Xylem cells cooperate in the control of lignification and cell death during plant vascular development.

Sacha Escamez
“[…] longitudinal capillary sap-vessels, through which rooted plants draw nourishment to every part from the earth.”

Vegetable Staticks: Or, An Account of some Statical Experiments on the Sap in VEGETABLES, etc. London: W. and J. Innys and T. Woodward, 1727

**Stephen Hales** (1677-1761), “The Newton of plant physiology”

**Sacha Escamez** pursued his higher education at the University of Caen in France. There, Sacha received a master’s degree in plant physiology for his work on sulphur nutrition in canola plants at the French Institute for Agronomical Research (UMR-INRA-950 Plant Ecophysiology, Agronomy and N, C, S Nutrition, University of Caen, France). Sacha has then been recruited as a PhD student at the Umeå Plant Science Centre, in order to conduct research on the development of the vascular tissue which conducts water in plants. This doctoral thesis presents the findings of his research, discusses its implications and tells about possible applications.
Xylem cells cooperate in the control of lignification and cell death during plant vascular development.

Sacha Escamez
“The acid test of empirical scientific content in an argument is to see what happens when you try to unpack it by stating its opposite and ask for an empirical test between the two. If no such test exists, then we are dealing either with sociological, polemical viewpoints, which can differ according to the stance of the speaker, or we are dealing with metaphor, and of course, we could be dealing with both since metaphor is a favourite recourse of polemicists.”

Denis Noble

In: The Music of Life: Biology Beyond the Genome, Oxford University Press, Oxford, UK (p. 14)
Papers
Published papers and manuscripts presented in the printed version of this thesis

Paper I (published):


Paper II (published):


Paper III (manuscript):


Paper IV (manuscript):

Other contributions from the author of this PhD thesis
(This list of contributions only includes work related to the thesis)

**Review article:**


**Book chapter:**


**Patent** (filed in August 2014 but not published yet):

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Simplified summaries in different languages

Umeå Plant science centre är ett center för experimentell växtbiologi. Centret består av forskargrupper från både Umeå universitet och Sveriges lantbruksuniveritet (SLU) och har forskare från över 40 olika länder. Detta internationella klimat som då bildas bidrar till att skapa ett mångkulturellt samarbete vilket gynnar forskningen på flera sätt. För att spegla en av dessa positiva mångkulturella effekter på forskningen har jag bett mina kollegor från olika delar av världen att översätta denna förenklade sammanfattningen av min forskning på deras respektive moderntal.

Enkel sammanfattning (Svenska /Swedish)

The Umeå Plant Science Centre is a dynamic institute for research on plant biology. This institute regroups both Umeå University and the Swedish University of Agricultural Sciences (SLU) and employs around two-hundred persons from over forty different countries. This very international environment gives a great opportunity for sharing different cultures, which in the end fosters research. To reflect this cultural wealth and its positive effect on research, I have asked colleagues from different parts of the world to translate the simplified summary of my research into their mother tongue.

**Simplified summary (English)**

Plants are living organisms which use solar energy to turn carbon dioxide and water into sugar. Hence, the plants that live on land surfaces have evolved a vascular tissue called xylem to draw water in the soil and to transport it within their body. In trees, the xylem tissue forms what is commonly called the wood, which means that the xylem represents an important source of biomass for materials, pulp and paper and biofuel production. The biological, ecological and economical importance of xylem have driven research endeavours to understand how this tissue is formed. The work presented in this thesis has studied the formation of the xylem cells that conduct the water. These water conducting cells build a thick wall around them which gets reinforced by a rigid polymer called lignin such that they can sustain the pressure from the water flow. I participated in discovering that the deposition of the lignin polymer requires the help from neighbouring cells, which demonstrates the existence of cooperation between different xylem cells. In addition, the water-conducting xylem cells must organize their own death so as to leave their walls empty, which forms hollow “tubes” in which the water can flow better. When they do so, the water conducting xylem cells use dangerous molecular “tools” to self-destruct. I was involved in discovering that during their self-destruction, these water conducting cells also implement measures to protect the surrounding cells, which constitutes another example of cellular cooperation during xylem formation.
Le Centre pour la Science des Plantes d’Umeå (Umeå Plant Science Centre, UPSC) est un institut dynamique de recherche biologique sur les plantes. Cet institut est un regroupement auquel participent l’Université d’Umeå (Umeå University) et l’Université Suédoise pour les Sciences Agronomiques (SLU). L’UPSC emploie environ deux-cents personnes venant de plus de quarante pays. Cet environnement résolument international représente une formidable opportunité de partage culturel, ce qui favorise grandement la recherche scientifique. Pour refléter cette richesse culturelle et son effet positif sur la recherche, j’ai demandé à nombre de mes collègues des quatre coins du monde de traduire le résumé simplifié de mes travaux dans leurs langues maternelles.

