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Mechanisms of leaf and fine root senescence in black cottonwood (*Populus trichocarpa* Torr. & Gray)

Mechanizmy procesu starzenia liści i korzeni chłonnych topoli kalifornijskiej (*Populus trichocarpa* Torr. & Gray)

Praca doktorska wykonana pod kierunkiem

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SPIS TREŚCI

STRESZCZENIE ROZPRAWY DOKTORSKIEJ

Proces starzenia stanowi końcowy etap w rozwoju ontogenetycznym roślin, bezpośrednio poprzedzający śmierć wybranej grupy komórek, tkanek, organów lub całych organizmów roślinnych. Pomimo, iż jest to proces destrukcyjny, postępuje w sposób uporządkowany i wysoce regulowany. Uznawany jest zazwyczaj za przejaw programowanej śmierci komórki (PCD), obejmujący szereg mechanizmów prowadzących do śmierci komórek w następstwie degradacji ich protoplastu. Znaczenie PCD dla przebiegu procesu starzenia zostało potwierdzone w liściach i płatkach kwiatów, jednakże mechanizmy regulujące starzenie korzeni chłonnych – organów równie efemerycznych jak liście bądź płatki korony, były dotąd nieznane. Formowanie korzeni chłonnych zachodzi szczególnie w okresach silnego zapotrzebowania na wodę i związki mineralne z gleby, tj. na początku sezonu wegetacyjnego oraz podczas intensywnego wzrostu. Jesienią, gdy zapotrzebowanie na te związki maleje, większość korzeni starzeje się i zamiera.

Nadrzędnym celem pracy doktorskiej było poszerzenie wiedzy dotyczącej starzenia i zamierania korzeni chłonnych. Sformułowano hipotezę główną, zakładającą istnienie analogii w przebiegu procesu starzenia liści i korzeni chłonnych poprzez wykształcenie wspólnych mechanizmów regulujących ten proces. Materiał do badań stanowiły liście i korzenie chłonne topoli kalifornijskiej (*Populus trichocarpa*), uprawianej w systemie ryzotronów. System ten umożliwiał obserwację wzrostu i funkcjonowania korzeni w warunkach *in situ*.

Przeprowadzone badania wykazały liczne podobieństwa pomiędzy procesem starzenia liści i korzeni chłonnych. Obejmowały one podobne zmiany na poziomie morfologicznym, anatomicznym i ultrastrukturalnym tj. zmiana barwy organów, modyfikacja kształtu komórek, a na końcowym etapie przerwanie ciągłości tonoplastu i degradacja protoplastu. W obu organach udokumentowano uruchomienie mechanizmów związanych z procesem autofagii: wzrost ekspresji genów *ATG* (*AuTophagy-related Genes*), zwiększoną zawartość białka ATG8, jak również obecność struktur związanych z tym procesem tj. autofagosomy i ciała autofagowe w starzejących się organach. Analiza ekspresji genów wykazała ponadto znaczne zmiany w ekspresji genów, związanych z sygnalizacją, biosyntezą oraz metabolizmem wielu fitohormonów w tym kwasu abscysynowego (ABA) i kwasu

jasmonowego (JA). Zwiększoną zawartość tych związków wykazano zarówno w liściach, jak i w korzeniach chłonnych. Uzyskane wyniki sugerują jednak odmienny sposób ich działania w obu organach. W korzeniach chłonnych, ABA i JA nie regulują bezpośrednio procesu starzenia, uczestniczą jednak w tym procesie pośrednio poprzez ochronę korzeni przed atakiem patogenów (JA) oraz zwiększenie oporności na niską temperaturę (ABA). Rola tych fitohormonów w liściach jest bardziej złożona i związana jest z regulacją wielu procesów poprzez ich wpływ na działanie czynników transkrypcyjnych. Starzenie zarówno liści, jak i korzeni chłonnych uruchamiało mechanizmy pozwalające na remobilizację azotu. Udokumentowano spadek zawartości tego pierwiastka w obu starzejących się organach, powiązany ze wzrostem ekspresji genów kodujących cytozolową formę syntetazy glutaminianowej (GS1), jak również ze wzrostem zawartości samego enzymu. Metabolizm węglowodanów różnicował natomiast oba organy, w korzeniach chłonnych były one bowiem magazynowane, podczas gdy w liściach skrobia była wycofywana ze starzejących się tkanek.

Uzyskane wyniki przyczyniły się do szczegółowego poznania biologii starzenia korzeni chłonnych oraz wykazały, iż pomimo różnic w budowie oraz odmiennego środowiska wzrostu, proces starzenia korzeni chłonnych i liści angażuje zbliżone mechanizmy aktywacji szlaków autofagii i remobilizacji azotu. Uzyskane wyniki sugerują, że starzenie korzeni chłonnych można sklasyfikować jako jeden z kolejnych przykładów programowanej śmierci komórki (PCD) podczas rozwoju roślin.

SUMMARY OF DOCTORAL THESIS

Senescence is a coordinated series of events that begins at a cellular level and then broadens to entire tissues, organs, or the whole organism. Despite its destructive character, senescence is a precisely controlled process that follows a prescribed order. This process is often associated with programmed cell death (PCD) as has been demonstrated in the senescence of leaves and petals. To date, however, there was a lack of comprehensive data on the process of senescence, and its mechanisms, in fine roots, organs that are as ephemeral as leaves and petals. Fine roots are formed at the beginning of the growing season to increase water and nutrient absorption. Then, begin to senesce and die in the autumn when demand for water and nutrients decreases.

The aim of this thesis was to broaden our knowledge of senescence process in fine roots by documenting its temporal occurrence and confirming the similarities that exist between the senescence of fine roots, leaves, and petals due to activation of a common mechanism. All experiments were performed on leaves and fine, absorptive roots of *Populus trichocarpa* grown in a rhizotron system that enabled *in situ* observations of roots.

Results of the investigation revealed numerous similarities between the senescence process that occurs in leaves and fine roots, including similar changes in morphology (color changes, wilting/shrinkage), anatomy (changes in cell shape,) and cytology (tonoplast rupture, degradation of protoplast). Importantly, the activation of mechanisms associated with autophagy was documented in senescing fine roots. Autophagy was indicated by the elevated expression of *ATG* genes (AuTophagy-related genes), increased levels of ATG8 protein, and the presence of autophagy-related structures in senescing organs. Molecular analyses revealed significant changes in gene expression, including those associated with signaling, biosynthesis, and the metabolism of several phytohormones, including abscisic acid (ABA) and jasmonic acid (JA). The increased levels of these phytohormones was confirmed in both leaves and fine roots. The data indicate, however, that ABA and JA in fine roots act indirectly in the regulation of senescence by contributing to the protection of these organs against pathogens (JA) and increasing their tolerance to low temperature (ABA). Activation of mechanisms related to nitrogen remobilization were documented in both senescing leaves

and roots. Decreases in nitrogen content, increased expression of genes encoding the cytosolic form of glutamine synthetase (GS1), and increased levels of GS1 were observed in both organs. In contrast, differences in the metabolism of carbohydrates were identified in the two studied organs. While carbohydrates accumulated in senescing fine roots, starch levels decreased in senescing leaves.

The results of this thesis investigation provide a deeper understanding of the senescence process in fine roots and also demonstrate that despite differences in structure, as well as different growth conditions, the senescence process in fine roots and leaves involve similar mechanisms, including changes in morphology and anatomy, and the activation of autophagy and nitrogen remobilization. Based on these results, it is suggested that fine root senescence should be classified as another type of programmed cell death (PCD) that occurs within the life cycle of plants.

WYKAZ PUBLIKACJI WCHODZĄCYCH W SKŁAD

ROZPRAWY DOKTORSKIEJ

1.) **Wojciechowska N**., Sobieszczuk–Nowicka E., Bagniewska–Zadworna A. (2017) Plant organ senescence – regulation by manifold pathways. *Plant Biology*, 20: 167:181. (IF*=2.159; 70 pkt MNiSW)

2.) **Wojciechowska N.,** Marzec–Schmidt K., Kalemba E., Zarzyńska–Nowak A., Jagodziński A., Bagniewska–Zadworna A. (2018) Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*. *BMC Plant Biology*, 18:260. (IF*=3.670; 140 pkt MNiSW)

3.) **Wojciechowska N.,** Wilmowicz E., Marzec-Schmidt K., Ludwików A., Bagniewska-Zadworna A. (2020) Abscisic acid and jasmonates are involved in the regulation of senescence in roots and leaves of *Populus trichocarpa*. *International Journal of Molecular Sciences* 21, 2042. (IF*=4.183; 140 pkt MNiSW)

4.) **Wojciechowska N.,** Marzec-Schmidt K., Kalemba EM., Ludwików A., Bagniewska-Zadworna A. (2020) Seasonal senescence of leaves and roots of *Populus trichocarpa* – is the scenario the same or different? *Tree Physiology*, doi: 10.1093/treephys/tpaa019 (IF*=3.477; 140 pkt MNiSW)

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WPROWADZENIE

Starzenie roślin jest procesem zachodzącym w ich rozwoju ontogenetycznym w sposób naturalny wraz z wiekiem, jak również może być indukowane przez czynniki endo- i egzogenne, takie jak ciemność, niedobór składników mineralnych, chłód czy susza (Lim i in. 2007, Guiboileau i in. 2012, Sobieszczuk-Nowicka i in. 2018). Starzenie może obejmować całą roślinę, poszczególne jej organy, bądź nawet grupy komórek; może też występować sezonowo w okresach narażenia na czynniki stresowe (np. przed zimą) jak również wynikać z progresywnego starzenia i zamierania starszych organów i tkanek oraz zastępowania ich nowopowstałymi. Najczęściej jednak starzenie jest utożsamiane z ostatnim, poprzedzającym śmierć, etapem w rozwoju ontogenetycznym roślin (Quirino i in. 2000). Pomimo destrukcyjnego charakteru, jest to proces wysoce regulowany i uporządkowany, postępujący zgodnie ze ściśle określoną sekwencją zdarzeń. Proces starzenia jest często uznawany za przykład programowanej śmierci komórki (PCD, *ang. Programmed Cell Death*). Różni się on jednak od pozostałych typów PCD – zachodzących znacznie szybciej niż starzenie m.in. ksylogenezy (Iakimova i Woltering 2017), tworzenia warstwy odcinającej (Bar-Dror i in. 2011) lub degradacji tapetum (Li i in. 2006). Wydłużenie przebiegu PCD podczas starzenia umożliwia proces remobilizacji, podczas którego ze starzejących się tkanek wycofywane zostają cenne dla rośliny pierwiastki (Avila-Ospina i in. 2014, 2015). Jednakże aspekt starzenia, w kontekście PCD, jest w dalszym ciągu zagadnieniem szeroko dyskutowanym. Część badaczy uważa, że PCD i starzenie to dwa niezależne procesy, w których PCD występuje po procesie starzenia (van Doorn i Woltering 2004). Postuluje się również, że PCD jest procesem odnoszącym się do śmierci pojedynczych komórek, podczas gdy starzenie dotyczy organów, a nawet całych organizmów roślinnych (Nooden 2003, Thomas 2003). Inne kryterium sugeruje, że starzenie można zaklasyfikować jako PCD dopiero po przekroczeniu pewnej granicy, gdy proces ten stanie się nieodwracalny, a zmiany, które zaszły w obumierającym organie uniemożliwiają odzyskania pełnej aktywności (Bagniewska-Zadworna i Arasimowicz-Jelonek 2016, Sobieszczuk-Nowicka i in. 2018). W naturze odwrócenie sezonowego starzenia zachodzi niezwykle rzadko, a przekroczenie wspomnianej granicy następuje natychmiast po rozpoczęciu tego procesu (van Doorn i Woltering 2004). Pomimo tych rozbieżności większość autorów przyznaje, że starzenie wiąże się z genetycznie zaprogramowanym „samozniszczeniem", prowadzącym do śmierci komórek i należy

je rozpatrywać w kontekście poszczególnych komórek, tkanek, organów lub całego organizmu.

Badania dotyczące liści i płatków kwiatów wykazały, że starzenie tych organów roślinnych można zaklasyfikować jako rodzaj PCD (Lee i Chen 2002, Shibuya i in. 2009). Jak odnotowano w pracy Wojciechowska i in. 2018 (*Publikacja 1*), w literaturze brakuje natomiast kompleksowego ujęcia przebiegu procesu starzenia korzeni chłonnych. Zgodnie z najnowszą klasyfikacją korzenie chłonne są jedną z grup funkcjonalnych składających się, razem z korzeniami transportowymi, na korzenie drobne (ø<2mm) charakteryzujące się brakiem budowy wtórnej, często obecnością mikoryzy oraz wysokim stosunkiem powierzchni do masy (McCormack i in. 2015). Cechy te stanowią przystosowanie do funkcji jaką te korzenie pełnią, czyli absorpcji wody i zawartych w niej związków mineralnych z gleby. Dane literaturowe podają, iż długość życia korzeni chłonnych jest cechą gatunkową i może się wahać od kilku tygodni do 2 lat (Wells i Eissenstat 2001, Xia i in. 2010), a w przypadku drzew z rodzaju *Populus* rzadko przekracza 95 dni (McCormack i in. 2012). Korzenie chłonne, stanowiące istotną składową biomasy gleby, w dużej mierze wpływają na przebieg procesów biologicznych w ekosystemach leśnych. Biorąc pod uwagę wielkość rocznej produkcji biomasy korzeni drobnych, rozważenie aspektu starzenia i zamierania korzeni chłonnych jest istotne również w perspektywie obiegu pierwiastków w przyrodzie (Gill i Jackson 2008, Brassard i in. 2009).

Starzenie liści, płatków oraz korzeni chłonnych, pomimo różnic w ich budowie, funkcji organów oraz środowisku wzrostu przejawia się występowaniem podobieństw w morfologii i anatomii *(Publikacja 1, Fig. 1)*. Zaliczyć do nich można m.in.: podobieństwa w morfologii i anatomii, jak: zmiana barwy, utrata turgoru, występowanie licznych obkurczeń i zmiany kształtu komórek. Ponadto, badania ultrastrukturalne wykazały, że w każdym z wyżej wymienionych organów, podczas starzenia obserwowano, obecność struktur związanych z procesem autofagii, a finalnie przerwanie ciągłości tonoplastu powodujące degradację protoplastu i śmierć komórki (Matile i Winkenbach 1971, Uzelac i in. 2016). Dla starzejących się liści i płatków przeprowadzone zostały liczne analizy na poziomie metabolomicznym, proteomicznym i transkryptomicznym (Porat i in. 1993, Guo i in. 2004, Langston i in. 2005, Gregersen i Holm 2007, Shibuya i in. 2009, Avila-Ospina i in. 2015, Salleh i in. 2016, Chen i in. 2018, Sobieszczuk-Nowicka i in. 2018, Yang i in. 2019, Bengoa Luoni i in. 2019), których wyniki

wskazują, iż pomimo destrukcyjnego charakteru, starzenie jest ściśle regulowanym procesem, co zostało również podkreślone i opisane w *Publikacji 1*. W przypadku korzeni chłonnych, wnikliwe analizy umożliwiające poznanie szlaków regulujących starzenie nie zostały dotychczas przeprowadzone, dlatego kwestia ich starzenia i zamierania w dalszym ciągu jest tematem słabo poznanym.

Nadrzędnym celem przedstawionej rozprawy doktorskiej było poszerzenie wiedzy dotyczącej starzenia i zamierania korzeni chłonnych oraz zweryfikowanie hipotezy głównej zakładającej istnienie analogii w przebiegu procesu starzenia liści i korzeni chłonnych poprzez wykształcenie wspólnych mechanizmów.

W celu weryfikacji hipotezy głównej, przeprowadzone zostały cztery zadania badawcze:

1) Identyfikacja morfologicznych, anatomicznych, cytologicznych oraz molekularnych markerów procesu starzenia w badanych organach roślinnych
2) Analiza procesu autofagii jako kluczowego mechanizmu uczestniczącego w degradacji struktur komórkowych podczas starzenia
3) Określenie czy starzenie korzeni chłonnych wykorzystuje te same szlaki związane z regulacją hormonalną, które są zaangażowane w regulację starzenia liści
4) Analiza procesu remobilizacji pierwiastków i związków organicznych podczas procesu starzenia.

OMÓWIENIE WYNIKÓW

Materiał do badań stanowiły liście i korzenie chłonne topoli kalifornijskiej (*Populus trichocarpa*). Gatunek ten, ze względu na szybki przyrost biomasy oraz zsekwencjonowany genom stanowi doskonały model badawczy spośród roślin drzewiastych. Rośliny uprawiano w systemie ryzotronów, który umożliwił bezpośrednią obserwację korzeni w warunkach *in situ*, bez konieczności wcześniejszego izolowania ich z rośliny macierzystej.

Pierwszym etapem badań pracy doktorskiej było wyselekcjonowanie różnych stadiów zachodzącego starzenia dla obu badanych organów roślinnych. Bazując na analizach morfologicznych i spadku poziomu chlorofilu na pierwszy etap starzenia liści wyznaczono liście żółknące (LS1) w których poziom chlorofilu obniżył się o 40%, natomiast liście żółte, gdzie poziom tego barwnika zmniejszył się o 60% określono jako drugi etap procesu starzenia (LS2) *(Publikacja 2; Fig. 1, Fig. 3A-C)*. W przypadku korzeni chłonnych, podobnie jak w liściach, jednym z morfologicznych symptomów procesu starzenia była zmiana barwy. Korzenie, początkowo białe, zmieniały barwę poprzez jasnobrązową, brązową (pierwszy etap starzenia; RS1), aż do ciemnobrązowej (drugi etap starzenia; RS2) *(Publikacja 2, Fig. 2A-C)*. Wyniki analiz żywotności (FDA) potwierdziły, że zmiana barwy koresponduje z utratą żywotności przez badane organy *(Publikacja 2, Fig. 2D-F; Fig. 3D-F).* Dodatkowych danych dotyczących symptomów starzenia, szczególnie tych związanych ze starzeniem korzeni chłonnych, dostarczyły przeprowadzone analizy anatomiczne. Wykazano, że podczas tego procesu znacząco zmniejsza się liczba komórek miękiszu kory pierwotnej, jak również średnica korzenia oraz walca osiowego *(Publikacja 1, Fig. 4A,B)*. Zmiany w anatomii starzejących się liści nie były tak wyraźne, aczkolwiek zaobserwowano statystycznie istotne zmniejszenie szerokości blaszki liściowej *(Publikacja 1, Fig. 4D)*. W obu badanych organach roślinnych, liczne zmiany wywołane przez proces starzenia, obserwowano na poziomie ultrastrukturalnym *(Publikacja 2, Fig. 5,6)*. Wśród nich wyróżniono m.in.: pojawienie się struktur związanych z procesem autofagii, zmianę kształtu komórek oraz finalnie przerwanie ciągłości tonoplastu i degradację protoplastu. W liściach obserwowano także liczne zmiany w obrębie morfologii chloroplastów, dotyczące zmiany kształtu tych organelli, zwiększenia liczby i wielkości plastoglobul *(Publikacja 2, Fig. 6D)* oraz rozdęcia błon tylakoidów *(Publikacja 2, Fig. 6G)*.

Ponadto, udokumentowano obecność struktur przypominających ciała RCB (ang. *Rubisco-Containing Bodies*) *(Publikacja 2, Fig. 6E)*, które uczestniczą w przenoszeniu na drodze autofagii białek chloroplastowych do wakuoli, gdzie następnie podlegają degradacji (Izumi i in. 2010).

Biorąc pod uwagę, że w obu badanych organach analizy ultrastrukturalne wykazały obecność struktur związanych z procesem autofagii, takich jak autofagosomy oraz ciała autofagowe, kolejny etap badań dotyczył analizy tego procesu. Zarówno w liściach, jak i w korzeniach chłonnych wykazano wzrost ekspresji wielu genów z rodziny *ATG* (ang. *AuTophagy-related Genes*) podczas procesu starzenia. Wzrost ten dotyczył zwłaszcza genów kodujących różne izoformy białka ATG8 *(Publikacja 2, Fig. 7,8)*. Wzory ekspresji badanych genów były jednak różne dla obu analizowanych organów roślinnych, wskazując na specyficzność organową ekspresji tych genów. Została ona już wcześniej potwierdzona także u *Arabidopsis* (Thompson i in. 2005). Mając na uwadze, że ATG8, odpowiadając za prawidłowe formowanie się autofagosomu, a także regulując jego wielkość, jest kluczowym białkiem dla procesu autofagii (Xie i in. 2008, Nakatogawa i in. 2009), dalsze badania dotyczyły detekcji i lokalizacji tego białka. Przeprowadzona analiza western blot wykazała wzrost zawartości tego białka (zarówno formy wolnej jak i związanej z fosfatydyloetanoloaminą [PE]) w starzejących się organach *(Publikacja 2, Fig. 9A, 10A)*. Co więcej, przy użyciu metod immunohistochemicznych potwierdzono, iż sygnał świadczący o obecności białka ATG8 lokalizowany był w tych tkankach, w których wcześniejsze analizy anatomiczne i ultrastrukturalne wykazały liczne zmiany spowodowane postępującym procesem starzenia *(Publikacja 2, Fig. 9E-J; Fig. 10F-N)*. Stanowiło to kolejny dowód na zaangażowanie autofagii w proces starzenia.

Aby jednoznacznie potwierdzić, iż proces starzenia korzeni chłonnych jest procesem regulowanym genetycznie i można klasyfikować go jako przykład PCD przeprowadzona została analiza mikromacierzy. Uzyskane wyniki wykazały znaczące zmiany w ekspresji genów podczas starzenia liści i korzeni chłonnych *(Publikacja 3, Fig. 2A,B)*. Odnotowano 1898 i 1348 genów, których ekspresja zmieniła się odpowiednio podczas starzenia korzeni chłonnych i liści. Co ciekawe, spośród tej puli jedynie wąska grupa genów była wspólna dla obu badanych organów roślinnych *(Publikacja 3, Fig. 2C)*. Przeprowadzone analizy funkcjonalne wykazały, że duża grupa genów o zmienionej ekspresji związana była z biosyntezą, metabolizmem oraz szlakami sygnałowymi wielu hormonów roślinnych

(Publikacja 3, Fig. 3A,B). Postanowiono zatem sprawdzić **czy starzenie korzeni chłonnych wykorzystuje te same szlaki związane z regulacją hormonalną, które są zaangażowane w regulację starzenia liści.** Pomimo, iż analiza mikromacierzy wykazały znaczące zmiany w ekspresji genów związanych z wieloma fitohormonami, dalsze analizy dotyczyły kwasu abscysynowego (ABA) i kwasu jasmonowego (JA) - dwóch hormonów roślinnych znanych jako dodatnie regulatory procesu starzenia liści *(Publikacja 1, Tab. 2)*. W korzeniach chłonnych geny związane z ABA, których ekspresja wzrastała podczas starzenia kodowały m.in. białka związane z odpowiedzią na stres chłodu - WCOR413 oraz COR413IM1. Ponadto, odnotowano obniżoną ekspresję wielu genów kodujących akwaporyny, jak również wykazano zmiany w ekspresji genów kodujących enzymy uczestniczące w szlakach sygnalizacyjnych tj. kinazy i fosfatazę *(Publikacja 3, Fig. 4A)*. Geny te nie kodują białek bezpośrednio zaangażowanych w regulację procesu starzenia, dlatego możliwe jest, że ABA wpływa na proces starzenia korzeni chłonnych w sposób pośredni poprzez regulację odpowiedzi na stres chłodu. W liściach duża grupa genów związanych z ABA dotyczyła tych kodujących czynniki transkrypcyjne (TF) m.in. MYB, bZIP oraz NAC. Ponadto, odnotowano wzrost ekspresji genów związanych z metabolizmem węglowodanów, lipidów, degradacją białek, a także geny kodujące fosfatazy i kinazy *(Publikacja 3, Fig. 4C)*. Analiza ilościowa poziomu ABA wykazała, że w obu badanych organach roślinnych jego zawartość wzrastała podczas procesu starzenia *(Publikacja 3, Fig. 5A,B)*. Ponadto, na podstawie immunocytochemicznej lokalizacji ABA wykazano zwiększoną koncentracje tego fitohormonu w tych tkankach korzeni chłonnych, w których uprzednio obserwowano pozostałe symptomy starzenia tj.: zmianę kształtu komórek, obecność struktur związanych z procesem autofagii, obecność białka ATG8 *(Publikacja 2, Fig. 9C-F vs Publikacja 3, Fig. 7C,D)*. W liściach najwyższy poziom ABA zarejestrowano w liściach żółknących (LS1), w których chloroplasty nie były jeszcze w pełni zdegradowane *(Publikacja 2, Fig. 6D-E)* i wciąż odnotowywana była autofluorescencja chlorofilu *(Publikacja 3, Fig. 8D-F)*. Wysoki poziom zawartości ABA oraz jego zwiększona lokalizacja w komórkach mezofilu *(Publikacja 3, Fig. 8D- F)*, są zgodne z ostatnimi ustaleniami dotyczącymi roli ABA w procesie chlorofagii (Zhuang i Jiang 2019).

Spośród genów związanych z JA w korzeniach chłonnych nie odnotowano genów bezpośrednio związanych z procesem starzenia. Udokumentowano jednak zwiększoną ekspresję genu kodującego białko ERECTA, zaangażowanego w regulację odpowiedzi

Arabidopsis thaliana na atak patogenów (Llorente i in. 2005, Häffner i in. 2014) oraz genu kodującego jeden z czynników transkrypcyjnych z grupy MYB *(Publikacja 3, Fig. 4B)*, których zwiększoną ekspresję wykazano podczas starzenia się liściach *Solanum* i *Arabidopsis* (Huang i in. 2015, Li i in. 2019). W liściach, oprócz zwiększonej ekspresji genów kodujących czynniki transkrypcyjne należące do rodziny MYB, odnotowano wzrost ekspresji genów kodujących czynnik transkrypcyjny zależny od etylenu RAP2-3 oraz TBF1. Co więcej, zauważono zwiększoną ekspresję genu kodującego białko JAZ1, który oddziałuje z czynnikiem transkrypcyjnym z grupy MYB w kaskadzie sygnalizacyjnej JA *(Publikacja 3, Fig. 4D)*. Analiza ilościowa wykazała, że zawartość JA zwiększa się w obu badanych organach roślinnych. Ponadto wzrost odnotowano również dla jasmonianu metylu (MeJA) *(Publikacja 3, Fig. 6)*, związku stymulującego starzenie liści owsa (*Avena sativa*) (Ueda i Kato 1980). Wyniki reakcji immunolokalizacji wykazały zwiększoną koncentrację JA w starzejących się organach, zwłaszcza w tych tkankach, w których wcześniej odnotowano inne symptomy procesu starzenia *(Publikacja 3, Fig. 9C-F, Fig. 10D-I)*. Wyniki te wskazują, że jasmoniany mogą odgrywać ważną rolę w pośredniej regulacji starzenia. W korzeniach chłonnych JA może być zaangażowany w reakcję na stres biotyczny i ochronę tych organów przed patogenami. Wydłużenie starzenia umożliwia remobilizację zmagazynowanych składników odżywczych, które uległyby utracie podczas szybkiej śmierci korzeni chłonnych. Rola JA w liściach wydaje się być bardziej złożona i wiąże się z regulacją wielu procesów poprzez wpływ JA na aktywację TF.

Biorąc pod uwagę wyniki badań Guiboileau i in. (2012, 2013), w których wykazano, że poza niezaprzeczalną rolą autofagii w degradacji struktur komórkowych, proces ten pełni również istotną rolę w procesie remobilizacji, ostatni etap badań dotyczył **sprawdzenia czy procesy relokacji cennych dla roślin substancji tj. pierwiastków (azot, węgiel) oraz związków organicznych (węglowodany) są uruchamiane podczas starzenia korzeni chłonnych, tak jak ma to miejsce w przypadku liści**. Aspekt ten był niezwykle interesujący, ponieważ w literaturze prezentowane są sprzeczne dane odpowiednio potwierdzające (Zadworny i in. 2015) oraz negujące (Kunkle i in. 2009) relokację azotu ze starzejących się korzeni. Analiza ilościowa zawartości azotu wykazała, że w obu badanych organach roślinnych poziom tego pierwiastka zmniejszał się podczas procesu starzenia *(Publikacja 4, Fig. 1A,B)*. W przeciwieństwie do azotu, zawartość węgla utrzymywała się na tym samym

poziomie przez cały sezon wegetacyjny *(Publikacja 4, Fig. 1C,D)*. Istotniejszym parametrem wpływającym na procesy związane z regulacją metabolizmu komórkowego jest jednak stosunek węgla do azotu (C:N) (Chen i in. 2015). W starzejących się liściach i korzeniach chłonnych *Populus trichocarpa* obserwowano wzrost tego parametru *(Publikacja 3, Fig. 1E,F).* Wzrost stosunku C:N w liściach i korzeniach chłonnych *P. trichocarpa* potwierdza, iż inicjacja starzenia jest wywołana wysoką zawartością węgla i niską dostępnością azotu w tkankach roślinnych (Wingler i in. 1998, Aoyama i in. 2014). Syntetaza glutaminianowa (GS) jest jednym z najważniejszych enzymów zaangażowanych w remobilizację azotu w starzejących się liściach (Avila-Ospina i in. 2014). U *P. trichocarpa* forma cytozolowa (GS1) kodowana jest przez trzy paralogi genu GS1: *GS1.1, GS1.2, GS1.3*, podczas gdy chloroplastowa izoforma (GS2) przez jeden gen *GS2* (Castro-Rodríguez i in. 2011). Aby sprawdzić czy podobny mechanizm związany z remobilizacją azotu wykorzystywany jest w obu badanych organach roślinnych, przeprowadzona została analiza ekspresji genów *GS1*, jak również immunodetekcja syntetazy glutaminianowej (GS) podczas sezonu wegetacyjnego. Wykazano, że zarówno w starzejących się liściach, jak i w korzeniach chłonnych wzrasta ekspresja genu kodującego cytozolową izoformę GS, jak również poziom tego białka *(Publikacja 4, Fig. 2A-D)*. Poza GS, w obu badanych organach roślinnych analiza mikromacierzy wskazuje na znaczące zmiany w ekspresji innych genów związanych z metabolizmem azotu tj. transportery aminokwasów czy geny związane z metabolizmem glutaminanu *(Publikacja 4, Fig. 4).*

Istotne różnice między badanymi organami zaobserwowano w metabolizmie węglowodanów podczas starzenia. W starzejących się korzeniach chłonnych, w przeciwieństwie do liści, stwierdzono akumulację rozpuszczalnych cukrów prostych oraz skrobi *(Publikacja 4, Fig. B,D)*. W liściach, zawartość rozpuszczalnych cukrów prostych nie wykazywała statystycznie istotnych zmian, podczas gdy poziom skrobi znacząco się obniżył *(Publikacja 4, Fig. A,C)*. Rezultaty te potwierdzają udokumentowany w transmisyjnym mikroskopie elektronowym spadek zawartości ziaren skrobi w starzejących się liściach *(Publikacja 2, Fig. 6)*. Źródła obserwowanych wzorów należy upatrywać w zmianach w ekspresji genów związanych z metabolizmem skrobi, w tym wzrostu ekspresji genu kodującego β-amylazę w starzejących się liściach *(Publikacja 4, Fig. 5)*. Oprócz genów zaangażowanych w metabolizm skrobi, w obu badanych organach roślinnych odnotowano

znaczące zmiany w ekspresji genów zaangażowanych w metabolizm sacharozy, heksoz oraz genów kodujących transportery cukrowe *(Publikacja 4, Fig. 5)*.

WNIOSKI

Na podstawie przeprowadzonych badań wyciągnięto następujące wnioski:

1. Proces starzenia korzeni chłonnych i liści *Populus trichocarpa* charakteryzuje się podobnymi symptomami na poziomie morfologicznym, anatomicznym i ultrastrukturalnym, do których zaliczyć można m.in.: zmianę barwy starzejących się organów, zmianę kształtu komórek oraz przerwanie ciągłości tonoplastu

2. W obu badanych organach roślinnych podczas starzenia uruchamiane są mechanizmy związane z procesem autofagii, na co wskazuje obecność ciał autofagowych i autofagosomów, wzrost ekspresji genów należących do rodziny *ATG* oraz zwiększony poziom białka ATG8

3. Zarówno starzenie korzeni chłonnych, jak i liści jest związane ze znaczącymi zmianami w ekspresji genów, co wraz z pozostałymi wynikami sugeruje, że proces ten można zaklasyfikować jako kolejny przykład PCD podczas rozwoju roślin

4. Wykazano, że czynnikami odpowiedzialnymi za inicjację i koordynację procesu starzenia są fitohormony. Analiza funkcjonalna wykazała, że wiele genów, których ekspresja zmienia się podczas starzenia liści i korzeni chłonnych związana jest właśnie z metabolizmem, sygnalizacją i biosyntezą hormonów roślinnych. Wykazano wzrost zawartości ABA, JA oraz MeJA. Jednakże, zmiany w ekspresji genów związanych z tymi fitohormonami wskazują, że w korzeniach chłonnych ABA i JA wpływają na proces starzenia w sposób pośredni poprzez regulację odpowiedzi na stres chłodu i obecność patogenów

5. Podczas procesu starzenia w obu organach roślinnych wykazano uruchomienie mechanizmów związanych z remobilizacją azotu, potwierdzone spadkiem jego

poziomu, wzrostem ekspresji genów kodujących syntetazę glutaminianową (GS1) oraz wzrostem zawartości GS1 w obu badanych organach roślinnych

6. Wykazano, że podczas procesu starzenia korzenie chłonne i liście prezentują odmienny metabolizm węglowodanów. Podczas gdy w liściach skrobia jest wycofywana ze starzejących się tkanek, w korzeniach wykazano akumulację tego wielocukru, jak również rozpuszczalnych cukrów prostych. Magazynowanie cukrów niestrukturalnych wpływa na zwiększenie ciśnienia osmotycznego w komórkach przez co może zapobiegać mechanicznym uszkodzeniom wywołanym niską temperaturą i przedłużać ich żywotność, tym samym umożliwiając przeprowadzenie procesu starzenia i remobilizację cennych dla roślin związków.

PODSUMOWANIE

Wyniki niniejszej rozprawy doktorskiej znacząco rozszerzyły dotychczasową wiedzę na temat starzenia korzeni chłonnych, do tej pory opierającej się w głównej mierze na ujęciu ekologicznym. Wykazano, iż pomimo różnic w budowie liści i korzeni chłonnych, a także zupełnie odmiennego środowiska wzrostu, procesy starzenia wykazują wiele analogii *(Fig. 1)*.

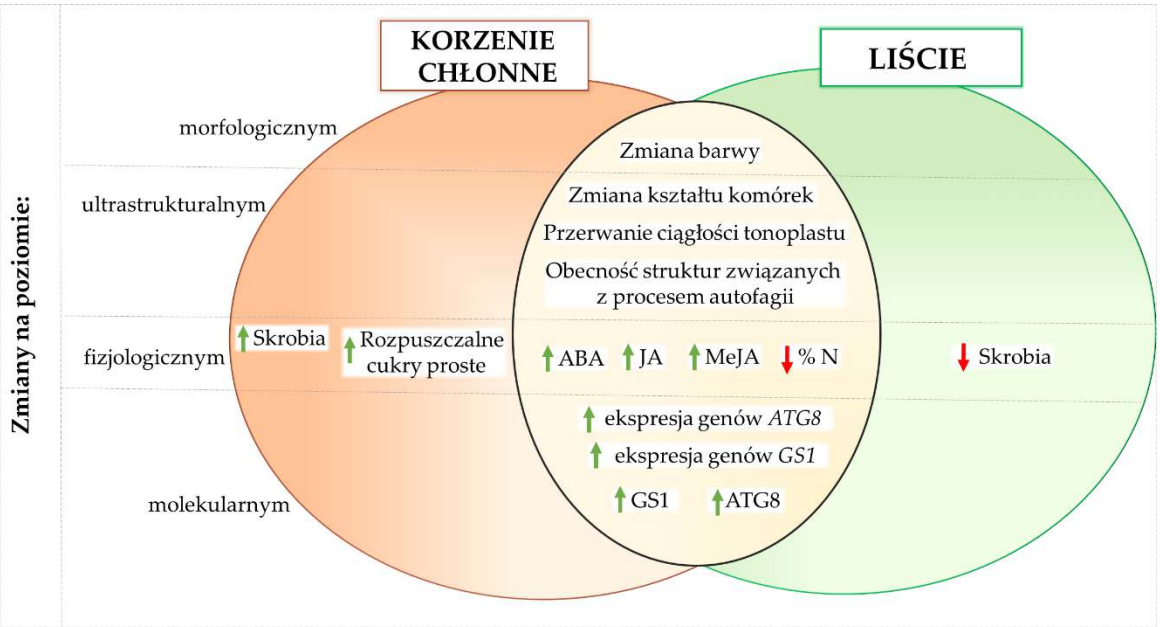

Fig. 1. Skonstruowana w oparciu o uzyskane wyniki, sekwencja zdarzeń wywołanych procesem starzenia w korzeniach chłonnych i liściach *P. trichocarpa*.

[↑wzrost, ↓ spadek, ABA – kwas abscysynowy, JA – kwas jasmonowy, MeJA – Jasmonian metylu, N – azot, *ATG8* – geny *ATG* (AuTophagy related Genes), ATG8 – białko ATG8, *GS1* – gen kodujący cytozolową izoforme syntetazy glutaminianowej, GS1 – syntetaza glutaminianowa, forma cytozolowa]

Przeprowadzenie badań na poziomie molekularnym umożliwiło potwierdzenie tezy o genetycznym podłożu procesu starzenia korzeni chłonnych, podobnie jak liści, stanowiąc kolejny przykład programowanej śmierci komórki (PCD) u roślin. Uzyskane wyniki stanowią cenne źródło informacji o mechanizmach związanych ze starzeniem efemerycznych organów

roślinnych. Dzięki wielopłaszczyznowym analizom, łączącym aspekt cytochemiczny, fizjologiczny i molekularny, stanowią innowacyjne podejście do poszerzenia wiedzy o czynnikach regulujących starzenie korzeni chłonnych. Długofalową konsekwencją starzenia i zamierania korzeni chłonnych jest uwolnienie nagromadzonych związków węgla i włączenie ich do obiegu pierwiastków. Biorąc pod uwagę wielkość biomasy korzeni chłonnych w ekosystemach leśnych, która często jest równa lub przewyższa biomasę liści, zrozumienie mechanizmów kontrolujących starzenie korzeni ma podstawowe znaczenie dla dalszych badań nad krążeniem pierwiastków w przyrodzie.

