). Leaves were sampled at the same position in the WT.

For determining the expression of *UGT72A2* in the WT by RT-qPCR, emerging leaves (apical zone, top 5 cm), expanding (sink) leaves (half the size of a mature leaf) and mature leaves (located at the basal third of the stem) were sampled. Expanding (sink) leaves were used to quantify the expression of *UGT72A2* in silenced lines as compared to the WT.

For dot blot, expanding (sink) leaves (half the size of a mature leaf), expanded (source) leaves and old leaves (20 cm from the soil) were pooled from 5-6 different trees.

Chlorophylls and carotenoids content

These pigments were quantified in green leaves, as well as in leaves starting to yellow in the *UGT72A2*-04 line. Fresh leaves were ground with MgCO_3 in liquid nitrogen. Approximately 100 mg were aliquoted with 10 ml acetone 80% in technical triplicate and agitated at 4 °C in

the dark overnight. After centrifugation at 4 °C, pigments were quantified spectrophotometrically by reading A470, A647 and A663 nm using the equations provided in Lichtenthaler (1987).

Lipid peroxidation products and total anthocyanin content

The protocol described by Bresson et al. (2018) was used. Ground frozen green leaf samples (25 mg) were homogenised with 0.5 ml 0.1% trichloroacetic acid (TCA) in technical duplicate. After centrifugation, 0.2 ml of supernatant were mixed either with i. 800 µl of 20% TCA spiked with 0.01% butylated hydroxytoluene, or, ii. with 800 µl of 20% TCA spiked with 0.5% (w/v) thiobarbituric acid. Both fractions were vortexed, incubated at 95 °C for 30 min and cooled quickly in an ice bath. Following centrifugation, A440, A532 and A600 nm were recorded and malondialdehyde equivalents as well as total anthocyanin content were calculated with equations provided by Bresson et al. (2018).

Total flavonoids, total phenolics, proanthocyanidins and lignin contents

Soluble phenolics were extracted from 200 mg of frozen green leaf material using 10 ml of 80% methanol in a 50-ml tube under shaking for 3 h at room temperature, followed by centrifugation. Total phenolics were measured with the Folin-Ciocalteu assay. Briefly, 1 ml of extract was diluted 10 times and allowed to react with 1 ml of Folin-Ciocalteu reagent for 5 min, before addition of 10 mL of 7% (w/v) Na₂CO₃. The total volume was brought to 25 ml with water and allowed to react for 60 min in the dark at room temperature. Total phenolics content was measured with A750 nm with gallic acid as standard (Wang et al. 2017). A374 nm was measured in a 3-fold dilution of the extract to calculate total flavonoids content with the equation provided in Granato et al. 2016.

Soluble and insoluble proanthocyanidins were quantified using a protocol adapted from Gourlay and Constabel (2019). Briefly, 100 mg of ground leaves were extracted twice with 100% methanol in an ultrasonication bath. The pooled methanolic extract (soluble proanthocyanidins) was reacted with the butanol-HCl reagent, using unheated mixture as blank for each reaction. Leaf pellets remaining after methanol extraction (insoluble proanthocyanidins) were dried, weighted and allowed to react with the butanol-HCl reagent. After heating both samples (95°C for 1h), A550 nm was recorded with a plate reader.

Lignin content was measured using the acetyl bromide method (Behr et al. 2018). Cell wall residue was digested in 25% acetyl bromide (in glacial acetic acid) at 55 °C for 135 min using a Hach LT200 system. The solution was then transferred in a tube containing 10 ml of glacial acetic acid and 12 ml of NaOH 2M. The tube was rinsed with glacial acetic acid and 1.75 ml of 0.5 M hydroxylammonium chloride was added. A280 nm was measured to determine lignin content with an extinction coefficient of $20.09 \text{ g}^{-1} \text{ l cm}^{-1}$.

Enzymatic assay with recombinant UGT72A2

Recombinant protein production, purification and enzymatic activities were essentially performed as previously described (Speeckaert et al. 2020). Briefly, full-length coding sequence of *UGT72A2* (GenBank MT181026) cloned into pGEX-4T-1 (GE HealthCare) was transferred into *E. coli* BL21(DE3)pLysS strain (Novagen). Protein production was induced by addition of IPTG following overnight incubation at 18 °C under agitation. After washing, cells were lysed, and the supernatant was applied to a column with GST• Bind resin (Novagen). After washing, proteins were eluted in consecutive fractions and their purity was checked with SDS-PAGE. The enzymatic assay was performed overnight at 30 °C with 5 µg of pure protein using UDP-glucose as sugar donor and cyanidin, naringenin, eriodictyol, quercetin, kaempferol, myricetin, esculetin and scopoletin as sugar acceptors. The reactions were analysed with a LC-UV-MS/MS

system (Agilent 1100 with variable wavelength detector coupled to Bruker Esquire 3000 Plus MS/MS).

Modelling of UGT72A2 3D structure and docking protocols

A BLAST search performed on the Protein Data Bank (PDB) database identified UGT72B1 from *Arabidopsis thaliana* as a potential template for building a UGT72A2 3D structural model. Five 3D models of UGT72A2 were built with Modeller 9v11 (Fiser and Šali 2003) using UGT72B1 complexed with UDP-glucose and trichlorophenol crystal structure as a template (PDB ID: 2VCE; Brazier-Hicks et al. 2007) and the sequence alignment carried out with ClustalW. The structural model having the lowest value of the MODELLER objective function and a reasonable stereochemistry value according to PROCHECK (Laskowski et al. 1993) was prepared using the Protein Preparation Wizard (Schrödinger 2017a, 2017b, 2017c, 2020).

The initial 3D structures of the ligands were generated using the LigPrep module (Schrödinger 2017d). The Epik program was used to predict the different protonation states of all ligands (Schrödinger 2017e). Docking of naringenin, eriodictyol, kaempferol, quercetin, myricetin and cyanidin was carried out in the prepared structural model using Glide (Schrödinger 2021) and performed in a delimited area ($19.6 \text{ \AA} \times 19.6 \text{ \AA} \times 19.6 \text{ \AA}$) based on the location of the bound trichlorophenol in the UGT72B1 structure. At most 10 poses were generated for each molecule. The Glide XP function was chosen to score the docking poses of the ligand.