Résumé simplifié (Français / French)

Les plantes sont des organismes qui utilisent l’énergie solaire pour fabriquer du sucre à partir de CO₂ et d’eau. De ce fait, les plantes qui peuplent les milieux terrestres ont évolué de manière à acquérir un tissu vasculaire appelé le xylème, qui transporte l’eau tirée du sol au sein du corps des plants. Chez les arbres, le xylème constitue le bois, ce qui signifie que le xylème représente une importante source de biomasse pour la production de matériaux, de patte à papier et de biocarburants. L’importance biologique, écologique et économique du xylème ont conduit à d’intenses efforts de recherche pour comprendre la formation de ce tissu. Le travail présenté dans cette thèse constitue une étude sur la formation des cellules qui conduisent l’eau au sein du xylème. Ces cellules conductrices d’eau construisent une paroi cellulaire épaisse renforcée par un polymère rigide appelé la lignine, permettant de supporter la pression exercée par le flux d’eau. J’ai participé à découvrir que la déposition de la lignine nécessite l’intervention des cellules avoisinantes, ce qui démontre l’existence d’une coopération entre les cellules du xylème. De plus, les cellules conductrices d’eau du xylème organisent leur propre mort de manière à ne laisser que leurs parois, qui une fois vides forment ainsi des « tubes » dans lesquels l’eau circule plus efficacement. Pour que cela se produise, les cellules conductrices d’eau utilisent des « outils » moléculaires dangereux afin de s’autodétruire. J’ai participé à découvrir que pendant leur autodestruction, les cellules conductrices de l’eau mettent également en place des mesures de protection pour épargner les cellules voisines. Ces mécanismes protecteurs constituent un autre exemple de coopération cellulaire lors de la formation du xylème.
Vereinfachte Zusammenfassung
(Deutsch /German)

Umeå Plant Science Center jest dynamicznie rozwijającym się instytutem skupiającym naukowców z dziedziny biologii roślin. Instytut składa się z dwóch jednostek: Umea University oraz Swedish University of Agricultural Sciences (SLU). Zatrudnionych jest tutaj ponad dwieście osób pochodzących z ponad czterdziestu krajów. Stanowi to bardzo międzynarodowe środowisko i daje ogromną możliwość wymiany kulturowej, która ostatecznie przyczynia się do rozwoju nauki. Dla odzwierciedlenia kulturowego bogactwa a era pozycywnego wpływu na naukę poprosiłem kolegów z różnych części świata o przetłumaczenie uproszczonego streszczenia na ich język ojczysty.

Uproszczone streszczenie (Polski/Polish)

مركز أوميا لعلوم النبات (UPSC) هو معهد فعال للبحث في بيولوجيا النبات. هذا المعهد يجمع بين كل من جامعة أوميا (Umeå) والجامعة السويدية للعلوم الزراعية (SLU) ويعمل فيه نحو مائتي شخص من أكثر من أربعين دولة مختلفة. تبعث هذه التشكيلة الدولية فرصه عظيمة جدا لتبادل الثقافات المختلفة وبالتالي هذه الثروة الثقافية لها تأثير إيجابي على تشجيع البحث العلمي.

وقد طلبت من الزملاء من مختلف أنحاء العالم لترجمة ملخص مبسط للبحث في اللغة الأم:

ملخص مبسط (اللغة العربية):

النباتات كائنات حية تستخدم الطاقة الشمسية لتحويل ثاني أكسيد الكربون والماء إلى سكر حتى تستطيع أن تعيش. وبالتالي فقد طورت النباتات التي تعيش على سطح الأرض أنسجتها الوعائية بحيث يقوم نسيج الخشب بسحب المياه من التربة ونقلها داخل الجسم النباتي.

في الأشجار تشكل نسبة الخشب ما يسمى عادة الغلاف، هو خليج مصنوع من مصادر الكتلة الحيوية للمواد الطبيعية (لب الورق والورق والغلاف الجلدي). فضلا عن الأهمية البيئية والبيولوجية للخشب، فهو يستخدم لمختلف التطبيقات.

تسعى العديد من البحوث لفهم كيفية تشكيل هذا النسيج. في هذه الدراسات تم دراسة كيفية تشكيل خلايا نسيج الخشب الذي يمثل الفناء الخاص لنقل المياه. هذه الخلايا الموصلة للمياه تتميز ببناء ديك مصبوغ من حولها بوساطة مادة دعائية تدعى بوليمر اللجنين. ونذكر يمكنها من تحمل الضغط الناتج من تدفق المياه.

شاركت في اكتشاف أن ترسب بوليمر اللجنين الذي يتطلب مساعدة من الخلايا المجاورة، مما يدل على وجود تعاون بين خلايا نسيج الخشب المختلفة. بالإضافة إلى ذلك يجب على خلايا الخشب الموصلة للمياه تنظيم عملية موقعة وذلك لترك جدرانها فارغة وبذلك تشكيل "أنايب" جوفاء نقل المياه وعليه تكون عملية تدفق المياه نحو أفضل.