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OŚWIADCZENIA WSPÓŁAUTORÓW

PUBLIKACJA 1

Wojciechowska N, Sobieszczuk-Nowicka E, Bagniewska-Zadworna A. (2018) **Plant organ senescence - regulation by manifold pathways.** *Plant Biology* 20(2):167-181.

Poznań, 26.03.2020

Natalia Wojciechowska
Zakład Botaniki Ogólnej
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

OŚWIADCZENIE

Oświadczam, że w publikacji:

Wojciechowska N, Sobieszczuk-Nowiska E, Bagniewska-Zadworna A. (2018) **Plant organ senescence - regulation by manifold pathways..** *Plant Biol.* 20(2):167-181.

mój udział obejmował:

- Napisanie rozdziałów (Introduction, Senescence-associated events, Hormonal regulation of senescence, Regulation of senescence by sugars),
- Przygotowanie schematów.

Natalia Wojciechowska

UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU
Wydział Biologii
Zakład Fizjologii Roślin

Poznań, 21.04.2020

Prof. UAM, dr hab. Ewa Sobieszczuk-Nowicka
Zakład Fizjologii Roślin
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji: Wojciechowska N, Sobieszczuk-Nowiska E, Bagniewska-Zadworna A. (2018) **Plant organ senescence - regulation by manifold pathways..** *Plant Biol.* 20(2):167-181 do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

- udział w opracowaniu koncepcji pracy,
- napisanie rozdziału „Regulation of senescence by polyamines",
- redakcje manuskryptu.

Z poważaniem
Ewa Sobieszczuk-Nowicka
Ewa Sobieszczuk-Nowicka

Poznań, 05.04.2020

Prof. UAM, dr hab. Agnieszka Bagniewska-Zadworna
Zakład Botaniki Ogólnej, Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
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61-614 Poznań

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Sobieszczuk-Nowicka E, Bagniewska-Zadworna A. (2018) **Plant organ senescence - regulation by manifold pathways.** *Plant Biology* 20(2):167-181.

do Jej rozprawy doktorskiej, oświadczam, że mój wkład w powstanie pracy obejmował: udział w opracowaniu i dyskusji koncepcji pracy oraz w przygotowaniu publikacji do druku.

A. Zadworna

PUBLIKACJA 2

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Zarzyńska-Nowak A, Jagodziński AM, Bagniewska-Zadworna A. (2018) **Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*.** *BMC Plant Biology* 18(1):260.

Poznań, 26.03.2020

Natalia Wojciechowska
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OŚWIADCZENIE

Oświadczam, że w publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Zarzyńska-Nowak A, Jagodziński AM, Bagniewska-Zadworna A. (2018) **Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*.** *BMC Plant Biol.* 18(1):260.

mój udział obejmował:

- Wysiewanie i prowadzenie uprawy topoli kalifornijskiej przed wysadzeniem roślin do ryzotronów,
- Zbiór materiału,
- Sporządzenie dokumentacji fotograficznej związanej z morfologicznymi, symptomami starzenia liści i korzeni chłonnych,
- Utrwalenie i zatopienie materiału przeznaczonego do analiz anatomicznych,
- Analizę anatomiczną korzeni chłonnych i liści,
- Przeprowadzenie analizy żywotności,
- Udział w analizach ultratrukturalnych (obserwacja w TEM, analiza mikrografii),
- Udział w optymalizacji warunków do izolacji RNA oraz reakcji odwrotnej transkrypcji,
- Udział w izolacji RNA,
- Przeprowadzenie analiz real-time PCR,
- Zatopienie materiału oraz przygotowanie preparatów do analiz immunolokalizacji białka ATG8,
- Przeprowadzenie reakcji immunolokalizacji białka ATG8,
- Udział w optymalizacji i przeprowadzeniu izolacji białek oraz reakcji Western-blot,
- Analizę statystyczną,
- Udział w interpretacji wyników i przygotowaniu publikacji.

Natalia Wojciechowska

Axvall, 22.04.2020

Dr inż. Katarzyna Marzec-Schmidt
Zakład Botaniki Ogólnej
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Uniwersytet im. Adama Mickiewicza w Poznaniu
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61-614 Poznań

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Zarzyńska-Nowak A, Jagodziński AM, Bagniewska-Zadworna A. (2018) **Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*.** *BMC Plant Biol.* 18(1):260.

do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

- Udział w zbiorze materiału,
- Udział w optymalizacji warunków do izolacji RNA, reakcji odwrotnej transkrypcji oraz łańcuchowej reakcji polimerazy w czasie rzeczywistym,
- Udział w izolacji RNA,
- Konsultacje naukowe,
- Redakcje manuskryptu.

Katarzyna Marzec-Schmidt

Poznań, 20.04.2020

dr hab. Ewa M. Kalemba
Pracownia Biochemii Nasion
Instytut Dendrologii PAN
ul. Parkowa 5
62-035 Kórnik

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Zarzyńska-Nowak A, Jagodziński AM, Bagniewska-Zadworna A. (2018) **Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*.** *BMC Plant Biol.* 18(1):260.
do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

- Optymalizację warunków izolacji białek i analizy Western blot,
- Udział w detekcji białka ATG8 za pomocą techniki Western blot,
- Konsultacje naukowe,
- Udział w redakcji manuskryptu.

Ewa Kalemba

Poznań, 20.04.2020

dr Aleksandra Zarzyńska-Nowak
Zakład Wirusologii i Bakteriologii
Instytut Ochrony Roślin-
Państwowy Instytut Badawczy
Ul. Wł. Węgorka 20
60-318 Poznań

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Zarzyńska-Nowak A, Jagodziński AM, Bagniewska-Zadworna A. (2018) **Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*.** *BMC Plant Biol.* 18(1):260.

do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:
- pokrojenie materiału do analiz ultrastrukturalnych,
- kontrastowanie materiału,
- pomoc w obserwacjach mikroskopowych,
- przygotowanie figur dotyczących zmian w ultrastrukturze podczas starzenia,
- redakcje manuskryptu.

A. Zarzyńska-Nowak

Poznań, 20 kwietnia 2020 roku

dr hab. inż. Andrzej M. Jagodziński, prof. ID PAN
Pracownia Ekologii
Instytut Dendrologii PAN
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62-035 Kórnik

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N., Marzec-Schmidt K., Kalemba E.M., Zarzyńska-Nowak A., Jagodziński A.M., Bagniewska-Zadworna A. (2018) **Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*.** *BMC Plant Biol.* 18(1):260.

do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

współudział w zaplanowaniu doświadczenia terenowego oraz wniesienie uwag do maszynopisu publikacji.

Poznań, 05.04.2020

Prof. UAM, dr hab. Agnieszka Bagniewska-Zadworna
Zakład Botaniki Ogólnej
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Uniwersytet im. Adama Mickiewicza w Poznaniu
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61-614 Poznań

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Zarzyńska-Nowak A, Jagodziński AM, Bagniewska-Zadworna A. (2018) **Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*.** *BMC Plant Biology* 18(1):260.

do Jej rozprawy doktorskiej, oświadczam, że mój wkład w powstanie pracy obejmował: opracowanie koncepcji badań, zaplanowanie i założenie eksperymentu, udział w analizie danych i interpretacji wyników oraz przygotowaniu publikacji do druku, a także opiekę merytoryczną podczas wykonywania badań.

PUBLIKACJA 3

Wojciechowska N, Wilmowicz E, Marzec-Schmidt K, Ludwików A, Bagniewska-Zadworna A. (2020) **Abscisic acid and jasmonate metabolisms are jointly regulated during senescence in roots and leaves of *Populus trichocarpa*.** *International Journal of Molecular Science* 17;21(6).

Poznań, 26.03.2020

Natalia Wojciechowska
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OŚWIADCZENIE

Oświadczam, że w publikacji:

Wojciechowska N, Wilmowicz E, Marzec-Schmidt K, Ludwików A, Bagniewska-Zadworna A. (2020) **Abscisic acid and jasmonate metabolisms are jointly regulated during senescence in roots and leaves of *Populus trichocarpa*.** *Int J Mol Sci 17;21(6)*

mój udział obejmował:

- Udział w opracowaniu koncepcji i planu badań,
- Wysiewanie i prowadzenie uprawy topoli kalifornijskiej przed wysadzeniem roślin do ryzotronów,
- Zbiór materiału,
- Udział w izolacji RNA,
- Udział w analizie danych uzyskanych z mikromacierzy (selekcja genów związanych z fitohormonami),
- Udział w analizach ilościowych badanych fitohormonów,
- Zatopienie materiału oraz przygotowanie preparatów do analiz immunolokalizacji, kwasu abscysynowego (ABA) i kwasu jasmonowego (JA),
- Przeprowadzenie reakcji immunolokalizacji ABA i JA,
- Przygotowanie schematu oraz figur,
- Analizę statystyczną,
- Udział w interpretacji wyników i przygotowaniu publikacji.

Natalia Wojciechowska

Toruń, 20.04.2020

dr hab. Emilia Wilmowicz, prof. UMK,
Katedra Fizjologii Roślin i Biotechnologii
Wydział Nauk Biologicznych i Weterynarii
Uniwersytet Mikołaja Kopernika
ul. Lwowska 1
87-100 Toruń

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Wilmowicz E, Marzec-Schmidt K, Ludwików A, Bagniewska-Zadworna A. (2020) **Abscisic acid and jasmonate metabolisms are jointly regulated during senescence in roots and leaves of *Populus trichocarpa*.** *Int J Mol Sci 17;21(6)*

do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

- Optymalizację warunków izolacji i ilościowej analizy kwasu abscysynowego (ABA) i kwasu jasmonowego (JA) w liściach i korzeniach chłonnych,
- Współudział w analizach ilościowych ABA, JA i MeJA
- Konsultacje naukowe
- Redakcje manuskryptu.

Emilia Wilmowicz

Axvall, 22.04.2020

Dr inż. Katarzyna Marzec-Schmidt
Zakład Botaniki Ogólnej
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Wilmowicz E, Marzec-Schmidt K, Ludwików A, Bagniewska-Zadworna A. (2020) **Abscisic acid and jasmonate metabolisms are jointly regulated during senescence in roots and leaves of *Populus trichocarpa*.** *Int J Mol Sci 17;21(6)*

do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

- Udział w zbiorze materiału,
- Udział w optymalizacji warunków do izolacji RNA, reakcji odwrotnej transkrypcji oraz łańcuchowej reakcji polimerazy w czasie rzeczywistym,
- Udział w izolacji RNA,
- Analizę i opis wyników eksperymentu z użyciem micromacierzy DNA (identyfikacja genów o zmienionej ekspresji, analizy porównawcze i funkcjonalne)
- Konsultacje naukowe,
- Redakcje manuskryptu.

Katarzyna Marzec-Schmidt

Poznań, 31.03.2020

Prof. UAM, dr hab. Agnieszka Ludwików
Zakład Biotechnologii
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji: Wojciechowska N, Wilmowicz E, Marzec-Schmidt K, Ludwików A, Bagniewska-Zadworna A. (2020) **Abscisic acid and jasmonate metabolisms are jointly regulated during senescence in roots and leaves of *Populus trichocarpa*.** *Int J Mol Sci* 17;21(6) do Jej rozprawy doktorskiej, oświadczam, że mój udział w powstaniu publikacji polegał na: przygotowaniu i wykonaniu eksperymentu z użyciem mikromacierzy DNA (hodowla i zbiór materiału roślinnego, izolacja tRNA do hybrydyzacji; analiza jakościowa tRNA do hybrydyzacji; analiza jakościowa danych; analiza statystyczna danych), konsultacjach naukowych i redakcji manuskryptu.

Poznań, 05.04.2020

Prof. UAM, dr hab. Agnieszka Bagniewska-Zadworna
Zakład Botaniki Ogólnej
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Wilmowicz E, Marzec-Schmidt K, Ludwików A, Bagniewska-Zadworna A. (2020) **Abscisic acid and jasmonate metabolisms are jointly regulated during senescence in roots and leaves of *Populus trichocarpa*.** *International Journal of Molecular Science 17;21(6)*

do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

opracowanie koncepcji badań i zaplanowanie eksperymentu, udział w analizie danych i interpretacji wyników oraz przygotowaniu publikacji do druku, a także opiekę merytoryczną podczas wykonywania badań.

PUBLIKACJA 4

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Ludwików A, Bagniewska-Zadworna A. (2020) **Seasonal senescence of leaves and roots of *Populus trichocarpa* - is the scenario the same or different?** *Tree Physiology* doi: 10.1093/treephys/tpaa019.

Poznań, 26.03.2020

Natalia Wojciechowska
Zakład Botaniki Ogólnej
Wydział Biologii
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ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

OŚWIADCZENIE

Oświadczam, że w publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Ludwików A, Bagniewska-Zadworna A. (2020) **Seasonal senescence of leaves and roots of *Populus trichocarpa* - is the scenario the same or different?** *Tree Physiol.* doi: 10.1093/treephys/tpaa019.

mój udział obejmował:

- Udział w opracowaniu koncepcji i planu badań,
- Wysiewanie i prowadzenie uprawy topoli kalifornijskiej przed wysadzeniem roślin do ryzotronów,
- Zbiór materiału,
- Analizę ilościową zawartości pierwiastków (N i C),
- Udział w izolacji RNA,
- Przeprowadzenie analiz real-time PCR,
- Udział w optymalizacji i przeprowadzeniu izolacji białek oraz reakcji Western-blot,
- Analizę ilościową zawartości cukrów,
- Analizę statystyczną,
- Udział w interpretacji wyników i przygotowaniu publikacji.

Natalia Wojciechowska

Axvall, 22.04.2020

Dr inż. Katarzyna Marzec-Schmidt
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OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Ludwików A, Bagniewska-Zadworna A. (2020) **Seasonal senescence of leaves and roots of *Populus trichocarpa* - is the scenario the same or different?** *Tree Physiol.* doi: 10.1093/treephys/tpaa019.

do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

- Udział w zbiorze materiału,
- Udział w optymalizacji warunków do izolacji RNA, reakcji odwrotnej transkrypcji oraz łańcuchowej reakcji polimerazy w czasie rzeczywistym,
- Udział w izolacji RNA,
- Analizę i opis wyników eksperymentu z użyciem micromacierzy DNA (identyfikacja genów o zmienionej ekspresji, analizy porównawcze i funkcjonalne)
- Konsultacje naukowe,
- Redakcje manuskryptu.

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Poznań, 20.04.2020

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W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Ludwików A, Bagniewska-Zadworna A. (2020) **Seasonal senescence of leaves and roots of *Populus trichocarpa* - is the scenario the same or different?** *Tree Physiol.* doi: 10.1093/treephys/tpaa019.

do Jej rozprawy doktorskiej, oświadczam, że mój udział obejmował:

- Optymalizację warunków izolacji białek i analizy Western blot,
- Udział w detekcji białka syntetazy glutaminianowej (GS) za pomocą techniki Western blot,
- Konsultacje naukowe,
- Udział w redakcji manuskryptu.

Ewa Kalemba

Poznań, 31.03.2020

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OŚWIADCZENIE

W związku z zamiarem włączenia przez mgr Natalię Wojciechowską publikacji:

Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Ludwików A, Bagniewska-Zadworna A. (2020) **Seasonal senescence of leaves and roots of *Populus trichocarpa* - is the scenario the same or different?** *Tree Physiol.* doi: 10.1093/treephys/tpaa019 do Jej rozprawy doktorskiej, oświadczam, że mój udział w powstaniu publikacji polegał na: przygotowaniu i wykonaniu eksperymentu z użyciem mikromacierzy DNA (hodowla i zbiór materiału roślinnego, izolacja tRNA do hybrydyzacji; analiza jakościowa tRNA do hybrydyzacji; analiza jakościowa danych; analiza statystyczna danych), konsultacjach naukowych i redakcji manuskryptu.

Poznań, 05.04.2020

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OŚWIADCZENIE

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Wojciechowska N, Marzec-Schmidt K, Kalemba EM, Ludwików A, Bagniewska-Zadworna A. (2020) **Seasonal senescence of leaves and roots of *Populus trichocarpa* - is the scenario the same or different?** *Tree Physiology* doi: 10.1093/treephys/tpaa019.

do Jej rozprawy doktorskiej, oświadczam, że mój udział polegał na:

opracowaniu koncepcji badań i zaplanowaniu eksperymentu, udziale w interpretacji wyników i przygotowaniu publikacji do druku, a także opiece merytorycznej podczas wykonywania badań.

A. Zadworna

KOPIE PUBLIKACJI WCHODZĄCYCH W SKŁAD

ROZPRAWY DOKTORSKIEJ

PUBLIKACJA 1

Wojciechowska N, Sobieszczuk-Nowicka E, Bagniewska-Zadworna A. (2018) **Plant organ senescence - regulation by manifold pathways.** *Plant Biology* 20(2):167-181.

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REVIEW ARTICLE

Plant organ senescence – regulation by manifold pathways

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Keywords

Autophagy; carbohydrates; fine roots; leaves; petals; phytohormones; programmed cell death; senescence.

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ABSTRACT

Senescence is the final stage of plant ontogeny before death. Senescence may occur naturally because of age or may be induced by various endogenous and exogenous factors. Despite its destructive character, senescence is a precisely controlled process that follows a well-defined order. It is often inseparable from programmed cell death (PCD), and a correlation between these processes has been confirmed during the senescence of leaves and petals. Despite suggestions that senescence and PCD are two separate processes, with PCD occurring after senescence, cell death responsible for senescence is accompanied by numerous changes at the cytological, physiological and molecular levels, similar to other types of PCD. Independent of the plant organ analysed, these changes are focused on initiating the processes of cellular structural degradation *via* fluctuations in phytohormone levels and the activation of specific genes. Cellular structural degradation is genetically programmed and dependent on autophagy. Phytohormones/plant regulators are heavily involved in regulating the senescence of plant organs and can either promote [ethylene, abscisic acid (ABA), jasmonic acid (JA), and polyamines (PAs)] or inhibit [cytokinins (CKs)] this process. Auxins and carbohydrates have been assigned a dual role in the regulation of senescence, and can both inhibit and stimulate the senescence process. In this review, we introduce the basic pathways that regulate senescence in plants and identify mechanisms involved in controlling senescence in ephemeral plant organs. Moreover, we demonstrate a universal nature of this process in different plant organs; despite this process occurring in organs that have completely different functions, it is very similar. Progress in this area is providing opportunities to revisit how, when and which way senescence is coordinated or decoupled by plant regulators in different organs and will provide a powerful tool for plant physiology research.

INTRODUCTION

Senescence is a universal feature of all living organisms and involves the gradual deterioration of function of multiple cells and tissues. Plant senescence is usually referred to as a process of developmental ageing; however, the term can relate to a specific group of cells, tissues or organs or an entire plant. On the basis of many substantive/semantic arguments (Thomas *et al.* 2003; van Doorn & Woltering 2004; Thomas 2004, 2013; van Doorn *et al.* 2011; Jones *et al.* 2014; Munné-Bosch 2015), the processes of senescence, ageing, lifespan and death should be considered separate in plants. At the level of the whole organism, senescence is the terminal phase of plant ontogenetic development, leading to death. In many cases, however, we focus on the description of cellular senescence and its progress.

Cellular senescence is highly regulated by environmental and autonomous factors. Many stimuli that induce senescence exist, such as shortened days in autumn, drought, frost and shading as well as ageing, phytohormone levels, higher-order epigenetic mechanisms and the expression of specific environment-dependent genes (Guo & Gan 2005; Ay *et al.* 2014). Senescence can also be induced by several treatments, including darkness (Kunz *et al.* 2009), nitrogen deficiency (Criado *et al.* 2007) and other biotic or abiotic stresses (Miller *et al.* 1999). However, compared with developmental senescence, induced senescence results in significantly different patterns of genetic regulation (van der Graaff *et al.* 2006).

Another subject of debate is whether programmed cell death (PCD) is involved in senescence or whether senescence is just an example of PCD. The term PCD indicates an active process of elimination of cellular components, which ultimately leads to death that occurs during development, sometimes in response to environmental conditions (Greenberg 1996). Some authors present a different opinion, suggesting that senescence and PCD are two separate processes, with PCD occurring after senescence (van Doorn & Woltering 2004). It is also recommended that senescence be classified as PCD when the process becomes irreversible and the cells cannot recover their full vitality (Bagniewska-Zadworna & Arasimowicz-Jelonek 2016). In nature, reversal of seasonal senescence is extremely rare, and crossing the point-of-no-return occurs instantaneously. In many cases, senescence starts when the programme leading to death has already been initiated (van Doorn & Woltering 2004). In contrast, Noode′n (2004) assumed that the term senescence describes

a process that leads to the death of organs or even entire plants, whereas PCD relates to the death of single cells. According to Barlow (1982), senescence in leaves must meet several criteria that allow the entire process to be classified as PCD: (i) cells die at a predetermined time and place; (ii) cell death enables survival of the whole organism; and (iii) information about death is encoded in the genetic material. Despite these discrepancies, most authors admit that senescence involves genetically programmed self-destruction that leads to cell death, and that senescence can apply to the ageing of particular cells, tissues, organs or the entire organism.

Thus, we support the notion that PCD during senescence is considered another example of developmental PCD (Olvera-Carrillo *et al.* 2015). However, other types of developmental PCD, such as xylogenesis, formation of the abscission layer and degradation of the tapetal and synergid cells during the development of male and female gametophytes, occur much faster than does senescence (Lim & Nam 2007). The prolonged course of PCD is a simple consequence of the remobilisation of valuable elements and their translocation to other parts of the plant (Lim *et al.* 2003). Several ultrastructural, physiological and molecular studies have shown that PCD is involved in the senescence of leaves and flower petals (Beers 1997; Rogers 2006). Moreover, studies performed by Bagniewska-Zadworna *et al.* (2014) have suggested that PCD can also participate in the seasonal senescence of fine roots. This phenomenon is confirmed by similar changes at the anatomical and ultrastructural level that are common features of the senescence of fine roots, leaves and petals. We would argue that separating PCD and senescence is not always needed, given that there are no traits allowing definition of the spatiotemporal boundary and unequivocal separation of these processes. Moving forward, it will be important for the community to adopt common – even basic – regulatory pathways and mechanisms that enable comparative linkages to be made between different plant organs and senescence.

Where meaningful patterns can be identified, senescence is a highly complex phenomenon that must be controlled strictly by different regulatory pathways and can potentially improve our understanding of the process at scales larger than those encompassing only cellular changes. It is not possible to indicate one constant mechanism regulating senescence; in many cases, these mechanistic pathways are interdependent and complement each other. However, in this review, we demonstrate the possible common mechanisms that are involved in controlling the senescence process in different ephemeral plant organs, and we provide highly valuable information on many broad aspects and combine these findings into a general picture. We take account of previous efforts to identify broad patterns for comparing the course of senescence in different plant organs (leaves, petals and fine roots). We specifically address questions that were not directly considered in previous reports. Does the senescence process occur in a universal manner for all ephemeral organs? Which links of these processes are common, and where are the most crucial differences in the case of leaves, petals and fine roots? Accurate analysis of the senescence processes of different organs allows demonstration of the potential differences associated with dissimilar functions in senescent plant organs or indication that senescence is not a tissue-specific process but progresses in a universal manner (Fig. 1, Table S1).

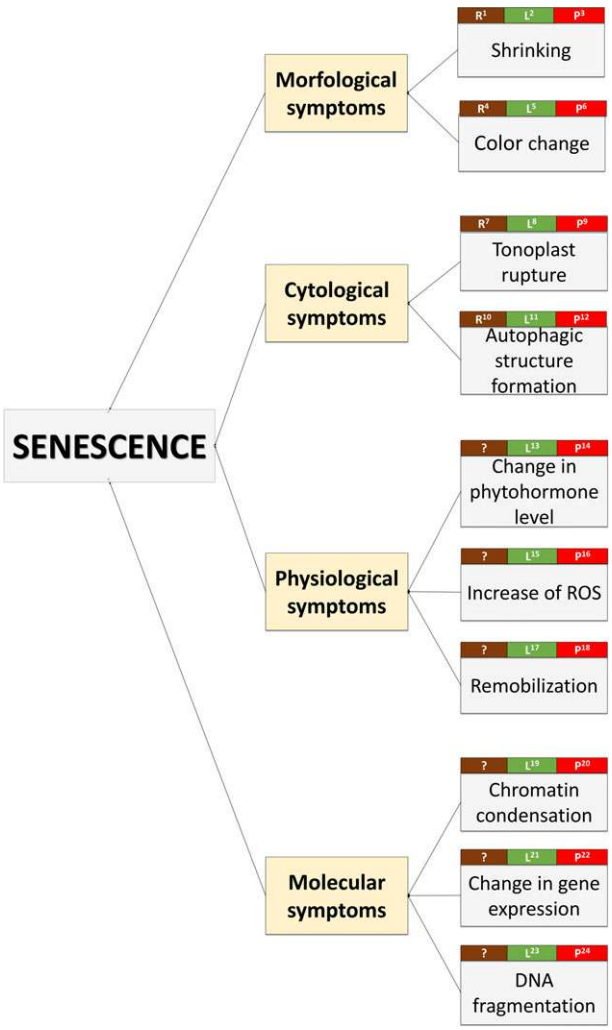

Fig. 1. Main symptoms of senescence in different ephemeral plant organs. Comparison of the main symptoms of senescence indicates the universality of the senescence process. Despite the differences in function of the analysed organs, the symptoms are analogous. R – fine roots; L – leaves; P – petals.

SENESCENCE-ASSOCIATED EVENTS

Despite its destructive character, senescence is a precisely controlled process that is regulated and conducted in a well-defined order. It entails many morphological, cytological, physiological and molecular changes, such as activation of the expression of specific genes, fluctuations in phytohormone levels and initiating the processes of cellular structural degradation. These intricate regulatory pathways are activated to enable the realization of one of the most important purposes of senescence, namely, the remobilisation process. This process is activated to withdraw and translocate valuable nutrients such as carbon, nitrogen and other minerals from senescing tissues to other parts of the plant (Lam 2004; Rogers 2006; Milla *et al.* 2007; Wang 2012).

In leaves, one of the initial signs of senescence is the degradation of chloroplasts as well as a reduction in photosynthetic efficiency; this degradation is a mutual feature of developmental and induced senescence. Chloroplasts are the first organelles that are degraded and represent a large pool of deposited

Table 1. Examples of up-regulated genes during senescence of plant ephemeral organs.

group of senescence-regulated genes	example of genes	organ	species	references
Genes involved in protein degradation	*Cysteine proteases*	Petals	*Alstroemeria pelegrina*	Wagstaff *et al.* 2002
			Hemerocallis hybrid	Valpuesta *et al.* 1995
			Petunia hybrida	Jones *et al.* 1995
			Narcissus pseudonarcissus	Hunter *et al.* 2002
		Leaves	*Arabidopsis thaliana*	Guo *et al.* 2004; Hensel *et al.* 1993
			Zea mays	Smart *et al.* 1995
			Brassica napus	Buchanan-Wollaston & Ainsworth 1997
			Populus tremula	Bhalerao *et al.* 2003
	Serine proteases	Petals	*Narcissus pseudonarcissus*	Hunter *et al.* 2002
		Leaves	*Petroselinum crispum*	Jiang *et al.* 1999
	Aspartic proteases	Petals	*Alstroemeria pelegrina*	Breeze *et al.* 2004
			Hemerocallis hybrid	Panavas *et al.* 1999
		Leaves	*Brassica napus*	Buchanan-Wollaston & Ainsworth 1997
Genes involved in phospholipids and fatty acids degradation	*Allene oxide synthetase*	Petals	*Hemerocallis* hybrid	Panavas *et al.* 1999
			Petunia hybrida	Xu *et al.* 2006
	Lipases	Petals	*Dianthus caryophyllus*	Hoeberichts *et al.* 2007
		Leaves	*Arabidopsis thaliana*	Guo *et al.* 2004
	Acyl-CoA dehydrogenase	Petals	*Iris x hollandica*	van Doorn *et al.* 2003
	Acyl-CoA oxidase	Petals	*Dianthus caryophyllus*	Hoeberichts *et al.* 2007
			Phalaenopsis orchids	Do & Huang 1997
	Phospholipase D	Leaves	*Ricinus communis*	Ryu & Wang 1995
	Lipooxygenases	Leaves	*Arabidopsis thaliana*	Guo *et al.* 2004
Genes involved in nucleic acids degradation	*BFN1*	Leaves	*Arabidopsis thaliana*	Matallana-Ramirez *et al.* 2013
	NUC1	Petals	*Petunia hybrida*	Langston *et al.* 2005
Autophagy related genes	*ATG18*	Leaves	*Arabidopsis thaliana*	Xiong *et al.* 2005
	ATG7, ATG8a, ATG 8e, ATG9	Leaves	*Arabidopsis thaliana*	van der Graaff *et al.* 2006
	ATG8	Petals	*Ipomoea nil*	Shibuya *et al.* 2009
Genes involved in remobilisation	*Glutamine synthetase*	Petals	*Alstroemeria pelegrina*	Breeze *et al.* 2004
		Leaves	*Oryza sativa*	Kamachi *et al.* 1991
			Arabidopsis thaliana	Bernhard & Matile 1994
		Leaves	*Lycopersicon esculentum Mill.*	Pérez-Rodríguez & Valpuesta 1996
Trancriptional factor	*NAC family*	Petals	*Narcissus pseudonarcissus*	Hunter *et al.* 2002
			Dianthus caryophyllus	Hoeberichts *et al.* 2007
		Leaves	*Arabidopsis thaliana*	Guo *et al.* 2004
	WRKY family	Leaves	*Arabidopsis thaliana*	Hinderhofer & Zentgraf 2001
			Panicum virgatum	Rinerson *et al.* 2015
		Petals	*Gardenia jasmonoides*	Tsanakas *et al.* 2014

nitrogen. Up to 75% of the total nitrogen in leaves resides in chloroplasts; thus, it is reasonable that this nitrogen must be recovered and transported to other parts of the plant as part of the remobilisation process (Peoples & Dalling 1988). A vesicle-based process associated with autophagy may be crucial for chloroplast degradation and selective nutrient recycling (Li & Viestra 2012; Avila-Ospina *et al.* 2014). The degradation of chloroplasts leads to one of the most commonly observed phenomena associated with senescence – change in leaf colour (Carrión *et al.* 2013). This change is associated with a decrease in the photosynthetic pigment chlorophyll. The loss of chlorophyll always occurs in the same manner – from the leaf margins towards the centre of the leaf (Gan & Amasino 1997). Thus, the senescence of individual cells is not synchronous: the cells that surround the vascular tissues age later to facilitate the mobilisation of nutrients from adjacent senescing cells (Gan & Amasino 1997).

Knowledge concerning the patterns and underlying mechanisms of the senescence of other organs is less advanced. However, morphological, anatomical, cytological and even physiological and molecular symptoms can show similar patterns despite dissimilar organ functions (Fig. 1, Table S1). Thus, senescence of other ephemeral organs (petals and fine roots) is also related to changes in colour (Withington *et al.* 2006; van Doorn & Woltering 2008; Bagniewska-Zadworna *et al.* 2014). During the senescence of *Hibiscus syriacus*, the petals become bluish because of an increased flavonoid to anthocyanin ratio, and this effect was reinforced by changes in cell sap pH (Kim *et al.* 1989). Similar results were obtained for the ageing of rose petals (*Rosa hybrida* L.), in which high pH levels caused a structural modification in anthocyanin pigments, resulting in their blue colour (Schmitzer *et al.* 2010). A change in pigmentation during senescence can also be observed in senescent fine roots. The colour of these organs changes from white to light brown, dark brown and finally black. Experiments with 2,3,5-triphenyl tetrazolium chloride and fluorescein diacetate have confirmed that a correlation exists between colour change and root shrivelling as cell viability

decreases (Fig. 1, Table S1; Comas *et al.* 2000; Bagniewska-Zadworna *et al.* 2014).

Aside from morphological changes such as colour changes in senescent plant organs, several other observations are related to this process, including the loss of water from ageing tissues, the leakage of ions, the generation of reactive oxygen species (ROS), an increase in membrane fluidity and lipid peroxidation (Tripathi & Tuteja 2007). The last two indicators are associated with the increased activity of lipid-degrading enzymes such as phospholipase D, phosphatidic acid phosphatase, lytic acyl hydrolase and lipoxygenase (Thompson *et al.* 1998, 2000). Similarly, the activity of enzymes involved in the hydrolysis of proteins to amino acids also increases (Lim & Nam 2007). Transcriptomic studies of leaf senescence in *Arabidopsis thaliana* have indicated that the expression of many enzymes involved in protein degradation is affected; among these enzymes, cysteine protease is most frequently implicated (Guo *et al.* 2004). The level of total RNA in leaves decreases rapidly during the initial phase of senescence as a consequence of increased RNase activity, as chloroplastic and cytoplasmic rRNA are degraded first (Wagstaff *et al.* 2003; Yamada *et al.* 2006a,b; Lim & Nam 2007). To control all the mechanisms that regulate the senescence process, both the nucleus and mitochondria remain fully active until the late stages (Quirino *et al.* 2000). This activity enables gene expression to continue and fulfils the constant demand for energy production for remobilisation and other processes. However, during the final stage of the senescence of leaves and petals, the nuclei also undergo changes, such as chromatin condensation and, ultimately, the degradation of nuclear DNA (nDNA) (Fig. 1, Table S1; Kołjek *et al.* 2007; Lim & Nam 2007; Shibuya *et al.* 2013). One of the most characteristic signs of leaf senescence is internucleosomal DNA fragmentation as visualized by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction or by nDNA laddering (Yen & Yang 1998; Lee & Chen 2002). Similar results have been obtained during petal senescence in the same species of flowers (*Gladiolus hybrida* and *Pisum sativum*; Orzáez & Granell 1997; Yamada *et al.* 2003). However, other studies have yielded inconclusive evidence and have shown that nDNA fragmentation is not always a good indicator of petal senescence: in *Petunia hybrida*, *Ipomoea nil*, *Argyranthemum frutescens* and *Alstroemeria pelegrina*, DNA laddering is not observed (Wagstaff *et al.* 2003; Yamada *et al.* 2006a,b).

The main process that involves the rearrangement of subcellular membranes is the sequestration of cargo, which is later delivered to vacuoles where the segregated material becomes degraded (Levine & Klionsky 2004). Autophagy can play a dual role, corresponding to the induction and execution stages of PCD (Minina *et al.* 2014), and can also be activated as pro-survival and pro-death processes; this phenomenon is most important not only in degradation but also in recovery during senescence. There are three types of autophagy in plants: micro-, macro- and megaautophagy. During the course of microautophagy, a small portion of the cytoplasm is trapped in a vacuole due to tonoplast invagination. This portion of cytoplasm becomes part of an intervacuolar vesicle called an autophagic body. After the vesicular membrane is digested, the contents of the vesicle are released into the vacuolar sap and degraded (Fig. 2; van Doorn & Woltering 2005). Macroautophagy involves the formation of double-membrane vesicles in the cytoplasm called autophagosomes. These transport larger protoplast fragments to the vacuole to be degraded by hydrolytic enzymes (Fig. 2; van Doorn & Woltering 2005). Megaautophagy involves the intense production of hydrolytic enzymes and their accumulation in the enlarging vacuole. The ultimate steps in this type of autophagy include tonoplast rupture, the release of hydrolytic enzymes and protoplast degradation (van Doorn & Papini 2013). In cells undergoing PCD, more than one type of autophagy can be observed at the same time (van Doorn & Woltering 2005; Bagniewska-Zadworna *et al.* 2012). Microscopy research of senescent leaves, petals and fine roots has confirmed the role of autophagy in protoplast degradation during senescence (Fig. 1, Table S1). Ultrastructural studies performed on senescent petals of *Ipomoea purpurea* (Matile & Winkenbach 1971) and *Dianthus caryophyllus* (Smith *et al.* 1992) have indicated the presence of numerous vesicles containing residues of cytoplasmic structures in vacuoles. Similar symptoms of vacuolar cell death were observed during an analysis of senescence processes in the fine roots of *Populus trichocarpa* (Bagniewska-Zadworna *et al.* 2014). These vesicles might have been formed during the course of micro- and/or macroautophagy. A unique type of macroautophagy can be observed in leaves during chloroplast degradation. The cytoplasm of ageing parenchymal cells can accommodate specific autophagosomes (Ishida *et al.* 2014). Given that these structures contain rubisco, they are called rubisco-containing bodies (RCBs). The RCBs are similar to autophagosomes and have double membranes that seem to be derived from the chloroplast envelope. These membranes fuse with the tonoplast and then are degraded by hydrolytic enzymes (Fig. 2; Ishida *et al.* 2014). The last recognisable stage of cellular senescence in leaves (Lim *et al.* 2007), petals (van Doorn & Woltering 2008) and fine roots (Bagniewska-Zadworna *et al.* 2014) is protoplast degradation *via* megaautophagy, during which the tonoplast is ruptured and the cell structures are eliminated by vacuolar hydrolytic enzymes.

Senescence is also characterised by substantial changes in gene expression (Fig. 2, Table 1). Genes that are up-regulated during the process are termed senescence-associated genes (*SAG*s), whereas genes that are down-regulated are defined as senescence down-regulated genes (*SDG*s; Noh & Amasino 1999; Simeonova & Mostowska 2001; Ay *et al.* 2014). The regulation of gene expression during the senescence of petals and leaves has been studied by analysing the transcriptomes and by profiling studies. These studies have revealed a massive reprogramming of gene expression (Buchanan-Wollaston 1997; Guo *et al.* 2004; van der Graaff *et al.* 2006; van Doorn & Woltering 2008; Tsanakas *et al.* 2014). The senescence of leaves and petals involves the same set of genes, and these genes can be classified into different subcategories: genes associated with the autophagy process; genes associated with the degradation of macromolecules (proteins, nucleic acids and lipids); genes involved in the remobilisation process; and genes that code for transcription factors (Table 1). Although the major pathways have been revealed during recent years, the direct incorporation of various molecular data into a broader framework is an important long-term goal.

As we attempt to scale regulatory pathways to different plant organs, we must understand the scaling relationships between particular regulators and the senescence of individual ephemeral organs. This area is still underappreciated.