Glutathione, NADP⁺ and NADPH measurement

The plate method designed by Queval & Noctor (2007) was used to quantify these molecules. Briefly, NADP⁺ and glutathione were measured in acidic extracts, while NADPH was quantified in basic extracts. Acidic and basic extractions consist in homogenisation of 100 mg

of ground frozen material with 1 ml of cold HCl solution (0.2 M) or 1 ml of cold NaOH solution (0.2 M), respectively. Both extractions and subsequent steps were performed in technical triplicates. Quantification of pyridine nucleotides relies on phenazine methosulfate-catalysed reduction of dichlorophenolindophenol. Quantification of glutathione relies on reaction of glutathione thiol group with 5,5'-dithiobis-(2-nitrobenzoic acid) to form 2-nitro-5-thiobenzoate. Concentrations were determined according to standard curves with three concentrations of reduced and oxidised glutathione, NADP⁺ and NADPH.

Soluble peroxidase activity

Soluble peroxidase activity was determined following the protocol described in Bindschedler et al. (2006). Ground leaves (100 mg) were homogenised in 500 µl of Tris-HCl (pH 7.2) containing 5 mM 2-mercaptoethanol and 0.25 M sucrose. After centrifugation, the supernatant was collected. The peroxidase activity was evaluated using ABTS (10 mM in sodium acetate buffer, pH 4.4). To 1 ml of ABTS solution, 50 µl of protein extract were added in technical quadruplicate. A blank test without protein extract was used as reference for each sample. When A405 nm was constant, 1.33 µl of H₂O₂ (0.3%) was added to start the reaction, which was monitored for 2 min. Enzyme activity was calculated using the maximum linear rate and normalised by the quantity of proteins (Bradford).

Immunolocalisation and dot blot

An antibody specific for UGT72A2 was previously produced and characterised to validate its specificity (Speeckaert et al. 2020). Leaf pieces were fixed in a solution of 4% formaldehyde and 0.1% Triton in 50 mM Pipes during 2 h at 4 °C. Leaf sections (100 µm) were incubated overnight at 4 °C with primary antibody diluted at 1% in 0.75x Odyssey blocking buffer (LI-COR Biosciences). Sections were washed thrice in 50 mM Pipes, incubated at 37 °C for 90 min

with anti-rabbit antibody conjugated to AlexaFluor 488 (diluted 1:500, Life Technologies), washed thrice in Pipes and observed with a Zeiss LSM710 confocal microscope. Chloroplast fluorescence was acquired at excitation/detection wavelengths of 594/621-700 nm and secondary antibody at 488/495-573 nm.

Soluble proteins were extracted from ground leaves by precipitation with an ice-cold solution of 20% (w/v) TCA and 0.1% (w/v) DTT in acetone (Behr et al. 2018). Ten volumes of solvent were added to one volume of leaf powder, vortexed and allowed to precipitate overnight at -20°C. After centrifugation, the pellet was washed thrice with ice-cold acetone, allowed to dry at room temperature and resuspended in PBS 0.8x with 2% SDS. Following two centrifugation steps, the supernatant was transferred to a new tube and the protein content was determined with a Bradford assay. A volume of 3 µl of protein extract (corresponding to *ca.* 4 µg) was slowly spotted on a nitrocellulose membrane, which was allowed to dry before immersion in Odyssey blocking buffer for 1 h at room temperature. Primary antibody specific to UGT72A2 was added to the blocking buffer (dilution 1:1000) and incubated overnight at 4°C under soft agitation. The membrane was washed thrice with TBS-Tween 20 (0.05% v/v), pH 7.5, before incubation for 1 h at room temperature with the secondary antibody (LI-COR IRDye 800CW goat anti-rabbit, 1:20000 in blocking buffer + 0.2% Tween 20). The membrane was finally washed thrice with TBS-T and once with PBS 1x to remove residual Tween 20. The signal was quantified with a LI-COR Odyssey imaging system and normalised by the protein input.

Cloning of *PtaMYB119*, *PtaMYB6* and transactivation

Coding sequences of *PtaMYB119* (GenBank MW762689) and *PtaMYB6* (GenBank MZ170960) (orthologous to Potri.017G125600 and Potri.001G005100, respectively) were amplified from young leaf cDNA and cloned with the Zero Blunt TOPO PCR Cloning kit (Life

Technologies). Subsequently both genes were cloned in the vector pK7WG2 (Karimi et al. 2002) by Gateway technology and Sanger-sequenced. These constructs were used as effector. The proximal 1878 bp promoter region of *UGT72A2* (*pUGT72A2*, GenBank MT193261) previously cloned in pKGWFS7 (promoter fused to *GUS* reporter gene, Speeckaert et al. 2020) was used as reporter. Expression clones were mobilised to *Agrobacterium tumefaciens* (strain C58RifPMP90). Agroinfiltration was performed in tobacco leaves with cultures displaying A600 nm around 0.5. A first batch of plants was inoculated with the reporter construct as control, while the second batch was infiltrated with a mix in equal amounts of the reporter and effector constructs (biological quadruplicate). Leaves were harvested two days after transfection, snap frozen in liquid nitrogen, ground and total proteins were extracted and kept on ice. The GUS activity of the extracts was assessed using 4-methylumbelliferyl- β -D-glucuronide (Duchefa Biochemie) as substrate in PBS (2 h at 37 °C) and quantified using the fluorescence of 4-methylumbelliferone (4-MU) at excitation/emission wavelengths of 365/447 nm (Cervera 2005) normalised by the protein content of each extract (Bradford).