أذ عندما يتم ذلك، فإن خلايا الخشب الموصلة للمياه تستخدم "أدوات "٪ أزيتينة% خطرة لفرض القبض بعملية التدمير الذاتي. وقد شاركت في اكتشاف أن خلال عملية التدمير الذاتي، تقوم هذه الخلايا الموصلة للمياه أيضا بتنفيذ تدابير لحماية الخلايا المحيطة بها، وهو ما يشكل مثالا آخر على التعاون الخلوي أثناء تشكيل الخشب.
Planten zijn levende organismen die met behulp van zonne-energie, koolstofdioxide en water omzetten in suiker. Om te overleven op het vaste land moesten de landplanten een vaatstelsel ontwikkelen (xyleem) om water uit de grond op te nemen en te vervoeren door de gehele plant. In bomen wordt het xyleem ook wel hout genoemd. Het hout kan gebruikt worden als bouwmateriaal, maar wordt voor een groot deel ook gebruikt voor de productie van pulp, papier en biobrandstoffen. Door de ecologische/biologische belangen en door het gebruik van het xyleem wordt er veel onderzoek gedaan naar de ontwikkeling van het xyleem. Deze thesis richt zich op het onderzoek naar de formatie van het xyleem dat water transporteert. Deze water transporterende cellen bouwen een dikke celwand om zich heen, wat wordt verstevigd door een stugge polymeer genaamd lignine. Door deze versteviging kunnen de water transporterende xyleem cellen grote druk weerstaan. Ik heb mede ontdekt dat er samenwerking nodig is tussen de omringende xyleem cellen om de depositie van lignine mogelijk te maken. Daarbij, moeten de water transporterende xyleem cellen hun eigen dood in werking stellen, wat de cel leeg maakt en water transport beter mogelijk maakt. Om deze cel dood in werking te stellen gebruiken de water transporterende xyleem cellen gevaarlijke moleculaire “gereedschappen” om zichzelf te doden. Ik was betrokken bij de ontdekking dat tijdens de cel dood, de water transporterende cellen, de omringende cellen beschermen. Dit laat nog eens zien dat er cellulaire samenwerking is tijdens de xyleem formatie.
瑞典于墨尔植物科学中心是一所充满活力的植物学科学研究所。该中心由墨尔大学及瑞典农业大学联合组建，拥有近两百位来自世界四十多个国家的植物科学家。来自世界各地的拥有不同文化背景的研究人员在这所国际化的研究中心内广泛交流，分享知识，并推动研究进展。基于这一文化财富及其产生的研究的推动力，我请来自不同地区的同事将我的博士论文摘要翻译成不同语言。

摘要（中文 /Chinese）

植物能有效的利用太阳能将二氧化碳和水转换为糖。因此，陆地植物进化出了维管组织，即木质部，将水份从土壤中运输到植物体的各个部分。作为重要的生物质来源，林木的木质部，即通常所称的木材，被用于材料，纸浆造纸和生物燃料。木材的生态学和生物学意义，以及其广泛的应用价值，使其研究工作主要致力于了解其组织的形成机制。本论文的研究对象为木材中运输水份的木质细胞。这些水份运输细胞具有环绕其自身的厚细胞壁。此类细胞壁由一类刚性聚合物，即木质素，参与组成，便于支撑由于水分流动产生的压力。我参与发现了邻近细胞对于木质素聚合的帮助作用，并证明了不同木质细胞的之间存在协同互作。此外，水份运输细胞通过自主的“死亡”，清除细胞内含物，形成便于水份流动的中空管状结构。这一“死亡”过程是细胞通过其“危险的分子工具”进行“自我摧毁”来完成的。我还发现“自我摧毁”过程中，这些水份运输细胞还能检测并保护其周围细胞，从而揭示了另一类在木材形成过程中细胞水平的协同作用。
Az Umeái Növénybiológiai Kutatóintézet (Umeå Plant Science Centre) egy nagyon dinamikus intézmény, mely egyesíti az Umeå Egyetemet (Umeå University) és a Svéd Agrártudományi Egyetemet (Swedish University of Agricultural Sciences), több mint kétszáz személyt foglalkoztatva, a világ több mint 40 országából. Ez a nemzetközi légkör kiváló lehetőséget biztosít különböző kultúrák megismerésére, ezáltal is gazdagítva a kutatást. Megkértem a munkatársaimat a világ különböző részeiről, hogy fordítsák le kutatásom rövid összefoglalóját a saját anyanyelvükre, ezzel is kifejezve a kulturális sokszínűség fontos szerepét a tudományban.

Rövid összefoglaló (Magyar/Hungarian)

A növények olyan élőlények, amelyek napenergia felhasználásával a szén-dioxidot és a vizet cukorrá alakítják. Az evolúció során a szárazföldön élő hajtásos növényekben kialakult a farész, más néven xilémmel, amelyen keresztül a víz eljut a talajból a növény különböző részeibe. A fák esetében ez a szövet alkotja a faanyagot, amely egy nagyon fontos bioszennyezett forrás, papír és bioüzemanyag előállításához. A farész biológiai és ökológiai jelentősége, valamint a széleskörű felhasználása miatt elengedhetetlen a közönséges növények kialakulásához megismerését célzó kutatások. Az itt bemutatásra kerülő doktori dolgozat a xilémet alkotó vízszállító sejtek vizsgálatát célozza meg.