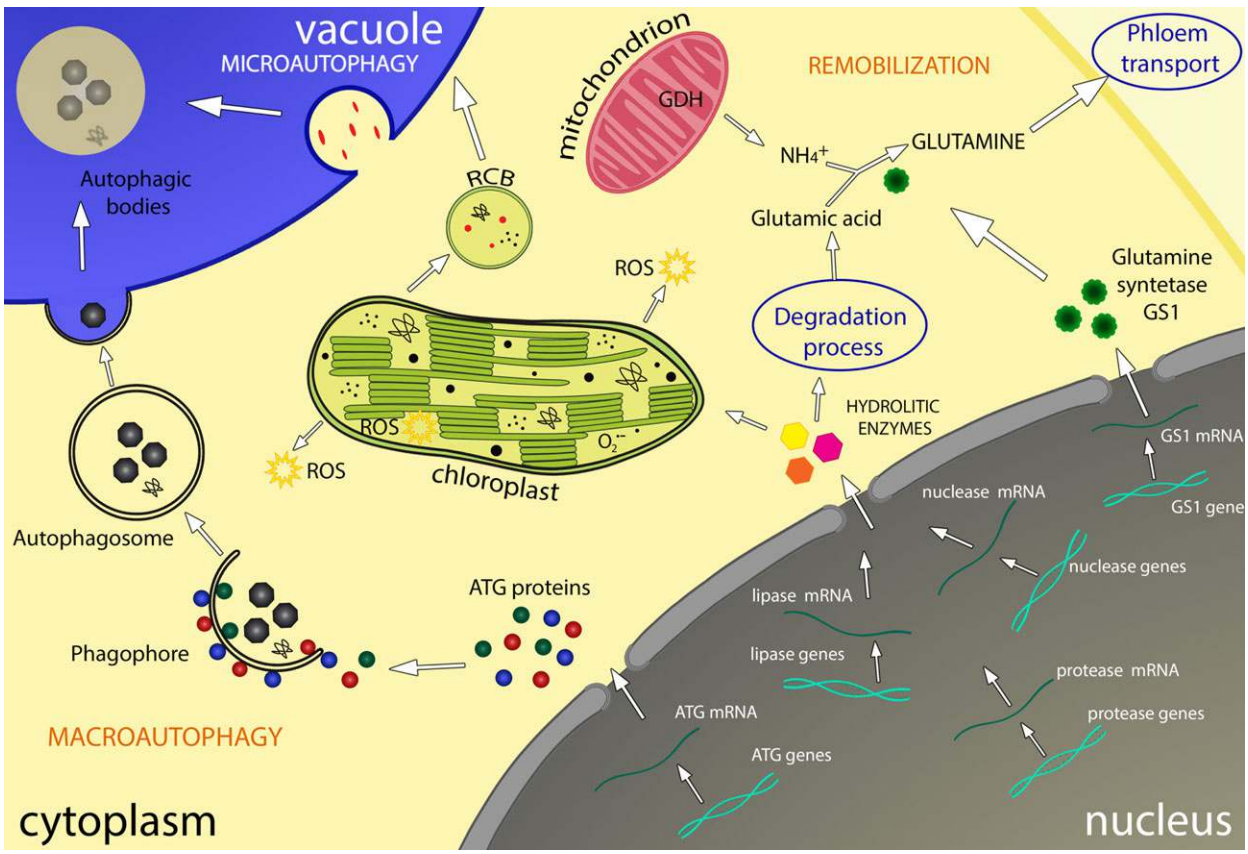

Fig. 2. Main processes that regulate leaf senescence. Multiple layers of the regulation of senescence are illustrated: micro- and macroautophagy, remobilization, ROS production and changes in gene expression. Senescence is presented as an adjustable complex process in which a plurality of processes are mutually complementary and regulate each other. Note: the figure is not drawn to scale. This figure was prepared based on the following reports: Avila-Ospina *et al.* (2014), Ishida *et al.* (2014) and Liu *et al.* (2008). GDH, glutamate dehydrogenase; ROS, reactive oxygen species; RCBs, rubisco-containing bodies.

HORMONAL REGULATION OF SENESCENCE

Phytohormones are among the most important regulators that control senescence (Tripathi & Tuteja 2007; Sarwat *et al.* 2013; Zhang & Zhou 2013; Khan *et al.* 2014). Studies carried out on leaves and petals have confirmed the important role of phytohormones in the regulation of senescence in those organs (Fig. 3, Table 2). Moreover, some of these phytohormones [*e.g.* ethylene, jasmonic acid (JA) and abscisic acid (ABA)] can promote senescence, whereas others [*e.g.* cytokinins (CKs)] can suppress it (Table 2; Tripathi & Tuteja 2007; Sarwat *et al.* 2013; Zhang & Zhou 2013; Khan *et al.* 2014). Despite many studies, the molecular mechanisms that underline the relationships between phytohormones and senescence have not been fully elucidated.

Cytokinins

The CKs play an important role in the regulation of many cellular processes, including senescence. In leaves and petals, CKs are considered negative regulators of senescence (Table 2). The first indications of this were observed during the 1970s when Sabater & Rodriguez (1978) demonstrated that decreasing levels of CKs were correlated with the loss of green colour in leaves. This phenomenon was confirmed in many subsequent studies. Several studies have been performed involving the application of exogenous CKs, introduction of genetic modifications to increase the endogenous levels of CKs or modification of the signalling pathways of CKs. These studies have confirmed the role of CKs as endogenous negative regulators during the senescence of leaves and petals (Fig. 3). Despite a well-documented relationship between the total level of CKs and the rate of senescence, the molecular mechanisms by which this regulation occurs are not well understood. Experiments on transgenic plants, *e.g. Nicotiana tabacum* (Gan & Amasino 1995) and *Petunia x hybrida* (Chang *et al.* 2003), provided a breakthrough that partly explained the regulation of senescence by CKs. The plants were transformed with the isopentenyl transferase (IPT) gene from *Agrobacterium tumefaciens*. The IPT gene encodes one of the enzymes involved in the biosynthesis of CKs. To avoid developmental abnormalities associated with the increased expression of this gene, the expression of IPT was under the control of the senescence-specific *SAG12* promoter. Under such control, increased CKs synthesis occurs only at the beginning of senescence. These experiments showed that the leaves of the transgenic plants remain green longer and that the senescence of leaves and petals is delayed. Other experiments have indicated that, during the senescence of cotton leaves, CKs can regulate the transcription of many genes associated with various metabolic pathways, such as flavonoid synthesis, the metabolism of arginine and proline, the glyoxal cycle and RNA degradation (Zhao *et al.* 2013). Disturbance in the

Table 2. Role of different regulators in plant organ senescence.

Phytohormone/regulator	Regulation	Effect
Cytokinins (CKs)	–	• Endogenous CK levels declined during senescence *(LS)* (Lim *et al.* 2007) • Reducing the level of CKs correlates with a decrease in the level of chlorophyll *(LS)* (Sabater & Rodriguez 1978) • Exogenous application of CKs delayed senescence *(LS, PS)* (Mayak & Halevy 1970; Hwang *et al.* 2012) • Transcription of genes involved in CK biosynthesis and signalling is repressed *(LS)*(Buchanan-Wollaston *et al.* 2005) • Expression of CK oxidase, an enzyme involved in CK degradation is induced *(LS)* (Buchanan-Wollaston *et al.* 2005)
Auxins (IAA)	–	• Decrease expression of genes from *SAG* family after exogenous applications of IAA *(LS)* (Sarwat *et al.* 2013)
	+	• Abundance of free, bioactive IAA increased twofold *(LS)* (Lim *et al.* 2007) • Increased expression of genes involved in the biosynthesis of auxins (*TSA1, AO1, NIT13*) *(LS)* (Hou *et al.* 2013) • Increased level of gene *SAUR 36*, which is a positive regulator of senescence process (*LS*) (Hou *et al.* 2013)
Ethylene	+	• Decrease expression of the gene encoding the ACC (*LS*) (Khan *et al.* 2014) • Increase expression of genes encoding proteins involved in the biosynthesis of ethylene *(PS)* (ten Have & Woltering 1997) • Influence of EIN2 protein on gene *ore1* transcription which affects the increase in the expression of multiple genes of the family *SAG (LS)* (Kim *et al.* 2009)
Abscisic acid (ABA)	+	• Exogenous application of ABA promotes senescence *(LS, LP)* (Wilkinson *et al.* 1997; Lim *et al.* 2007) • Genes involved in the key steps of ABA biosynthesis and signalling are up-regulated during senescence *(LS)* (Buchanan-Wollaston *et al.* 2005) • Increase the level of ABA during the first symptoms of senescence *(PS)* (Hunter *et al.* 2002) • Regulation of gene expression of the *SAG* family by ABA *(LS)* (Zhang *et al.* 2012)
Jasmonic (JA)	+	• Induced expression of a number of key enzymes involved in the degradation of chlorophyll *(LS)* (Reinbothe *et al.* 2009) • Regulation of ACC level by JA *(LS)* (Porat *et al.* 1995) • Increase expression of genes encoding proteins involved in the biosynthesis of JA *(LS)*(Seltmann *et al.* 2010a) • Repression and degradation of rubisco activase by JA *(LS)*(Shan *et al.* 2011)
Polyamines (PAs)	+	• PA accumulation upon senescence is linked to up-regulation of PA biosynthesis gene and consequently to increase in the corresponding enzymatic activities. • Transcript levels and corresponding activation of PA catabolic enzymes increase also during senescence. • Putrescine conjugate accumulation in the senescing leaf (*LS*) (Sobieszczuk-Nowicka *et al.* 2016) • Spermidine and spermine production, their transport into the apoplast, where they produce H_2O_2 and diaminopropane, both of which can participate in senescence-dependent degradation processes *(LS)* (Sobieszczuk-Nowicka *et al.* 2016). • Dark-induced leaf senescence corresponds to a wide contribution of PAs within chloroplast: inhibition of photosynthesis, cell death, chloroplast-to-gerontoplast conversion and chloroplasts disintegration, where PAs can be transported or synthesised *de novo (LS)*(Sobieszczuk-Nowicka *et al.* 2015) • *Arabidopsis* polyamine back-conversion oxidase mutants deficient in the conversion of spermine to spermidine have delayed entry into dark-induced leaf senescence *(LS)* (Sequera-Mutiozabal *et al.* 2016). • Treatment with exogenous spermine is effective in delaying the progression of senescence (*PS*) (Bagni & Tassoni 2006).

(continued)

Table 2. (Continued)

Phytohormone/regulator	Regulation	Effect
SUGARS	+	• High level of sugar was reported in petals showing visible symptoms of senescence *(PS)* (van Doorn 2004) • Overexpression of HXK1 gene accelerates senescence *(LS)* (Dai *et al.* 1999) • In hexokinase-1 *gin2-1* mutant indicated delayed senescence *(LS)* (Moore *et al.* 2003)
	–	• Accumulation of sugars can block CKs effect *(LS)* (Wingler *et al.* 1998) • Application of sugar to cut flowers delayed visible symptoms of senescence (*PS*) (van Doorn 2004) • After sugar feeding protein degradation and *SAG* genes expression are delayed (*PS*) (Eason *et al.* 1997) • Decrease of glucose content delaying senescence *(LS)* (Balibrea *et al.* 2004) • Sugars rapidly decreased the amount of transcript *SAG*12 *(LS)* (Noh & Amasino 1999)

LS, leaf senescence; PS, petal senescence, (–), inhibit senescence; (+), promote senescence.

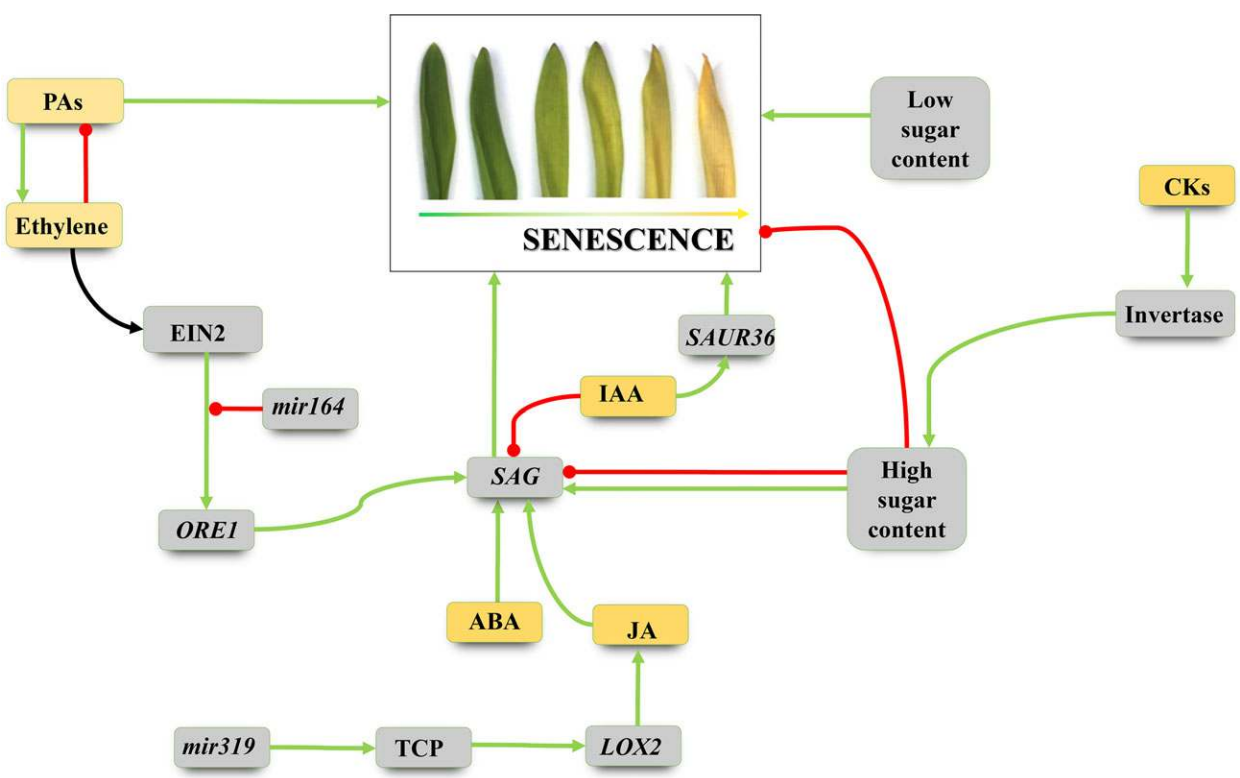

Fig. 3. Signalling pathways leading to the onset of leaf senescence. Note: green arrows indicate positive regulation and red bars show negative regulatory roles. This figure was prepared based on the following reports: Khan *et al.* (2014) and Wingler *et al.* (1998, 2006). ABA, abscisic acid; CKs, cytokinins; EIN2, Ethylene Insensitive 2; IAA, indole-3-acetic acid; JA, jasmonic acid; PAs, polyamines; *SAG*s, senescence-associated genes; TCPs, teosinte branched/cycloidea/PCF transcription factors.

expression of these genes leads to a reduction in antioxidants and ethylene and also carries the risk of degradation of RNA, lipids and proteins. All these factors promote the senescence of plant organs (Zhao *et al.* 2013). Studies using transgenic plants have also demonstrated that CKs can delay leaf senescence *via* the control of invertase activity (Balibrea *et al.* 2004). Invertase catalyses the hydrolysis of sucrose to glucose and fructose. This reaction enables the conversion of sucrose from phloem cells to hexose molecules, which are transported to other plant cells. Transgenic plants with increased biosynthesis of CKs show increased invertase activity. However, if invertase activity is inhibited, the effect of CKs on delaying senescence is eradicated. This phenomenon suggests that CKs inhibit expression of the invertase inhibitor. Consequently, CKs delay leaf senescence by affecting both invertase activity and the accumulation of hexose molecules, thus inducing plant organs to become

sugar sinks instead of initiating the remobilisation of sugars, which is associated with senescence. This mechanism relies on a combination of signalling pathways that both regulate ageing and are controlled by phytohormones and sugars (Zwack & Rashotte 2013); however, progress in drawing these connections has been slow.

Auxins

The role of auxins in senescence is widely debated, as these phytohormones can both stimulate and inhibit senescence. The hypothesis that auxins promote senescence originates from studies on senescent leaves. These studies have shown increased expression of key genes involved in the biosynthesis of auxins, including tryptophan synthase (*TSA1*), indole-3-acetic acid (IAA) oxidase (*AO1*) and nitrilase (*NIT1-3*), leading to increases in the total pool of auxins (van der Graaff *et al.* 2006). Furthermore, Hou *et al.* (2013) indicated that auxins are regulators of the *SAUR36* gene, which is highly up-regulated in senescing leaves (Fig. 3). Loss of *SAUR36* function results in delayed senescence, but increased expression of *SAUR36* leads to premature leaf senescence. In contrast, several studies have suggested that auxins are negative regulators of senescence and have demonstrated that exogenous application of auxins down-regulates some *SAG* genes (Fig. 3; Noh & Amasino 1999; Hong *et al.* 2000; Jones *et al.* 2010; Kim *et al.* 2011). How auxins affect petal senescence is not well understood. Studies performed on cut flowers have indicated that exogenous application of IAA stimulates ethylene production, wilting and the senescence of some ethylene-sensitive flowers (Van Staden 1995). Numerous studies have investigated the involvement of auxins in the regulation of senescence, focusing on understanding the role of auxin response factor 2 (ARF2) during this process. The protein ARF2 is a transcription factor that binds to auxin-responsive elements in the promoters of auxin-regulated genes (Lim *et al.* 2010). Studies performed on *arf2* mutants of *A. thaliana* have indicated that ARF2 is necessary for various auxin-mediated developmental processes such as increased growth of aerial organs and increased seed size due to extra cell division, inhibition of floral bud opening, abscission of floral organs and delays in silique ripening during flowering and leaf senescence (Ellis *et al.* 2005; Okushima *et al.* 2005; Schruff *et al.* 2006). This involvement of *ARF2* has been confirmed by microarray analyses, showing that *ARF2* expression is increased in senescent leaves during developmental and dark-induced senescence (Buchanan-Wollaston *et al.* 2005; Ellis *et al.* 2005; Lim *et al.* 2010). Furthermore, the expression of other auxin response factor genes (*ARF7* and *ARF19*) is also induced during senescence (Ellis *et al.* 2005; Okushima *et al.* 2005). However, mutation of these genes does not affect the phenotype of senescent leaves but does increase the effect caused by mutations in the *ARF2* gene, delaying the senescence process (Ellis *et al.* 2005). In summary, despite the availability of vast amounts of information concerning the regulation of senescence by auxin, the role of this phytohormone appears to be complex and requires further investigation. Indeed, accurate estimation of specific auxin roles in the promotion or inhibition of senescence continues to be a serious unresolved problem.

Ethylene

Numerous studies carried out on plant organs (leaves and flower petals) have shown an important role of ethylene in the promotion of the senescence process. Much of the available information concerning this regulation originates from studies with genetic mutants (*etr1-1*, *ein2-1* and *ein1-1*) that exhibit disturbances in the ethylene-signalling pathway. These mutants are characterised by the delayed expression of senescence markers, including *SAG1*, *SAG2* and *SAG12*, and the resultant delayed onset of leaf senescence (Grbić & Bleecker 1995). Molecular studies have subsequently shown that the EIN2 protein regulates the transcription of the *ORE1* gene, which encodes a transcription factor required to initiate the expression of genes associated with senescence, such as *SAG*. In young plant organs, the amount of mRNA encoding *ORE1* is regulated by microRNA (miR)-164. During senescence, the level of mir-164 decreases, positively affecting the expression of *ORE1* and the progression of senescence (Fig. 3; Kim *et al.* 2009). However, molecular studies have shown that plants continuously overproducing ethylene age in a similar manner to wild-type plants. This phenomenon indicates that, in addition to ethylene, other factors might play a crucial role in the senescence process (Lim *et al.* 2007).

Several studies have focused on the influence of ethylene on the wilting and senescence of flower petals. The results of these analyses showed that the examined flower species could be divided into two groups with respect to senescence: an ethylene-dependent group (*Petunia*, *Arabidopsis* and *Ipomoea*) and an ethylene-independent group (*Alstroemeria*, *Iris* and *Sandersonia*; Woltering & van Doorn 1988). In the first group, ethylene is the major factor that determines the time of senescence and is a major regulator of this process. In the second group, the senescence of petals does not depend on ethylene, but despite many studies, the regulation of senescence in these plants is not yet fully understood (Shibuya *et al.* 2013). In ethylene-dependent flowers, senescence is accompanied by a surge in ethylene production (O'Neill 1997), which is associated with increased expression of 1-aminocyclopropane-l-carboxylic acid (ACC; van Doorn & Woltering 2008). Moreover, the promotive role of ethylene has been confirmed in studies performed on transgenic flowers (*Petunia hybrida* and *Dianthus caryophyllus*). These plants are characterised by the presence of a mutation in the ethylene receptor gene. In agreement with the dysfunction of the ethylene signalling pathway, petal senescence is delayed in these plants (Wilkinson *et al.* 1997; Bovy *et al.* 1999; Shaw *et al.* 2002).

Abscisic acid

Abscisic acid is also involved in the control of senescence, and similar to ethylene, ABA is a positive regulator of the senescence process. Application of exogenous ABA accelerates senescence in leaves (Lim *et al.* 2007) and flower petals (Wilkinson *et al.* 1997). Studies carried out on *A. thaliana* have shown that in senescent leaves there is a significant increase in the expression of genes that encode enzymes involved in the biosynthesis of ABA (Buchanan-Wollaston *et al.* 2005). Moreover, ABA regulates the expression of *SAG* genes, including *SAG113* (Fig. 3; Zhang *et al.* 2012). This gene encodes a protein that belongs to the phosphatase PP2C family and is located in the Golgi

apparatus. Induced overexpression of the *SAG113* gene results in reduced stomatal sensitivity to ABA and thus increased water loss by the cell, leading to premature leaf senescence (Zhang *et al.* 2012). In plants insensitive to ethylene, ABA is the primary hormone regulator of flower petal senescence. Exogenous application of ABA prematurely up-regulates events that occur during natural senescence, such as the loss of differential membrane permeability, increases in lipid peroxidation and the induction of proteinase and RNase activities (Panavas *et al.* 1998). Studies performed on *Iris* and *Hemerocallis* hybrids have confirmed the role of ABA in the positive regulation of petal senescence (Zhong & Ciafré 2011). Exogenous application of ABA stimulates the appearance of morphological, biochemical and molecular changes that are typical for the natural senescence of *Iris* petals, such as increased total protease activity, phospholipid degradation and the up-regulation of *RNase* and *UBQ-E2* gene expression. However, ABA inhibits the expansion of flag petals, even during the opening stage (Zhong & Ciafré 2011).

Jasmonic acid

Many studies have indicated that JA is a positive regulator of the senescence process. However, the involvement of this phytohormone in the regulation is not as evident as previously thought (Taylor & Whitelaw 2001; Seltmann *et al.* 2010a,b). The first report on the role of jasmonates in senescence originated from the observation that an isolated compound from wormwood (*Artemisia absinthium*) causes the rapid loss of chlorophyll in oats (*Avena sativa*). This compound was identified as methyl jasmonate (MeJa), a volatile derivative of JA (Ueda & Kato 1980). The same effect has also been observed during the natural senescence process in *A. thaliana*, in which senescent leaves, compared with non-senescent leaves, are characterised by four-fold higher levels of jasmonates (He *et al.* 2002). Elevated levels of JA are associated with the increased expression of genes encoding proteins involved in the biosynthesis of JA (Seltmann *et al.* 2010a). Indirectly, the high levels of JA that occur during senescence are also associated with the activity of miR-319, which regulates the teosinte branched/cycloidea/PCF (TCP) transcription factors. These factors control expression of the *LOX2* gene, which encodes a key enzyme involved in the biosynthesis of JA (Fig. 2; Schommer *et al.* 2008). The exogenous application of MeJA causes increased expression of *SAG* genes such as *SEN4* and *SEN5*, which stimulate leaf senescence (Park *et al.* 1998; Xiao *et al.* 2004; Shan *et al.* 2011). The hormone JA also has a stimulating effect on petal senescence. Application of MeJA hastens senescence in *Petunia hybrida*, *Dendrobium* and *Phalaenopsis*, presumably by elevating ACC and thereby stimulating ethylene production (Porat *et al.* 1993, 1995). However, some authors think that JA does not play an important role in the senescence process or could even be a by-product of this process. Moreover, many studies have suggested that JA is a secondary by-product of the breakdown of macromolecules, explaining why the level of JA increases during senescence (Seltmann *et al.* 2010a).

REGULATION OF SENESCENCE BY POLYAMINES

Numerous studies have linked polyamines (PAs) to the regulation of plant cell senescence. Major PAs in plants include putrescine (Put), spermidine (Spd) and spermine (Spm). These PAs have been implicated in the prolonged survival of excised organs or senescing organs *in vivo*, namely, leaves, flowers and fruits (Cai *et al.* 2015). However, contradictions exist concerning whether PA levels increase or decrease during senescence (Cai *et al.* 2015).

The biosynthesis, catabolism, conjugation, interconversion and transport of PAs contribute to PA homeostasis (reviewed in Moschou & Roubelakis-Angelakis 2013; Sobieszczuk-Nowicka 2017). Transformations between individual PAs might contribute to responses to environmental changes, and this phenomenon has been highlighted in senescing barley leaves (Sobieszczuk-Nowicka *et al.* 2016). The accumulation of PAs upon senescence is linked to the up-regulated expression of genes involved in the biosynthesis of PAs and, consequently, to increases in the corresponding enzymatic activities. Transcript levels and activation of the PA catabolic enzymes diamine oxidase (DAO) and PA oxidase (PAO) increase during developmental and dark-induced senescence; therefore, these enzymes are considered important components of senescence-related mechanisms (Ioannidis *et al.* 2014; Sobieszczuk-Nowicka *et al.* 2016). Inhibiting PAO activity substantially increases levels of Spd and Spm, which decrease during senescence. This phenomenon is expected, but remarkably, the increase also slows down the senescence-associated loss of chlorophyll. As such, *Arabidopsis* PA back-conversion oxidase mutants, in which the conversion of Spm to Spd does not occur, show delayed initiation of dark-induced senescence (Sequera-Mutiozabal *et al.* 2016). In these mutants, delayed leaf senescence is associated with higher levels of Spm and nitric oxide, together with reduced production of ROS. Taken together, these data suggest that Spm is a signalling metabolite that provides protection against stress *via* metabolic conversions that involve modifications to the ascorbate/dehydroascorbate redox state, changes in sugar and nitrogen metabolism, cross-talk with ethylene biosynthesis and modulation of the mitochondrial electron transport chain (Sequera-Mutiozabal *et al.* 2016).

In senescent leaves, Put dominates within the free PA fraction and initially accumulates to high levels before decreasing. This decrease in free Put is accompanied by the formation of Put conjugates (Sobieszczuk-Nowicka *et al.* 2016). The senescence-dependent flow of remobilised nitrogen and carbon might contribute to PA conjugation. The sensing of PAs as organic nitrogen by plant cells and PA stimulation of nitrogen molecule turnover has been discussed previously (Mattoo *et al.* 2006, 2010).

Another interesting facet of PA metabolism is the involvement of a DAO-mediated Put oxidation process during the production of γ-aminobutyric acid (GABA). Microarray-based profiling of the expression of the glutamate decarboxylase gene suggests that synthesis of GABA from glutamate is gradually suppressed in dark-induced senescing leaves (Sobieszczuk-Nowicka *et al.* 2016). Oxidation of Put could contribute to the alternative source of GABA and possibly also to signalling pathways. As described above, hormone regulation of plant senescence involves ethylene. In this regard, PAs seem to act as anti-senescence regulators by inhibiting ethylene, and conversely, ethylene inhibits the biosynthesis of PAs (Fig. 2; Anwar

et al. 2015 and references therein). A possible temporal relationship between PAs and ethylene during plant development has been presented, wherein competition for *S*-adenosylmethionine (SAM), which is an early precursor of both PAs and ethylene, has been discussed (Fluhr & Mattoo 1996; Cassol & Mattoo 2003; Harpaz-Saad *et al.* 2012). In addition, the simultaneous biosynthesis of PAs and ethylene, first presented in studies on tomato fruit (Mehta *et al.* 2002), is corroborated by studies on dark-induced leaf senescence in barley (Sobieszczuk-Nowicka *et al.* 2016).

The metabolism of PA during senescence is linked to many intracellular metabolic pathways, including signalling molecules and metabolites that are associated with cellular responses to environmental changes (Sobieszczuk-Nowicka 2017). The findings indicate that the internal pool of PAs undergoes regulation in senescing leaves. More information is becoming available on how PA metabolism is linked to physiological changes that ultimately lead to cell death and the nature of changes in the levels of free, conjugated and bound forms of PAs. Processes that are interlinked with an increase or decrease in PA titre during senescence and the ability of plants to control senescence in relation to their ability to metabolise PAs are slowly being elucidated.

Flower petal senescence and death are other highly regulated developmental phases controlled by PAs (Lee *et al.* 1997; Serafini-Fracassini *et al.* 2002; Bagni & Tassoni 2006; Della Mea *et al.* 2007a). PA modulation of flower senescence has been studied in model species that are differentially sensitive to ethylene, including carnation, *Nicotiana* and *Gerbera*. Aliphatic PAs share with ethylene the common precursor SAM. In 'ethylene-sensitive' carnation flowers, treatment with exogenous Spd effectively delayed the progression of senescence; this delay was accompanied by a marked increase in free Spd and Put, suggesting that the PA could then bind and stabilise essential molecules (*e.g.* DNA fragmentation was delayed) of the corolla cells (Bagni & Tassoni 2006). PAs were also found conjugated to proteins, as reported in *Nicotiana* by Serafini-Fracassini *et al.* (2002). This system represents a good flower corolla model, and the senescence and death have been studied *in planta* or in flowers excised at different growth stages (Serafini-Fracassini *et al.* 2002; Della Mea *et al.* 2007a,b). To evaluate the anti-senescence effects of PAs, detached *Nicotiana* flowers were treated with exogenous Spm and with silver thiosulphate (an inhibitor of ethylene action); the treated flowers showed delayed senescence, slowed DNA fragmentation and vacuole damage, and prolonged chloroplast viability, together with visible preservation of chlorophyll content (Serafini-Fracassini *et al.* 2002). Spm taken up was also converted back to Put and Spd; both were found either in free or TCA-soluble form. In *Nicotiana*, these conjugates are mainly hydroxycinnamoyl derivatives, which are known to increase during flowering (Martin-Tanguy *et al.* 1996). However, no evidence has been reported regarding their involvement in senescence.

REGULATION OF SENESCENCE BY SUGARS

The role of carbohydrates in the regulation of plant organ senescence remains debatable. Abundant evidence indicates that carbohydrates can induce and inhibit the senescence process in leaves and petals (van Doorn 2008, 2004). During leaf senescence, the rate of photosynthesis decreases, which suggests that a low carbohydrate content induces senescence (Thimann *et al.* 1977; Quirino *et al.* 2000). Confirmation of this claim originates from experiments performed in the 1970s in which segments of oat leaves were floated on a solution of glucose or sucrose in the dark. It was demonstrated that yellowing was inhibited in the infiltrated segments of leaves because of the continued presence of sugars in leaf tissues (Thimann *et al.* 1977). Grbić & Bleecker (1995) reported a positive correlation between low carbohydrate content in leaves and increased production of ethylene, which is a senescence-inducing phytohormone.

Carbohydrates also have a crucial impact on the regulation of senescence-related gene expression. The *SAG12* gene, which encodes a cysteine protease, is expressed during late leaf senescence. Paul & Pellny (2003) determined that late *SAG*s such as *SAG12* are repressed by sugar, whereas early *SAG*s are induced by sugar. Noh & Amasino (1999) indicated that sugars rapidly reduce the amount of *SAG12* transcripts in *Arabidopsis*. During petal senescence, new evidence was found indicating that sugar starvation can stimulate this process. In general, the exposure of cut flowers to sugars delays the visible symptoms of senescence (van Doorn 2004). Changes in ultrastructure and gene expression during petal senescence are similar to those in sugar-starved organs. Studies performed on *Sandersonia aurantiaca* have shown that after petals are fed with sugar, protein degradation and the expression of several genes related to senescence are delayed (Eason *et al.* 1997). Carbohydrates also constitute an important factor controlling senescence in roots. One of the hypotheses for the regulation of root lifespan assumes that depletion of starch due to its use during root formation and cellular respiration determines root viability (Adams 2014).

However, some authors claim that carbohydrates both stimulate senescence and provide opposing evidence to that described previously. In poplar, the level of soluble carbohydrates is significantly lower in brown or dead roots than in white fine roots (Kosola *et al.* 2002). One of the arguments that supports this hypothesis is the occurrence of high levels of carbohydrates found in leaf tissues at the beginning of senescence (Quirino *et al.* 2001; Stessman *et al.* 2002; Diaz *et al.* 2005; Pourtau *et al.* 2006; Wingler *et al.* 2006; Agüera *et al.* 2010). It is also unknown why sugars accumulate while photosynthetic activity decreases. This issue has not been fully explained. One probable explanation is that the accumulation of non-structural carbohydrates is associated with both the hydrolysis of starch present in the cells and the loss of the structural and functional integrity of cellular membranes, resulting in the intensified catabolism of membrane lipids and the formation of sugars during the course of gluconeogenesis. Experiments in which wheat (Feller & Fischer 1994) and barley (Parrott *et al.* 2005) phloem export was prevented by girdling treatments at the base of the leaves have shown that the accumulation of sugar is associated with earlier leaf yellowing and (in barley) increased protease activity. High levels of carbohydrates were also reported in petals that showed visible signs of senescence, which suggests a senescence-inducing role of sugars (van Doorn 2004).

Many experiments related to the regulation of senescence by sugars have been performed on *A. thaliana*. Leaf senescence of growing plants can be stimulated in the presence of 2% glucose in combination with low concentrations of nitrogen (Pourtau

et al. 2006; Wingler *et al.* 2006). The most important evidence about the role of sugar signalling in the regulation of senescence comes from genetic studies. Moore *et al.* (2003) have demonstrated that the *Arabidopsis* hexokinase-1 mutant *gin2-1* experiences delayed senescence. In contrast, overexpression of the HXK1 gene accelerates senescence (Dai *et al.* 1999). Hexokinase-1 is a sugar sensor that is involved in sugar signalling during senescence; hexoses do not accumulate in the senescing leaves of the mutant. Compared with wild-type plants, plants in which HXK1 is overexpressed accumulate higher concentrations of hexoses. Additional evidence supporting the hypothesis that the accumulation of sugar is one of the factors that induces senescence comes from changes in gene expression that occur in the sugar-accumulating *pho3* mutant (Lloyd & Zakhleniuk 2004). Many genes whose expression increases during developmental senescence are also induced in the *pho3* mutant.

Successful understanding of the cooperation between different regulators, explanation of how sugar induces senescence and the interaction between phytohormones and sugar signalling are crucial. Balibrea *et al.* (2004) showed that CKs could induce extracellular invertase activity, which increases the use of sugar and, surprisingly, reduces the accumulation of glucose, resulting in delayed senescence. However, according to Wingler *et al.* (1998), the accumulation of sugars during senescence can block the effects of CKs, especially under low-light conditions, and this blockage can induce senescence. Nevertheless, we must be aware that the accumulation of sugar might be a consequence of only age-dependent changes in metabolism and is not directly involved in the regulation of leaf senescence. Substantial efforts should be made to synthesise existing information regarding senescence regulation by sugars to determine which of these dual roles is more crucial.

SUMMARY, PERSPECTIVES AND RECOMMENDATIONS

In summary, the regulation of senescence involves a complicated network of dependencies. It is not possible to distinguish one leading trail. Senescence is regulated by a number of different processes that are also complementary to each other. For this reason, a large network of mutual regulation occurs wherein all these processes acting together lead to the death of organs. As such, it is interesting how many similarities in the senescence process occur between various ephemeral organs that perform completely different functions and are characterized by completely different structures. The senescence processes of plant organs are quite similar, and similarities are seen both at the physiological and molecular levels. Leaves and petals are characterised by many similarities during senescence at the morphological, physiological and molecular levels. In the case of senescence of fine roots, no molecular studies exist to confirm a common mechanism of this process. However, taking into account the conducted cytological analyses, similar changes within the fine roots could possibly be discerned.

Despite studies in previous decades providing valuable information about plant hormones/regulators involved in signalling or regulatory pathways during individual plant organ senescence, a broader framework of this process as a universal route for every ephemeral organ and/or the whole plant is less well founded. We propose a few priorities and goals to help guide future research on both senescence and its regulatory pathways. Outstanding tasks that should provide new insight into the universality of senescence include the following issues:

1 Unlike that concerning aboveground leaf and petal traits, there is a lack of information on fine root senescence mechanisms and regulatory pathways.
2 Joint assessments of plant hormone/regulator crosstalk, as well as the identification of receptors and other upstream signalling components, are needed.
3 Long-term and complex studies at multiple time points of the senescence of long-lived species that develop organs with limited lifespan are indispensable.
4 Better scaling of plant senescence requires a holistic research approach and explanation of why many exceptions, if they occur, do not fit the expected patterns.

In conclusion, senescence as an evolutionarily acquired developmental strategy is a complex and complicated process that leads to death, is prolonged and maintains its own dynamics. Given that senescence is a key process that enables the valuable remobilisation of nutrients from senescent organs/tissues, it is surprising that the physiological mechanisms that regulate senescence are still poorly understood. This poor understanding is due to the difficulty in distinguishing one main mechanism that is responsible for the regulation of senescence or a single precisely defined pathway for every organ among the above- and belowground parts of the plant. In contrast, versatility and universality of this process exist regardless of the organ or species analysed. Given that the wide range of questions to which regulatory pathways of senescence can be applied, identifying which individual symptoms are most crucial is challenging and perhaps not always meaningful. The involvement of regulators of plant growth and sugars in senescence enables this process to be initiated and to progress at a beneficial time for the function of the entire organism. We postulate that several processes that are interdependent and complement each other are involved, and that this involvement results in a large regulatory network that ensures the appropriate course of the senescence process. Therefore, despite the development of molecular biology methods, the composite study of this process is not straightforward, and many questions remain unanswered, which makes senescence an important subject for further research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Supplement to Fig. 1, with detailed information not included in the schema.