Gene expression analysis

Total RNA was extracted from poplar leaves using Agilent Plant RNA Isolation Mini Kit and retrotranscribed with Promega GoScript™ Reverse Transcription Mix Oligo(dT). Gene amplification efficiency (E) was calculated used a serial dilution of pooled cDNA from leaves. qPCR analysis was performed on biological triplicate (in technical triplicate) with Luna Universal qPCR MasterMix (New England BioLabs) and gene specific primers (Table S1) on a LightCycler 480 system (Roche Life Science), complemented by a melt curve to assess the specificity of the amplification. Relative quantities were normalised by the expression of *CYC063* and *CDC2* (Baldacci-Cresp *et al.*, 2016) as reference genes with the $E^{\Delta\Delta C_t}$ method (Hellemans et al. 2007).

Experimental setup and statistical analysis

In each growing period (usually two a year), the poplar lines (6-7 plantlets each) were mixed in the same growing facilities and moved every week to homogenise received light and correct for possible differences in temperature. Trees with comparable development stage and phenotype of oxidative damages were used for the analyses. We performed either Student *t*-test or ANOVA with Bonferroni post-hoc test to assess the significance of the results. Unless stated otherwise, all experiments were performed with five biological replicates for each poplar line.

Results

Production of transgenic poplar lines downregulated for *UGT72A2* and global phenotype

A functional characterisation of *UGT72A2* was conducted using transgenic poplar lines carrying a RNAi construct designed to silence *UGT72A2*. A preliminary RT-qPCR screening performed on 30 independent in vitro transformants evidenced 12 downregulated lines, from which six were selected, propagated in vitro and transferred to soil in phytotron for further characterisation (Figure 1). In the WT, *UGT72A2* expression decreases from apical leaves, exhibiting the highest expression, to old leaves located at the basal third of the stem (Figure 1a). We therefore determined the expression of *UGT72A2* in RNAi lines in expanding (sink) leaves, after 12 weeks of growth (Figure 1b). All lines displayed a substantial reduction of gene expression (residual expression between 21% and 75%) as compared to the WT. A dot blot with a specific polyclonal antibody was also performed, highlighting a decreased abundance of *UGT72A2* in the sink leaves of the RNAi line (Figure S2). The three lines that showed the lowest *UGT72A2* expression level (Figure 1b), including *UGT72A2*-04, *UGT72A2*-48 and *UGT72A2*-50, were selected for functional analysis.

After three months of growth, the most striking feature of the *UGT72A2* silenced lines was a leaf yellowing and necrosis (Figure 1d,e) under standard growing conditions. This phenotype appeared around the 14th leaf from the stem apex. At first, a yellowing was observed in areas of the leaf blade around the main vein, followed by the appearance of a brown coloration spreading to leaf margins and then leaf abscission (Figure 1e). The whole process lasted between one to two weeks. It is noteworthy that the detection of the first yellowing leaf depends on the level of *UGT72A2* residual expression. It was first detected in 8 to 9-week-old *UGT72A2*-04 line (21% residual expression), then two to three weeks later in *UGT72A2*-48 (45% residual expression) and *UGT72A2*-50 (53% residual expression). *UGT72A2*-67, with the highest residual expression level (75% of WT), did not show any leaf symptoms, as noticed for WT. As expected, total chlorophylls and carotenoids content decreased as the leaf turns to yellow, but significantly lower concentrations were already recorded in the green leaves above the yellowing ones of the *UGT72A2*-04 line, as compared to leaves of the WT sampled at the same developmental stage (Figure 1c). Altogether, these data demonstrate that this phenotype is a consequence of *UGT72A2* silencing.

The leaf phenotype was restricted to the middle part of the stem, while leaves located in the top and bottom thirds were not affected. We may hypothesise that the observed oxidative damages result from an imbalance between pro- and antioxidants. Expression of *UGT72A2* being negligible in WT basal leaves (Figure 1a), it is likely that this gene is not involved in protection against oxidative damages in these leaves. Instead, other photoprotection mechanisms are activated (Juvany et al. 2013). Middle mature (source) leaves moderately express *UGT72A2* and they have higher metabolic and photosynthetic activities so possibly more ROS are generated than in mature leaves, and silencing *UGT72A2* causes the phenotype. Finally, emerging and expanding leaves are protected against oxidative damages by antioxidant

enzymes and molecules such as anthocyanins, carotenoids and xanthophyll (Juvany et al. 2013), possibly preventing oxidative damages in relation with silencing of *UGT72A2*.

Consistent with premature leaf yellowing and abscission, height and stem diameter of *UGT72A2*-04 and *UGT72A2*-48 lines were significantly reduced as compared to the WT (Figure 1b). Leaf sampling for following experiments was performed when the three transgenic lines displayed yellowing leaves (typically 3 months after transfer to soil).

Phenolic profiles are altered in *UGT72A2* silenced poplar lines

We next investigated whether the silencing of *UGT72A2* was associated with an alteration in flavonoids and total phenolics contents. Flavonoids include flavanones, isoflavones, flavones, flavan-3-ols, flavonols (such as quercetin, kaempferol and myricetin), anthocyanidins (such as cyanidin) and anthocyanins. The contents in total anthocyanin and total flavonoids were significantly decreased in transgenic lines (Figure 2a-b), as well as total soluble phenolics (Figure 2c). However, a significant increase in both soluble and insoluble proanthocyanidins (resulting from polymerisation of flavan-3-ols) was noted in *UGT72A2*-04 and *UGT72A2*-48 (Figure 2e-f). Lignin was similar in all lines, with silenced poplars displaying a slightly lower content (Figure 2d).

UGT72A2 silencing is associated to oxidative damages

We quantified lipid peroxidation products (Figure 3a) and glutathione (Figure 3b) as indicators of oxidative stress. All three transgenic lines were characterised by a higher accumulation of malondialdehyde equivalents and glutathione in green leaves. This trend was more pronounced in *UGT72A2*-04, displaying the lowest residual *UGT72A2* expression level. The peroxidation of lipids from cell membrane systems (i.e., chloroplasts, mitochondria and plasma membranes) may result from the imbalance in the redox system in the transgenic lines.

Imbalance in redox scavenging systems in *UGT72A2* silenced poplar lines

In addition to phenolics, several enzymes and electron donors regulate cellular redox homeostasis. Therefore, we investigated whether some of these redox mechanisms were affected in transgenic lines, accounting for their phenotypical and physiological traits.