Lignin szilárdító polimér épül a sejtfalokba, így a vízszállító sejtek (tracheidák) sejtfala még jobban megvastagszik, ezáltal képesek ellenállni a vízszállításból fakadó nyomásnak. Bebizonyítottuk, hogy a lignin lerakódása a sejtfalban a szomszédos sejtek hozzájárulásával történik, bizonyítván a különböző xilémsejtek közötti kooperációt. Továbbá, a vízszállító sejtek véghez kell végződjen egy „öngyilkossági programot”, melynek eredményeképpen a sejt tartalma felszámolódik, az üres csövekben (tracheák) a víz akadálymentesen tud áramlani a növényben. A sejtek veszélyes molekuláris eszközök arzenálját használják fel az önmegsemmisítéshez. Kutatómunkám során részt vettem annak a feltárásában, hogy ezek a sejtek miközben önpusztítást visznek véghez, intézkedéseket tesznek a szomszédos sejtek megóvásáért, mely egy újabb példa a farész kialakulása során alkamazott együttműködési stratégiáira.
要旨 (Japanese)

植物は太陽エネルギーを利用し二酸化炭素と水を糖に変換する生物体である。ゆえに、地表に住む植物は地中から水を汲み上げ、植物内部で輸送するために木部と呼ばれる維管束組織を進化させた。樹木において、木部組織はいわゆる木材を形成し、それは木質材料、パルプ・製紙、バイオ燃料となる重要なバイオマス原料となっている。生態学的、生物学的、さらにはその多様な利用価値の重要性から、この組織形成を解明する研究の努力がなされてきた。本博士論文には、水の通道に関わる木部細胞の形成の研究が記されている。これら水を通道する細胞は、水流による圧力に耐えるため、リグニンと呼ばれる硬いポリマーによる強化された厚い細胞壁を形成する。本稿では、リグニンポリマーの沈着は隣接した細胞からの助けが必要であり、そしてそれは異なる木部細胞間での連携が存在するということの発見を示す。加えて、通道木部細胞は通水するために細胞を空洞化、つまり自身の死を組織せねばならない。その死が起こる際には通道木部細胞は自身を破壊するための危険な分子道具を使用する。この自己破壊の間、これらの通道細胞はまた周囲の細胞を保護するための方策を実行し、つまりそれは木部形成における細胞間の連携が存在するという別の事象の発見を本稿でさらに示した。
Sumário simplificado (Português /Portuguese)

As plantas são organismos que utilizam a energia solar para converter o dióxido de carbono em açúcares. Desta forma, as plantas terrestres evoluíram no sentido de desenvolver um tecido vascular denominado xilema que tem as funções de absorver a água do solo, transportando-a seguida para todo o corpo da planta. Nas árvores, o tecido xilémico forma o que é normalmente denominado de “madeira”. Daqui resulta que o xilema constitui uma importante fonte de biomassa para produção de materiais, como papel e aglomerados, tal como para a produção de biocombustíveis. A relevância biológica e económica do xilema, bem como as suas várias aplicações, impulsionou os esforços de investigação para compreender como se forma este tecido. O trabalho apresentado nesta tese estuda a formação das células que, no xilema, são as responsáveis pela condução da água na planta. Estas células segregam uma espessa parede celular que é reforçada pela deposição de um polímero rígido, a lenhina, e cuja principal função é suportar a pressão hidrostática. O presente estudo demonstrou que a deposição de lenhina nas células condutoras necessita da ajuda das células adjacentes, evidenciando assim a existência de cooperação entre os diferentes tipos de células que constituem o xilema. Adicionalmente, as células condutoras necessitam de programar a sua própria morte para que se possam formar os “tubos” ocos através dos quais o fluxo de água é facilitado, sendo que estas células usam “ferramentas” moleculares perigosas para se autodestruirem. Estive igualmente envolvido no estudo que demonstrou que, durante a sua morte programada, estas células implementam também medidas de protecção das células vizinhas, o que constitui um outro exemplo da cooperação celular que existe durante a formação do xilema.
Umea Plant Science Centre es un dinámico instituto de investigación en biología vegetal. Este instituto cuenta con alrededor de 200 personas de más de 40 diferentes nacionalidades pertenecientes a la Universidad de Umea o a la Universidad Sueca de Agricultura (SLU). Este ambiente internacional propicia el intercambio cultural, lo que repercute en la investigación. Para reflejar esta riqueza cultural y su efecto positivo en la investigación, he pedido a varios compañeros de diferentes nacionalidades que traduzcan el resumen de mi tesis doctoral a su lengua materna.