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Autophagy counteracts instantaneous cell death during seasonal senescence of the fine roots and leaves in *Populus trichocarpa*

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Abstract

Background: Senescence, despite its destructive character, is a process that is precisely-regulated. The control of senescence is required to achieve remobilization of resources, a principle aspect of senescence. Remobilization allows plants to recapture valuable resources that would otherwise be lost to the environment with the senescing organ. Autophagy is one of the critical processes that is switched on during senescence. This evolutionarily conserved process plays dual, antagonistic roles. On the one hand, it counteracts instantaneous cell death and allows the process of remobilization to be set in motion, while on the other hand, it participates in the degradation of cellular components. Autophagy has been demonstrated to occur in many plant species during the senescence of leaves and flower petals. Little is known, however, about the senescence process in other ephemeral organs, such as fine roots, whose lifespan is also relatively short. We hypothesized that, like the case of seasonal leaf senescence, autophagy also plays a role in the senescence of fine roots, and that both processes are synchronized in their timing.

Results: We evaluated which morphological and cytological symptoms are universal or unique in the senescence of fine roots and leaves. The results of our study confirmed that autophagy plays a key role in the senescence of fine roots, and is associated also with the process of cellular components degradation. In both organs, structures related to autophagy were observed, such as autophagic bodies and autophagosomes. The role of autophagy in the senescence of these plant organs was further confirmed by an analysis of *ATG* gene expression and protein detection.

Conclusions: The present study is the first one to examine molecular mechanisms associated with the senescence of fine roots, and provide evidence that can be used to determine whether senescence of fine roots can be treated as another example of developmentally programmed cell death (dPCD). Our results indicate that there is a strong similarity between the senescence of fine roots and other ephemeral organs, suggesting that this process occurs by the same autophagy-related mechanisms in all plant ephemeral organs.

Keywords: Autophagy, *ATG* genes, ATG8 protein, Senescence, Leaves, Fine roots

Background

Senescence, as the final, inevitable stage of development before death, can occur in a select group of cells, tissues, organs, or even an entire plant. Seasonal senescence of organs is an adaptation that allows plants to adapt to a yearly change in environmental conditions. Regardless of the reason, senescence is a precisely regulated process that follows well-defined steps, clearly reflected by distinct physiological, cytological, and transcriptomic events [1, 2]. The precise control of senescence is necessary to allow the process of remobilization to occur, which is the main goal of prolonged senescence instead of rapid death [3]. During senescence, the degradation of cellular components is accelerated. The remobilization process allows those degraded components, that are still valuable for plants, to be transformed into forms that can be transported in the phloem and relocated to other parts of the plant e.g. to developing seeds or other

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storage organs [4–8]. There is also a body of evidence which demonstrates that autophagy plays a significant role in nutrient recycling during the senescence of plant organs [9–12].

Autophagy is an evolutionarily conserved, intracellular pathway in eukaryotic cells for the massive degradation of cytoplasmic components in a lytic compartment within cells [13]. It is responsible for the turnover of cytoplasm [14], scavenging of unnecessary cellular components [15], formation of some tissues [16–18], and biotic [19–23] and abiotic stress responses [24–28]. Thus, autophagy helps to preserve cell homeostasis. Microscopic observations of cells can distinguish three types of autophagy: micro-, macro-, and mega-autophagy [12, 29, 30]. During microautophagy, a small fragment of sequestered cytoplasmic constituents is incorporated into the vacuole by invagination of the tonoplast membrane [14, 31]. In macroautophagy, cellular material, or even entire organelles, intended for degradation are encapsulated in double-membrane vesicles called autophagosomes which are then transported to the vacuole. After fusion of the autophagosome and tonoplast membranes, the cytoplasmic cargo, contained a single membrane vesicle structure (autophagic body) is delivered into the vacuolar lumen [31]. Mega-autophagy, the third type of autophagy, begins with an intensive synthesis of hydrolytic enzymes, which results in enlarged vacuoles and increased tonoplast permeability. Finally, when the tonoplast is ruptured, the protoplast of the cell becomes acidified which leads to cell death [31].

The first evidence that autophagy plays a significant role in the controlled senescence of plant organs came from microscopic studies of senescing leaves of *Triticum aestivum*. Wittenbach et al. [32] observed that whole chloroplasts were present in the central vacuole which was filled with lytic hydrolases. In senescing petals of *Ipomoea purpurea* [33] and *Dianthus caryophyllus* [34], numerous vesicles containing fragments of degraded protoplast were observed. Similarly, in senescing fine roots of *Populus trichocarpa*, numerous autophagy-related structures have been observed [29]. As molecular tools developed, a plethora of mechanisms associated with autophagy were reported. In genetic screens of *Saccharomyces cerevisiae* for autophagy-defective yeast mutants, a number of *ATG* (*AuTophaGy*) genes required for autophagy were identified as being indispensable for the formation of autophagosomes during macroautophagy [13, 35]. The *ATG* genes and their protein products are also highly conserved in plants [14] and their occurrence and activity have been described in detail in *Arabidopsis* [36–38], rice [39], and maize [40]. The central core of autophagy machinery, which is necessary for autophagosome assembly, consists of 18 ATG proteins. These proteins can be divided into four groups based on their function: *(1)* the ATG1 protein kinase complex, which is necessary for induction and coordination of autophagy; *(2)* the PI3 kinase complex that is involved in the recruitment of the ATG18–ATG2 complex to PI3P in the autophagic membrane through an interaction between ATG18 and PI3P *(3)* the ATG9 complex which plays a role in delivering lipids to the pre-autophagosomal structure, and *(4)* two ubiquitination-like systems involved in elongation and enclosure steps during autophagosome formation (ATG12, ATG8) [41]. Analyses of gene expression indicated a significant increase in the expression of *ATG* genes during the senescence of leaves and flower petals [12, 42–44]. A significant role of autophagy in the senescence process was also confirmed in studies utilizing *Arabidopsis* mutants that displayed early and fast leaf senescence phenotypes [9]. In that study, the authors also indicated an intriguing role for autophagy in the remobilization process. The *atg* mutants are characterized by hypersensitivity to nitrogen, reduced seed production, and inhibition in the formation of Rubisco-containing bodies (RCB) [9]. Similar to leaves and flower petals, most fine roots, in contrast to pioneer roots, are short-lived [45]. Despite all the information that has been forthcoming on senescence, autophagy, and remobilization in leaves and flower petals, a similar level of understanding of the process of senescence in fine roots is lacking.

The most recent classification scheme classifies fine roots as first, second, and third order roots with a diameter < 2 mm [46]. They are characterized by a lack of secondary structure, the frequent presence of mycorrhizae, and a high surface to weight ratio [46]. These properties make them efficient in the absorption of water and minerals from the soil [47]. Fine roots, similar to leaves and flower petals, senesce and die after performing crucial functions that support plant growth and development. Root senescence and death have received a great deal of research interest over many years due to the importance of roots as a component of soil biomass and their effect on biological processes in forest ecosystems. The annual biomass production of fine roots is equal to or greater than the biomass of leaves, thus, the senescence and death of fine roots represent an important aspect of the cycling of chemical elements [48, 49].

In the present study, focus was placed on developing a more complete understanding of the process of fine root senescence relative to the same process in leaves. Despite the number of published root studies, few overall generalizations pertaining to the senescence process in roots have been established. This is perhaps principally because no conceptual framework exists for how root lifespan is constrained and controlled by cell or tissue physiology and genetics. While some theories to explain the control of fine root lifespan have been forwarded, very little data is available to evaluate these theories. Although this knowledge is crucial, obtaining high-quality data on this subject can be difficult and problematic. In the present study, we hypothesize that autophagy is an integral aspect of the senescence process in

fine roots, as it is in seasonal leaf senescence, and that both processes are synchronized in their timing. We have conducted a significant amount of research to determine which morphological and cytological symptoms of root and leaf senescence are characteristic and either universal or unique to each organ. A molecular analysis of fine root senescence was also conducted, which provides the first evidence to support the premise that the senescence of fine roots can be seen as another example of developmentally programmed cell death (dPCD).

Results

Structure of senescing fine roots and leaves of *P. trichocarpa*

Fine roots and leaves were systematically monitored during the growing season to detect the first visible/measurable symptoms of senescence. Therefore, several morphological, anatomical, and cytological features were identified. Chlorophyll levels were also measured in leaves (Fig. 1). After an analysis we classified the material studied into six groups and these groups were used as experimental variants in other studies. The six classified groups were designated as: green leaves - control (LC); two stages of senescing leaves - yellowing leaves (LS1) and yellow leaves (LS2); white fine roots - control (RC); and two stages of senescing roots - light brown roots (RS1) and dark brown roots (RS2).

Morphological symptoms of senescence and cell viability in senescing organs

The pigmentation of both fine roots and leaves changed as the senescence process progressed (Fig. 2a-c; Fig. 3a-c). Fine roots changed in color from white to light brown to dark brown or black. A significant shrinkage in dark brown and black roots was also observed (Fig. 2b, c, h, i). Color changes in leaves were associated with decreases in chlorophyll content (Fig. 1; Fig. 3a-c). A viability assay was conducted to determine if the changes in color were associated with a loss of cell viability in leaves and fine root tissues. A fluorescent signal was observed in the majority of cells of white fine roots (RC) and green leaves (LC) (Fig. 2d; Fig. 3d); indicating a high level of cell viability. The number of cells with a fluorescent signal in the light brown roots (RS1) and yellowing leaves (LS1), however, decreased relative to the signal levels in control samples (Fig. 2e; Fig. 3e). Lastly, the fluorescent signal in dark brown roots (RS2) and yellow leaves (LS2) was very low and was not present in many of the analyzed sections of tissues (Fig. 2f; Fig. 3f).

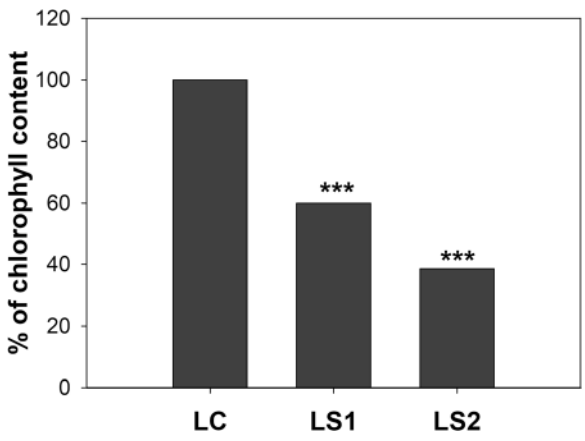

Fig. 1 Changes in chlorophyll level in leaves during the growing season

Anatomical characteristics of senescence

An anatomical analysis using light microscopy was conducted to identify anatomical changes that were characteristic of the senescence process in two organs (leaves and fine roots). Pronounced, progressive changes were observed in fine roots. At the beginning of the growing season, fine roots (RC) were white and their morphology was round and regular (Fig. 2g). Internally, their cells had the appearance of features reflective of full turgor without any evidence of damage. The layer of cortical parenchyma cells was characterized by the presence of a large number of cells (Fig. 4a) without any evident signs of senescence. In the next two sampling periods (October and November), an increasing number of senescing roots were harvested. The most apparent characteristic in senescing fine roots (RS1 and RS2) were changes in their shape. Due to the occurrence of folded cell walls in cortical parenchyma cells, the morphological shape of the fine roots was not consistently round and regular, as had been observed in the RC root samples (Fig. 2h, i). This was confirmed by diameter measurements where a statistically significant decrease was apparent in RS1 and RS2 fine roots, relative to RC fine roots (Fig. 4b). Furthermore, many of fine roots collected at the RS2 stage were already dead and their overall structure was completely destroyed (Fig. 2i).

In contrast to fine roots, anatomical symptoms of senescence in leaves were not as readily evident (Fig. 3g-i). Significant changes in the shape of mesophyll cells were not observed, but the number of mesophyll cells was significantly lower relative to the control leaves (Fig. 3g-i). Measurements did not show statistically significant differences in the width of palisade mesophyll, however, a decrease in the leaf width occurred during the senescence process (Fig. 4c, d).

Cytological analyses of senescing fine roots and leaves

Based on the morphological and anatomical observations made of senescing leaf and fine root organs, cytological analyses focused on the cortical parenchyma cells of fine roots (Fig. 5) and the palisade and spongy mesophyll cells in leaves (Fig. 6). Cortical parenchyma cells in white, fine roots (RC) exhibited a regular shape with thin cell walls (Fig. 5a).

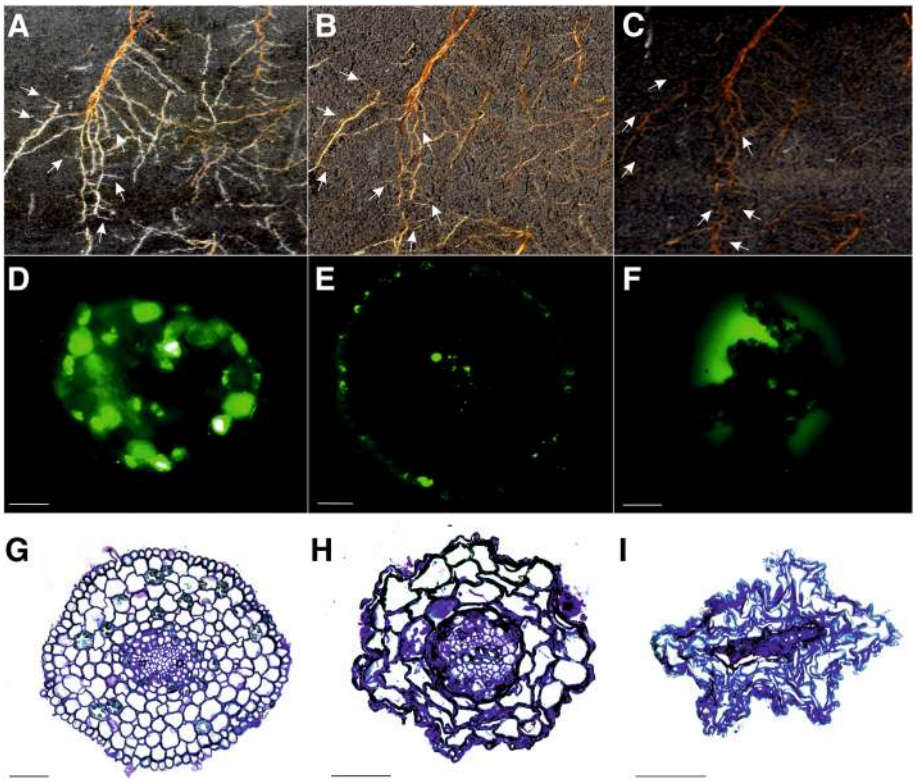

Fig. 2 Senescence-related changes in fine roots (**a**-**c** – changes in morphology; **d**-**f** – changes in cell viability; **g**-**i** – changes in anatomy). Bars, 50 μm

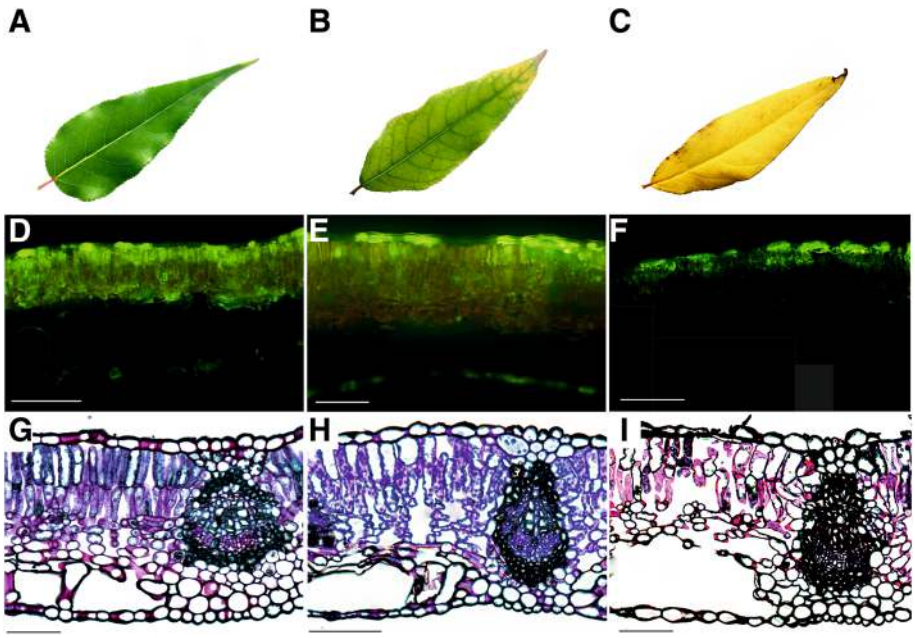

Fig. 3 Senescence-related changes in leaves (**a-c** – changes in morphology; **d-f** – changes in cell viability; **g**-**i** – changes in anatomy). Bars, 100 μm

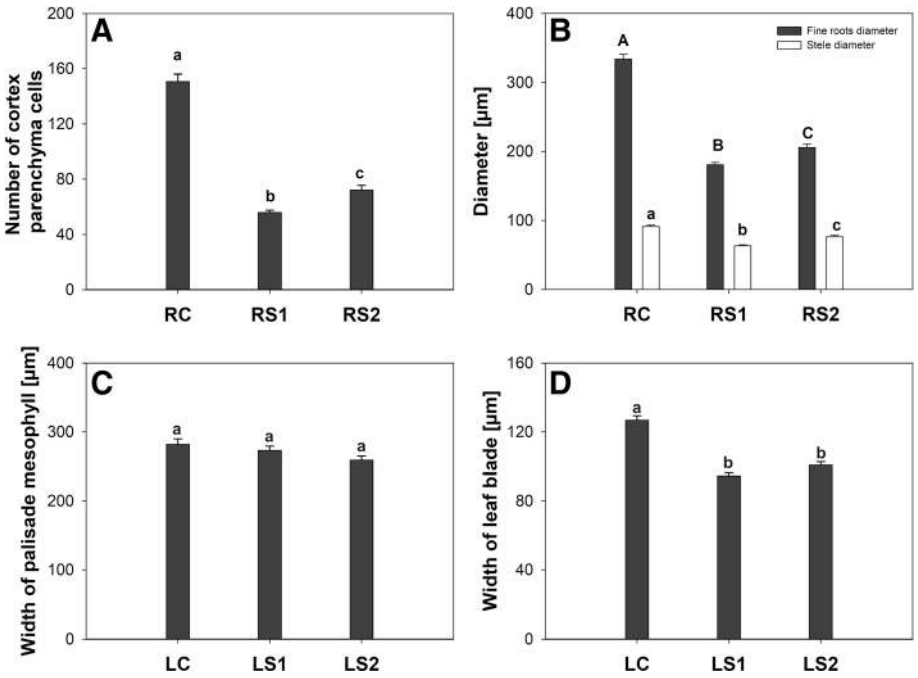

Fig. 4 Changes in the structure of fine roots and leaves in relationship to the senescence process. **a** – Number of cortical parenchyma cells per section of fine roots. **b** – Changes in the diameter of roots and the stele during senescence. **c** – Width of the palisade mesophyll in leaves of *P. trichocarpa*. **d** – Width of the leaf lamina in *P. trichocarpa*. Bars sharing the same letter are not significantly different ($P = 0,05$). Values represent the mean ± SE (standard error)

A centrally located vacuole occupied most of the entire cell. The cytoplasm with its organelles was present as a thin band along the periphery of the cell wall (Fig. 5b). Tannins were observed in vacuoles of several cortical parenchyma cells, usually in close vicinity of the tonoplast (Fig. 5c). In contrast, evidence of senescence was readily observed in light brown (RS1) and dark brown (RS2) fine roots. The majority of cortical parenchyma cells in RS1 fine roots exhibited structures related to autophagy (Fig. 5d-f). Vesicles with cytoplasmic residues were observed in numerous cells. Those structures were similar to the vesicles present in cells undergoing microautophagy (Fig. 5d, e). Moreover, in RS2 cortical parenchyma cells, autophagic bodies inside vacuoles were also detected (Fig. 5g). Furthermore, the cell shape in the majority of RS2 cortical cells was more irregular than the oval shape of cells that were observed in RC and RS1 samples (Fig. 5h, i). Notably, cell walls were folded and the tonoplast was ruptured in cells that appeared to be in the last stage of senescence before dying. Furthermore, numerous microorganisms were observed in the external cortex of RS2 fine root samples (Fig. 5i).

Many changes in leaf ultrastructure related to the senescence process were also visible (Fig. 6). Control cells from LC were characterized by the presence of plenty organelles (mitochondria, endoplasmic reticulum and chloroplasts) with a normal appearance. Moreover, a significant number of starch granules were observed in both palisade (Fig. 6a) and spongy mesophyll (Fig. 6b) cells. In contrast, the appearance of the majority of the cells from yellowing leaves (LS1) was distinctly different (Fig. 6d-f). Among the different organelles, the first and most rapid alterations in response to senescence were observed in chloroplasts where the internal structure was greatly modified (Fig. 6d). Ultrastructural analysis revealed the disintegration of thylakoids, with a concomitant massive formation of plastoglobules that were mostly located between the thylakoids within the senescing chloroplasts (Fig. 6d). Moreover, spherical bodies separating themselves from chloroplasts were observed in several cells, including Rubisco-containing bodies (RCB) (Fig. 6e). Furthermore, several different autophagy-related structures were observed in the cytoplasm, including autophagic bodies in the vacuole lumen (Fig. 6d) and autophagosomes (Fig. 6e). Evidence of the formation of these structures was also observed, seen as the joining of several tubules and vesicles (Fig. 6f). Many cells in yellow leaves (LS2) exhibited more advanced senescence-related changes (Fig. 6g-i). The structure of chloroplasts was more visibly altered, an increasing number and size of plastoglobules were observed (Fig. 6g), as well as more distended thylakoids. Ruptured tonoplasts were observed in several cells, which resulted in the degradation of all cellular structures due to the acidification of the cytoplasm that occurred once the tonoplast was ruptured (Fig. 6h, i).

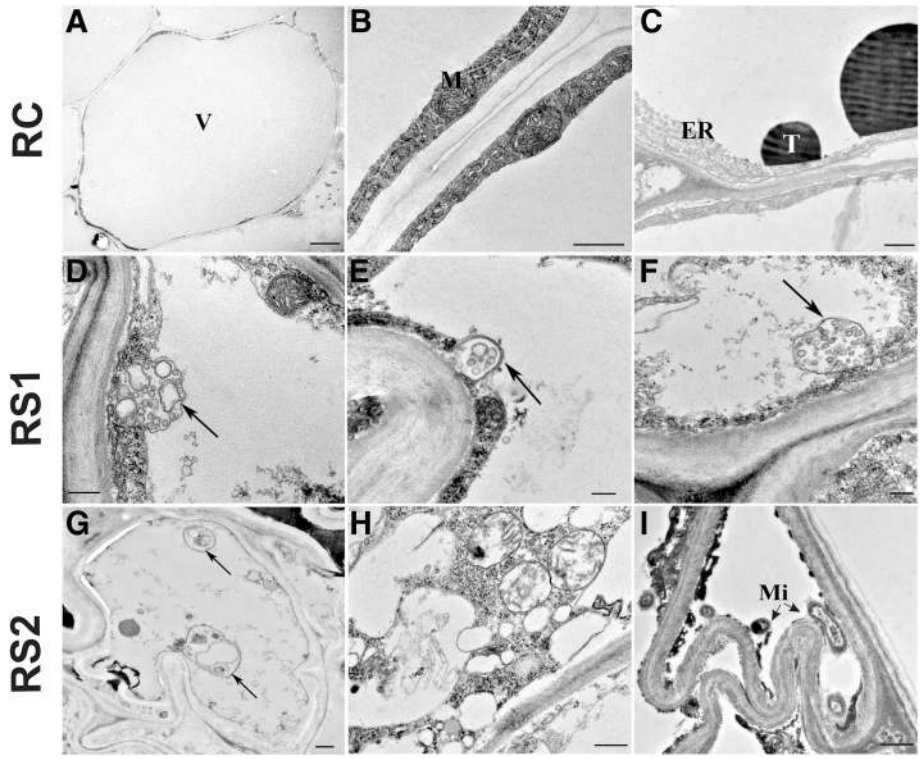

Fig. 5 Changes in ultrastructure of cortical parenchyma cells in fine roots during the course of senescence. **a**-**c** - white fine roots - control (RC); **d**-**i** - two stages of senescing roots - light brown roots (RS1, **d**-**f**) and dark brown roots (RS2, **g**-**i**). Abbreviations: *V* vacuole, *ER* endoplasmic reticulum, *M* mitochondria, *T* tannins, *Mi* microorganism. Arrows indicate autophagy-related structures. Bars, 0,5 μm

Expression of *ATG* genes during senescence

The analysis of *ATG* genes expression revealed significant differences in gene expression between control and senescing leaf and fine root tissues (LC vs LS and RC vs RS). The expression of *ATG7*, *ATG8c*, *ATG8d*, *ATG8g*, *ATG8h*, *ATG11*, and *ATG18* were examined (Fig.7; Fig. 8). Statistically significant changes in the expression of majority *ATG8* genes (*ATG8c*, *ATG8d*, *ATG8g*) were observed in fine roots (Fig. 7). Expression of all of these genes increased at the first stage (RS1) of senescence and then decreased in the second stage (RS2) of senescence (Fig. 7). In contrast, a slightly different pattern of expression was observed in leaf tissues. In contrast to roots, the expression of all of the examined *ATG* genes was upregulated in leaf tissues in both stages (LS1 and LS2) of senescence (Fig. 8). The largest increase in expression level was observed in the second stage (LS2) of senescence.

Distribution and localization of ATG8 protein

Based on the significantly increased expression of *ATG* genes in both roots and leaves, the amount and localization of ATG8 protein, which is necessary for appropriate autophagosome formation, was examined by immunoblot (Western blot) and immunolocalization analyses. ATG8 protein can be detected either as a protein conjugated to phosphatidylethanolamine (PE) on an autophagosomal membrane or as a free protein without PE. The level of ATG8 protein in both fine roots and leaves changed over the course of the growing season (Fig. 9a; Fig. 10a). Results indicated that the amount of ATG8 protein exhibited a similar pattern to changes in *ATG8* gene expression.

The level of ATG8 was relatively low in viable, white roots (RC) (Fig. 9a). ATG8 was located mainly in xylem tissues or cells of the rhizodermis (Fig. 9b-d). A significant increase in the level of ATG8 was observed in the first stage (RS1) of senescence when fine roots appeared brownish (Fig. 9a). Both forms of ATG8 (free and conjugated to PE) were detected. ATG8 was localized in the majority of cortex parenchyma cells and in xylem tissues (Fig. 9e-g). ATG8 was detected in the cytoplasm, near the cell wall, or more concentrated in spherical bodies (Fig. 9e-g, arrows). Subsequently, when roots became dark brown (RS2), a slight decrease in the level of ATG8 was observed in fine root tissues. ATG8 conjugated to PE was the main form observed in RS2 fine root cells. The level of free protein was clearly lower in RS2 than in RS1 fine root cells (Fig. 9a). The localization of the conjugated and free protein did not appear to significantly change between the RS1 and RS2 stages of senescence (Fig. 9h-j).

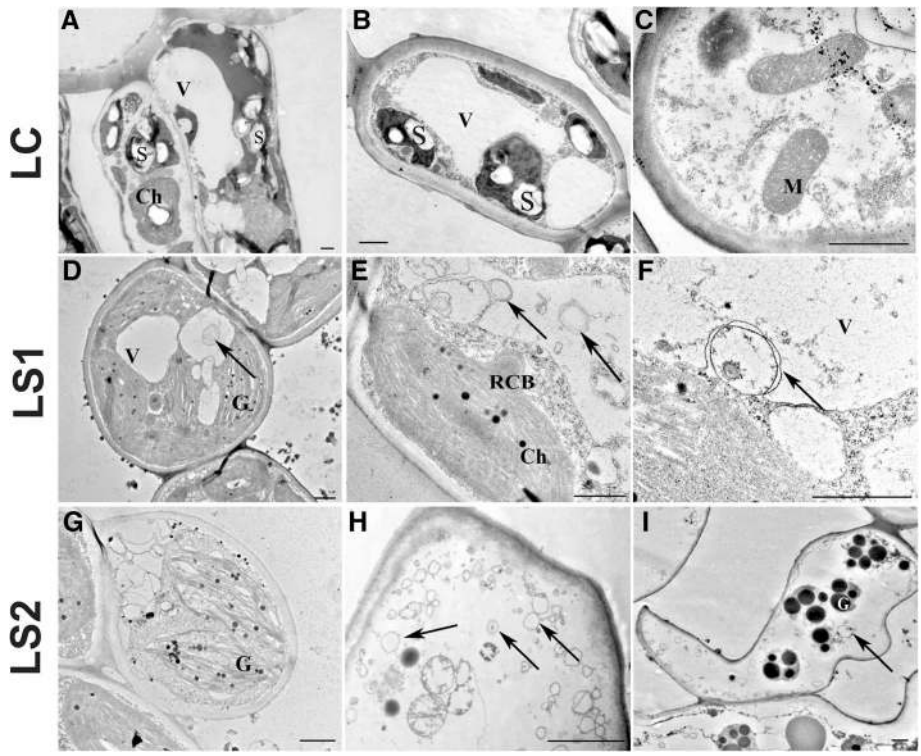

Fig. 6 Changes in ultrastructure of palisade and spongy mesophyll leaf cells during the course of senescence. **a**-**c** - green leaves - control (LC); **d**-**i** - two stages of senescing leaves - yellowing leaves (LS1, **d**-**f**) and yellow leaves (LS2, **g**-**i**) Abbreviations: *V* vacuole, *S* starch, *M* mitochondria, *RCB* Rubisco containing bodies, *G* gerontoplast, *Ch* chloroplast. Arrows indicate autophagy-related structures. Bars, 1 μm

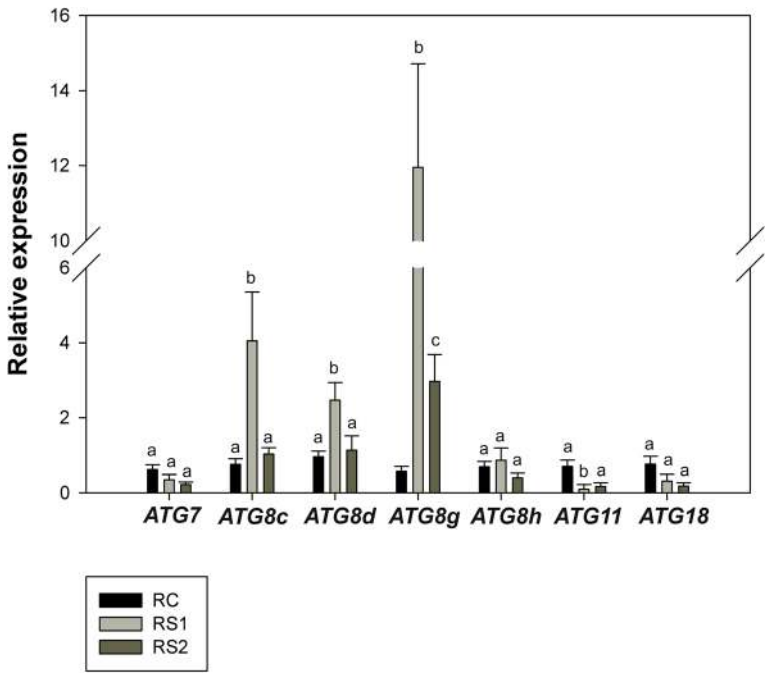

Fig. 7 Relative expression of *ATG* genes in fine roots (RC – root control, RS1 –first stage of root senescence, RS2 –second stage of root senescence) of *Populus trichocarpa*. Bars sharing the same letter are not significantly different ($P = 0{,}05$). Values represent the mean ± SE (standard error)

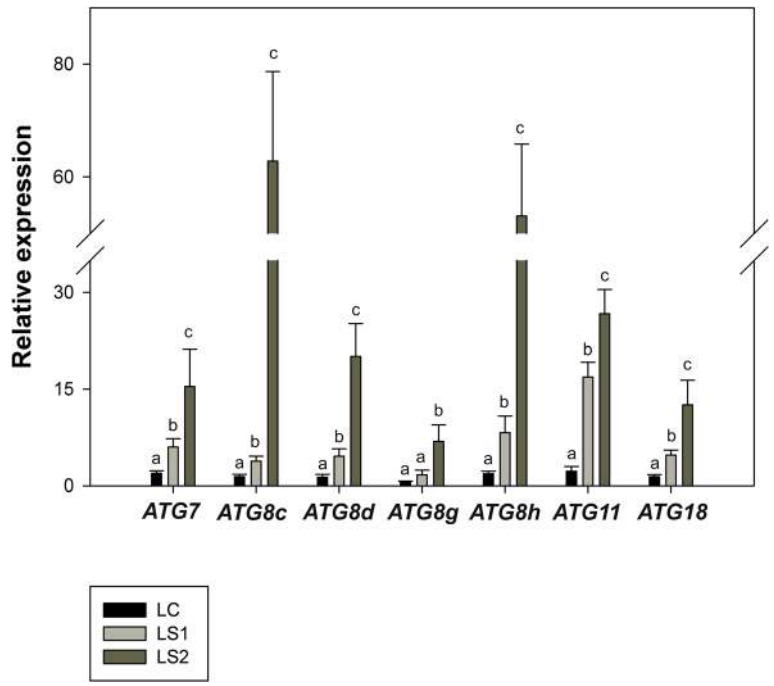

Fig. 8 Relative expression of *ATG* genes in leaves (LC – leaf control, LS1 –first stage of leaf senescence, LS2 – second stage of leaf senescence) of *Populus trichocarpa*. Bars sharing the same letter are not significantly different ($P = 0{,}05$). Values represent the mean ± SE (standard error)

The level of the free form of ATG8, as well as the form in which ATG8 is conjugated to PE, was very low in green leaf (LC) tissues (Fig. 10a). A positive localization signal was mainly observed within the vascular bundle in xylem cells (Fig. 10b, c). In palisade and spongy mesophyll cells, ATG8 was localized in several cells but the signal level was relatively low (Fig. 10b-e). The level of ATG8 noticeably increased in yellowing leaves (LS1) and was mainly the form in which ATG8 is conjugated to PE (Fig. 10a). The signal was localized in epidermal cells, as well as the spongy and palisade mesophyll (Fig. 10f-i). ATG8 protein was mostly localized in spherical bodies (Fig. 10h, i, arrow) which were located in proximity to the cell wall. A significant level of localization also occurred in xylem vessels (Fig. 10f). A notable increase in the ATG8 level was observed in yellow leaves (LS2) (Fig. 10a). Microscopic analysis also revealed a strong localization of ATG protein in most cells (Fig. 10j-n). ATG8 protein was localized in cells of the epidermis, spongy and palisade mesophyll, and in xylem vessels. The distribution of ATG8 was similar in both the LS1 and LS2 stages of senescence, where it was concentrated in spherical bodies (Fig. 10j, m, n, arrow) but also dispersed in the cytoplasm (Fig. 10j-n).

Discussion

In this work, we emphasize the universality of senescence, which occurs in all ephemeral organs, and further indicate that regardless of the organ being examined, some aspects of senescence are common to all aging processes. Despite copious research conducted on programmed cell death in plants, a detailed understanding of the mechanisms underlying autophagy, which occurs during the senescence of all organs and tissues, is still insufficient. A key question is whether common mechanisms can be identified that are responsible for senescence in different plant organs? While the very precisely controlled death of specific cells during early development has been well described, the process of senescence in plants is well described only for leaves, fruits, and flower petals. Although senescence also occurs in below ground plant organs, this process is barely understood in root systems due to the difficulty in harvesting of roots. In spite of, or perhaps because of, these limitations, the present study compared the natural senescence process that occurs in two different plant organs: leaves and fine roots. We were interested to determine whether organs that serve completely different functions and possess a completely different structure undergo senescence in a similar manner. Morphological, anatomical, cytological, and molecular characteristics were used to analyze this question.

Utilizing the FDA viability tests, both fine roots and leaves were confirmed to undergo a gradual decrease in cell viability, along with morphological symptoms of senescence. Moreover, the decrease in viability observed in leaves and fine roots was synchronized in its timing, indicating that senescence in these two organs is induced by the seasonal change in environmental conditions. Our results are consistent with those obtained by Comas

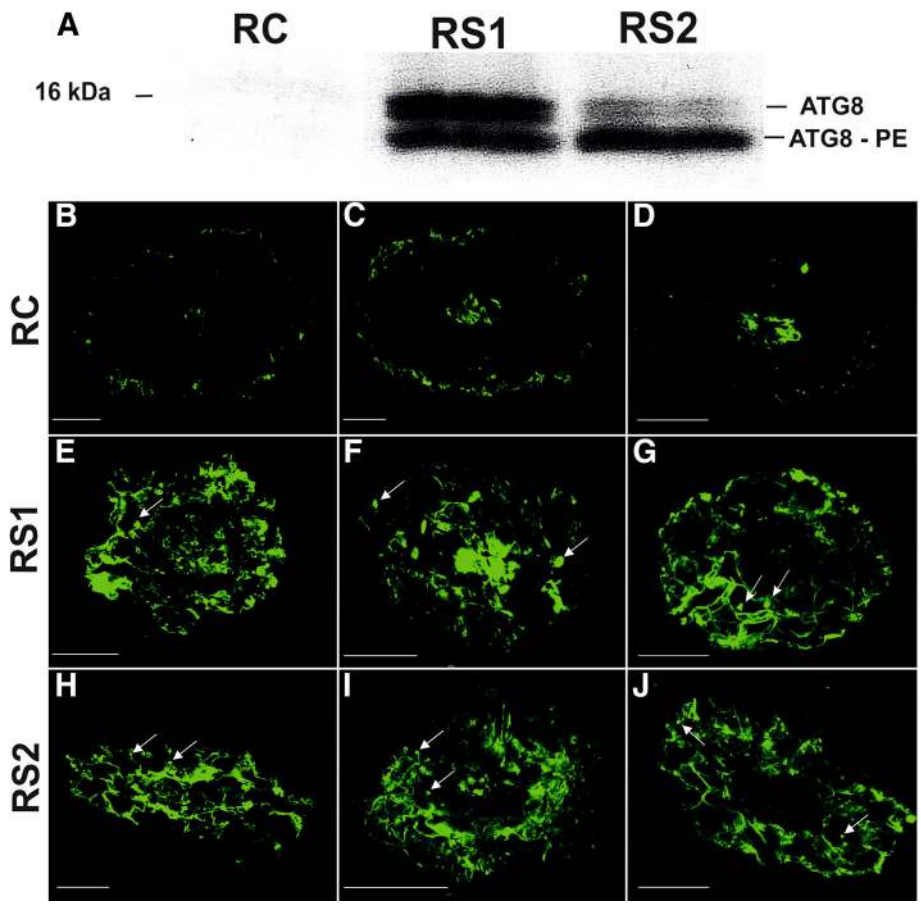

Fig. 9 ATG8 protein levels (**a**) and the immunolocalization of ATG8 protein (**b-j**) in fine roots (RC – root control, RS1 – first stage of root senescence, RS2 – second stage of root senescence) during the growing season. Bars, 50 μm

et al. [50] and Bagniewska–Zadworna et al. [29], who investigated the senescence of roots in *Vitis labruscana* and *Populus trichocarpa,* respectively. These studies also observed a progressive decrease in cell viability during the senescence process. The morphological features that were observed in our study were associated with a change in the pigmentation of the senescing organs and a shrinkage of the entire organ. Indeed, the first noticeable similarity in the senescence of fine roots and leaves was a change in color. The change in leaf color, which results from the degradation of chlorophyll, has been often described in numerous plant species, including *Glycine max* and *Arabidopsis thaliana* [51], *Chenopodium quinoa* [52], *Gossypium hirsutum* [53]. Chlorophyll degradation during leaf senescence exposes carotenoids [54], and is the cause of the change in leaf color that occurs in autumn in deciduous trees. The color change of flower petals, another ephemeral organ, has also been documented in several plant species from different plant families, including *Ipomoea nil* [55, 56], *Nicotiana mutabilis* [57], *Antirrhinum majus* [58], *Argyranthemum frutescens* [58], and *Petunia hybrida* [59]. The senescing petals of *Hibiscus syriacus* become blueish when the ratio of flavonoids and anthocyanins changes and alters the pH of the cytoplasm [60].