Soluble peroxidases reduce cellular H_2O_2 to limit the deleterious effects of this oxidant molecule (Smirnoff and Arnaud 2019). Soluble peroxidase activity was reduced by a factor of 3 in *UGT72A2*-04, with similar decreases in *UGT72A2*-48 and *UGT72A2*-50 (Figure 4a), possibly leading to a higher H_2O_2 accumulation in transgenic lines.

NADPH is an electron donor, which is notably used in the chloroplasts within the ascorbate peroxidase system (Smirnoff and Arnaud 2019). The observed diminution in the NADPH to $NADP^+$ ratio in *UGT72A2*-04 and *UGT72A2*-48 (Figure 4b) may therefore indicate impaired reduction of $NADP^+$ to NADPH, preventing efficient RS enzymatic removal with ascorbate- and glutathione-dependent scavenging systems.

Silencing *UGT72A2* improves tolerance to methyl viologen

Considering the oxidative damages and the impairment of several redox homeostasis systems reported in RNAi-*UGT72A2* lines, we hypothesize a higher sensitivity to exogenous oxidative stress. We therefore challenged leaves from the three transgenic lines with methyl viologen (50 μM), a xenobiotic enhancing the production of superoxide ion in the chloroplasts, and observed their tolerance as compared to WT through monitoring the maximum quantum yield of photosystem II (F_v/F_m) after 6h and 24h exposure. Unexpectedly, *UGT72A2*-04 line was the most tolerant to the herbicide, followed by *UGT72A2*-48 (Figure 5). A twenty-four-hour exposure slightly decreased the F_v/F_m of *UGT72A2*-04 (from 0.81 to 0.69), while the WT was drastically impacted, from 0.82 to 0.14.

Cis-elements related to putative functions of UGT72A2 in chloroplast

We previously demonstrated using stable transgenic poplar lines expressing a 35S-UGT72A2-eGFP construct that UGT72A2 localises in chloroplasts (Speeckaert et al. 2020). The presence of a transmembrane alpha helix domain (DLFGSEAMMVAD) may explain this localisation, consistent with the prediction of the BUSCA server (Savojardo et al. 2018) suggesting an allocation within organelle membranes. Such a domain was evidenced in REDOX-ACTIVE RUBREDOXIN 1, a protein with an essential role in photosystem II assembly and repair (García-Cerdán et al. 2019).

We investigated the presence of *cis*-elements related to photomorphogenesis, chloroplast biogenesis and flavonoid biosynthesis (Hartmann et al. 2005, Lee et al. 2007, Pfeiffer et al. 2014, Yuan et al. 2017, Behr et al. 2019, Wang et al. 2019) in *pUGT72A2*. Overall, 29 non-overlapping GT-1 elements, 1 Phytochrome-Interacting bHLH transcription Factors (PIF) binding element (PBE) box, 4 ACGT containing element (ACE), 4 G-box elements and 7 GATA boxes were retrieved. Three AC-motifs related to the MYB transcriptional activation of flavonoid biosynthesis were also detected (Table S2). The presence of these *cis*-elements, as well as the chloroplastic subcellular location of UGT72A2, suggest that this enzyme is part of the molecular network regulating chloroplast development and/or metabolism.

Expression of *UGT72A2* is regulated by PtaMYB119

We investigated whether MYB transcription factors involved in phenylpropanoid / flavonoid biosynthesis were able to regulate the expression of *UGT72A2*. In poplar, MYB119 is known to induce the expression of flavonoid/anthocyanin biosynthesis genes (Cho et al. 2016), while MYB6 partakes in the tuning of flavonoid and monolignol biosynthesis (Wang et al. 2019). We therefore used PtaMYB119 and PtaMYB6 to explore the transcriptional regulation of

UGT72A2 using a transactivation assay. The result indicates that the expression of UGT72A2 is induced by PtaMYB119 but not modified by PtaMYB6 (Figure 6).

Phylogeny and molecular docking suggest UGT72A2 flavonoid glycosylation

The phenotype of the RNAi lines and the chloroplastic location of UGT72A2 prompted us to determine its possible substrates. In order to narrow this investigation, we performed a phylogenetic analysis of UGT72s from several species whose in vitro substrates are already known (Figure 7). This analysis highlights the presence of five groups with high branch support value (> 95%). UGT72A2 was found within group II, together with UGT72AM1 from *C. sinensis*, UGT72AZ2 from *P. tremula* x *P. alba*, UGT72C1 and UGT72D1 from *Arabidopsis* and UGT72V3 from *Lotus japonicus*. CsUGT72AM1 glycosylates coniferaldehyde, cyanidin, eriodictyol, kaempferol, myricetin, naringenin and quercetin (Zhao et al. 2017). PtaUGT72AZ2 glycosylates ferulic acid and sinapic acid (Speeckaert et al. 2020). AtUGT72C1 glycosylates esculetin and scopoletin while AtUGT72D1 glycosylates catechin, dihydroquercetin, esculetin, kaempferol, luteolin, quercetin and scopoletin (Yang et al. 2018). Finally, LjUGT72V3 glycosylates daidzein, genistein, kaempferol, lutiolin, myricetin and quercetin (Yin, Shen, Chang, et al. 2017).

The 3D structure of UGT72A2 would be most helpful in understanding the determinants of its substrate specificity. In the absence of such 3D data, five 3D modelled structures of UGT72A2 were built by comparative modelling using UGT72B1 from *A. thaliana* in complex with UDP-2-deoxy-2-fluoro glucose (UDP-2FGlc) and trichlorophenol (TCP) as a template and the two most representative were used for docking analysis. Some of the in vitro substrates previously described, including naringenin, eriodictyol, kaempferol, quercetin, myricetin and cyanidin, were docked in a pocket whose location was defined on the basis of the binding site of TCP in the UGT72B1 structure.