Resumen simplificado (Español /Spanish)

Las plantas son organismos vivos que usan la energía solar para convertir dióxido de carbono y agua en carbohidratos. Por esta razón, las plantas que viven en el medio terrestre han desarrollado un tejido vascular llamado xilema para captar el agua del suelo y transportarla por el interior. En los árboles, el xilema forma lo que comúnmente se conoce como madera, hecho que lo dota de gran importancia al representar una fuente muy importante de biomasa para la producción de materiales, pulpa, papel y biocombustibles. La importancia biológica y ecológica del xilema, así como su uso en varias aplicaciones ha impulsado la investigación para entender cómo este tejido se forma. El trabajo presentado en esta tesis doctoral contribuye al estudio de la formación de las células del xilema que conducen el agua. Estas células en particular forman una pared gruesa alrededor de ellas mismas que es reforzada por un polímero rígido llamado lignina, lo que les permite soportar la presión asociada al flujo de agua desde la raíz a la hoja. He participado en el descubrimiento de que la deposición de lignina requiere de la ayuda de células vecinas, lo que demuestra la existencia de cooperación entre diferentes células del xilema. Además, las células conductoras de agua del xilema deben orquestar su propia muerte de forma que vacíen sus paredes para dar lugar a “tubos” en los que el agua puede fluir más fácilmente. Cuando llevan a cabo este proceso, las células conductoras de agua del xilema usan peligrosas “herramientas” moleculares para autodestruirse. Estuve también involucrado en el descubrimiento de que durante su autodestrucción, estas células conductoras de agua también realizan acciones para proteger a las células del alrededor, lo que constituye un ejemplo más de la cooperación que se da durante la formación del xilema.
Özet (Türkçe /Turkish)

گیاهان موجودات زنده ای هستند که با استفاده از انرژی خورشید، دی اکسید کربن و اب را تبدیل به قند می‌کنند. بنابراین گیاهانی که روی زمین زندگی می‌کنند یک بافت وحشی تشکیل داده اند به نام بافت چوبی که اب درون خاک را به درون خود منتقل می‌دهد. در درختان، این بافت چوبی چوب نامیده می‌شود که نقش عضدهای در تولید بیوماس، خمیر چوب، کاغذ و زیست‌سوزخت دارد.

احتمالاً اکتولوژیکی و زیستی اوند جویی به توجه به کاربرد ان در زمینه‌های گوناگون، نقش چشمگیری در زمینه‌ی تحقیقات علمی یبدا کرده‌تا بتوان دریافت که این بافت چوب‌شکل می‌گیرد.

این پایان نامه ی تحقیقاتی چگونگی شکل‌گیری سلول‌های بافت چوبی و هدایت کنتنه‌ی را بررسی می‌کند. سلول‌های هدایت گونه‌ای اب یک دیوار ضخیم به نام لیگنین به دور خود تشکیل می‌دهند که به واسطه یا ان نواحی پادربی‌ها در برای فشار آب را دارند. من به این موضوع پی برم که تغییر فرم لیگنین به کمک سلول‌های مجاور نیاز دارد که مبتنی بر وجود یک لی ارتباطی بین سلول‌های چوبی می‌باشد. همچنین بافت چوبی هدایت کنتنه‌ی اب به دلیل خاوی انگشت دیواره‌ی چوب و میسر تموین جریان اب باعث مرگ خود به وسیله یا بک ابزار خطرناک می‌شود.

سهمی از در این زمینه تحقیقاتی علاوه بر پی بردن به این روشن که هم‌دانستن این نکته که سلول‌های هدایت کنتنه‌ی باعث حفاظت از سلول‌های اطراف می‌شوند که خود نمونه‌ای دیگر از این پل ارتباطی سلول‌ها در چگونگی ایجاد بافت چوبی می‌باشد.
Abstrakt (Svenska)

Abstract (English)

The evolutionary success of land plants was fostered by the acquisition of the xylem vascular tissue which conducts water and minerals upwards from the roots. The xylem tissue of flowering plants is composed of three main types of cells: the sap-conducting tracheary elements (TE), the fibres which provide mechanical support and the parenchyma cells which provide metabolic support to the tissue. Both the TEs and the fibres deposit thick polysaccharidic secondary cell walls (SCWs), reinforced by a rigid phenolic polymer called lignin. The cell walls of TEs form efficient water conducting hollow tubes after the TEs have undergone programmed cell death (PCD) and complete protoplast degradation as a part of their differentiation. The work presented in this thesis studied the regulation of TE PCD by characterizing the function of the candidate PCD regulator METACASPASE9 (MC9) in Arabidopsis thaliana xylogenic cell suspensions. These cell suspensions can be externally induced to differentiate into a mix of TEs and parenchymatic non-TE cells, thus representing an ideal system to study the cellular processes of TE PCD. In this system, TEs with reduced expression of MC9 were shown to have increased levels of autophagy and to trigger the ectopic death of the non-TE cells. The viability of the non-TE cells could be restored by down-regulating autophagy specifically in the TEs with reduced MC9 expression. Therefore, this work showed that MC9 must tightly regulate the level of autophagy during TE PCD in order to prevent the TEs from becoming harmful to the non-TEs. Hence, this work demonstrated the existence of a cellular cooperation between the TEs and the surrounding parenchymatic cells during TE PCD. The potential cooperation between the TEs and the neighbouring parenchyma during the biosynthesis of lignin was also investigated. The cupin domain containing protein PIRIN2 was found to regulate TE lignification in a non-cell autonomous manner in Arabidopsis thaliana. More precisely, PIRIN2 was shown to function as an antagonist of positive transcriptional regulators of lignin biosynthetic genes in xylem parenchyma cells. Part of the transcriptional regulation by PIRIN2 involves chromatin modifications, which represent a new type of regulation of lignin biosynthesis. Because xylem constitutes the wood in tree species, this newly discovered regulation of non-cell autonomous lignification represents a potential target to modify lignin biosynthesis in order to overcome the recalcitrance of the woody biomass for the production of biofuels.
Preface