Another morphological characteristic that occurs universally during the senescence of every ephemeral organ is shrinking and/or wilting [2]. In the present study, shrinkage was evident during the senescence of most fine roots of *Populus trichocarpa* in the second stage (RS2) of senescence. This observation is similar to the reports in earlier root studies [29, 50]. In contrast, a decrease was observed in the width of the leaf blades of *Populus trichocarpa* during leaf senescence. This may have been related to a loss in cell turgor, which makes the leaves appear withered.

A common sequence of events during the senescence of fine roots and leaves was also observed at an ultrastructural level. In both organs, the shape of cells became irregular and altered during the senescence process, which was in sharp contrast to the regular outline of cell shape observed at the beginning of the growth season. It is plausible that the change in cell shape may have been induced by an impairment of the cytoskeleton [61]. Early degradation of the lattice formed by cortical microtubules

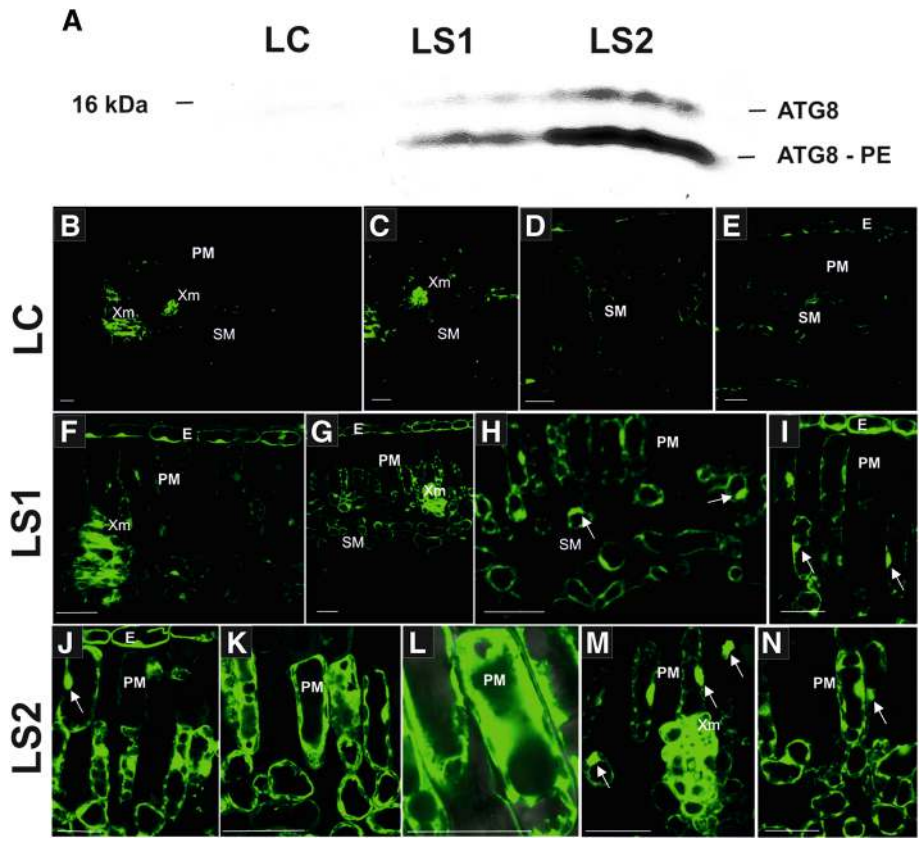

Fig. 10 ATG8 protein levels (**a**) and the immunolocalization of ATG8 protein (**b-n**) in leaves (LC – leaf control, LS1 – first stage of leaf senescence, LS2 – second stage of leaf senescence) during the growing season. (Abbreviations: *Xm* xylem vessels, *PM* palisade mesophyll, *SM* spongy palisade, *E* epidermis). Bars, 25 μm

was reported to occur during both natural and dark-induced senescence of *Arabidopsis* leaves [61]. The expression of genes related to the cytoskeleton, such as α-, β-, and γ-tubulins, were also reported to be repressed during leaf senescence [42].

Another common feature of the senescence process appears to be the occurrence of autophagy, which has been observed to occur at the beginning of senescence, evidenced by the accumulation of a large number of vesicles in senescing cells. These vesicles most likely formed through micro and/or macroautophagy, as evidenced by their localization and appearance, which indicated vesicle formation. The formation of multiple vesicles by the fusion of several tubules was evidence of macroautophagy according to van Doorn and Papini [62]. Spherical bodies separated from chloroplast, and remaining Rubisco-containing bodies (RCB) were also observed in leaf cells in the present study. RCB bodies are double-membrane vesicles which contain chloroplast proteins such as Rubisco and Gln synthetase [63, 64]. The presence of numerous double membraned vesicles was also observed in senescing petals of *Ipomoea purpurea* [33] and *Dianthus caryophyllus* [34]. These observations indicate that autophagy plays an important functional role during the senescence of all ephemeral organs, where it is equally responsible for degradation of cellular components and the selective recycling and remobilization of chemical constituents.

Autophagy is a universal mechanism in cells that is responsible for the degradation of aberrant proteins and damaged organelles so that cellular homeostasis is maintained [65, 66]. Autophagy is typically accompanied by the process of programmed cell death (PCD). This relationship has been confirmed during various developmental events in plants, such as xylogenesis [67], anther development [16], tapetum degradation [16], and the hypersensitive response (HR) [21]. Similar mechanisms may regulate cell death during the senescence of leaves and flower petals [43, 55, 64, 68, 69]. A significant knowledge gap still exists, however, regarding the presence of autophagy in the senescence of fine roots and details of its functional role.

Similar to the senescence process in leaves and petals, autophagy in fine roots is also involved in the disintegration of membranes, and delivering unwanted cytoplasmic material, such as targeted proteins, carbohydrates, and lipids, to vacuoles for breakdown; thus replenishing the supply of nutrients

needed for normal cell function. Therefore, autophagy often plays a dual antagonistic role as executioner and as a mediating, dilatory factor in senescence. Ultrastructural studies performed on senescing leaves and fine roots of *P. trichocarpa* in the present study provided many general observations. Many autophagy-related structures were observed in the cytoplasm and vacuole lumens of both leaf and fine root cells. To provide evidence supporting the origin of these vesicles and their association with autophagy in both organs, *ATG* gene expression and ATG protein levels were analyzed. The expression of the selected *ATG* genes increased in both leaf and fine root tissues during senescence. The highest increase in expression among the analyzed genes was observed for *ATG8* genes. ATG8, is a ubiquitin-like peptide tag which is necessary for formation of autophagosomes and is responsible for regulating their size [13, 70, 71]. ATG8 is conjugated to phosphatidylethanolamine (PE) on an autophagosomal membrane by a bond between the carboxyl-terminal glycine (Gly) of ATG8 and PE [12]. In various studies, ATG8 and its homologs (LC3 in mammals) was used as a reliable marker for the induction and progression of autophagy. Several *ATG8* genes have been identified in the plants [72]. The expression of *ATG8* genes observed in the present study in senescing organs (leaves and fine roots) of *P. trichocarpa* was slightly different in the two organs. In leaves, *ATG8c* and *ATG8h* exhibited the highest level of expression, while *ATG8g* was the most upregulated in fine roots. Tissue-specific expression of *ATG8* genes has also been observed in *Arabidopsis* [72]. During developmental and dark-induced senescence, *ATG8* expression has been reported to increase in leaves of *Arabidopsis thaliana* [42] and *Hordeum vulgare* [44], as well as in senescing petals of *Petunia hybrida* and *Impomea nil* [12, 65]. The involvement of autophagy in senescing fine roots was convincingly confirmed in our study by protein analysis, which indicated that the level of ATG8 protein significantly increased in senescing roots. ATG8 protein was localized in the cytoplasm and highly concentrated in specific, membrane bound structures. A similar observation was reported by Thompson et al. [72], who detected ATG8 fused with GFP in hypocotyl cells of young seedlings during N starvation.

Only a few studies can be identified where meaningful evidence of the dual role of autophagy during the senescence of plant organs has been provided. Although the dual role of autophagy as both a pro-survival and pro-death process was recently discussed [41], most studies have only focused on its role in the process of degradation. In the 1980s, electron microscopy provided visual evidence of chloroplast degradation and the presence of degraded chloroplast components in vacuoles [32]. Later, Ishida et al. [63] reported the accumulation of small bodies, which were designated as Rubisco containing bodies (RCB) based on their composition, in senescing leaves of *Triticum aestivum*. Plants constitutively expressing stroma-targeted GFP demonstrated that the accumulation of the GFP signal was localized in the vacuolar lumen of cells treated with concanamycin A, a drug that inhibits the degradation of autophagic bodies in the vacuole. Interestingly, RCB bodies were not observed in the cells of *atg5* mutants, suggesting that the autophagy-dependent process is responsible for the degradation of chloroplasts. A similar result was obtained with *Arabidopsis* mutants, *atg4a, atg4b-1*, which exhibit autophagy disorders and where RCB bodies were also not detected [73]. Degradation of chloroplasts by autophagy was unequivocally confirmed in studies where co-expressed stroma-targeted RFP and ATG8 fused with GFP colocalized in the vacuole of leaves [63, 74]. Autophagy plays the role of an executioner in the last stage of senescence process when increased permeability and eventual rupturing of the tonoplast membrane; resulting in the release of hydrolytic enzymes which cannibalize the protoplast and cause cell death [66]. Rupture of the tonoplast membrane represents the point-of-no-return and the described sequence of events has been observed in senescing leaves [75], flower petals [33], and fine roots [29].

Much less attention has been paid to the role of selective autophagy in the remobilization process [9–11]. Additionally, knowledge concerning the mechanisms and function of autophagy in nutrient availability and recycling in plants is less advanced for roots than it is for leaves. The first evidence for the role of autophagy in remobilization was provided in studies of *Arabidopsis* leaf senescence [9]. Genetic and molecular analyses utilizing mutants with impaired *ATG* genes help to document the biological function of autophagy in remobilization. Using wild-type (WT) and *atg* mutants of *A. thaliana* treated with $15NO_3^-$, the level of 15 N was evaluated. Results indicated that remobilization was significantly lower in the *atg* mutants than in WT plants [9]. Interesting results regarding the relationship between autophagy and remobilization during senescence came from a study of maize *atg12* mutants [11]. This study demonstrated that 15 N remobilization to seeds was altered in *atg12* autophagy-defective mutants. Surprisingly, the relocation of nitrogen to newly-formed leaves was greater in the *atg12* autophagy-defective mutants as compared to WT. Remobilization of nutrients is also observed during the senescence of flower petals. Quantitative analysis of nitrogen in *Petunia hybrida* flowers demonstrated that the level of N changed before and after pollination-induced senescence in the examined parts of a flower. Nitrogen content decreased in petals and increased in the ovaries of pollinated flowers [12].

The role of autophagy in remobilization is also essential in fine roots. Fine roots are characterized by a short lifespan which typically does not exceed two years [76, 77]. In *Populus*, the life-span of fine roots is usually

< 95 days [78]. Considering that the biomass of fine roots is equal to or greater than the biomass of leaves, remobilization is an important subject when discussing the cycling and recycling of chemical elements [48, 49]. Our current study indicated that the autophagy machinery is present and active in senescing fine roots, and suggests that substantial amounts of the elements stored in fine roots are remobilized to other parts of the plant. How large a portion is released to the soil may be also regulated by autophagy. Identifying the reason and underlying mechanism for the induction of autophagy and its biological function during the time period prior to the final death of fine roots will require additional studies.

Conclusion

The senescence of plant organs, despite its destructive character, is a genetically controlled process that follows a well-defined sequence of events and is regulated by multiple pathways [2]. Cell viability is also essential for the initiation and progression of cell senescence. As long as a cell is viable, autophagic processes can be utilized to continue the process of degradation and remobilization in a controlled manner without crossing the point-of-no return and the final result, cell death. Our study comparing the senescence process in fine roots and leaves, helps to establish a cohesive model of the process of senescence in ephemeral organs. The combination of current and long-established information, clearly indicates that autophagy is a multifaceted system that plays a role in both the degradation of unwanted, unneeded cellular material, and the remobilization of valuable nutrients. How autophagy regulates cell survival and death however, is still not well understood and should be a priority for future research.

Methods

Plant material and growth conditions

All experiments were performed on fine roots and leaves of *Populus trichocarpa* (Torr. & Gray) growing at an experimental field site at the Institute of Dendrology, Polish Academy of Sciences in Kórnik (52°14′40″N and 17°06′27″E).

Seeds were obtained from the FLORPAK Młynki Seeds Store, Poland. Seedlings were initially grown in a plant growth chamber (*Conviron GR96*) at 18 °C day/14 °C night and a 16 h day/8 h night photoperiod. After 3 months, plants were transferred into rhizotrons. The rhizotrons (50x30cm) were constructed of two transparent polycarbonate plates held 3 cm apart by thick-walled plastic tubing to provide sufficient growing space. The rhizotrons were placed in an underground chamber. They combine the controlled conditions of laboratory experiments with the advantages of a natural field setting. Waterlogging was avoided by providing a drainage hole in the bottom of each rhizotron. This permitted soil aeration and drainage of excess water. An automated system was used for the watering of individual plants. Plants were grown in rhizotrons consisting of clear-walled chambers filled with natural soil that allow shoots to grow above the soil surface. Rhizotrons were installed in a semi-open, foil greenhouse, to prevent flooding and heat stress. The rhizotrons provide the ability to collect root growth measurements over time without disturbing aboveground plant growth and without the need for destructive sampling of roots until deemed necessary based on the experimental design.

Senescent leaves were identified based on chlorophyll measurements (Fig. 1) and senescent roots were identified based on symptoms as defined by Comas et al. [50]. Additional data obtained on anatomy, cytology and a viability test were also taken into account when interpreting the collected data.

Samples were collected three times during a growth season. The first collection was considered as a control and was collected in early summer (July 7–15) when leaves and the root system were fully developed and functional. Control leaf samples were designated as LC and control fine root samples were designated as RC. The second group of leaf and root samples were harvested in early autumn (October 1–7) when chlorophyll levels in leaves had decreased by approximately 40% (Fig. 1) and when fine roots had changed in color from white to brown. The first stage of leaf senescence was designated as LS1 and the first stage of fine root senescence was designated as RS1. The third group of samples were harvested in the middle of autumn (November 2–9) when chlorophyll levels in leaves decreased by approximately 65% (Fig. 1) and fine roots were dark brown or black color. The second stage of leaf senescence was designated as LS2 and the second stage of fine root senescence was designated as RS2.

Morphological studies

Photographic documentation of leaves was collected along with chlorophyll measurements to better illustrate the relationship between the two parameters during the senescence process. Chlorophyll levels were measured several times during the growth season using a CCM-200 plus Chlorophyll Content Meter (Opti-Sciences). Changes in the morphology of fine roots were examined several times during the growth season. This was done by removing the rhizotrons from the chamber and taking photos of the root systems, and immediately returning them back into the chamber. The same 30 plants were analyzed each time.

Viability test using a fluorescein diacetate (FDA) staining assay

The viability of cells in fine roots and leaves was assessed with fluorescein diacetate (FDA)(Sigma). After harvesting, fine roots and leaves were cut into 35 μm thick

cross-sections using a Leica VT1200S vibratome (Leica Biosystems, Nussloch, Germany). The sections were transferred to 100 μl of a diluted stock solution of FDA (stock solution 5 mg FDA in 1 ml of acetone, stock solution diluted 1:250 in Phosphate-buffered saline (PBS) (Sigma). After a 15 min incubation period at room temperature (RT), sections were rinsed three times in PBS buffer. Fluorescence was only observed in live cells due the conversion of non-fluorescent fluorescein diacetate into fluorescein. Fluorescence was induced by exposure to a wavelength of 470 nm (blue excitation and green fluorescence) under an Axioscope A1 microscope (Zeiss, Jena, Germany). Fluorescence images were digitally captured.

Anatomical studies

The harvested samples of fine roots and leaves were immediately fixed in a mix 2% (*v*/v) glutaraldehyde (pH 6.8; Polysciences, Warrington, USA) and 2% (v/v) formaldehyde (pH 6.8; Polysciences, Warrington, USA). After an overnight incubation in fixative solution, the samples were rinsed three times with a cacodylate buffer (0.05 M; pH 6.8; Polysciences) and then dehydrated in a graded ethanol series (10–100%, v/v). Subsequently, the samples were incubated in a series of ethanol:Technovit 7100 resin mixture (Heraeus Kulzer, Wehrheim, Germany) with ratios of 3:1, 1:1, 1:3, and finally in pure Technovit 7100 resin. Cross-sections were cut with a Leica RM2265 Fully Automated Rotary microtome (Leica-Reichert, Bensheim, Germany) at a thickness of 10 μm. The cross-sections were stained with 1% (m/v) aniline blue and examined under a light microscope (Axioscope A1, Carl Zeiss, Jena, Germany).

Cytological studies

For cytological studies, the fragments of fine roots and leaves were fixed in 2% (v/v) glutaraldehyde (pH 6.8; Polysciences, Warrington, USA) and 2% formaldehyde (v/v) (pH 6.8; Polysciences, Warrington, USA) at 4 °C overnight. Subsequently, the samples were rinsed three times with a cacodylate buffer (0.05 M; pH 6.8, Polysciences) and postfixed in 1% (v/v) osmium tetroxide (Polysciences) at RT for 2 h. The double fixed material was counterstained for 1 h with 2% uranyl acetate (Polysciences) and embedded in low viscosity resin using the method described by Zenkteler and Bagniewska Zadworna [79]. Ultrathin sections (70 nm) were cut on a Leica EM UC7 (Leica-Reichert, Bensheim, Germany) ultramicrotome using a diamond knife and cut sections were collected on formvar-coated copper grids. The sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi HT7700 transmission electron microscope (Hitachi, Tokyo, Japan) operating at an accelerating voltage of 80 kV.

Protein extraction, gel electrophoresis, and western blot analysis

Total protein was extracted from the collected samples according to the method described by Szuba et al. [80], which is based on phenol extraction. After extraction, proteins were solubilized in a buffer containing 7 M urea, 2 M thiourea, 40 mM dithiothreitol (DTT), 0.5% carrier ampholytes, and 4% CHAPS. Protein concentration was measured with a 2-D Quant Kit (GE Healthcare, Piscataway, USA). Proteins were separated by SDS-PAGE on 12% polyacrylamide gels, with an equal amount of protein (20 μg) in each lane. The western blot analysis was performed according to the method described by Kalemba and Litkowiec [81]. A primary antibody - anti-ATG8 (Agrisera) was diluted 1:1000. The presence of reactive protein was visualized on a membrane using an alkaline phosphate substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Sigma Aldrich, St. Louis, USA).

RT-qPCR analysis of gene expression

RNA isolation was performed with a Ribospin Plant kit (GeneAll Biotechnology Co., Ltd., Korea) according to the manufacturer's recommendations. RNA was suspended in nuclease free water and stored at − 80 °C. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific Inc., USA) following the protocol supplied by the manufacturer. Reverse transcription – quantitative PCR (RT-qPCR) was carried out using a SYBR Green Master Mix kit (Applied Biosystems, Thermo Fisher Scientific Inc., USA). All analyses of gene expression by RT-qPCR utilized three technical replicates from three biological replicates of each experimental variant. Analyses were conducted in 96-well plates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA) utilizing the following amplification program: denaturation by a hot start at 95 °C for 10 min, followed by 40 cycles of a two-step program (denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min). Primers used in this study were designed using Primer3 software (The Whitehead Institute for Biomedical Research, Cambridge, MD, USA). The sequences of the primer pairs are listed in Table 1. Several reference genes (such as: *GADPH*, *Actin*, *18S rRNA*, *ß-Tubulin*, *PKFE*, *EF1a*, *NADH*, and *Ubiquitin*) were utilized. *ß-Tubulin*, *GAPDH*, and *Ubiquitin* were selected as housekeeping genes and for normalization of expression values because they exhibited the lowest sample to sample variation and high stable expression in all samples types and time points.

Table 1 List of primer sequences used for RT-qPCR analyses

Gene	Primer Sequences
ATG7	F - 5′-GGAATCGAATTCCTGCTTCA-3′ R - 5′-TGTCTCATCATCCCAGTCCA-3′
ATG8c	F - 5′-TGCCTGTGTTACGGATCTTG-3′ R - 5′-ACCCCAAATGTGTTCTCACC-3′
ATG8d	F – 5′-GCCAACAGTGAGATCAGCAG-3′ R – 5′-GGGACTTTGTGAGGTGTGCT-3′
ATG8g	F - 5′-CGTTGCCTCAAACAGCAAGT -3′ R – 5′-AGAAAGGATGATACAGCTTAGCCA-3′
ATG8h	F - 5′-TAGAGAGGTGGTTGGGTGCT-3′ R – 5′-CCTGCTTCTGACCCTTCTTG-3′
ATG11	F- 5′- AGAGCTGCTTGACAAGTACCCA-3′ R- 5′-CTTTCCTTGTTTGCCTGCTTCT-3′
ATG18	F - 5′-GACAATGACGAGCCAGGATT-3′ R – 5′- AGAGTTCGAGTGGCTGGAGA-3′
ß-TUBULIN	F – 5′-TTCTCCTGAACATGGCAGTG-3′ R - 5′-CCACACAACGTGAAATCCAG-3′
GAPDH	F - 5′-CAATGAATGGGGCTACAGGT-3′ R – 5′-CATGAATCAGCTGCACATCC-3′
UBIQUITIN	F - 5′-AGGAACGCGTTGAGGAGAAG -3′ R – 5′-TATAABCAAAAACCGCCCCTG -3′

F forward primer, *R* reverse primer

Data analyses were performed according to the method described by Bagniewska-Zadworna and Stelmasik [82]. The average cycle threshold (Ct) values of the reference genes were subtracted from the corresponding Ct value of each gene to obtain a $^{\Delta}$Ct value, and the relative expression levels were calculated using the $^{\Delta\Delta}$Ct method.

Immunodetection of ATG8 using a tyramide signal amplification (TSA) assay

A tyramide signal amplification (TSA) technique was used to assess the localization of ATG8 protein due to its high level of sensitivity. The TSA technique is approximately 1,000× more sensitive than the standard protocol for immunolocalization.

Pieces of fine roots and leaves were fixed in 2% (v/v) glutaraldehyde (pH 6.8; Polysciences, Warrington, USA) and 2% (v/v) formaldehyde (pH 6.8; Polysciences, Warrington, USA) for 12 h and then rinsed three times in 1xPBS (Sigma) buffer. Immunolocalization in leaf samples utilized 32 μm thick sections, which were obtained using a Leica VT 1200S (Leica Biosystems, Nussloch, Germany) vibratome. Fine root samples were dehydrated in a graded ethanol series (10–100%) and then infiltrated and embedded in Paraplast Extra (melting point – 57.8 °C; Sigma, St Louis, MO, USA). Fine root sections (20 μm) were obtained using a Leica RM2265 (Leica Biosystems, Nussloch, Germany) microtome.

For immunolocalization, the material was incubated in 3% hydrogen peroxide solution for 1 h at RT to quench endogenous peroxidase activity. Subsequently, the material was rinsed three times in 0,01 M PBS buffer and blocked with 2% bovine serum albumin (BSA, Sigma) for 20 min. A primary ATG8 rabbit antibody (Agrisera) was used for immunolocalization of ATG8 proteins. The primary antibody was diluted 1:1000 in 0.2% BSA (Sigma) and the sectioned material was incubated with the primary antibody at 6 °C overnight. The material was rinsed five times in PBS buffer and then incubated with poly-HRP-conjugated secondary antibody (Thermo Fisher Scientific Inc., USA, attached to TSA Super Boost kit) for 1 h at 36 °C. The antibodies were rinsed from the samples five times with PBS and then the samples were exposed to a working solution of tyramide for 8 min at RT. The working solution of tyramide was prepared according to the manufacturer's directions (Thermo Fisher Scientific Inc., USA). The reactions were arrested by the addition of 100 μl of a stop reagent (Thermo Fisher Scientific Inc., USA). After rinsing in PBS buffer, the sectioned samples were mounted in Prolong Gold (Life Technologies). Results of the immunolocalization assay were recorded with a Leica TCS SP5 confocal microscope (Leica Biosystems, Nussloch, Germany). Negative control reactions produced an undetectably low signal compared with the standard reactions (Additional file 1, Figure S1).

Statistical analysis

Statistical analyses (ANOVA and Tukey's test) were performed using Statistica 12.0 software (StatSoft Poland Inc., Tulsa, OH, USA).

Additional file

Additional file 1: Figure S1. Comparison of ATG8 immunolocalization reactions with a negative control. Figure. 1a, b – The localization of ATG8 in senescence leaf. Fig. 1c, d – The negative control reaction performed omiting the primary antibody. (TIF 42774 kb)

Abbreviations

ATG: Autophagy related genes; BSA: Bovine serum albumin; GFP: green fluorescent protein; LC: Control (green leaves); LS1: First stage of senescence (yellowing leaves); LS2: Second stage of senescence (yellow leaves); PBS: Phosphate-buffered saline; PE: Phosphatidylethanolamine; RC: Control (white fine roots); RCB: Rubisco-containing bodies; RFP: Red fluorescent protein; RS1: First stage of senescence (brown fine roots); RS2: Second stage of senescence (dark brown or black fine roots); RT: Room temperature; TSA: Tyramide signal amplification; WT: Wild type

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NW collected material and performed the analyses with contributions from KMS, EK, AZN, AMJ; ABZ conceived the original concept and research plan, designed the experiments and oversaw the study; NW and ABZ analyzed the data, NW wrote the first draft of the manuscript with critical comments and supervision provided by ABZ; all authors discussed the results, read, and approved the final version of the manuscript.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors. The ethics approval is unnecessary for our study.

Consent for publication

All authors read the manuscript and approved its final version.

Competing interests

Trade names or commercial products mentioned in this publication are only to provide specific information and do not imply any recommendation or endorsement by the authors.

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Abscisic Acid and Jasmonate Metabolisms Are Jointly Regulated During Senescence in Roots and Leaves of *Populus trichocarpa*

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Abstract: Plant senescence is a highly regulated process that allows nutrients to be mobilized from dying tissues to other organs. Despite that senescence has been extensively studied in leaves, the senescence of ephemeral organs located underground is still poorly understood, especially in the context of phytohormone engagement. The present study focused on filling this knowledge gap by examining the roles of abscisic acid (ABA) and jasmonate in the regulation of senescence of fine, absorptive roots and leaves of *Populus trichocarpa.* Immunohistochemical (IHC), chromatographic, and molecular methods were utilized to achieve this objective. A transcriptomic analysis identified significant changes in gene expression that were associated with the metabolism and signal transduction of phytohormones, especially ABA and jasmonate. The increased level of these phytohormones during senescence was detected in both organs and was confirmed by IHC. Based on the obtained data, we suggest that phytohormonal regulation of senescence in roots and leaves is organ-specific. We have shown that the regulation of ABA and JA metabolism is tightly regulated during senescence processes in both leaves and roots. The results were discussed with respect to the role of ABA in cold tolerance and the role of JA in resistance to pathogens.

Keywords: senescence; phytohormones; abscisic acid; jasmonate; absorptive roots; leaf senescence; microarrays analyses

1. Introduction

Senescence is a coordinated series of events that begins at a cellular level and then broadens to whole tissues, organs, and in monocarpic plants, the whole organism [1]. In crops, this process usually overlaps with the reproductive phase and might influence reducing crop yield when it is induced prematurely or/and under unfavorable environmental conditions [2]. The onset of senescence may be related to age [3,4] or can be stimulated by internal factors, such as reactive oxygen species (ROS) or changes in phytohormone level, as well as external factors such as photoperiod, temperature, nutrient deficiency, or shading [5–8]. While the process of senescence appears to be outwardly destructive, the fact that it is a highly regulated process allows plants to relocate a significant portion of valuable nutrients from senescing organs to other tissues so that they can be re-utilized rather than lost [9,10].

One of the most characteristic features of senescence is an increase of catabolic metabolic reactions in relation to anabolic ones [11]. This shift leads to the degradation of individual organelles and eventually

the rupture of the tonoplast, protoplast acidification, and cell death [8,12–14]. Chloroplasts, which contain approximately 80% of total leaf nitrogen, are one of the first organelles to be degraded [15,16]. Massive degradation of chloroplast proteins as well as other macromolecules initiates the main goal of senescence: remobilization [17]. Contrary to chloroplasts, the nucleus, which is essential to coordinate senescence progression by gene transcription, and mitochondria, which supply energy, remain unharmed up to the last stages of senescence [18,19]. Several studies have indicated that abscisic acid (ABA) [7,20], jasmonic acid (JA) [21,22], ethylene (ET) [23–25], and salicylic acid (SA) [26,27] promoted senescence in leaves and flower petals before they were ceased, while cytokinins (CKs) [28,29] delayed this process. This has been confirmed by the analysis of phytohormone levels during senescence, as well as by molecular studies in which the expression of genes related to the biosynthesis of phytohormones was found to increase in senescing organs [30]. The expression of a wide range of genes is also modulated during senescence. Specifically, genes that are up-regulated during senescence are termed *Senescence-Associated Genes* (*SAGs*), while genes that are down-regulated are called *Senescence down-regulated genes* (*SDGs*) [31]. Functional analyses have revealed that among *SAGs* are those genes encoding proteolytic enzymes, ATG proteins, nitrogen-metabolizing enzymes, and several transcriptional factors (TFs) such NAC or MYB [17,32]. In contrast, *SDGs* encode proteins related to photosynthesis or oxidative enzymes, such as catalase [33].

Programmed cell death (PCD) is a cellular process that is often associated with senescence, and such relation was confirmed for leaves and flower petals [8,14,34]. Recent studies have provided evidence that PCD is also involved in the senescence of fine roots [12,13]. Fine, absorptive roots constitute an important component of soil biomass and play a significant role in biogeochemical cycling in forest ecosystems [35,36]. Based on recent studies, it is apparent that fine roots, with a diameter < 2 mm, should not be considered as a homogeneous entity because they include both absorptive and transport roots. The first two to three root orders are classified as absorptive, fine roots. These roots are characterized by high absorption and respiration rates, and they are often colonized by mycorrhizae [13,35–38]. The life span of fine roots is species-specific and may range from a few weeks to as long as two years [39,40]. However, the average life span of fine roots in *Populus* is typically < 95 days. New insights on root senescence suggest that it is not a passive process but rather is genetically regulated [12,13]. Similar to flower petals and leaves, changes in morphology (color changes, wilting/shrinkage) and cytology (tonoplast rupture, changes in cell shape) occur, including the activation of autophagy. Despite this cursory information, a comprehensive outlook on the senescence of underground organs is still lacking. Considering the total biomass of fine, absorptive roots, a better understanding of senescence and death in these organs is essential to understanding chemical element circulation in forest ecosystems.

In the present study, we report on significant changes that occur in gene expression during the senescence of leaves and fine, absorptive roots in *Populus trichocarpa*. We focused on checking which changes accompanied senescence of two organs that play completely different roles in plant physiology and metabolism, and they also exist under completely different growth conditions. Comparison of these two organs was conducted to determine if the senescence process, despite significant differences in structures among others related to the presence of a photosynthetic apparatus in leaves, followed the same or independent pathways. The results obtained from transcriptomic and physiological analyses as well as microscopic localization provided new information that ABA and JA may contribute to senescence processes in leaves and fine roots and confirmed that the senescence of fine, absorptive roots should be classified as another example of PCD. We suggest that the senescence of both studied organs is a hormonally regulated process, but this regulation in both organs is different, which indicates organ-specific hormonal regulation.

2. Results

2.1. Changes in Gene Expression During the Senescence of Leaves and Roots

Before carrying out the analyses, morphological and anatomical characteristics of the plant material were performed, as described previously [13]. In addition, basic physiological parameters for leaf senescence, such as chlorophyll level, were examined, and the ratio of fresh mass (FW) / dry mass (DW) was calculated (Supplementary Figure S1A). On this basis, sampling moments related to control variants and senescence stages were estimated. The main senescence-related feature that was taken into account was the change of color. For leaves, it was associated with a decrease in the chlorophyll level (Supplementary Figure S1B); for roots, in addition to color change, shrinkage was also included. The microarray analyses were conducted at three time points for each organ and included viable organs without any symptoms of senescence (LC, RC) as well as early (RS1, LS1) and advanced stages of senescence (RS2, LS2) (Figure 1).

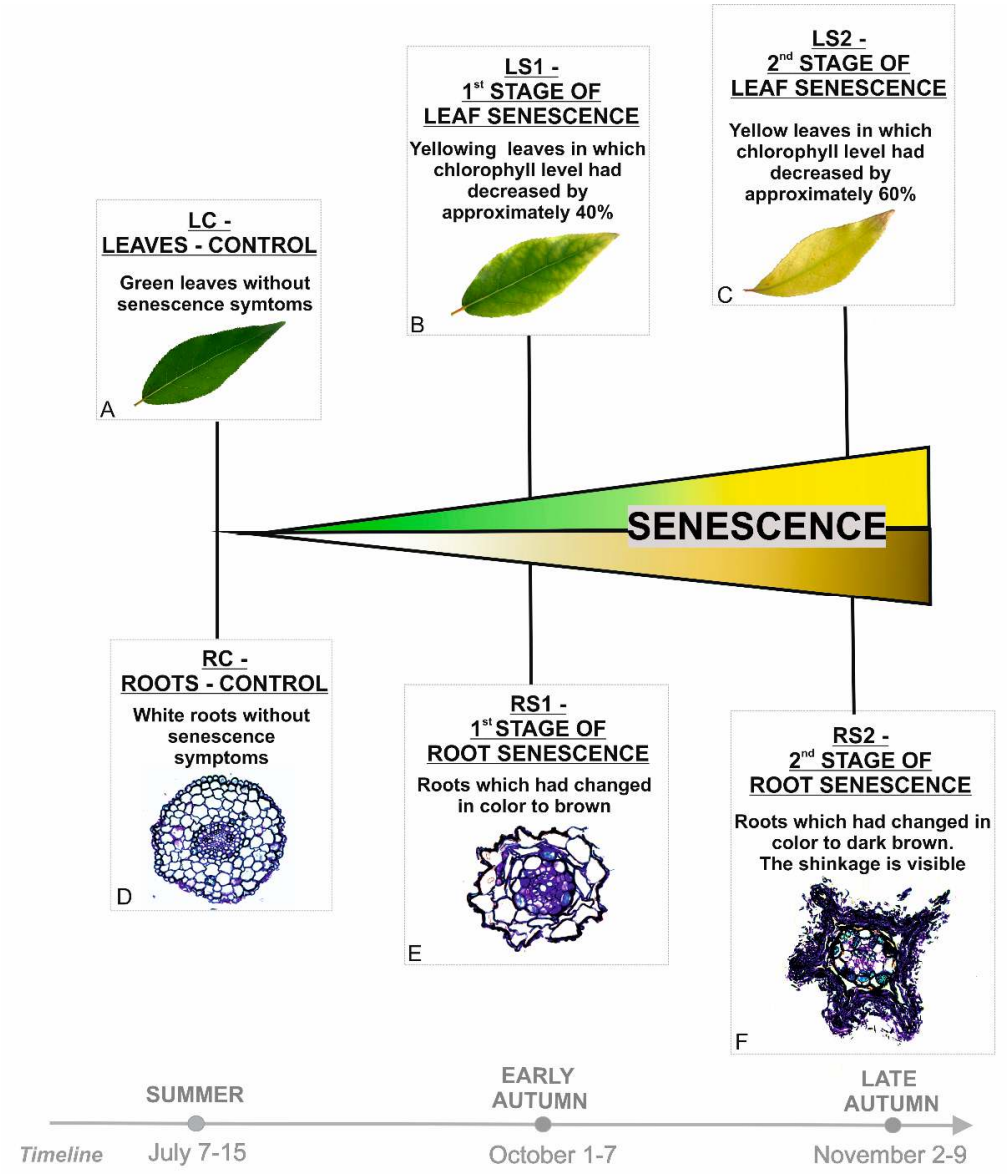

Figure 1. Stages of leaves (**A**–**C**) and fine, absorptive roots (**D**–**F**) selected for analyses based on morphological and anatomical changes observed during the vegetative season.

Microarray analyses revealed significant changes in gene expression during the course of senescence of roots and leaves.

In roots, a total of 1898 differentially expressed genes (DEGs) were identified (One-way ANOVA corrected *p*-value cut-off = 0.001, post-hoc Tukey HSD and Benjamini–Hochberg correction, fold change ≥ 2) in the three stages of senescence that were analyzed. Most of the DEGs exhibited down-regulation in the early and late stages of senescence. A total of 924 DEGs were down-regulated in RS1 and 1169 in RS2. In contrast 556 DEGs were up-regulated in RS1 and 692 in RS2 (Figure 2A).