In the first model, the ranking order is as follows: myricetin>quercetin>eriodictyol>cyanidin>kaempferol>naringenin with myricetin showing the most favourable score (Figure S3). In the second model, the ranking order is maintained for the compounds except for cyanidin which comes first (Figure S3). In both models, the comparison of strong (myricetin or cyanidin) versus weak (naringenin) predicted binders indicates that electrostatic and hydrogen bond contributions favour the binding of myricetin/cyanidin over that of naringenin whereas the van der Waals energies does the opposite. The hydrophobic contribution is similar for both ligands.

Docking calculations performed on the first structural model of UGT72A2 (Figure S3) particularly support the possibility that the enzyme is capable of catalysing the glycosylation of flavonoids. Several features from the analysis of the docked positions of all 6 ligands in the catalytic site point to a possible transfer of glucose to the acceptor flavonoid. One is the location of one attacking hydroxyl oxygen at a distance of about 4 Å from the anomeric C1 carbon of glucose, positioned for nucleophilic attack (Figure 8 and Figure S3). Additionally, the His21 residue, known to act as a Brønsted base, hydrogen bonds with a hydroxyl oxygen of the flavonoid molecule and could proceed to proton abstraction to generate a stronger nucleophile (Teze et al. 2021). π - π interaction is formed between the aromatic ring of Phe120 and the A-ring of all six tested flavonoids (Figure 8b,c and Figure S3). In the best-score pose, the UDP-Glc glucose moiety hydrogen bonds to Gln395 (Figure 8c).

The corresponding residues to His21, Phe120 and Gln395 are His19, Phe119 and Gln389 in UGT72B1 (Brazier-Hicks et al. 2007). In UGT72B1, His19 acts as a Brønsted base to deprotonate the sugar acceptor and the H19A mutant loses the glucosylating activity; Phe119 is part of the hydrophobic site enclosing TCP; Gln389 interacts with the glucose moiety (Brazier-Hicks et al. 2007). Asp118 (corresponding to Asp117 in UGT72B1) that is part of a reminiscent catalytic triad together with His21, although not observed to interact directly with

tested flavonoids, is positioned close to His21 such that it could stabilise this residue and neutralise its charge after deprotonating the sugar acceptor. Accordingly, the D117A mutation in UGT72B1 suppresses the glucosylating activity (Brazier-Hicks et al. 2007). These four residues critical for glucosylating activity are also conserved in UGT72AM1 (Figure S4), glucosylating myricetin and quercetin among other flavonoids (Zhao et al. 2017), further pointing flavonoids as potential substrates of UGT72A2.

Overall, predictions of molecular docking with one of the two modelled structures are consistent with UGT72A2 glycosylating flavonoids, as highlighted by their interactions with amino acid residues crucial for enzymatic activity.

In light of the predictions from phylogeny and molecular docking, we investigated whether recombinant UGT72A2 glucosylates in vitro cyanidin, naringenin, eriodictyol, quercetin, kaempferol, myricetin, esculetin and scopoletin. Unfortunately, in our experimental conditions, UGT72A2 does not show any measurable enzymatic activity towards these molecules.

Discussion

This article depicts the functional role of *UGT72A2* in the context of the maintenance of redox status in poplar. By silencing the expression of *UGT72A2* at variable degrees, we demonstrate that this gene has a role in the protection of leaves against oxidative damages under standard growing conditions. Phylogenetic analysis of UGT72s from various species (Figure 7) and the prediction from molecular docking (Figure S3) suggest that UGT72A2 glycosylates flavonoids.

UGT72A2 is connected to the flavonoid biosynthesis pathway

We detected a significant decrease of the anthocyanin content in silenced *UGT72A2* lines (Figure 2). Comparable results were obtained in the *ugt78d1 ugt78d2 Arabidopsis* mutant, which is deficient in flavonol glycosylation (Yin et al. 2012), implying that glycosylation reaction is a major regulatory step for the biosynthesis of flavonoids in both herbaceous and woody species. Similarly, overexpression in poplar of *UGT84A17* glycosylating phenolic acids results in higher accumulation of (hydroxyl) cinnamate/benzoate glucose esters as compared to the WT (Babst et al. 2014), further pointing to glycosylation-mediated modulation of specialised metabolism.

Flavonoid biosynthesis is partly controlled at the transcriptional level (Cho et al. 2016). This regulation relies on the presence of AC-motifs activated by MYBs in *pUGT72A2* (Table S2). Accordingly, we demonstrated that PtaMYB119 activates the expression of *UGT72A2* (Figure 6). As *UGT72A2*, poplar *MYB119* is preferentially expressed in young expanding leaves (Sundell et al. 2015), suggesting that this transcription factor induces the expression of *UGT72A2 in planta*. In *P. trichocarpa*, overexpression of *MYB119* activates the expression of genes from the anthocyanin biosynthesis pathway, comprising *UFGT1* from the *UGT78* family (Cho et al. 2016). In addition, PtaMYB119 displays two motifs conserved in anthocyanin-promoting MYBs (Cho et al. 2016), ANDV and KPRPWRF. On the opposite, PtaMYB6 was not able to activate the expression of *UGT72A2*. These two MYBs do not belong to the same phylogenetic clade (Wang et al. 2019) and their overexpression does not lead to the same phenotype. Poplar overexpressing *MYB6* strongly accumulate anthocyanins and proanthocyanidins at the cost of lower lignification and secondary cell wall formation. Leaves of poplar overexpressing *MYB119* turn violet due to accumulation of cyanidin-3-*O*-glucoside while no repression of the secondary cell wall *CELLULOSE SYNTHASE 4* was observed. Finally, PtrMYB119 positively regulates the transcription of *CHALCONE SYNTHASE 1* and

two *ANTHOCYANIDIN SYNTHASES* in a transactivation assay (Cho et al. 2016). The PtaMYB119-driven transactivation of *UGT72A2* therefore suggests a role in the biosynthesis of anthocyanins.