This PhD thesis is a book that tells a story, yet it is no fiction. It is no novel, but it reports novelty. It describes the discovery of new lands in the field of biology. It is a logbook written by this peculiar type of explorer called a “scientist”, who uses knowledge, ideas and experiments to navigate in an ocean of unknown in a quest for the coasts of discovery. Once found, the coasts of discovery must be precisely placed on the map of universal knowledge so that others can find this piece of information, verify its validity and ultimately use it as a resource. The map of universal knowledge is drawn with words and any addition to it must be a text, an article, a thesis, or more generally, a story. But not any story. Alfred Hitchcock once asked “what is drama but life with the dull parts cut out?” Science is no drama. In biology, a scientific story is life with the uncertainly true and certainly untrue parts cut out. Should it often seem dull, it at least ought to be true.

Finding the truth, however, is no easy task. The coasts of discovery may sit still but the search to find them can be troublesome. Science is no drama, but research can turn to be dramatic. Navigating the ocean of unknown requires great skills, hard work, patience, organization and cooperation. I must therefore prove with this thesis that I possess such abilities. This book thus tells another story: The story of my actions as a researcher, which can only be read between the lines. This unwritten chapter of personal adventures, sometimes on the edge of tragedy, often reminiscent of a comedy, always complex and confusing, has at least made me grow. This period of personal learning and development shall now find a place on the map of my life. If I were to imagine the text of my biography, I hope that I could name this period with only three letters: P, h and D.
Abbreviations (1/3)
(including abbreviations used in the manuscripts)

4CL#: 4-coumarate coenzyme A ligase protein number “#”
ACL5: ACAULIS5 protein
Arabidopsis: Arabidopsis thaliana
At: Arabidopsis thaliana
ATG#: autophagy related protein number “#”
ATP: adenosine triphosphate
bHLH#: basic-helix-loop-helix protein number “#”
C3H#: p-coumarate 3-hydroxylase number “#”
C4H: cinnamate 4-hydroxylase
CAD#: cinnamyl alcohol dehydrogenase number “#”
CCoAOMT#: caffeoyl-Coenzyme A O-methyltransferase number “#”
CCR#: cinnamoyl-CoA reductase number “#”
CESA#: cellulose synthase protein number “#”
ChIP: chromatin immunoprecipitation
ChIP-qPCR: chromatin immunoprecipitation followed by qPCR
CHS: chalcone Synthase
cLSM: confocal laser scanning microscopy
CoA: Coenzyme A
Co-IP: co-immunoprecipitation
COMT: caffeic acid O-methyltransferase
CSL: cellulose synthase-like protein
CSC: cellulose synthesizing complex
DIC: differential interference contrast
DNA: deoxyribonucleic acid
EXO70: exocyst subunit 70
F5H#: ferulate 5-hydroxylase number “#”
FDA: fluorescein diacetate
FT-IR: Fourier transform infrared
GFP: green fluorescent protein
GRI: GRIM REAPER peptide
GSK3: glycogen synthase kinase 3
GUS: β-glucuronidase
H2Bub1: histone H2B monoubiquitination mark
H3K4me3: lysine 4 trimethylation of histone H3 mark
H3K36me3: lysine 36 trimethylation of histone H3 mark
HA: human influenza hemagglutinin derived-tag (attached to a protein of interest)
HCT: p-hydroxycinnamoyl-Coenzyme A shikimate/quinate p-hydroxycinnamoyltransferase
**Abbreviations (2/3)**
(including abbreviations used in the manuscripts)

**His**: histidine  
**HUB#**: HISTONE MONOUBIQUITINATION protein number “#”  
**IP**: immunoprecipitation  
**IRX#**: IRREGULAR XYLEM (when knocked-out) protein number “#”  
**Leu**: leucine  
**MC #**: metacaspase protein number “#”  
**MS**: Murashige and Skoog medium  
**MYB**: protein containing a DNA binding domain similar to that of the human MYB proto-oncogene protein (c-myb), called after the avian myelocytomatosis virus protein b (MYB)  
**MYC**: human c-myc protein derived tag (attached to a protein of interest), called after the avian myelocytomatosis virus protein c (MYC)  
**NST#**: NAC SECONDARY WALL THICKENING PROMOTING FACTOR protein number “#”  
**OE**: overexpressor  
**OPLS-DA**: orthogonal projections of latent structures-discriminant analysis (also known as: orthogonal partial least squares-discriminant analysis)  
**PA**: piperonylic acid  
**PAL#**: phenylalanine ammonia-lyase protein number “#”  
**PCD**: programmed cell death  
**PCR**: polymerase chain reaction  
**PI**: propidium iodide  
**PLCP**: papain-like cysteine protease  
**PP2AA3**: PROTEIN PHOSPHATASE 2A SUBUNIT A3  
**PRN#**: PIRIN protein number “#”  
**Py-GC/MS**: pyrolysis-gas chromatography/mass spectrometry  
**qPCR**: real-time quantitative PCR  
**RNA**: ribonucleic acid  
**ROS**: reactive oxygen species  
**SCW**: secondary cell wall  
**SND#**: SECONDARY WALL-ASSOCIATED NAC DOMAIN protein number “#”  
**SSH**: suppression subtractive hybridization  
**STS**: silver thiosulfate  
**T-DNA**: transfer-DNA
Abbreviations (3/3)
(including abbreviations used in the manuscripts)