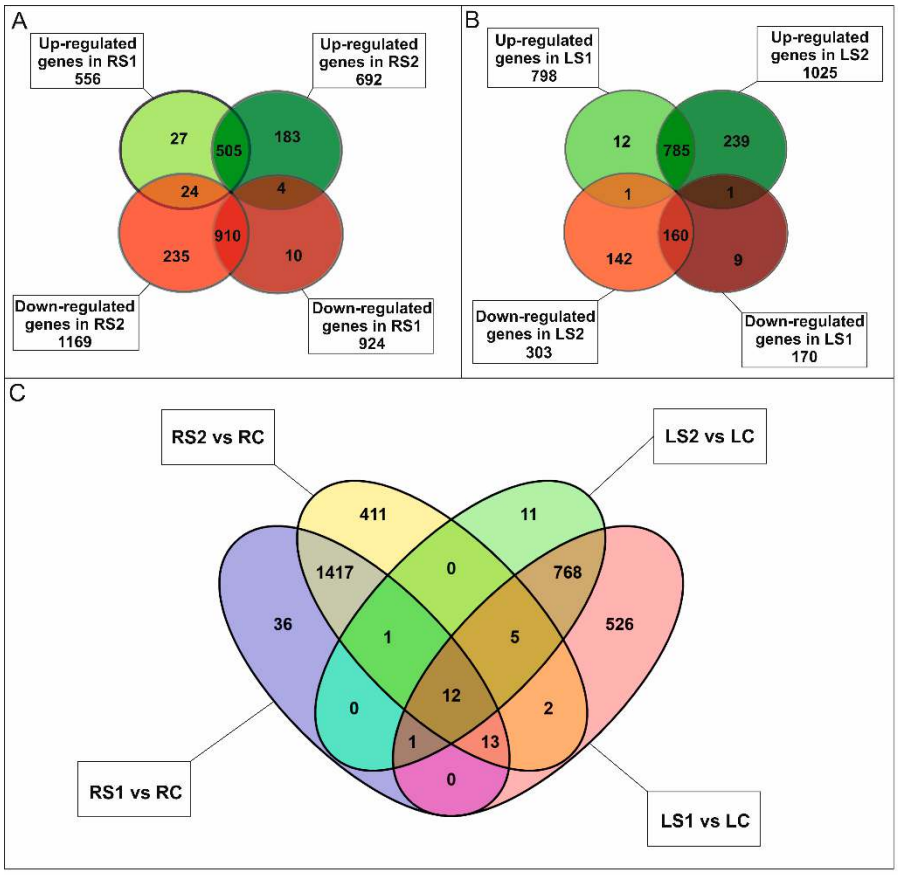

Figure 2. Transcriptomic analysis of fine, absorptive roots and leaves during senescence. (**A**) Venn diagrams showing the expression pattern of 1898 statistically significant differentially expressed genes (DEGs) in fine roots at two time points during senescence and the overlap in expression among them. (**B**) Venn diagrams showing the expression pattern of 1348 statistically significant genes in leaves at two time points during senescence and the overlap in expression among them. (**C**) Venn diagram showing the expression pattern of DEGs in roots and leaves at two time points during senescence and the common genes for both organs.

Most DEGs were either down- or up-regulated in both RS1 and RS2 stages (Figure 2A); however, there was a significant number of DEGs that either decreased or increased in their expression only in the final stage (RS2) of senescence. A total of 1500 DEGs were annotated, and 101 clusters and 77 functional categories were identified using the DAVID database [41,42]. The majority of the annotated genes encoded proteins located in extracellular regions (35 genes), the cytosol (34 genes), cell wall (31 genes), or plant-type cell wall (22 genes), or as an integral component of the plasma membrane (27 genes) (Supplementary Figure S2A). Gene Ontology (GO) enrichment analysis revealed that the most abundant categories were carbohydrate metabolic process (33 genes), ROS, and oxidative stress (response to oxidative stress, 15 genes; hydrogen peroxide catabolic process, 14 genes), cell wall (cell wall organization, 15 genes; plant-type cell wall organization, 14 genes; pectin catabolic process, 14

genes; xyloglucan metabolic process, 8 genes; cell wall biogenesis, 8 genes; cellulose catabolic process, 5 genes; xylan biosynthetic process, 4 genes; S-adenosylmethionine biosynthetic process, 3 genes; S-adenosylmethionine cycle, 2 genes), and microtubules (microtubule-based movement, 18 genes; microtubule-based process, 11 genes) (Supplementary Figure S2B). Notably, many genes (90) associated with phytohormones were also identified (Figure 3A).

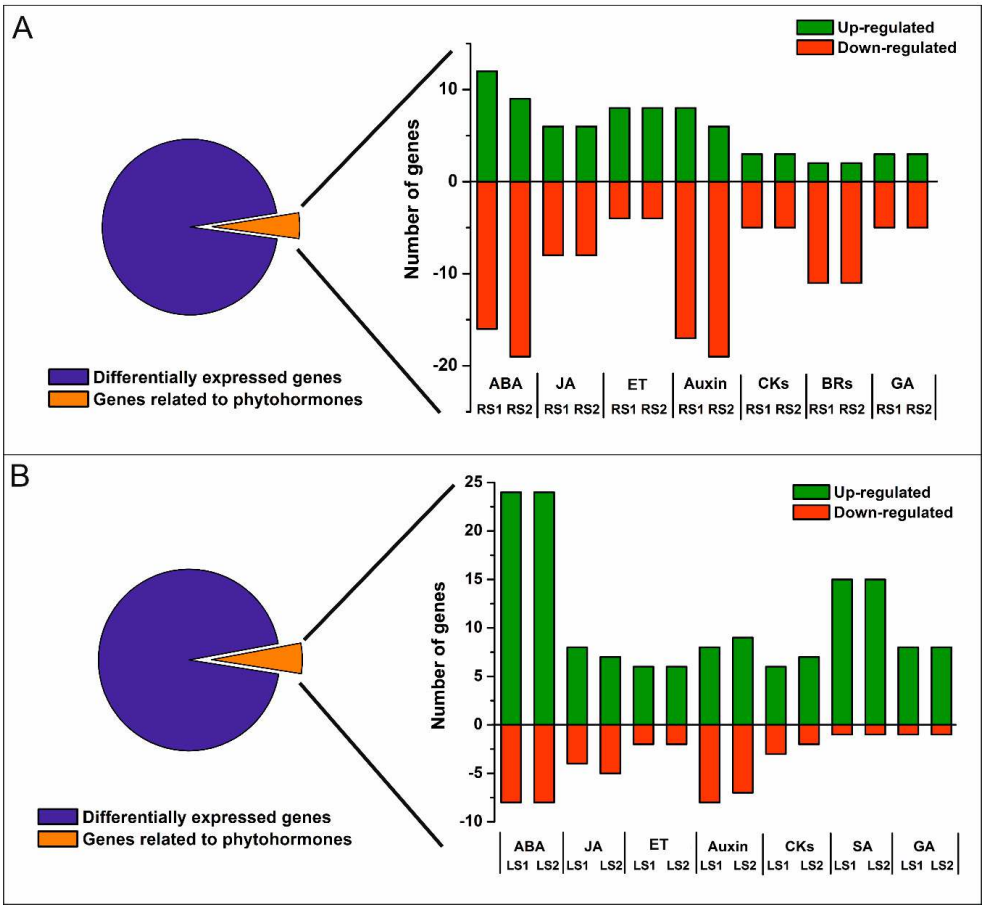

Figure 3. Number of up- and down-regulated phytohormone-related genes in roots (**A**) and leaves (**B**). Abbreviations: ABA, abscisic acid; JA, jasmonic acid; ET, ethylene; CKs, cytokinins; BRs, brassinosteroids; SA, salicylic acid; GA, gibberellic acid.

In leaves, a total of 1348 DEGs (One-way ANOVA corrected *p*-value cut-off = 0.005, post-hoc Tukey HSD and Benjamini–Hochberg correction, fold change ≥ 2) were identified over the course of the senescence process. The majority of the DEGs were up-regulated, 798 in LS1 and 1025 in LS2 stage, while 170 DEGs were down-regulated in LS1 and 303 in LS2 (Figure 2B). Of all DEG, only a small group of genes have been identified as common to both organs (Figure 2C). A total of 1063 DEGs were annotated, and 63 clusters and 42 functional categories were identified using the DAVID database [41,42]. Most of the annotated DEGs encoded proteins assigned, among others, to the nucleus (114 genes), cytoplasm (81 genes), and intracellular (27 genes) categories (Supplementary Figure S3A). GO enrichment analysis revealed that the most abundant enriched categories were those related to DNA-templated transcription (40 genes), protein degradation (proteasome-mediated ubiquitin-dependent protein catabolic process, 15 genes; protein ubiquitination involved in ubiquitin-dependent protein catabolic process, 13 genes; ubiquitin-dependent protein catabolic process, 8 genes), signaling (intracellular signal transduction, 11 genes; small GTPase-mediated signal transduction, 9 genes; Wnt signaling pathway, 4 genes), vesicle-mediated transport (8 genes), endocytosis (4 genes), as well as a plethora of categories related to phytohormones (77 genes) (Figure 3B, Supplementary Figure S3B).

2.2. Genes Associated with Phytohormones

Genes associated with phytohormone pathways were identified among the DEGs of both organs using Gene Ontology (GO) within the Biological Process (BP) category. These included genes related to abscisic acid (ABA), jasmonic acid (JA), brassinosteroids (BRs), cytokinins (CKs), auxin (IAA), ethylene (ET), gibberellin (GA), and salicylic acid (SA) (Figure 4A–D, Supplementary Figures S4 and S5). Further analyses focused on genes related to ABA and JA in both organs since the microarray analysis indicated significant changes in the expression of ABA- and JA-related genes and because these two phytohormones are known as positive regulators of leaf senescence.

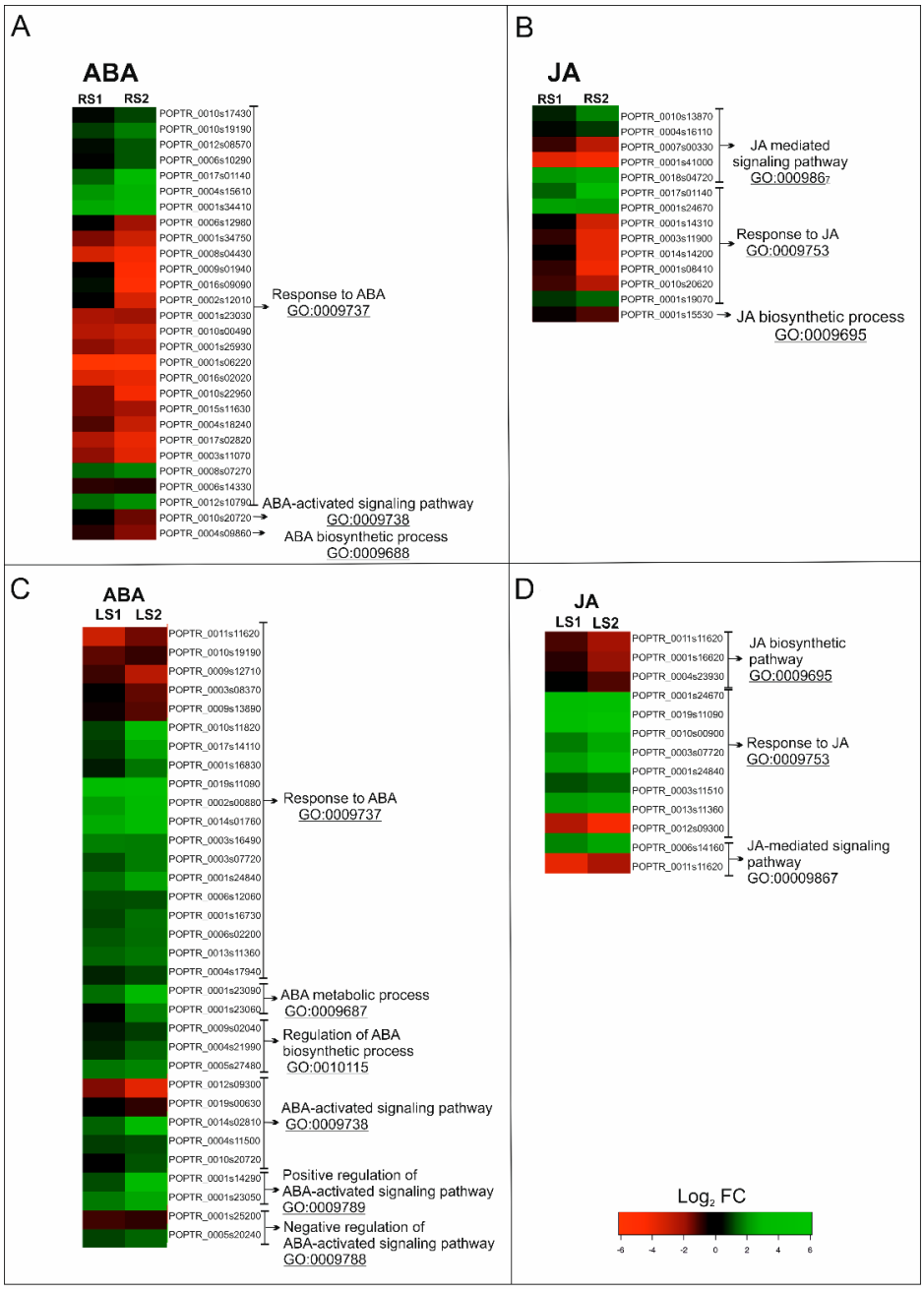

Figure 4. Changes in the expression of phytohormone-related genes in roots (**A**,**B**) and leaves (**C**,**D**) during senescence. (**A**,**C**) Heatmap illustrating the expression profiles of ABA-related genes. (**B**,**D**) Heatmaps illustrating the expression profiles of JA-related genes. Abbreviations: ABA, abscisic acid; JA, jasmonic acid; FC, fold change.

2.2.1. ABA-Related Genes

In roots, genes associated with ABA belonged to three subcategories within the GO BP, with "response to ABA" containing the greatest number of DEGs (Figure 4A). A total of 28 genes fell into this subcategory of BP, among which 12 were up-regulated in RS1 and 10 in RS2 (Figure 3A, Figure 4A; Supplementary Table S1). Concomitantly, 16 genes in this subcategory were down-regulated in RS1 and 18 in the RS2 stage of senescence. Among the identified up-regulated DEGs were genes encoding proteins related to stress response, including cold stress—*WCOR413* (POPTR_0004s15610) and *COR413IM1* (POPTR_0001s34410)—or oxidative stress—the precursors of ferritin (POPTR_0008s07270, POPTR_0010s19190). The down-regulated DEGs included several genes encoding aquaporin-related proteins (POPTR_0008s04430, POPTR_0010s22950, POPTR_0004s18240, POPTR_0009s01940). Several DEGs encoding proteins involved in signaling such as kinases (POPTR_0017s02820, POPTR_0001s23030, POPTR_0010s00490) or phosphatase (POPTR_0010s20720) were also found to be down-regulated (Supplementary Table S1).

In leaves, genes associated with ABA were placed in six subcategories of GO BP. Similar to fine roots, the GO BP subcategory "response to ABA" contained the largest number of DEGs; however, "ABA-activated signaling pathway" was also significantly represented (Figure 4B). Among the 33 ABA-related DEGs, 25 were up-regulated and 8 were down-regulated during both stages (LS1 and LS2) of senescence (Figure 3B; Figure 4C). Among the genes up-regulated by senescence were genes encoding transcription factors (TFs), e.g., MYB (PtrMYB168 POPTR_0019s11090, POPTR_0013s1136), bZIP (POPTR_0014s02810), and NAC (NAC034 POPTR_0005s20240, NAC052 POPTR_0003s16490). Up-regulated expression was also observed for genes related to carbohydrate metabolism (POPTR_0001s23090, POPTR_0001s23060), lipid metabolism (POPTR_0001s14290), as well as genes associated with the protein degradation process (POPTR_0004s17940, POPTR_0012s09300, POPTR_0005s27480). Notably, genes encoding proteins associated with ABA signal transduction, such as phosphatase 2C (POPTR_002s00880, POPTR_001s25200, POPTR_0010s20720) or kinases—SNF1-related protein kinase KIN10 (POPTR_0004s11500) and calcium-dependent protein kinase 1 (POPTR_0019s00630)—were also observed. Similar to the situation in fine, absorptive roots, down-regulation of DEGs encoding an aquaporin-related protein (POPTR_0009s13890) and a precursor of ferritin (POPTR_0010s19190) were also observed (Supplementary Table S2).

2.2.2. JA-Related Genes

In roots, genes associated with JA were placed in three subcategories of GO BP, with "response to JA" and "JA-mediated signaling pathway" being the subcategories containing the greatest number of DEGs (Figure 4B). A total of 14 DEGs, among which 6 were up-regulated and 8 were down-regulated, were identified in both stages of root senescence (Figure 3B; Figure 4B). Increased expression of DEGs was identified for genes encoding MYB TF (POPTR_0001s19070), SWI/SNF complex-related (POPTR_0017s01140), ERECTA-like protein (POPTR_0004s16110), and mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein (POPTR_0001s24670). Down-regulated DEGs included, among others, those related to flavonoid metabolism (POPTR_0014s14200, POPTR_0003s11900, POPTR_0001s14310, POPTR_0001s08410) and lipid metabolism (POPTR_0001s15530) (Supplementary Table S1).

In leaves, DEGs associated with JA were placed in the same three categories as the DEGs for fine roots. However, in the case of leaves, the subcategory of GO BP "JA biosynthetic pathway" contained the second greatest number of DEGs (Figure 4D). A total of 12 genes were identified, 8 and 7 of which were up-regulated in the LS1 and LS2 stages, respectively (Figure 3B; Figure 4D). Concomitantly, 4 DEGs were down-regulated in LS1 and 5 in LS2. The greatest increase in expression was observed for DEGs encoding transcriptional factors, such as an ethylene-responsive transcription factor RAP2-3 (POPTR_0010s00900), TBF1 (POPTR_0001s24840), and MYBs (PtrMYB180 POPTR_0013s11360 and PtrMYB168 POPTR_0019s11090). In addition, increased expression was observed for a gene similar to JAZ1, which encodes a jasmonate ZIM-domain protein (POPTR_0006s14160) (Supplementary Table S2).

These results indicate that the process of senescence in both leaves and fine, absorptive roots is not a passive process but rather a genetically regulated process that is accompanied by significant changes in gene expression, including a large group of genes associated with phytohormone synthesis and signaling pathways.

2.3. ABA, JA, and MeJA Levels During Senescence

Based on microarray analyses and literature data about the role of ABA and JA during leaf senescence, further analyses were carried out for these two phytohormones in order to check whether organs with different functions used similar mechanisms or affected the metabolism of these compounds to the progression of senescence process.

Quantitative analyses of ABA, jasmonic acid (JA), and methyl jasmonate (MeJA) revealed significant differences in the concentration of these phytohormones during the senescence of leaves and fine absorptive roots (Figure 5, Figure 6). The results of changing the phytohormone concentration were statistically significant ($p < 0.05$) (Supplementary Table S3). The level of ABA in roots increased during senescence, with the highest level observed in the second stage of senescence (RS2) (Figure 5A). In contrast, the highest level of ABA in leaves was observed in LS1, when leaves were yellowing (Figure 5B).

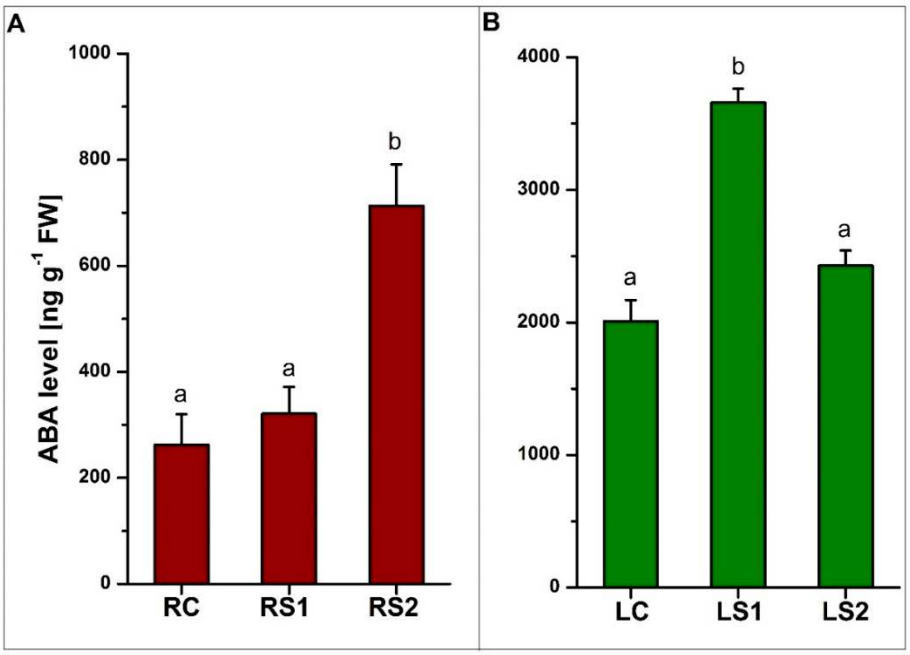

Figure 5. Quantitative analysis of abscisic acid (ABA) levels in fine absorptive roots (**A**) and leaves (**B**) during senescence. Means designated by different letters indicate statistically significant differences according to ANOVA and Tukey's post hoc test ($p < 0.05$). Values represent the mean ± SE (standard error).

Quantitative analyses of jasmonates (JA and MeJA) were also conducted. The concentration of both JA and MeJA in both organs increased during senescence, with the highest levels observed in the second stage of senescence (LS2, RS2) (Figure 6A,B). All of the observed changes in jasmonate levels were statistically significant at a p-value < 0.05.

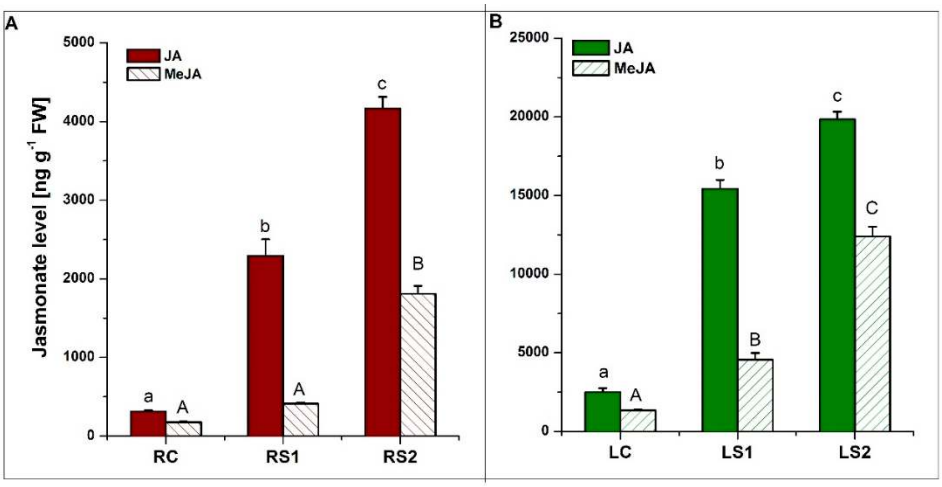

Figure 6. Quantitative analysis of jasmonic acid (JA) and methyl jasmonate (MeJA) levels in fine absorptive roots (**A**) and leaves (**B**) during senescence. Means designated by different letters indicate statistically significant differences according to ANOVA and Tukey's post hoc test ($p < 0.05$). Values represent the mean ± SE (standard error).

2.4. Immunolocalization of ABA and JA During Senescence

Based on the significantly increased concentrations of ABA and jasmonate in both leaves and fine, absorptive roots in the different stages of senescence, immunohistochemical detection of ABA and JA was conducted to determine their cellular and tissue distribution.

2.4.1. ABA Localization

In roots, ABA was localized in the rhizodermis, cortical parenchyma cells, and vascular tissue (mainly in xylem) of control, viable roots (RC) (Figure 7A,B). A signal for ABA was detected in the peripheral cytoplasm of cortical parenchyma cells, which had a large central vacuole (Figure 7A,B; arrows). In the first stage of root senescence (RS1), the ABA signal was observed in the same tissues as in RC, but the cellular distribution was different in cortical parenchyma cells. In this case, the signal was not only concentrated in of the peripheral cytoplasm, but it was also evident in the vacuole (Figure 7C,D; arrowheads). The majority of cortical parenchyma cells were folded and irregular in shape in RS2, which made determining the precise distribution of ABA in these cells problematic. Nevertheless, an intense fluorescent signal was detected in the folded cortical parenchyma cells, as well as in xylem tracheary elements (Figure 7E,F).

In leaves, the highest intensity of ABA signal in green, control leaves (LC) was detected in vascular bundles (Figure 8A–C; arrowheads). A weak signal was also observed in epidermal cells (Figure 8A,B), as well as in a few mesophyll cells (mainly in the palisade layer) (Figure 8C). Chloroplasts were readily defined due to the strong autofluorescence emitted by chlorophyll (Figure 8A–C). ABA in yellowing leaves (LS1) was detected in the majority of palisade and spongy mesophyll cells (Figure 8D–F). The signal was localized in the cytoplasm of these cells and sometimes in spherical spots inside the vacuole (Figure 8F, arrow). Chlorophyll autofluorescence was still visible (Figure 8D–F), whereas it decreased significantly in the second stage of senescence (LS2) (Figure 8G–I). The distribution of the ABA signal in LS2 leaf tissues, however, was different than it was in LS1; the signal was detected as different-shaped spots in the majority of mesophyll cells (Figure 8G–I, arrowheads).

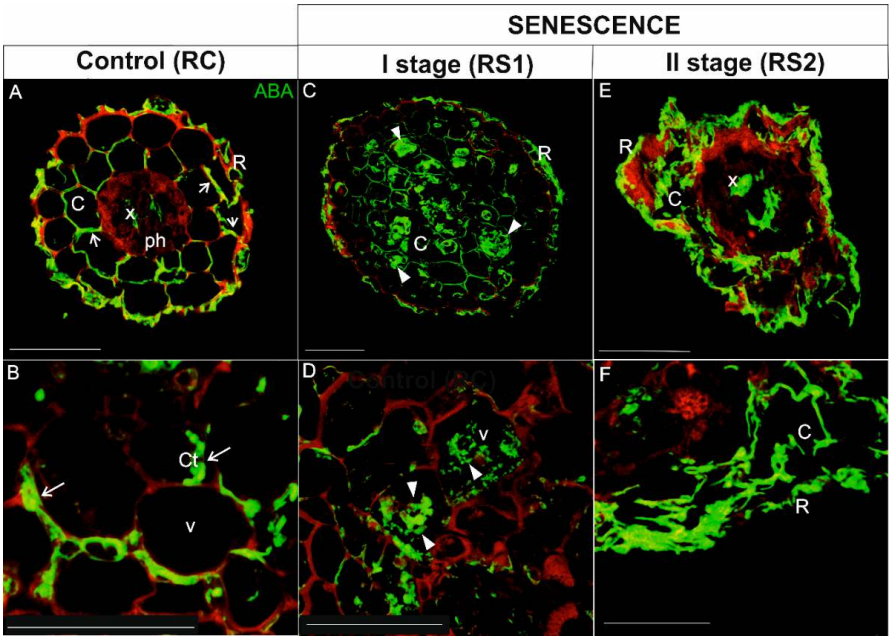

Figure 7. Immunolocalization of ABA (green fluorescence) during senescence process of fine, absorptive roots. (**A**,**B**) Root control (RC); (**C**,**D**) The first stage of root senescence (RS1); (**E**,**F**) The second stage of root senescence (RS2). Autofluorescence (red) of the cell wall was registered in order to visualize the cell/organ shape. Abbreviations: R, rhizodermis; C, parenchyma cortex cells; Ct, cytoplasm; Ph, phloem, X, xylem. Scale bars = 50 μm.

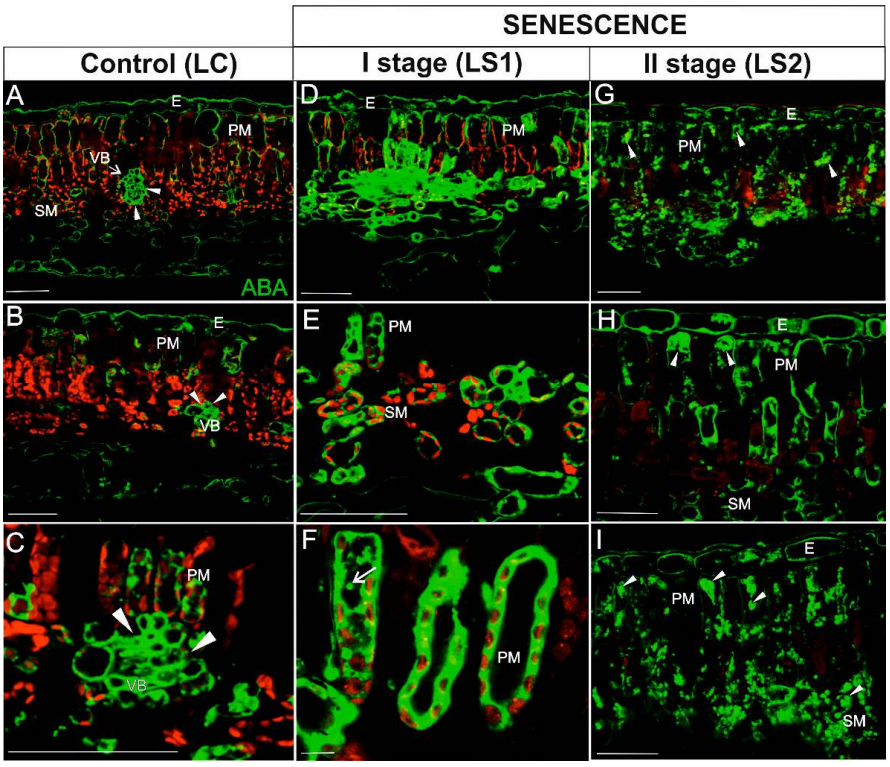

Figure 8. Immunodetection of ABA (green fluorescence) and red autofluorescence of chlorophyll during leaf senescence. (**A**–**C**) Leaves control (LC); (**D**–**F**) The first stage of leaf senescence (LS1); (**G**–**I**), The second stage of leaf senescence (LS2). Abbreviations: VB, vascular bundle; PM, palisade mesophyll; SM, spongy mesophyll; E – epidermis. Scale bars = 50 μm.

2.4.2. JA Localization

In roots, the JA signal in viable, control roots (RC) was concentrated mainly in phloem cells (Figure 9A,B). The JA signal was also detected in several cortical cells (Figure 9A,B). In the first stage of root senescence (RS1), the pattern of the JA signal was quite different; the JA was detected in the majority of cortical parenchyma cells, but in contrast to the signal in RC samples, the JA signal was observed throughout the cell rather than just in the peripheral cytoplasm of cells with large vacuoles (Figure 9C,D; arrowheads). In the second stage of senescence (RS2), the anatomical structure of roots was greatly degraded by the senescence process; however, the intensity of the signal was still very high and localized mainly in the folded cortical parenchyma cells (Figure 9E,F) as well as in phloem cells (Figure 9E).

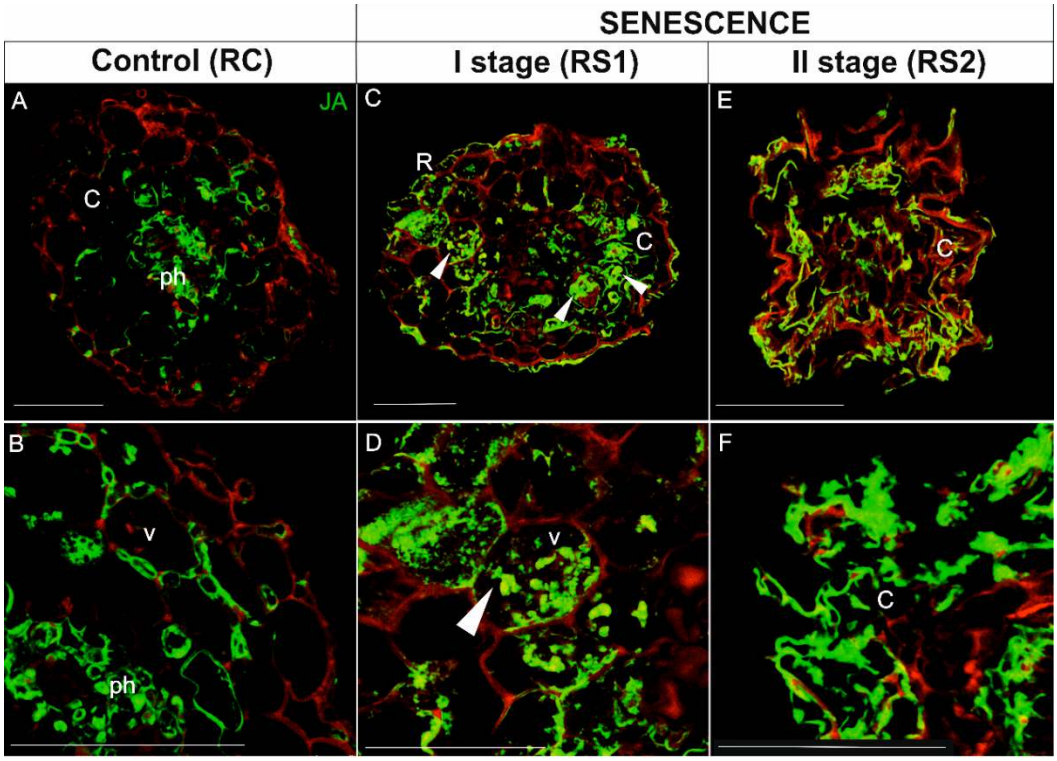

Figure 9. Immunolocalization of JA (green fluorescence) in fine, absorptive roots during senescence. (**A**,**B**), Root control (RC); (**C**,**D**) The first stage of root senescence (RS1); (**E**,**F**) The second stage of root senescence (RS2). Autofluorescence (red) of the cell wall was recorded to visualize the cell and/or organ shape. Abbreviations: R, rhizodermis; C, parenchyma cortex cells; Ph, phloem; X, xylem. Scale bars = 50 μm.

In leaves, the JA signal in green, control leaves was weak and was detected in cells within the vascular bundle (Figure 10A–C), the epidermis (Figure 10A), and a few mesophyll cells (Figure 10C). In mesophyll cells, JA was localized mainly in close proximity to chloroplasts (Figure 10C, arrow). In yellowing leaves (LS1), the JA signal was observed in the majority of cells (Figure 10C–E). The distribution of the signal in mesophyll cells, however, was different than in the LC stage. In addition to areas near the chloroplast, the signal appeared to be distributed throughout the cytoplasm (Figure 10D,F; arrowheads). Notably, an intense signal was still visible in the cells within the vascular bundles (Figure 10E). In the second stage of leaf senescence (LS2), JA was detected in the same tissues as in LS1, but the pattern of localization was different. In this case, the JA signal was observed as spherical spots within palisade mesophyll cells (Figure 10G,H, arrows) or it was concentrated in the peripheral cytoplasm against the cell wall of some cells (Figure 10I). As in the previous stage, the JA signal was

still observed within cells of the vascular bundles (Figure 10G). Chlorophyll autofluorescence was very low in the LS2 stage of leaf senescence (Figure 10G–I).

Negative control reactions had an undetectably low signal relative to the standard reactions (Supplementary Figure S6). The results obtained for ABA and JA indicated that these phytohormones might play an important role in the senescence process in both leaves and fine, absorptive roots. This was indicated by changes in the expression of many genes associated with ABA and JA, as well as quantitative analyses of phytohormone concentrations. In addition, the localization of the studied phytohormones showed that signal distribution accumulated in tissues with visible signs of senescence (shape changes, lowering of the chlorophyll autofluorescence level).

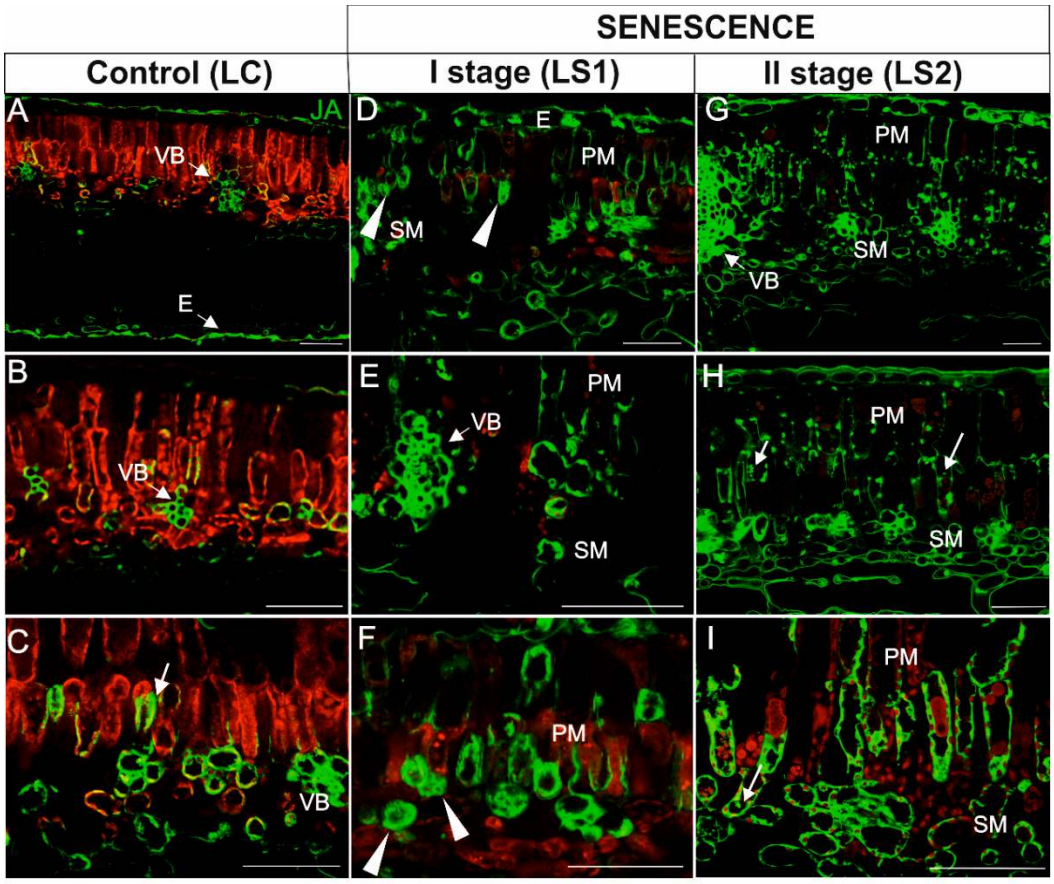

Figure 10. Immunodetection of JA (green fluorescence) and red autofluorescence of chloroplasts in leaves during senescence. (**A**–**C**) Leaves control (LC); (**D**–**F**) The first stage of leaf senescence (LS1); (**G**–**I**), The second stage of leaf senescence (LS2). Abbreviations: VB, vascular bundle; PM, palisade mesophyll; SM, spongy mesophyll; E, epidermis. Scale bars = 50 μm.