Flavonoids to protect plant cells against oxidative damages

The potential of flavonoids to protect plant cells from oxidative damages is long-time acknowledged (Landry et al. 1995). Dihydroxy B-ring substituted flavonoids such as quercetin and luteolin efficiently scavenge $^1\text{O}_2$ in the chloroplastic compartment (Agati et al. 2007, 2013). We may suggest that silencing *UGT72A2* induces a disturbed balance between the aglycone and the glycosylated forms of some flavonoids, such as anthocyanins. Two hypotheses may be raised to explain how this perturbation leads to oxidative damages observed in silenced lines. First, impaired glycosylation of the substrate(s) of *UGT72A2* leads to accumulation of a toxic aglycone molecule, causing the phenotype of leaf necrosis. For instance, (-)-catechin induces the production of ROS in *Arabidopsis* roots, which may cause the death of the root system (Bais et al. 2003). In *Arabidopsis*, *trans*-chalcone inhibits root growth and upregulates several genes associated with oxidative stress (Díaz-Tielas et al. 2019). However, flavonoid biosynthesis is under strong regulation at the transcriptional and post-transcriptional levels. Over-accumulation of flavonoid aglycone feedback-represses the expression and/or the activity of upstream genes and proteins, as demonstrated with phenylalanine ammonia-lyase (Zhang and Liu 2015), implying that plants are unlikely to accumulate toxic aglycones at concentrations detrimental to their life cycle. In the second hypothesis, the perturbation in the aglycone/glycosylated ratio may repress the global biosynthesis pathway, as illustrated by the lower total flavonoids content measured in silenced *UGT72A2* lines (Figure 2) and in accordance with the documented feedback repression of phenylalanine ammonia-lyase. Furthermore, poplar lines overexpressing

UGT72A2 showed the opposite trend, with an increased accumulation of total phenolics and total flavonoids (Figure S5).

The importance of flavonoid subcellular localisation regarding the regulation of their biosynthesis has also been documented. In *Arabidopsis*, anthocyanins are transported in vitro by MULTIDRUG AND TOXIN EXTRUSION TRANSPORTER 2 (MATE2), a tonoplast protein also mediating the vacuolar uptake of other flavonoid glycosides (Zhao et al. 2011). Loss of MATE2 decreases the level of flavonoid aglycones and glycosides in leaves, possibly through feedback into the biosynthesis pathway or turnover of incorrectly stored molecules (Zhao et al. 2011). Relatedly, ratios between scopoletin, quercetin, kaempferol, cyanidin and their corresponding glycosides partially regulate the biosynthesis of these phenylpropanoids because the translocation of glycosides in the vacuole derepresses their corresponding biosynthesis pathway in the cytoplasm (Taguchi et al. 2000, Gachon et al. 2004, Li et al. 2017, 2018). Such glycosylation and differential compartmentalisation may originate from exogenous factors. For instance, cold, salt and drought stresses induce the expression of *UGT79B2* and *UGT79B3* (Li et al. 2017). Corresponding overexpressing *Arabidopsis* lines were found to be more tolerant to these stresses through glycosylation of cyanidin and cyanidin 3-*O*-glucoside. The opposite phenotype was noted in lines with reduced (RNAi) or null (CRISPR-Cas9) expression of these genes. Similar results are obtained by manipulating the expression of transcription factors regulating the biosynthesis of specific families of specialized metabolites: accumulation of flavonols and anthocyanins resulting from overexpression of *MYB75/PAP1* (orthologous to poplar *MYB119*) in *Arabidopsis* enhances tolerance to drought stress and exposure to methyl viologen (Nakabayashi et al. 2014). Boosting the soluble proanthocyanidins content by overexpression of poplar *MYB115* and *MYB134* protects poplar leaves from methyl viologen-induced oxidative damages (Gourlay and Constabel 2019), consistent with our data (Figures 2 and 5). Altogether, these data support the hypothesis of flavonoid deficiency causing

the phenotype of oxidative damages in transgenic lines with low *UGT72A2* expression. Oxidative stress may induce the biosynthesis of proanthocyanidins at the expense of other flavonoids, in line with the conclusion of Gourlay and Constabel (2019) pointing these metabolites as an adaptive response to oxidative stress.

Exogenous application of various flavonoids upregulates gene expression and protein activity of several antioxidant enzymes. For instance, naringenin protects chloroplasts from oxidative damages through activation of chloroplastic H₂O₂ scavenging mechanisms, including superoxide dismutase, peroxidase, and glutathione S-transferase activities (Yildiztugay et al. 2020). This upregulation of the antioxidant machinery resulted in higher tolerance to salt and PEG-induced osmotic stresses in bean leaves, as measured by malondialdehyde equivalents accumulation (Yildiztugay et al. 2020, Ozfidan-Konakci et al. 2020). Exposure of tobacco seedlings to quercetin and epicatechin positively impacts on the activity of APX and SOD (Mahajan and Yadav 2013). The observed decrease in soluble peroxidase activity in silenced *UGT72A2* lines (Figure 4) might therefore be partially caused by the modified flavonoid homeostasis in these lines. Elucidating the substrate of *UGT72A2* will allow to experimentally challenge this hypothesis and will bring new insights in the regulation of the redox maintenance system in poplar and other species.

Silencing *UGT72A2* results *per se* in oxidative damages

Unlike our transgenic lines, plants with decreased expression of *UGTs* involved in redox homeostasis usually require challenging conditions to display an oxidative stress phenotype. For instance, in maize, two knock-out mutants in *UFGT2* exhibit H₂O₂ content similar to the WT under standard conditions. When exposed to 300 mM NaCl, 300 μ M H₂O₂ or 20% PEG, both *ufgt2* mutants accumulate significantly higher H₂O₂ amounts than the WT (Li et al. 2018). Rice lines knocked-out for *UGT90A1* displayed similar electrolyte leakage as the WT at 22 °C,

but higher plasma membrane degradation was recorded after a chilling treatment at 4 °C for 4 days (Shi et al. 2020). Oxidative damages were also reported after pathogen infections in *Arabidopsis ugt73b3* and *ugt73b5* mutants and in transgenic tobacco lines with down-regulation of two *UGT73* genes (Chong et al. 2002, Simon et al. 2014). This phenotypic difference may originate from the chloroplastic localisation of UGT72A2 (Speeckaert et al. 2020 and Figure S6), in contrast with most other glycosyltransferases mainly found within the Golgi, the cytosol, the ER and plasma membrane (Lao et al. 2014), such as UGT90A1 detected in the cytosol, plasma membrane and tonoplast but absent from chloroplasts (Shi et al. 2020). The chloroplastic localisation of UGT72A2 suggests a role in redox balance in this compartment producing relatively high amounts of ROS as compared to other organelles hosting glycosyltransferases.