**TDIF**: tracheary element differentiation inhibitory factor
**TE**: tracheary element
**TED4**: TE differentiation-associated protein 4
**Tryp**: tryptophan
**UBC#**: UBIQUITIN CARRIER protein number “#”
**UBQ10**: (poly)ubiquitin 10
**VE**: vessel element
**VND#**: vascular-related NAC domain protein number “#”
**VNI2**: VND-INTERACTING protein 2
**WT**: wild-type
**XCP#**: XYLEM CYSTEINE PROTEASE number “#”
**XF**: xylem fibre
**XND1**: xylem NAC domain 1
**ZEN1**: Zinnia endonuclease 1
**Zinnia**: *Zinnia elegans*
I. Introduction

I.1. General context, scope and relevance of the research

In one of his correspondences, Darwin (1879) lamented that “[t]he rapid development [...] of all the higher plants within recent geological times was an abominable mystery”, which seemed to challenge his view that evolution is a slow and gradual process (Darwin, 1859; Friedman, 2009). Later studies showed that the diversification of the flowering plants and of other types of vascular plants had all proceeded at a similarly high pace (Crepet and Niklas, 2009; Silvestro et al., 2015). Indeed, the different groups of vascular plants that appeared throughout history have all been evolutionarily successful thanks to one of their most defining features: their xylem vascular tissue (Ligrone et al., 2000), which allows for efficient conduction of water and minerals from the roots to the leaves (Raven, 1977; Brodribb, 2009), and which has therefore enabled plants to colonize most (dry) land habitats (Raven, 1977; Bateman et al., 1998). Nowadays, plants cover a majority of the global land surface (Latham et al., 2014) and over ninety percent of the extant plant species are vascular plants (Crepet and Niklas, 2009). Vascular plants thus have a tremendous ecological importance, especially when considering that their vascular xylem represents an important link between soil and atmosphere within the global water cycle (Van den Honert, 1948).

How xylem transports water upwards in a way that seemingly defies gravity has long been the subject of numerous reflections and investigations (for a historical perspective see Brown, 2013). Most scientists have come to recognize the Cohesion-Tension theory (attributed to Böhm, 1893; Dixon and Joly, 1894, 1895; and Askenasy, 1896) as the explanation for the rise of the xylem sap (Angeles et al., 2004; Brown, 2013). This theory of xylem sap conduction has inspired engineers to contemplate the possibilities of creating synthetic xylem-like designs to transport water under pressure at a null energetic cost for various applications (Stroock et al., 2014). However, such applications are not yet possible as further research is needed to reproduce xylem design via understanding better the biology and the development of the xylem (Stroock et al., 2014). Studying xylem biology and
development has also become increasingly important because xylem forms wood (xylem comes from the Greek “xylon” which signifies “wood”) in trees, and therefore represents an important source of biomass. Woody biomass is already used as construction material and in the pulp and paper industry, and is predicted to represent an important source of biofuels, biochemicals and even food in the future (Pauly and Keegstra, 2010; Ragauskas et al., 2014; for an optimistic review see Percival Zhang, 2013).

This PhD thesis presents new discoveries and potential applications related to xylem biology and development in higher plants. More precisely, the work presented here adds to the basic knowledge of how xylem vessel cells develop, and identifies genes involved in xylem biomass composition as potential targets to improve biomass properties for production of biofuels. This thesis does not pretend to solve Darwin’s “abominable mystery”, nor does it explain the world’s ecology or does it solve the food and energy crisis. This work is merely a demure contribution to solving the theoretical and practical problems evoked above. If understanding the vascular xylem of plants paves the way towards great scientific and technical progresses, this thesis is a single nonetheless necessary pavement. Or to use of more biological metaphor, the work presented here is nothing more, and nothing less, than a single cell within a biological tissue.

I.2. Xylem differentiation in higher plants

I.2.1. Xylem cell types

I.2.1.a. Vascular stem cells

**Procambium and cambium**

During normal development, all xylem cells differentiate from a vascular meristem called procambium over primary growth, and cambium over secondary growth (Torrey et al., 1971; Larson, 1994; Lucas et al., 2013). The (pro)cambial cells maintain a meristematic identity via intercellular signalling, thus defining the (pro)cambium as a stem cell niche (Hirakawa et al., 2010; Hirakawa et al., 2011), while another intercellular signalling is responsible for specification of xylem cell differentiation (For review see Hirakawa et al., 2011; Lucas et al., 2013; Milhinhos and Miguel, 2013). The specified xylem cells are of three types:
xylem parenchyma cells, xylem fibres and sap conducting vessels (Figure 1) and tracheids (Torrey et al., 1971; Fukuda, 1996; Raven et al., 2005).