3. Discussion

Over the past decade, extensive genetic, molecular, and physiological studies have revealed the intricate network controlling the process of senescence. The majority of studies have focused on elucidating the senescence process in leaves, which are a fundamental site for capturing energy through photosynthesis [1,19,43]. The onset of leaf senescence is definitely easier to monitor than in the case of the belowground organ. Moreover, in chloroplasts there is a large pool of nitrogen stored; therefore, these organs represent an ideal model to study nutrient remobilization, which is one of the critical aspects of senescence [9,17,44]. Much less attention has been focused on other organs. Recent studies have drawn attention, however, on the senescence of other ephemeral organs, such as fine, absorptive roots [12]. The senescence process in fine roots has many similarities to leaf senescence,

among which ultrastructural changes and/or the activation of autophagy-related mechanisms that have been emphasized [13]. Fine, absorptive roots also possess a high concentration of nitrogen, which, along with their large total biomass, provide an interesting model for studying the remobilization of nutrients [45]. There is still, however, a lack of information pertaining to the senescence process of underground organs, especially at the physiological and molecular levels.

In the present study, details regarding the genetic and phytohormonal regulation of the senescence of roots have been presented, along with a comparison of the senescence process in leaves vs. fine absorptive roots. Microarray data indicated that the senescence process of both of the studied organs is accompanied by significant changes in gene expression. GO classification of the DEGs revealed a plethora of genes associated with phytohormones. Previous studies have documented the importance of plant hormones in the regulation of leaf senescence [8,26,30]. Phytohormones can either act as inhibitors or accelerators of senescence. Many of the identified DEGs were those related to ABA. This phytohormone, in addition to its significant role in developmental processes or responses to environmental stresses [46–49], is also a well-known positive regulator of plant organ senescence [7,20]. The relationship between ABA and leaf age has been known for a long time. In fact, an increase in the level of ABA in yellowing leaves was first noted in the 1980s [50]. In addition to the effect of increasing levels of endogenous ABA [51–54], exogenous application of ABA has also been shown to accelerate the yellowing of leaves [53,55]. In the present study, a higher level of endogenous ABA in yellowing leaves of *Populus trichocarpa* was also observed. However, the same relationship was observed in senescing roots. Therefore, based on previous studies of leaves and flower petals [56–58], and the results observed in the present study, we suggest that the ABA similarly as in other ephemeral organs might also contribute to senescence process in fine roots.

Since ABA also plays a role in several physiological processes in non-senescing organs, it was not surprising that ABA was also detected in viable fine roots and leaves. The immunohistochemical detection of ABA in non-senescent organs, however, was mainly localized to the vascular tissue. The vascular localization may have been associated with ABA transport, which can be transported in both xylem (from roots to shoots) and phloem (from leaves to roots) [59]. An accumulation of ABA was observed in both the peripheral cytoplasm and central vacuole of cortical parenchyma cells of senescing roots. Earlier, such localization in mesophyll cells has also been described in *Arabidopsis* where an abscisic acid glucosyl ester (ABA-GE) was stored in the vacuole, and in response to abiotic stress, ABA-GE can be rapidly converted to the free form of ABA using vacuolar b-glucosidases [60]. In our study, vacuolar localization was not clearly visible in leaves where the ABA signal was primarily detected in the cytoplasm of cells. In the latter stage of senescence (RS2), the structure of cortical parenchyma cells in roots was highly disrupted, making the determination of ABA localization difficult. Nonetheless, a strong signal was observed in the vascular cylinder and within folded cortical parenchyma cells. In contrast, a much lower signal was observed in leaves in the latter stage of senescence (LS2), where the signal appeared as small spherical spots in the cytoplasm of both palisade and spongy mesophyll cells. In LS2, chlorophyll autofluorescence was barely visible, suggesting that the majority of these organelles had been degraded. These observations are in agreement with recent findings on the role of ABA in chlorophagy [61,62]. In that study, the stromule number increased in response to an ABA treatment, whereas treatment with a specific inhibitor of ABA synthesis prevented the formation of stromules by mannitol [61]. Moreover, it has been suggested that proteins belonging to kinase family (SnRK2, CK2), which are activated by the ABA signaling pathway, might participate in phosphorylation of chloroplast membrane proteins or the ATG proteins and activate chlorophagy [62,63].

The analysis of gene expression during senescence, especially ABA-related gene expression, did not reveal many similarities between leaves and fine roots. In fact, the majority of the DEGs were organ-specific. A previous comparative transcriptomic analysis of senescing flower petals and leaves also noted a lack of similarity in the transcriptome of these two organs [64]. In the present study, up-regulated DEGs encoding transcriptional factors (TFs) related to ABA, such as MYB, bZIP, and

NAC, were identified in senescing leaves. Similar results were reported in a transcriptomic analysis of senescing leaves of other species, further confirming that senescence is highly regulated by multifold networks [11,65–68]. The up-regulation of TFs, especially NAC family TFs, during organ senescence has also been documented in numerous crop species [16]. The NAC factor AtNAP has been demonstrated to play a crucial role in the integration of abscisic acid (ABA) signaling and leaf senescence. AtNAP binds to the promoter of phosphatase 2C (PP2C) family genes and activates their expression. One of those PP2C genes encodes a SAG113 protein, which inhibits stomatal closure and, thus, promotes water loss and accelerates leaf senescence [69]. We also detected three genes in senescing leaves of *Populus* encoding phosphatase 2C proteins that are associated with ABA, among which two were up-regulated. Collectively, the data suggested that the PP2C identified in our transcriptome analysis could be involved in the ABA-dependent regulation of leaf senescence.

Among the up-regulated DEGs related to ABA that were identified in senescing roots, two genes were associated with cold acclimation: *COR413* and *COR314*. The up-regulation of these genes can be induced by both environmental conditions (low temperature) and ABA [70]. COR proteins have been implicated in increasing plant tolerance to low temperature by affecting the metabolism of fatty acids, sugars, and purines. In age-related developmental senescence, preparation for cold may be one of the factors that induces the senescence of fine roots and the up-regulation of genes to enable the acquisition of increased low-temperature tolerance. Cold tolerance would enable the organs (fine roots) to complete the process of senescence and remobilize their nutrients to storage organs rather than just die outright due to low temperatures. Down-regulated genes related to ABA were also identified in both leaves and fine roots, including a gene encoding a PIP aquaporin, which plays a key role in radial water transport in roots and leaves and maintains water homeostasis during the plant response to environmental stress [71]. The expression of aquaporin genes is regulated by a variety of factors, including the concentration of ABA. Jang et al. [72] demonstrated that the expression of the majority of studied *PIP* genes was up-regulated in response to ABA treatment, while several *PIP* genes were down-regulated in response to cold treatment. Throughout the course of our study, plants were exposed to decreasing temperatures that typically occur in autumn and may generally cause a decrease of pressure and reduced sap flow [73]. It is plausible that these conditions may have induced the down-regulation of genes encoding aquaporins, despite the presence of a high level of ABA. Our results indicate that ABA content is tightly regulated during senescence in leaves and roots, possibly at the transcriptional level, and its accumulation may contribute to senescence processes in both organs. We suggest that ABA may increase cold tolerance in fine roots, while it acts as a signal molecule in leaves. As a result, it likely induces the expression of a variety of TFs that contribute to the coordination of several physiological processes, such as the regulation of water loss via the regulation of stomatal aperture.

In addition to ABA-related genes, numerous JA-related genes were also identified in our microarray analyses. JA not only plays a role in the adaptation of plants to biotic and abiotic stresses and the regulation of several developmental events (root inhibition, anthocyanin accumulation, trichome initiation, male fertility, etc.). It also plays a role in the positive regulation of senescence [21,22,74–76]. The first report documenting that jasmonate affects senescence was the observation that treatment with methyl jasmonate (MeJA) resulted in a rapid loss of chlorophyll [77]. Although this observation was made a considerable time ago, the molecular mechanisms underlying leaf ageing are still not fully understood. Similar to *Arabidopsis* [22], a significant increase in JA and MeJA levels was observed in senescing leaves of *P. trichocarpa*. The impact of JA on senescence of other ephemeral organs, however, is not so clear. Exogenous application of JA to flower petals of *Phalaenopsis* promoted senescence [78]. In contrast, endogenous levels of JA did not increase during the senescence of *Lilium* flower petals [79], and JA levels decreased while MeJA levels increased in senescing cotyledons of *Ipomoea nil* [80]. There are still insufficient data to confirm the promotive role of JA in root senescence; however, our results indicating variations of JA and MeJA content in senescent roots of *Populus* suggest that these phytohormones can be a metabolic signature of senescence. The localization of JA in viable organs

was concentrated primarily in the vascular bundle and might be due to several factors other than senescence, such as the synthesis of JA [81], modulations of the level of transfer cell wall ingrowths in the phloem [82], or JA transport [83]. The highest JA signal in senescing roots was observed in the cortical parenchyma cells, in which significant changes associated with senescence, such as the presence of autophagic-related structures and changes in cell shape, were already observed in RS1 [13]. Ultrastructural analyses also revealed the presence of microorganisms inside cells during the latter stages of senescence (RS2) [12,13]. Thus, the high concentration of JA in senescent roots may have been related to protecting these organs from pathogens, allowing the progress of senescence and, more importantly, nutrient remobilization instead of rapid death. Jasmonates have been reported to induce defense responses against microorganisms that cause plant diseases [84–86]. In this regard, *fad3–2*, *fad7–2*, and *fad8* mutants in *Arabidopsis*, in which JA accumulation is disrupted, exhibited roots that were more susceptible to root rot caused by a fungal root pathogen than the roots of wild-type plants. Notably, exogenous application of MeJA reduced this effect [85].

As found with ABA-related DEGs, the microarray analysis conducted in the present study did not identify JA-related DEGs that were common to both leaves and fine roots. In fact, there was a distinct lack of a significant number of JA-related DEGs in fine roots related to the course of senescence. Increased expression of a gene encoding an ERECTA protein, which functions in the regulation of immune responses and resistance to pathogens, however, was documented [87,88]. Collectively, these results, along with a previous report [12], appear to indicate that JA is not directly involved in the senescence process in roots. JA, however, may indirectly affect senescence by modulating the resistance response to pathogens that would prevent the rapid death of roots due to pathogen invasion. Consequently, this would allow sufficient time for root cells to complete the senescence processes in a prescribed manner, including remobilization, autophagy, etc. The expression of genes encoding MYB TFs was noted in both leaves and roots. MYB TFs are a large family of proteins that are a crucial element in regulatory networks controlling plant development, metabolism, and responses to biotic and abiotic stresses [89]. The up-regulation of several MYB proteins was documented in senescing leaves of *Arabidopsis* [90] and *Solanum* [91]. MYB TFs are also involved in the jasmonate signaling cascade by interacting with JAZ proteins [76,92], which are known as repressors of JA-responsive genes. The function of JAZ proteins in leaf senescence and the regulation of cell death during host and non-host interactions, however, has also been postulated [93,94]. Our results indicate that jasmonate might play an important role in the direct or indirect regulation of senescence in both leaves and roots; however, similar to ABA, the regulatory effect differs in the two organs. We suggest that jasmonate might be involved in the response to biotic stress in senescing roots, while its role in leaves seems to be more complex and overlap with the regulation of several other processes through the influence of jasmonate on activation of TFs. However, confirmation of the exact role of JA in the senescence of plant organs requires further research.

Summarizing, the senescence of plant organs involves an intricate network of episodes that, despite considerable research, still remains insufficiently understood. Particularly enigmatic is the senescence of underground ephemeral plant organs. Thus, in the current study we primarily focused on examining the senescence process in fine absorptive roots. We also sought to determine if the underlying mechanism regulating senescence was universal for all plant organs by comparing the senescence of fine roots with leaf senescence. Our results indicated that the senescence of both organs was accompanied by significant changes in gene expression. Many of the identified DEGs in both organs were associated with a variety of phytohormones. The quantitative analyses of the senescing organs also revealed that the levels of ABA, JA, and MeJA increased in both leaves and roots during senescence. Despite these similarities, however, our analysis of phytohormone-related genes indicated that the function of ABA and jasmonates may differ in the two organs during senescence. We suggest that phytohormones in roots do not directly regulate the progression of senescence but rather act indirectly by regulating other processes such as cold acclimatization (ABA) and resistance to soil microorganisms (JA). Although these regulatory processes are not directly associated with the

progression of senescence, they are essential to prevent premature cell death and allow senescence and the processes associated with it (such as remobilization) to occur in a prescribed manner rather than be terminated by premature cell death. In contrast, ABA and JA appear to have a direct role in the regulation of leaf senescence. Our study suggests that the regulatory effect of phytohormones on senescence is organ-specific. The exact mechanism regulating the senescence of leaves, and especially fine, absorptive roots, is still not well understood and should be a topic of future research.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

All experiments were performed on *Populus trichocarpa* (Torr. & A. Gray ex Hook.). Seeds were germinated on 1% agar. Seedlings (about 1–2 cm in length) were planted in soil in a seed-starting system. Plants were grown in a growth chamber (Conviron GR96) at 18 °C day/14 °C night temperature and a 16 h day/8 h night photoperiod. After two months, plants were transferred to rhizotrons as described in Wojciechowska et al. [95]. The material for the study was sampled during the first vegetative season at three time points based on morphological markers of senescence. For leaves, senescence-related stages were distinguished based on chlorophyll content. Chlorophyll measurements have been performed using a CCM-200 plus Chlorophyll Content Meter (Opti-Sciences, Hudson, NH, USA) in two places for one leaf (on both sides of each leaf blade analyzed). Such measurements were performed for 30 plants each time, throughout the vegetative season. The average result for green leaves (LC) was defined as 100% chlorophyll content. In yellowing leaves (LS1), material was collected in which the chlorophyll content dropped about 40%, and for yellow leaves (LS2) about 60% (Supplementary Figure S1B). For roots, senescence-related stages were also distinguished based on changing their color. Moreover, a viability test, as well as anatomical and cytological analyses, confirmed that color change was associated with the senescence process. The senescence-related stages have been broadly described in Wojciechowska et al. [13] and are presented in Figure 1.

4.2. Microarray Analysis

Total RNA was extracted from three biological replicates of leaves and roots using an RNeasy Plant Mini kit (Qiagen, Germantown, MD, USA). RNA quantity and quality were assessed using a NanoDrop1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). cDNA synthesis and microarray hybridization to an Affymetrix GeneChip Poplar Genome Array (A-AFFY-131) were performed according to the provided Affymetrix protocol. A complete microarray dataset was submitted to the Gene Expression Omnibus database (accession number GSE143559). The raw image data from a total of three A-AFFY-131 arrays were normalized with Robust Multi-Array Average (RMA). The normalized data were statistically analyzed using GeneSpringGX7 13.1 (Agilent Technologies Inc., Santa Clara, CA, USA) software. Data were subjected to a one-way ANOVA with a corrected p-value cut-off = 0.05 and a Benjamini–Hochberg correction. DEGs were annotated using Phytozome JGI database, BLAST, Ensembl Genome, and KAGIANA. Heatmaps were generated using MATLAB (The MathWorks Inc., Natick, MA, USA). The Venn diagram of DEGs was drawn by VENNY2.1 (https://bioinfogp.cnb.csic.es/tools/venny/).

4.3. Measurement of Endogenous Abscisic acid (ABA) and Jasmonates (JA and MeJA)

GC-MS was used to determine the concentrations of endogenous ABA, JA, and MeJA. Material for phytohormone concentration measurement was collected from nine plants, and measurements were performed in three technical replicates. After the plant material (~0.5 g) was homogenized, ABA and jasmonate levels were measured using the optimized protocols described by Wilmowicz et al. [96,97]. GC-MS-SIM was performed by monitoring m/z 134, 162, and 190 for measuring endogenous ABA and 138, 166, and 194 for [6-$^2H_3$] ABA according to the method described by Vine et al. [98]. GC/MS-selected ion monitoring was used to measure jasmonates by monitoring m/z 193, 195, 224, and

226. Statistical analyses (ANOVA with a corrected p-value = 0.05 and Tukey's post-hoc test) were performed using Statistica 12.0 software (StatSoft Poland Inc., Tulsa, OH, USA).

4.4. Immunolocalization of JA and ABA

Samples of roots and leaves were fixed in 3% (*v*/*v*) *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) for 2h and a mixture of 2% glutaraldehyde (pH 6.8; Polysciences, Warrington, USA) and 2% (*v*/*v*) formaldehyde (pH 6.8; Polysciences, Warrington, USA) for 12 h at 4 °C. The fixative was then discarded, and the samples were rinsed three times in 1x PBS (phosphate-buffered saline) (Sigma, St Louis, MO, USA) buffer. Leaf samples (5 × 5mm) were sectioned (30 µm) using a vibratome Leica VT 1200S (Leica Biosystems, Nussloch, Germany), while root samples were dehydrated in a graded ethanol series (10%–100%) and then infiltrated and embedded in Paraplast Extra (melting point, 57.8 °C; Sigma, St Louis, MO, USA). Root samples were sectioned (20 µm) using a Leica RM2265 (Leica Biosystems, Nussloch, Germany) rotary microtome. JA was localized using primary anti-JA rabbit antibodies (Agrisera, Sweden, catalogue number AS11 1799) at a 1:500 dilution. ABA was detected using a primary, anti-ABA rabbit antibody (Agrisera, Sweden, catalogue number AS09 446) at a 1:500 dilution. Immunolocalization assays were performed as described by Wojciechowska et al. [13]. Results of the localization were viewed and recorded with a Leica TCS SP5 confocal microscope (Leica Biosystems, Nussloch, Germany) using the following lasers: 405 diode-emitting light at a wavelength of 405 to observe chlorophyll fluorescence (in leaves) or cell wall fluorescence (in roots to observe the shape of the cells) and an argon laser-emitting light at a wavelength of 488 to observe fluorescence of Alexa 488 (ABA, JA). Negative control reactions consisting of samples processed without exposure to the primary antibodies were utilized for both ABA and JA (Supplementary Figure S6).

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/21/6/2042/s1. Figure S1: Characteristics of the material used in the study. (A)FW/DW ratio in roots and leaves during senescence. (*B*)Changes in chlorophyll content during the growing season. Means designated by different letters indicate statistically significant differences according to ANOVA and Tukey's post hoc test ($p < 0.05$). Values represent the mean ± SE (standard error); Figure S2: The functional classification based on (A) Gene Ontology Cellular Compartment (B) Gene Ontology Biological Process during root senescence; Figure S3: The functional classification based on (A) Gene Ontology Cellular Compartment (B) Gene Ontology Biological Process during leaf senescence; Figure S4: Heatmaps showing expression profiles of genes associated with (A) Ethylene, (B) Auxin, (C) Cytokinins (CKs), (D) Brassinosteroids (BRs), Gibberellic acid (GA) during root senescence, Figure S5: Heatmaps showing expression profiles of genes associated with (A) Ethylene, (B) Auxin, (C) Cytokinins (CKs), (D) Salicylic acid (SA), Gibberellic acid (GA) during leaf senescence; Figure S6: Representative images of negative control reactions with omitting primary antibody and reaction with antibodies anti-JA in leaves and fine roots, Table S1: DEGs with annotations associated with various phytohormones during root senescence; Table S2 DEGs with annotations associated with various phytohormones during leaf senescence; Table S3: Data related to the quantitative analysis of phytohormones.

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Abbreviations

ABA	Abscisic acid
ATG	Autophagy-related genes
CKs	Cytokinins
DEG	Differentially Expressed Genes
EDAC	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
FC	Fold change
GO	Gene Ontology
JA	Jasmonic acid
LC	leaves control, without any symptoms of senescence
LS1	first stage of leaf senescence
LS2	second stage of leaf senescence;
MeJA	Methyl jasmonate
PBS	Phosphate-Buffered Saline;
PCD	Programmed Cell Death;
RC	roots control, without any symptoms of senescence;
ROS	Reactive Oxygen Species;
RS1	first stage of fine, absorptive root senescence;
RS2	second stage of fine, absorptive root senescence;
SA	Salicylic acid;
SAGs	Senescence-Associated Genes;
SDGs	Senescence Downregulated Genes;
TFs	Transcriptional Factors

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Research paper

Seasonal senescence of leaves and roots of *Populus trichocarpa*—is the scenario the same or different?

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The remobilization and resorption of plant nutrients is considered as a crucial aspect of the seasonal senescence of plant organs. In leaves, the mechanisms responsible for the relocation of valuable compounds are well understood while the related processes in roots are still being debated. Some research indicates that remobilization in roots occurs, while other studies have not found evidence of this process. Considering that the total biomass of fine roots is equal to or greater than that of leaves, clarifying the conflicting reports and ambiguities may provide critical information on the circulation of chemical elements in forest ecosystems. This study provides new information concerning the basis for remobilization processes in roots by combining physiological data with gene expression and protein levels. We suggest that, as in leaves, molecular mechanisms involved in nitrogen (N) resorption are also activated in senescent roots. An analysis of N concentration indicated that N levels decreased during the senescence of both organs. The decrease was associated with an increase in the expression of a glutamine synthetase (GS) gene and a concomitant elevation in the amount of GS—one of the most important enzymes in N metabolism. In addition, significant accumulation of carbohydrates was observed in fine roots, which may represent an adaptation to unfavorable weather conditions that would allow remobilization to occur rather than a rapid death in response to ground frost or cold. Our results provide new insights into the senescence of plant organs and clarify contentious topics related to the remobilization process in fine roots.

Keywords: carbohydrates, fine roots, glutamine synthetase, nitrogen, *Populus trichocarpa*, remobilization, seasonal variation.

Introduction

Senescence is the last stage of plant ontogenetic development and can have a major influence on a wide spectrum of ecological processes, ranging from litter formation to nutrient cycling. The senescence process is largely mediated by programmed cell death (PCD), regardless of whether the organs in question are leaves, roots or petals (van Doorn and Woltering 2004, Sobieszczuk-Nowicka et al. 2018, Wojciechowska et al. 2018*a*). The PCD, however, is thought to represent an adaptation to the intermediate state between living and dead cells (Thomas et al. 2003). The irreversible senescence that occurs in leaves is associated with dramatic changes in gene expression, the degradation of macromolecules and a decrease in protein synthesis (Liu et al. 2008, Avila-Ospina et al. 2014). The global changes in gene expression vary and are represented by both increases and decreases in transcript levels. Mechanisms that regulate the network responsible for proper management of the degradation and remobilization of cellular

material during leaf senescence have been identified. Thus, plant constituents are preserved rather than lost. In order to maximize resource efficiency across many growing seasons, elements (especially nitrogen [N]) are translocated from the senescing tissues to developing seeds, newly formed leaves or storage organs (Buchanan-Wollaston and Ainsworth 1997, Avila-Ospina et al. 2014, 2015).

In plants, the senescence process is coordinated at both a structural and physiological level that can explicitly link plant traits to changes in vitality. For example, morphologically, yellowing is commonly associated with leaf senescence. It is directly related to chlorophyll levels and the conversion of chloroplasts into gerontoplasts (Avice and Etienne 2014, Sobieszczuk-Nowicka et al. 2018). Considering that more than 70% of all leaf proteins are present in chloroplasts, degradation of those structures is then associated with the release of a large pool of N (Liu et al. 2008). Hence, changes in chloroplast structure are highly correlated with the initial stages of senescence, when mechanisms associated with remobilization are activated. Increasing evidence suggests that this activation includes the release of free amino acids during the process of protein degradation and conversion into ammonia. Due to the toxicity of that compound, it is rapidly incorporated into glutamate by the amination of 2-oxoglutarane. This reaction is catalyzed by glutamate dehydrogenase (GDH) (Liu et al. 2008). Subsequently, glutamate is transformed into glutamine, which has the potential to be mobilized and relocated through the phloem sap. Glutamine synthetase (GS), one of the most important enzymes in N metabolism, is involved in this reaction (Liu et al. 2008, Avila-Ospina et al. 2015). Based on cellular localization, there are two main isoforms of GS: cytosolic (GS1) and chloroplastic (GS2) (Zhang et al. 2017). GS1 plays a fundamental role in glutamine synthesis, which enables the relocalization of N from senescent tissues to locations where it can be preserved or utilized (Liu et al. 2008, Castro-Rodríguez et al. 2011, Zhang et al. 2017). In contrast, GS2 is required for the reassimilation of ammonia generated during photorespiration (Miflin and Habash 2002, Liu et al. 2008, Castro-Rodríguez et al. 2011, Zhang et al. 2017). Although a well-defined set of steps is involved in N remobilization from leaves, the pattern, magnitude and factors involved in N remobilization from roots during senescence are weakly defined and supported by research.

Among all compounds that are relocated and recycled during senescence, N and carbon (C) (due to the high concentration in plant tissues) are critically important. There are reports indicating that starch is degraded and transformed into sucrose, the main form in which C is transported in plants (Cerasoli et al. 2004). Despite the importance of carbohydrate redistribution for nutrient conservation, the molecular mechanism of C translocation from absorptive roots undergoing senescence has received little attention.

In contrast to the considerable progress that has been made in elucidating the senescence process in leaves and flower petals (Agüera et al. 2010, Shibuya et al. 2011, 2013, 2014, Shibuya 2012, Avila-Ospina et al. 2015, Springer et al. 2015, Sobieszczuk-Nowicka et al. 2018), much less attention has been given to the belowground component of plant biomass. As the annual biomass production of fine roots is equal to or even greater than the biomass of leaves, the senescence and death of fine roots is important from the standpoint of the cycling of chemical elements (Gill and Jackson 2008, Brassard et al. 2009). Though traditionally defined as roots <2 mm in diameter, it is increasingly recognized that fine roots are not a homogeneous entity as they include both absorptive roots and transport roots (Bagniewska-Zadworna et al. 2012, 2014, McCormack et al. 2015, Zadworny et al. 2015, Wojciechowska et al. 2018*b*). The absorptive roots belong to the first two orders of roots, which are characterized by the highest absorptive capacity, high N concentration and respiration rate, and often mycorrhizal colonization (Eissenstat et al. 2000, Pregitzer et al. 2002, McCormack et al. 2015). Thus, the senescence process of the short-lived absorptive roots may provide important information for nutrient recycling, as well as understanding of plant adaptation to autumn and winter seasons. There is a premise that the death of absorptive roots may be a passive process where the provision of sugars and defense compounds is stopped, making the roots an easy target for pathogens (Yanai and Eissenstat 1997, Eissenstat and Volder 2005). Our recent studies, however, have indicated that the senescence and death process in these roots is active and genetically regulated, and represents another example of PCD in plants (Bagniewska-Zadworna et al. 2014, Wojciechowska et al. 2018*b*). The fact that absorptive roots undergo a genetically regulated death process emphasizes the premise that their nutrients, which are either limited in the environment or difficult to absorb, would be targeted for relocation so that they can be recycled. No details on the mechanisms associated with relocation and remobilization of valuable compounds from senescing absorptive roots are available, and the information that does exist is variable or conflicting (Kunkle et al. 2009, Zadworny et al. 2015). Identification of the mechanisms responsible for the senescence of absorptive roots represents the first step to understanding how roots die and how nutrient resorption from the entire root system can be incorporated into the measurement of nutrient turnover at the whole-plant level.

Therefore, an experiment was designed to analyze the senescence process in leaves and absorptive fine roots, with particular emphasis placed on the regulation of nutrient remobilization. We specifically expected that N and C concentrations would decline with senescence and the same dominant genetic cues (i.e. GS) would be closely associated with changes of N in both leaf and root.

Table 1. Senescence stages of roots and leaves.

Variant	Abbreviation	Characteristic features
Control leaves	LC	Green leaves without senescence symptoms
First stage of leaf senescence	LS1	Yellowing leaves in which chlorophyll level had decreased by ~40%
Second stage of leaf senescence	LS2	Yellow leaves in which chlorophyll level had decreased by ~60%
Control roots	RC	White roots without senescence symptoms
First stage of root senescence	RS1	Roots which had changed in color from white to brown
Second stage of root senescence	RS2	Roots which had changed in color from brown to dark brown or almost black. Shrinkage was also visible in most fine roots

Materials and methods

Plant material and growth condition

All experiments were performed on *Populus trichocarpa* (Torr. & A. Gray ex Hook.). Seeds, provided by the FLORPAK Młynki Seed Store, Poland, were placed on 1% agar. After germination, the seedlings (~1–2 cm in length) were planted in soil in a seed-starting system and grown for 2 months in a plant growth chamber (Conviron GR96) at 18 °C day/14 °C night temperature and a 16 h day/8 h night photoperiod. The 2-month-old plants were then removed from the seed-starting system, along with a clod of dirt in order to prevent injuring the root system, and re-planted in rhizotrons consisting of underground boxes (50 × 30 cm) made from two transparent polycarbonate plates held 3 cm apart by thick-walled plastic tubing to provide sufficient room for root growth. The rhizotrons were filled with soil obtained from a forest where *Populus* species naturally grow. The plants were watered with an automated system. The bottom of each rhizotron contained a drainage hole to avoid hypoxic, flooding conditions and to ensure that the soil was aerated. Rhizotrons were placed in containers, in a semi-open, greenhouse located at the Institute of Dendrology, Polish Academy of Sciences, in Kórnik, Poland (52°14′40″N and 17°06′27″E).

Leaf and root samples for each biological replicate were harvested from at least three individual plants. During sampling, the rhizotron windows were pulled out, opened and then the harvested roots were divided into individual orders using a steel scalpel, taking into account that tip-ended roots are first order (Pregitzer et al. 2002). Leaf and root samples were collected three times during the growing season based on morphological and anatomical indications of senescence as described by Wojciechowska et al. (2018*b*). The senescence stages of leaves and roots selected for analysis are presented in Table 1.

Quantitative determination of N, C and carbohydrates

The analysis of N, C and carbohydrates was conducted during the course of the growing season on leaves and the first three root orders. Fine roots were divided into three groups based on their order (first, second—absorptive roots, without secondary growth, third—transport roots, longer and mostly with secondary structure) immediately after harvesting. Root order was assigned according to the morphometric approach where distal roots represent first-order roots (Pregitzer et al. 2002). This grouping was used to assess the relocation of the studied elements from lower to higher order roots. The material (Table 1) was collected at the same time of day (morning) to avoid any daily fluctuations in the level of the studied elements/compounds. The samples were dried at 65 °C for 3 days and ground to a powder in a Retsch MM 200 mill (Retsch, Haan, Germany). Quantitative determination of N and C concentrations was performed using an Elemental Combustion System CHNS-O 4010 (Costech Instruments, Pioltello/Valencia, Italy/USA).

Carbohydrate levels were measured as described by Oleksyn et al. (2000), and the level of soluble carbohydrates and starch was analyzed. Sugars were extracted from the ground material with a solution of methanol–chloroform–water. Starch concentration was measured by converting the starch to glucose with amyloglucosidase followed by oxidation with a peroxidase–glucose oxidase complex. A UV-1700 Pharma Spec (Shimadzu, Kyoto, Japan) spectrophotometer was used to determine the concentration of soluble carbohydrates (wavelength of λ = 625 nm) and starch (wavelength of λ = 450 nm). The concentration of the studied carbohydrates was determined using glucose standards and is presented as a percentage of dry mass.

Protein extraction, gel electrophoresis and immunoblots

Protein extraction was performed according to the method described by Szuba et al. (2013). Proteins were dissolved in a buffer containing 7 M urea, 2 M thiourea, 40 mM dithiothreitol, 0.5% carrier ampholytes and 4% CHAPS, and protein concentration was measured with a 2-D Quant kit (GE Healthcare, Piscataway, NJ, USA). Proteins were separated by SDS-PAGE on 4–20% Mini-PROTEAN TGX precast gels (Bio-Rad Laboratories, Inc., Grand Junction, CO, USA), with an equal amount of protein (20 µg) loaded in each lane. Protein transfer from the gel to a polyvinylidene fluoride (PVDF) membrane was conducted using

a Trans-Blot® Turbo™ (Bio-Rad). Rabbit, anti-GS antibodies (AS08 295, Agrisera, Vännäs, Sweden) were used. The GS antibodies were able to bind to both GS1 and GS2 isoenzymes. Antibodies were diluted 1:10,000 in 2% skimmed milk powder. Incubation with primary antibodies was carried out overnight at 4 °C. Antibodies were washed from the PVDF membrane with phosphate-buffered saline (PBS) (Sigma-Aldrich St. Louis, MO, USA), followed by PBS with Tween-20 (PBST) (Sigma-Aldrich). Incubation with secondary antibodies was conducted using antibodies conjugated to horseradish peroxidase, goat, anti-rabbit (Agrisera) diluted 1:10,000 in 2% skimmed milk powder. After 1 h incubation with secondary antibodies, the PVDF membranes were washed in PBS and PBST and then incubated in Clarity western ECL substrate chemiluminescent detection reagent (Bio-Rad) for 5 min prior to image acquisition in a G-BOX CHEMI XR5 (Syngene, Cambridge, UK).

Reverse transcription-quantitative PCR analysis of gene expression

RNA isolation was performed using leaves and first-order roots. RNA isolation and cDNA synthesis were performed as described by Wojciechowska et al. (2018*b*). All of the reverse transcription-quantitative PCR (RT-qPCR) analyses of gene expression were conducted utilizing three technical replicates from each of independent three biological replicates of each variant. RT-qPCR analyses were conducted in 96-well plates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following amplification program: denaturation by a hot start at 95 °C for 10 min, followed by 40 cycles of a two-step program (denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min). The DNA sequences for GS1.1, GS1.2, GS1.3 and GS2 and primer design were selected based on the sequences reported by Castro-Rodríguez et al. (2011). Primers for the amplification of references genes were designed in Primer3 software (The Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The sequences of the primer pairs used in the RT-qPCR analyses are listed in Table 2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-TUBULIN and UBIQUITIN were selected as reference genes as they exhibited the most stable expression in all sample types and time points. The method utilized to determine the relative level of expression was described by Bagniewska-Zadworna and Stelmasik (2015).

Microarray analysis

Total RNA was isolated in triplicate from each sample of first-order roots and from leaves using an RNeasy Plant Mini kit (Qiagen, Germantown, MD, USA). RNA quantity and quality were measured using a NanoDrop1000 (ThermoFisher Scientific, Carlsbad, CA, USA). cRNA synthesis and microarray hybridization to an Affymetrix GeneChip Poplar Genome Array (A-AFFY-131) were performed at the Laboratory of Microarray Analysis (Institute of Biochemistry and Biophysics Polish Academy of Science, Warsaw, Poland) according to the provided Affymetrix protocol. The normalized data were statistically analyzed using GeneSpringGX7 13.1 software (Agilent Technologies, Inc., Santa Cara, CA, USA). Statistical analysis was performed using a one-way ANOVA with a corrected *P*-value cut off $\leq$0.05 and a Benjamini Hochberg correction.

Table 2. Sequence of gene-specific primers used in the RT-qPCR analysis.

Gene	Primer
GS1.1	F-5′ATGGTTGTCTGTCAATTTGTTTGCC-3′
	R-5′CCAGCAAGAGTTTTATTTAGATTAG-3′
GS1.2	F-5′GGAATTGAGTATTGGAAGATGATGG-3′
	R-5′TATGTTCATAAATGATCAACAGCC-3′
GS1.3	F-5′TGGAAACCATAAGAGATCACCACC-3′
	R-5′GAAGAGGCAATTCTTGTACCAAG-3′
GS2	F-5′GGAGCATCACTTGGATCTAGATGG -3′
	R-5′CAAAACCCAAGAGTAAAAAGGTCC-3′
β-Tubulin	F-5′TTCTCCTGAACATGGCAGTG-3′
	R-5′CCACACAACGTGAAATCCAG-3′
GAPDH	F-5′CAATGAATGGGGCTACAGGT-3′
	R-5′CATGAATCAGCTGCACATCC-3′
Ubiquitin	F-5′AGGAACGCGTTGAGGAGAAG-3′
	R-5′TATAABCAAAAACCGCCCCTG-3′

Statistical analysis

Statistical relationships were considered significant at $P \leq 0.05$. Root and leaf biochemical traits were $\log_{10}$ transformed to meet the assumption of normality. However, figures show the non-transformed data. In the quantitative analyses of N, C and C:N, as well as in the analyses of GS gene expression, one-way ANOVA and Duncan's post-hoc test were performed using Statistica 12.0 software (StatSoft Poland Inc., Tulsa, OH, USA).

Results

Quantitative assessment of N, C and the C:N ratio

Considering the ambiguous data on N relocation during root senescence, we determined the N and C concentration [%] to dispel doubts regarding the relocation of two main elements. Moreover, to check that remobilization is another universal feature of senescence, we also performed those and the rest of the analyses for leaves. Over the course of the growing season, there was a general decrease in N concentration in both leaves and the first three root orders (Figure 1A and B). In contrast to N, no significant seasonal or senescence effect on the level of C was observed in either leaves or roots (Figure 1C and D). However, due to the changes in N, there was a distinct increase in the C:N ratio in senescing leaves and the first three orders of fine roots ($P \leq 0.05$; Figure 1E and F). Our results demonstrate that N was relocated during senescence in both studied organs.