Leaves from *UGT72A2*-silenced lines show higher lipid peroxidation and glutathione content (Figure 3) and lower amount of total chlorophyll and carotenoids (Figure 1c). Lipid peroxidation is a hallmark of membrane degradation during oxidative stress in plants, causing further oxidative damages if it remains at a high level for a sustained period (Bresson et al., 2018; Morales & Munné-Bosch, 2019). Accumulation of total and oxidised glutathione is a suitable marker of ROS accumulation (Noctor et al. 2016). Chlorophyll degradation has been monitored in several species facing oxidative stress, such as pea (Szafrńska et al. 2017), *Arabidopsis* (Cui et al. 2019) and poplar (Ding et al. 2018), possibly through chlorophagy (Nakamura and Izumi 2018). Carotenoids prevent the accumulation of $^1\text{O}_2$ and are part of a redox signalling pathway aiming at preventing oxidative damages (Noctor et al. 2016, 2018). The phenotype observed in silenced *UGT72A2* lines is probably not a direct effect of gene silencing but rather results from the RS imbalance in leaves caused by the reduction of total flavonoids accumulation and impairment of ROS scavenging systems, as further explained in the next paragraph.

UGT72A2-silenced lines showed lower NADPH to NADP⁺ ratio (Figure 4), which may result from a deficient NADP⁺ reduction. NADPH is involved in the regeneration of oxidised ascorbate and glutathione pools, it reduces cytosolic and mitochondrial thioredoxins, contributes to maintain high photosynthetic performance and partakes in detoxification of lipid peroxides (Noctor 2006, Gómez et al. 2019). Backed to this redox couple, most of the enzymes active in the phenolic/peroxidase/ascorbic acid/glutathione system were functionally characterised in various plant species. In chloroplast, the glutathione redox cycle, relying on the activity of glutathione reductase, is a powerful mechanism to balance ROS and maintain efficient photosynthesis (Foyer et al. 1995). The removal of H₂O₂ by poplar thylakoid APX (tAPX) supports chloroplast development, at least during callus development (Li et al. 2021). The decrease in soluble peroxidase activity observed in silenced lines might therefore explain the leaf yellowing and the decrease in chlorophylls and carotenoids by net degradation of photosynthetic systems.

Transcriptional regulation of *UGT72A2* suggests a role in leaf core metabolism

Glycosylation of several classes of molecules, such as phenylpropanoids, occurs in plants facing adverse conditions (Le Roy et al. 2016). Accordingly, several *UGTs* have been highlighted to be induced in the framework of plant response to oxidative stress (reviewed by Behr et al. 2020). For instance, genes such as *UGT73B4*, *UGT73B5*, *UGT73C4*, *UGT74E1* and *UGT74E2* are upregulated in *cat2* mutant grown under conditions favouring oxidative stress (He et al. 2018a). *UGT74E2* is also upregulated by osmotic, oxidative, ultraviolet-B and salt stresses (Tognetti et al. 2010). Tea *UGT78A14* and *UGT91Q2* are induced by cold and glycosylate kaempferol and nerolidol, respectively (Zhao et al. 2019, 2020). We investigated in published datasets whether the leaf expression of *UGT72A2* and corresponding homologs in different poplar species is associated to such abiotic factors. Cossu and colleagues provide an expression value of 63.4

RPKM in unstressed poplar fully expanded leaves (6-8 internodes from apex), sensibly higher to the median expression of genes expressed in leaves (1.5 RPKM), thus *UGT72A2* is constitutively expressed at a rather high level in leaves (Cossu et al. 2014). Exposure of poplar to enhanced ozone concentration does not significantly modify the expression of *UGT72A2* (Zhang et al. 2019), suggesting that oxidative stress does not affect its transcription. Exposure to methyl jasmonate, salicylic acid (Luo et al. 2019), moderate and severe drought (Cossu et al. 2014), salt (Filichkin et al. 2018), heat (Ren et al. 2019) and short-term and prolonged cold (Filichkin et al. 2018) significantly repress the expression of *UGT72A2*, most often with fold-changes > 5. These comparisons suggest that *UGT72A2* is not stress-inducible in leaves. Instead, its transcriptional regulation by PtaMYB119 (Figure 6), as well as the strong phenotype of the RNAi lines (Figure 1) and the presence of *cis*-elements related to fundamental biological processes, i.e. photomorphogenesis and chloroplast biogenesis (Table S2), indicate that *UGT72A2* is a core enzyme of cell redox homeostasis in poplar.

Collectively, several elements link the phenotype of silenced lines and the role of *UGT72A2* in leaf redox homeostasis together. *UGT72A2* possibly regulates the metabolic flux to the biosynthesis of flavonoids. Flavonoids such as quercetin, kaempferol and anthocyanins act as a reservoir of electron donors which are able to scavenge H₂O₂ in a peroxidase-dependent mechanism (Yamasaki et al. 1997, Ferreres et al. 2011). Resulting flavonoid radicals are then reduced back by ascorbic acid / glutathione cycle in a reactional cascade involving NADPH as final reducing agent (Ferreres et al. 2011, Waszczak et al. 2018, Dumont and Rivoal 2019). Lower total flavonoids content coupled to lower soluble peroxidase activity and NADPH to NADP⁺ ratio in the *RNAi-UGT72A2* lines may therefore reasonably account for the observed leaf phenotype. The regulation of *UGT72A2* by MYB119 suggests that this gene is related to flavonoid biosynthesis, pushing to determine the substrate(s) of this enzyme.

In conclusion, we emphasize that a complex biochemical and developmental landscape takes place to account for the occurrence of the leaf phenotype at a precise developmental stage and in specific leaves. Since no investigations were performed to precisely explain this phenotype, this question remains open for future hypotheses and experiments.

Data and Materials Availability

The data that supports the findings of this study are available in the supplementary material of this article.

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Authors' Contributions

Study conception and design: MBe, MEJ, MBa. Methodology implementation: MBe, EK, MP, JR, WS, MBa. Experiment execution: MBe, NS, EK, MP, OM, AM, NMA. Data analysis/interpretation: MBe, NS, EK, WS, MEJ, MBa. Manuscript writing/revision: all authors.

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Figure 1 Global phenotype of *RNAi-UGT72A2*-transformed *Populus tremula* x *P. alba* lines aged of 3 months, as compared to the WT. (a), Relative expression of *UGT72A2* in emerging, expanding and mature leaves (WT). Data are mean $\pm$ SE, n=3. Different letters indicate significant differences with ANOVA ($P < 0.01$) and Bonferroni post-hoc test. (b), characteristics of *RNAi-UGT72A2* poplar lines, data are mean (SE). Stem diameter was measured 5 cm above the ground level. *t*-test significant at $P < 0.05^*$, 0.01^{**} and 0.001^{***} as compared to the WT; n.d., not determined. (c), concentration of photosynthetic pigments: chlorophylls and carotenoids content ($\mu\text{g mg}^{-1}$ FW) measured in green (GL) and yellowing leaves (YL) of UGT72A2-04 as compared to WT (mean $\pm$ SE, n=5). Different letters indicate differences significant at $P < 0.05$ (Bonferroni-Holm posthoc test). (d), general aspect of the UGT72A2-04 and UGT72A2-48 lines. This phenotype has been monitored in 5 independent experimental repeats. Scale bar: 20 cm. (e), representative progression of the leaf phenotype in *RNAi-UGT72A2* poplar lines, here illustrated with UGT72A2-04, starting with yellowing (left picture) followed by appearance of yellow patches and necrotic lesions, before abscission (right picture). Scale bar: 5 cm.

Figure 2 Phenolic profile of green leaves of RNAi-UGT72A2-transformed *Populus tremula* x *P. alba* lines and WT (3 months). (a), total anthocyanin content (nmol ml⁻¹). (b), total flavonoids (expressed as mg Quercetin Equivalent g⁻¹ FW); (c), total phenolics (expressed as mg Gallic Acid Equivalent g⁻¹ FW). (d), lignin content (% cell wall residue); (e) soluble proanthocyanidins (% WT); (f) insoluble proanthocyanidins (% WT). Data are mean $\pm$ SE, n=5. *t*-test significant at $P < 0.05^*$, 0.01^{**} and 0.001^{***} as compared to the WT.

Figure 3 Markers of oxidative stress in *RNAi-UGT72A2 Populus tremula* x *P. alba* lines and WT (3 months). Lipid peroxidation expressed as malondialdehyde (MDA) equivalents (nmol ml⁻¹) (a) and reduced (white) and oxidised (black) forms of glutathione (pmol ml⁻¹) (b). Green leaves were analysed for three RNAi lines. Data are mean $\pm$ SE, n=5. *t*-test significant at *P* <0.05* and 0.001*** as compared to the WT.

Figure 4 Antioxidant systems in green leaves of *RNAi-UGT72A2*-transformed *Populus tremula* x *P. alba* lines and WT (3 months). (a), soluble peroxidase activity, expressed as mmol of oxidised ABTS μg^{-1} soluble protein extract. (b), NADPH : NADP⁺ ratio. Data are mean $\pm$ SE, n=5. *t*-test significant at *P* <0.05* as compared to the WT.

Figure 5 Maximum quantum yield of photosystem II during exposure of leaves from *RNAi-UGT72A2*-transformed *Populus tremula* x *P. alba* lines and WT to methyl viologen (50 μM). Petiole was plunged into a methyl viologen solution at T=0h and F_v / F_m was measured using a non-destructive method (Handy PEA, Hansatech) 6 and 24 hours after the beginning of exposure. Leaves were continuously lighted during the experiment. Data are mean $\pm$ SE, n=10.

At 6h and 24h, different letters indicate differences significant at $p < 0.05$ (Bonferroni-Holm posthoc test).

Figure 6 Transactivation of *pUGT72A2*-driven *GUS* reporter gene by PtaMYB119 and PtaMYB6. The *GUS* activity in tobacco (*Nicotiana benthamiana*) leaves transfected with the reporter construct only was used as control and set to 1. *t*-test significant at $P < 0.001$ for PtaMYB119. Data are means of biological quadruplicate $\pm$ SE.

Figure 7 Phylogenetic analysis of UGT72s. This tree was generated online (phylogeny.fr) by the approximate likelihood-ratio test method (Anisimova and Gascuel 2006, Dereeper et al. 2008). UGT72A2 (group II) is indicated in blue. For the sake of clarity, the originated species of each UGT72 is indicated: At *Arabidopsis thaliana*, Lj *Lotus japonicus*, Glyma / Gm *Glycine max*, Pta *Populus tremula* x *P. alba*, Pt *P. tomentosa*, Cs *Camellia sinensis*, Mt *Medicago*

truncatula, SI *Solanum lycopersicum*. Scale bar: expected numbers of amino acid substitutions per site.

Figure 8 Molecular docking of myricetin in a 3D structural model of *Populus tremula* x *P. alba* UGT72A2. (a), representation of a 3D structural model of UGT72A2 in complex with UDP-Glc and myricetin. The protein is described as a purple ribbon. UDP-Glc is depicted as atom colour type sticks. The best-score docked structure of myricetin is shown in the active site region. (b, c), close-up view on the best-score docked position of myricetin (in turquoise) in the binding site of a 3D structural model of UGT72A2 in complex with UDP-Glc. Myricetin is represented as a molecular surface (b) or as sticks (c). It forms a hydrogen bond with His21 (yellow dashed line) and a π - π interaction with Phe120 (cyan dashed line), respectively. Glc hydrogen bonds to Gln395.