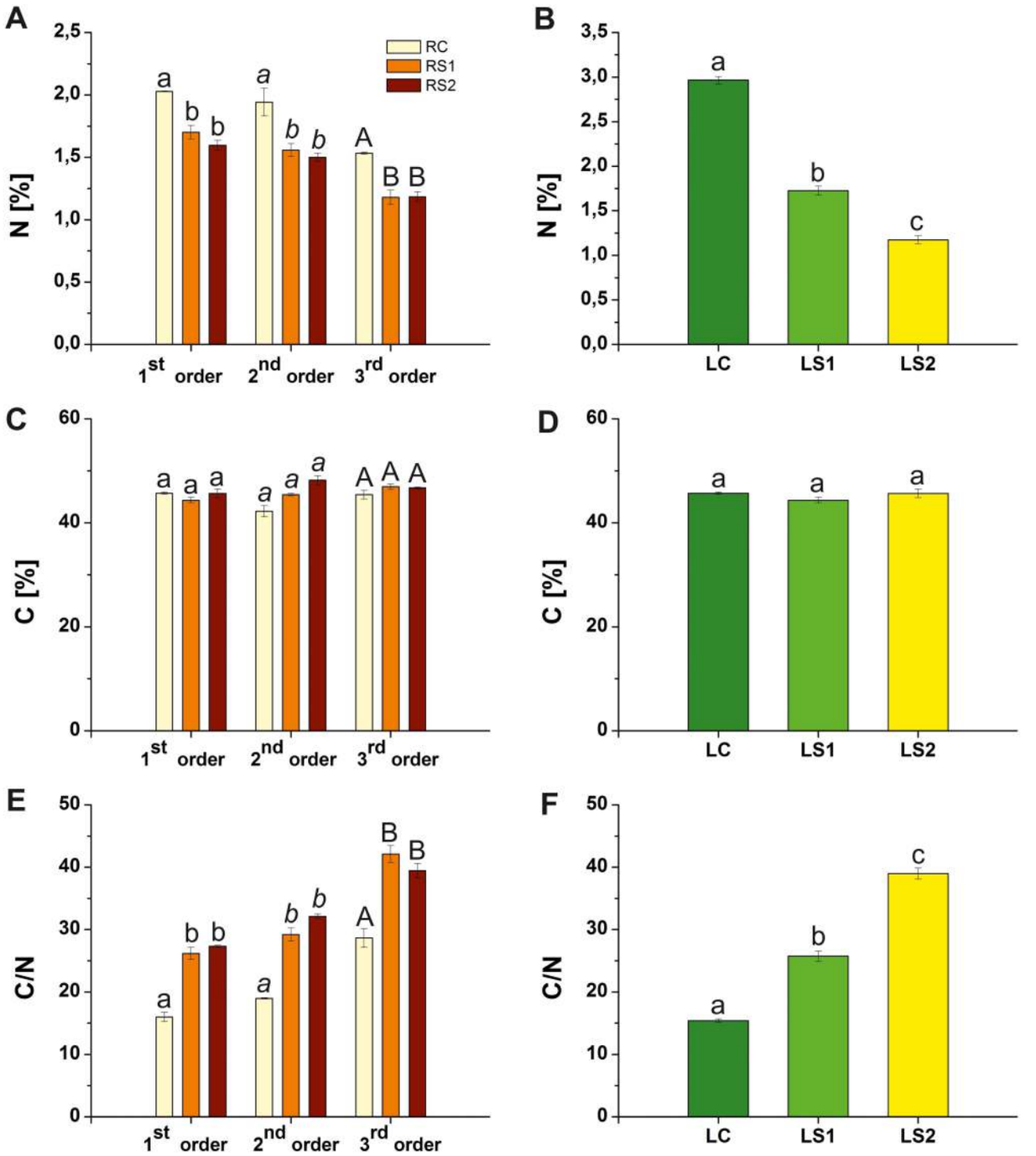

Figure 1. Quantitative analysis of N and C concentrations and the C:N ratio in absorptive roots (A, C, E) and leaves (B, D, F) of *P. trichocarpa* during the course of the growing season. (A, B) Quantitative analysis of N concentration [%]; (C, D) quantitative analysis of C concentration [%]; (E, F) quantitative analysis of the C:N ratio (RC: root control, RS1: first stage of root senescence, RS2: second stage of root senescence, LC: leaf control, LS1: first stage of leaf senescence and LS2: second stage of leaf senescence). Bars sharing the same letter are not significantly different ($P \leq 0.05$). Values represent the mean ± standard error (SE).

Expression of genes involved in N remobilization

The effect of the senescence processes on the expression of genes involved in N metabolism was studied by analyzing the expression of three genes (*GS1.1*, *GS1.2* and *GS1.3*) encoding a cytosolic form of GS. In addition, a gene encoding a chloroplastic isoform of the GS2 protein was also examined in leaves

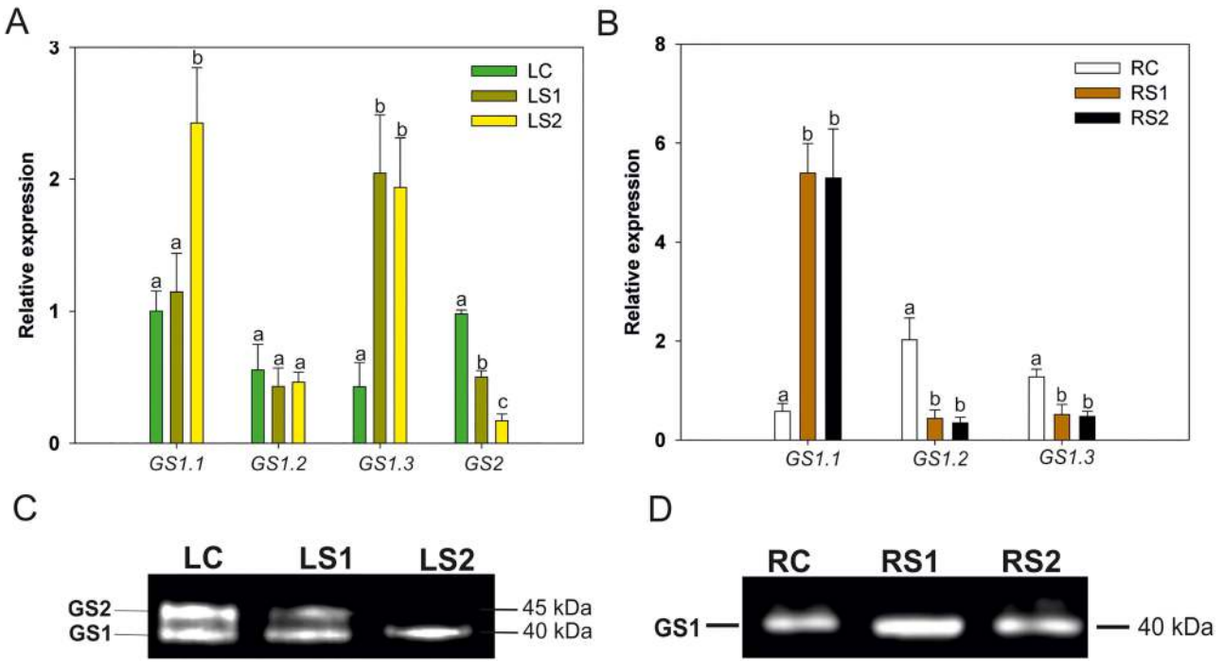

Figure 2. Analysis of the expression of GS. (A, B) Relative expression of GS in leaves (A) and roots (B) of *Populus trichocarpa*; (C, D) distribution of GS in leaves (C) and fine roots (D) (LC: leaf control, LS1: first stage of leaf senescence, LS2: second stage of leaf senescence, RC: root control, RS1: first stage of root senescence and RS2: second stage of root senescence). Bars sharing the same letter are not significantly different ($P \leq 0.05$). Values represent the mean ± SE.

(Figure 2A and C). Results of the RT-qPCR analyses indicated that leaf senescence induced the expression of GS1.1 and GS1.3. A statistically significant increase in GS1.1 expression, relative to non-senescent leaves, was observed only during the second stage of senescence (LS2: yellow leaves), whereas GS1.3 was up-regulated in both the first (LS1: yellowing leaves) and second stage of senescence (Figure 2A). GS1.2 exhibited a different pattern of expression than GS1.1 and GS1.3 (Figure 2A). GS2 expression in leaves decreased over the course of the growing season and senescence (Figure 2A). A significant increase in GS1.1 expression was observed at the first stage of root senescence (RS1: brown roots), and the elevated expression was maintained at the second stage of this process (RS2: dark brown roots with shrinkage) (Figure 2B). In contrast, the expression of GS1.2 and GS1.3 exhibited the opposite trend to GS1.1, with the highest level of expression was observed in non-senescent, control roots (RC: white roots, without any visible symptoms of senescence) (Figure 2B).

Immunoblot analysis of glutamine synthetase

We have checked also whether the level of GS is increased during senescence. Using immunoblots, we detected the changed content of this protein over the course of the growing season in both leaves and roots (Figure 2C and D). Two forms of GS were detected in leaves, cytosolic (GS1, 40 kDa) and chloroplastic (GS2, 45 kDa). Both isoforms were observed in green leaves, with the level of GS2 being slightly greater than that of GS1 (Figure 2C). In the LS1 stage of senescence, the level of GS2 significantly decreased while the level of GS1 was only slightly lower than in the LC stage. In fully yellow leaves (LS2), the GS2 form was almost undetectable, whereas the level of GS1 increased significantly, relative to the LC stage (Figure 2C). In contrast, a trend in the level of GS1 increasing was observed at the first stage of root senescence (RS1), relative to non-senescent, control roots (RC), but its level was significantly lower at the second stage of senescence (RS2) (Figure 2D). This experiment together with the GS expression analysis provides evidence of the presence a similar process involved in the relocation of N in both examined organs.

Quantitative assessment of carbohydrate levels during the growing season

We tested whether similar to how N is relocated during senescence, plants also remobilize C from starch and soluble carbohydrates in order not to lose those compounds, which may be used in the future as an energy source. The concentration of starch decreased significantly in senescing leaves, relative to non-senescent leaves (Figure 3A). In contrast, the concentration of starch increased in all examined orders of senescent roots, relative to non-senescent roots (Figure 3B). In addition, differences in carbohydrate levels were also observed between leaves and roots. Statistically significant increases in soluble carbohydrate concentration were observed in the first three orders of fine roots (Figure 3D), whereas soluble carbohydrate levels were

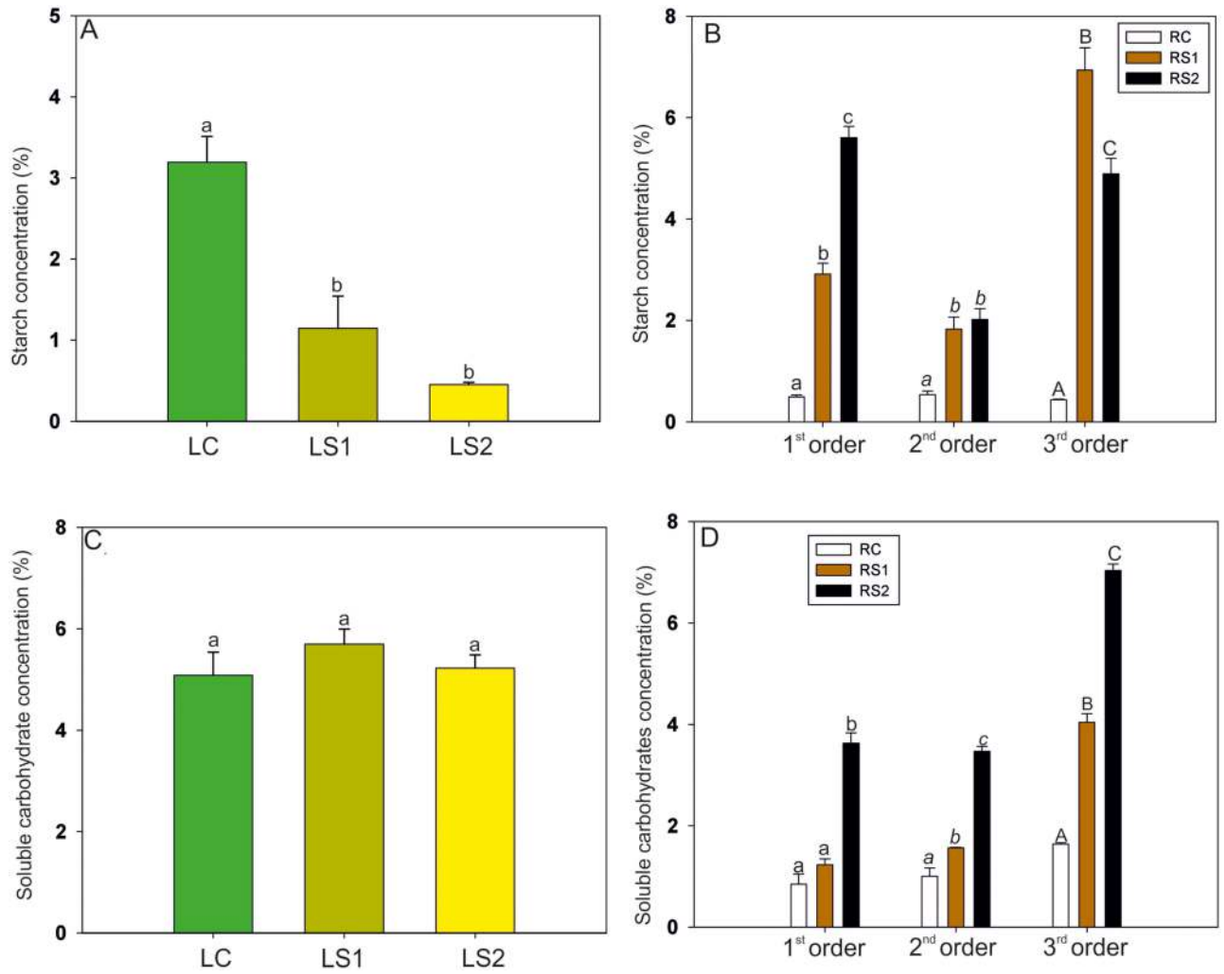

Figure 3. Quantitative analysis of carbohydrate levels in leaves (A, C) and in fine roots (B, D) of *P. trichocarpa* during the course of the growing season. (A, B) Quantitative analysis of starch concentration [%]; (C, D) quantitative analysis of the concentration of soluble carbohydrate [%] (LC: leaf control, LS1: first stage of leaf senescence, LS2: second stage of leaf senescence, RC: root control, RS1: first stage of root senescence and RS2: second stage of root senescence). Bars sharing the same letter are not significantly different ($P \leq 0.05$). Values represent the mean ± SE.

stable in leaves and no statistically significant changes were observed during the senescence process (Figure 3C).

Analysis of the expression of genes related to N remobilization and carbohydrate metabolism

Microarray analyses were performed to assess the expression of genes related to the process of the remobilization of chemical elements and nutrients during senescence of leaves and the first-order fine roots. This was done to identify genes other than GS1 that are involved in N remobilization. Additionally, due to the unexpected results obtained in the quantitative assessment of carbohydrate levels, it was deemed important to characterize the expression of genes involved in sugar metabolism in senescing leaves and first order of fine roots. Within the 56,055 transcripts present in the microarray, a total of 1348 differentially expressed genes (DEGs) were identified during the senescence process in leaves (one-way ANOVA, $P \leq 0.001$, fold change ≥ 2). A total of 1898 DEGs were identified in first-order roots during the course of senescence (one-way ANOVA, $P \leq 0.001$, fold change ≥ 2).

Among this large pool of DEGs, only these involved in N remobilization were subjected to further, in-depth analysis (Figure 4). In addition to GS1, the expression of which was confirmed by RT-qPCR analysis to be significantly increased during leaf senescence, an additional 20 genes were examined (Figure 4). Among those 20 selected genes, the majority of them were associated with amino acid transport (16 genes) and were up-regulated. A gene encoding GDH was also found to increase in expression during senescence. In contrast, two genes encoding glutamate synthetase were down-regulated, an NADH-dependent glutamate synthetase and a ferredoxin-dependent glutamate synthetase (Figure 4). In the case of roots, the number of genes associated with N remobilization was less pronounced (Figure 4). In addition to GS1, two genes encoding

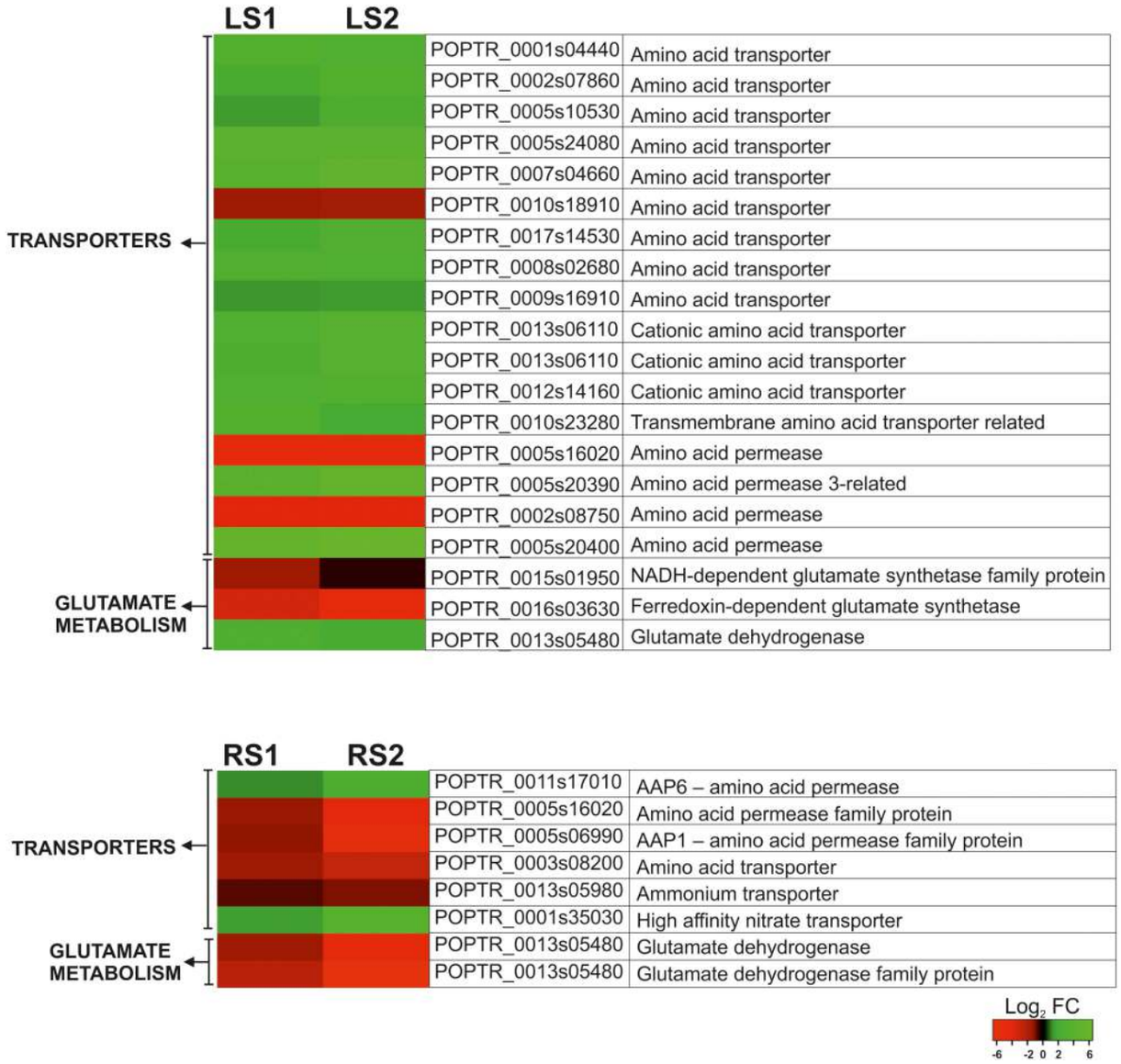

Figure 4. Heat map illustrating the fold changes (log2 basis) in the expression of the selected genes associated with N remobilization during leaf and root senescence.

GDH were identified and both were down-regulated. The expression of six genes that encode N compound transporters (amino acid transporters—four genes, ammonium transporter—one gene and nitrate transporter [NRT]—one gene) were also examined. Results indicated that a gene encoding a high-affinity NRT and another gene encoding an amino acid permease were both up-regulated, while the others were down-regulated (Figure 4).

To gain insight into which genes related to carbohydrate metabolism are different, regulated functional classification of the DEGs was performed using the database for annotation, visualization and integrated discovery. The most abundant gene ontology (GO) categories identified in senescing roots were those related to carbohydrate metabolic process (GO: 0005975). Thus, genes involved in sugar metabolism during the senescence of roots and leaves were examined further.

A total of 25 genes related to carbohydrate metabolism were identified, whose expression changed during leaf senescence. Among these DEGs, 13 were up-regulated and 12 were down-regulated (Figure 5). Similarly, the senescence of first-order absorptive roots was associated with the differential expression of 20 genes involved in carbohydrate metabolism. Among these DEGs, 11 were up-regulated and 9 were down-regulated (Figure 5). The DEGs could be divided into four

functional groups in both leaves and roots—starch metabolism, sucrose metabolism, sugar transporters and hexose metabolism (Figure 5).

Overall microarray analysis results indicate a large change in expression of genes associated with the remobilization of N and carbohydrates. These changes are in agreement with the quantitative analyses of N and sugars and confirm the N relocations and also indicate a different carbohydrates economy.

Discussion

The seasonal death of ephemeral plant organs is generally studied as a sequence of senescence-associated processes. The majority of research thus far has concentrated on elucidating this process in leaves or flower petals (Pérez-Rodríguez and Valpuesta 1996, Otegui et al. 2005, Liu et al. 2008, Agüera et al. 2010, Guiboileau et al. 2012, Shibuya 2012, Avila-Ospina et al. 2015). As a result, the ultrastructural, physiological and molecular changes associated with leaf and petal senescence have been identified and described as being a typical PCD (Yen and Yang 1998, Quirino et al. 2000, van Doorn and Woltering 2008, Shibuya et al. 2011, Sobieszczuk-Nowicka et al. 2018, Wojciechowska et al. 2018*a*). Despite the numerous studies on senescence that have been reported in the literature, only a small number of them have focused on the senescence process in absorptive roots (Bagniewska-Zadworna et al. 2014, Wojciechowska et al. 2018*b*).

Given that senescence is characterized by the intensification of catabolic and a decrease in anabolic processes, questions regarding the degradation and remobilization of valuable macromolecules and nutrients need to be addressed (Guo et al. 2004). Macromolecules are hydrolyzed and reduced to smaller, more mobile components that can be transported through the conductive tissue to other parts of the plant, such as developing seeds (Lemaıˆtre et al. 2008, Guiboileau et al. 2012). Remobilization mechanisms are activated at the beginning of the senescence to avoid losing these valuable macromolecules and their constituent elements. Mechanisms related to recycling N are well understood in senescent leaves (Liu et al. 2008). Studies in herbaceous plants (Diaz et al. 2008, Agüera et al. 2010, 2012, Avila-Ospina et al. 2015) have described a sequence of events associated with remobilization; including a decrease in chlorophyll, reduction in protein and N levels, and an increase in GS1 or AS activity. The occurrence of these events suggests that N can be translocated from senescent tissues through phloem sap to other plant organs (Lemaıˆtre et al. 2008, Guiboileau et al. 2012); however, the regulation of N remobilization and transfer in tree roots is poorly understood and it is possible that the mechanism may be different.

In the present study, we examined and compared factors regulating the translocation process in both leaves and absorptive roots of *P. trichocarpa*. Quantitative analyses showed that in both organs, concentration of N decreased during senescence. In leaves, this result is in line with literature, which showed such a relationship for *Arabidopsis thaliana* (Diaz et al. 2008), *Hordeum vulgare* (Avila-Ospina et al. 2015) and *Helianthus annuus* (Agüera et al. 2010, 2012). Remobilization of N in the absorptive roots of trees is more ambiguous. The quantitative analysis of N concentration in fine roots of different species that has been conducted previously provided contradictory information. Results of studies performed on the fine roots of *Quercus robur* are in agreement with the results obtained in the present study, suggesting that remobilization of N from fine roots may occur at the end of the growing season (Zadworny et al. 2015). This premise is supported by the observed decrease in N concentration in the first three orders of fine roots with a simultaneous significant increase of N in higher orders (from the fourth to sixth) of roots. Higher order roots exhibit a less ephemeral nature and have a longer lifespan than lower order roots (Xia et al. 2010, Jia et al. 2011, Zadworny et al. 2015). Nambiar (1987), however, suggested that N translocation is not a crucial process in trees, while Kunkle et al. (2009) reported an increase in N levels in dead roots of *Populus tremuloides*, *Betula alleghaniensis*, *Acer rubrum* and *Acer saccharum*, relative to the levels in living roots of these species. Results obtained by Kunkle et al. (2009) suggest that the relocation of N during senescence does not occur. However, their conclusion may arise from the colonization of dead roots by microorganisms containing high levels of chitin or due to the existence of significant variability in the classification of fine roots (e.g. as all roots with diameter lower than 2 mm), without dividing them based on their function as transport or absorptive roots (McCormack et al. 2015). Data may also have been collected without identifying senescence stages based on morphological or anatomical factors, as was described by Wojciechowska et al. (2018*b*). Considering the total biomass of fine, absorptive roots, the lack of active mechanisms related to N remobilization would result in the loss of a huge amount of N (Jackson et al. 1997).

To obtain additional evidence confirming remobilization in studied organ, additional molecular analysis has been conducted. A genetic analysis performed by Castro-Rodríguez et al. (2011) identified in *Populus* three groups of duplicated genes that encode GS1—*GS1.1*, *GS1.2* and *GS1.3*—and one gene encoding GS2. These genes were shown to exhibit organ-specific and seasonal-dependent patterns of expression (Castro-Rodríguez et al. 2011). In our study, *GS1.1* was up-regulated in both senescent organs—leaves and absorptive roots. Moreover, in leaves, expression of *GS1.3* was also increased. According to the literature, those duplicated genes may be involved in N metabolism, suggested function of *GS1.1* is glutamine biosynthesis, whereas the role of protein encoding by *GS1.3* is N remobilization (Castro-Rodríguez et al. 2011). The other isoform, *GS1.2* exhibited their highest expression in

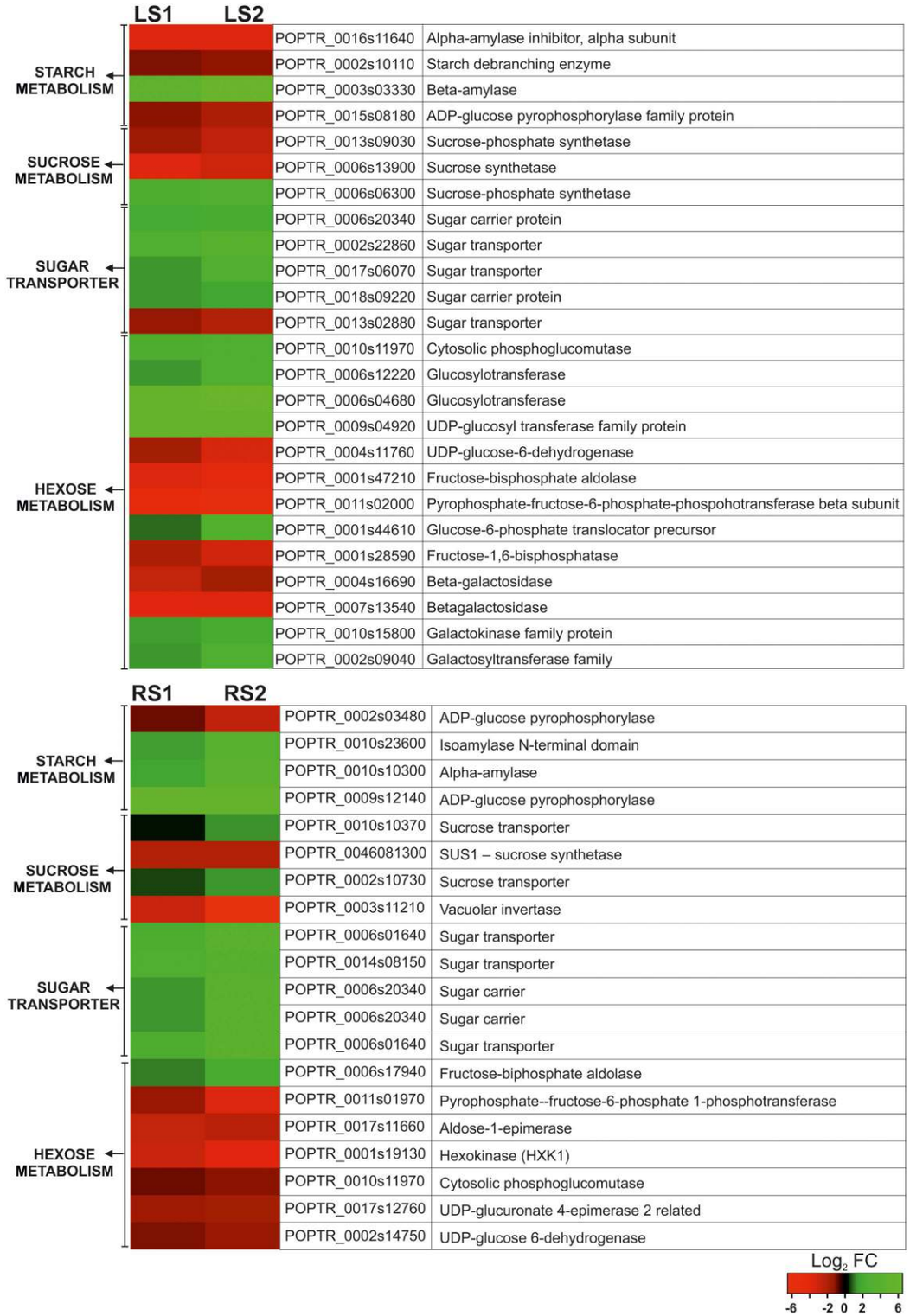

Figure 5. Heat map illustrating the fold changes (log2 basis) in the expression of the selected genes associated with carbohydrate metabolism during leaf and root senescence.

roots at the beginning of the growth season (RC) that could be associated with the primary assimilation of N from soil and/or lignin biosynthesis (Castro-Rodríguez et al. 2011). In addition to *GS1*, the chloroplastic isoform of GS (GS2) was principally expressed in green leaves (LC). This result is consistent with our previous results, where observed chloroplasts began to transform into gerontoplasts in the LS1 stage of senescence, and most of these organelles were in advanced stages of degradation at the LS2 stage of senescence (Wojciechowska et al. 2018*b*). Analyses of gene expression are compatible with protein analyses where increased GS1 was observed during senescence both organs. Such results during leaf senescence were observed in several species; however, for fine roots, this is the first such documentation, which supports hypothesis about resorption of N from the most distal senescent absorptive roots in *Populus*.

In addition to genes that encode GS1, the microarray analyses identified the differential expression of other genes related to N metabolism. Among these genes, N compound transporters constituted a large group, which was identified in both organs. In leaves, the majority of those genes were up-regulated, whereas in roots, only two genes encoding N transporters were characterized by increased expression. This included a gene that encodes a protein belonging to a large family of high-affinity NRT that may play multifunctional roles in nitrate uptake and transport throughout the plant (Bai et al. 2013). Studies performed on *Arabidopsis* indicated that NRT1.7 and NRT2.5 NRTs have contributed to the remobilization of nitrate from the source leaves to the sink organs (Fan et al. 2009, Lezhneva et al. 2014, Wu et al. 2014). Moreover, there is a premise that other NRTsiNRT1.6 and NRT1.5 — that are up-regulated during leaf senescence may play a role in senescence and they could also be involved in nitrate and ammonium remobilization (Have et al. 2017). Given the broad range of roles of NRT, which may act as either a nitrate sensor, a signal transducer or a transporter, it is hard to interpret which role it may play in senescent roots, and this issue requires further analysis. Moreover in leaves, up-regulation of the gene encoding GDH, which functions in the transfer of remobilized N, was noticed. A similar finding was reported in *Arabidopsis* and *Nicotiana* (Bernhard and Matile 1994, Masclaux et al. 2000, Guo et al. 2004, Li et al. 2017).

The mechanisms underlying N translocation in trees are not well understood. Guiboileau et al. (2012) reported that autophagy is an essential aspect of the relocation of N from senescing leaves to developing seeds. Studies with *Arabidopsis* mutants double mutants *atg* + *salicylic acid defective lines* have demonstrated that plants with impaired autophagy machinery accumulate N in leaves, especially under low nitrate availability. In the previous article (Wojciechowska et al. 2018*b*), we documented an increase in processes associated with autophagy in senescing roots and leaves of *P. trichocarpa*. Up-regulation of autophagy genes, as well as presence of micro- and macroautophagy, was observed in the same stage of fine roots senescence as increasing expression of GS1 genes and decreasing concentration of N. Based on this research, it seems likely that autophagy may also be involved in N remobilization in both leaves and roots of *P. trichocarpa*.

In contrast to N, C concentrations in senescing leaves and roots remained stable in our study. A slight decrease in C concentration was observed, however, during leaf senescence in *H. vulgare* (Avila-Ospina et al. 2015). A more important parameter related to the regulation of cellular metabolism during the senescence of plant organs is the C:N ratio (Chen et al. 2015). Neither C nor N alone, but rather the C:N ratio, plays a crucial role in several processes in *Arabidopsis* such as the regulation of seedling growth, remobilization of storage lipids, the expression of photosynthetic genes and natural senescence (Martin et al. 2002). A similar change in the C:N ratio during the vegetative season was observed in both leaves and roots, while the C:N ratio increased during senescence. An initiation of senescence has been reported to be induced by high C and low N availability in plant tissues (Wingler et al. 1998, Aoyama et al. 2014).

Significant differences between the examined organs were observed in carbohydrate metabolism during senescence. In the present study, starch concentration decreased rapidly in leaves during senescence. A previously reported ultrastructure analysis also revealed a decreasing number of starch granules in yellowing leaves of *P. trichocarpa* (Wojciechowska et al. 2018*b*). Similarly, senescing leaves of *H. annuus* (Agüera et al. 2012), *Oryza sativa* (Muthukumaran and Rao 2013) and *A. thaliana* (Diaz et al. 2008) were also characterized by a decrease in starch levels. Studies of starch degradation suggest that autophagy may play an important role in the breakdown of this carbohydrate. Wang et al. (2013) demonstrated that silencing of *ATG* genes reduces leaf starch degradation, resulting in an excessive accumulation of starch in *atg* mutants. These observations support the premise that the increase of autophagy activity observed in senescing leaves and roots of *P. trichocarpa* may play a multifunctional role in degradation, N remobilization and starch degradation (Wojciechowska et al. 2018*a*, 2018*b*, Guiboileau et al. 2012, Wand et al. 2013). Microarray analysis in senescent leaves revealed several genes associated with starch metabolism, which is in line with quantitative analyses of starch concentration, e.g. significant up-regulation of the gene encoding a β-amylase, which is associated with starch degradation (Lin et al. 1988, Kaplan and Guy 2004). In addition to β-amylase, the down-regulation of genes encoding starch debranching enzymes and ADP-glucose pyrophosphorylase was observed, indicating that starch biosynthesis is inhibited or completely stopped during senescence (Kubo et al. 1999, Ballicora et al. 2004). An inverse relationship is observed in absorptive roots, where the concentration of starch increased. This was surprising, however, it may be

elucidated by the fact that N relocation is dependent on an adequate supply of carbohydrates to provide energy for active senescence processes and for the conversion of N to glutamine. In contrast to leaves, expression of the ADP-glucose pyrophosphorylase gene was strongly up-regulated during absorptive root senescence. Similarly, expressions of genes encoding α-amylase and the isoamylase N-terminal domain, which regulate starch degradation, were also increased. In addition to initiating starch degradation, α-amylases are also involved in abiotic stress response, such as cold acclimation—a process that includes an increase in soluble sugars and hexose-phosphates in the cytosol (Ristic and Ashworth 1993, Hurry et al. 1995). A large body of research has demonstrated that sugars accumulate in cells and tissues to increase osmotic pressure and cold tolerance (Sasaki et al. 1996, Klemens et al. 2013, Tarkowski and Van den Ende 2015). An increase in sugar levels could help to prevent damage to roots by temporarily occurring freezing temperature conditions (even in fall), and thus extend their life span, thereby prolonging the senescence process until nutrients can be transferred to higher root orders. Microarray analyses also revealed several genes associated with sucrose metabolism in both organs. The primary mobile sugar in plants—sucrose—is transported from its source (synthesizing) organs to sink organs through the phloem, even at times over long distances (Lemoine 2000). Sucrose is known to inhibit the onset of senescence, which has been documented in *Brassica oleracea* branches (Irving and Joyce 1995), *Lilium* and *Dianthus caryophyllus* petals, and *Asparagus officinalis* spears (Hoeberichts et al. 2007, Arrom and Munné-Bosch 2012, Park 2016). During leaf senescence, several genes involved in the sucrose metabolism were identified. Sucrose synthetase is involved in the reversible conversion of sucrose and UDP into fructose and UDP-glucose. Similar to our results, changes in the expression levels of genes that encode a sucrose synthetase were also documented during the senescence of leaves of *Nicotiana tabacum* (Li et al. 2017). In senescent roots, the sucrose synthetase (*SUS1)* gene was down-regulated. A gene encoding a vacuolar invertase, which is responsible for converting sucrose into glucose and fructose inside a vacuole, was also down-regulated. However, the up-regulation of genes encoding sucrose transporters was observed, as well as the elevated expression of five genes encoding sugar carriers, collectively implying an increase in sugar relocation. In senescent leaves, the microarray analysis identified five genes encoding sugar transporters. Expression of four of them was up-regulated, which suggests that sugars are actively translocated out of senescing cells and tissues.

Conclusion

The senescence of plant organs is a precisely controlled process that allows plants to relocate valuable nutrients from senescent organs to other locations rather than lose them to the environment. The results of the present study provide evidence that in both leaves and roots, the process of N resorption is activated during senescence. This premise is supported by the analysis of N concentration and the molecular analysis of GS levels, a key enzyme in N remobilization. To our knowledge, this is the first confirmation that relocation of N during the senescence of absorptive fine roots is regulated at the molecular level. Significant changes in carbohydrate metabolism–gene expression were observed in both leaves and roots during senescence. Only in fine roots, however, was the accumulation of sugars observed. This may be related to the need to cold acclimate and increase the tolerance of roots to freezing temperatures, so that the remobilization of nutrients to higher order roots can be completed—something that could not occur if the roots died quickly due to freezing injury. The specific mechanisms responsible for the remobilization of nutrients during senescence and the functional role of carbohydrates during this process are not well understood and remain a critical priority for future research.

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Conflict of interest

None declared.

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