Figure 1: The xylem vascular tissue in the model plant species Arabidopsis thaliana and in an experimental system whereby cells in suspension can differentiate into TEs and parenchyma cells in vitro. All micrographs were acquired by the author of this thesis while the illustration of the Arabidopsis thaliana plant was modified after www.abcam.com
I.2.1.b. Xylem sap-conducting cells: tracheary elements

**Vessels and tracheids**
The vessels (in Angiosperms) and tracheids (in Gymnosperms) transport the xylem sap under negative pressure, which they can sustain because they deposit patterned secondary cell walls (SCWs), reinforced by a rigid lignin polymer (Smart and Amrhein, 1985; Eriksson et al., 1988; Yoshinaga et al., 1992). These SCWs represent an important fraction of the woody biomass of conifer trees, which do not possess fibres but only tracheids (Bailey and Tupper, 1918; Raven, 1977). To become functional, tracheids and vessels undergo programmed cell death (PCD), protoplast autolysis and partial degradation of their top and bottom cell walls, called perforation plates, thus forming longitudinally connected hollow tubes (von Mohl, 1851; O'Brien and Thimann, 1967; O'Brien, 1970; Torrey et al., 1971; Fukuda, 1996; Kuriyama and Fukuda, 2002; Motose et al., 2004; Turner et al., 2007). The tubular arrangement and the ribbed appearance of vessels and tracheids was found reminiscent of the insect’s respiratory trachea, leading Malpighi (1675) to collectively name tracheary elements (TEs) both the vessels and the tracheids. TEs represent one of the best characterized cell types in plants (Halperin, 1969; Turner et al., 2007), and their observation yielded most of the current knowledge on xylem cell specification and differentiation.

I.2.1.c. Cells specialised in mechanical support

**Fibres**
Differentiation of xylem fibres occurs in Angiosperms and also involves PCD and autolysis (Stewart, 1966; Courtois-Moreau et al., 2009; Déjardin et al., 2010; Bollhöner et al., 2012), although some species such as the model herbaceous plant *Arabidopsis thaliana* possess functional living fibres (Bollhöner et al., 2012). The function of xylem fibres is to provide mechanical support to the tissue, and subsequently to the entire plant body, thanks to their lignified SCWs (von Mohl, 1851; Bailey and Tupper, 1918; Raven, 1977; Bateman et al., 1998; Zhong et al., 2006; Zhong et al., 2011). It is worth noting that the fibres constitute the main source of polysaccharidic biomass in angiosperm trees, in great part due to their SCWs.
I.2.1.d. Cells specialised in metabolic support

Parenchyma cells
In contrast to TEs and fibres, the xylem parenchyma cells do not always deposit a SCW and they can remain alive during up to several years in trees (Stewart, 1966; O’Brien and Thimann, 1967; Srivasta and Singh, 1972; Nakaba et al., 2006; Donaldson et al., 2015). Xylem parenchyma cells are thought to provide metabolic support to the tissue (Larson, 1994; De Boer and Wegner, 1997 and references therein). More recently, a body of evidence has accumulated to suggest the involvement of xylem parenchyma in modulating the flow of xylem sap in the TEs (for review see Ménard and Pesquet, 2015). The cell wall edification of TEs, and possibly of fibres, is also influenced by xylem parenchyma cells (Ryser and Keller, 1992; Fukuda, 1996; Ros Barceló, 2005; Pesquet et al., 2013; Smith et al., 2013; Paper III). Overall, xylem parenchyma cells have been mostly studied in relation to their functions and interactions with other xylem cell types and little is known about the molecular and cellular aspects of their differentiation. Interestingly, xylem parenchyma cells can trans-differentiate into TEs upon adverse conditions such as pathogen infection (Reusche et al., 2012), or wounding (Vöchting, 1892; Simon, 1908) which thus represents an experimental system to induce and study TE differentiation (Sinnott and Bloch, 1945).

I.2.2. Specification of xylem cell fate

I.2.2.a. Molecular factors involved in xylem specification

Mobile molecular factors specifying xylem cell fate
The specification of xylem cell differentiation involves a variety of (mobile) molecular factors including micro RNAs (Emery et al., 2003; Carlsbecker et al., 2010), class III homeodomain leucine zipper transcription factors (Zhong and Ye, 1999; Emery et al., 2003; Ohashi-Ito et al., 2005; Carlsbecker et al., 2010), arabinogalactan proteins (Motose et al., 2004; Kobayashi et al., 2011), signal peptides (Matsubayashi et al., 1999; Ito et al., 2006; Kondo et al., 2006; Kondo et al., 2011; Kondo et al., 2014) and the plant hormones ethylene (Pesquet and Tuominen, 2011), gibberellins (Eriksson et al., 2000; Biemelt et al., 2004; Tokunaga et al., 2006), brassinosteroids (Iwasaki and Shibaoka, 1991; Yamamoto et al., 1997; Cañó-Delgado et al., 2004), cytokinins (Fosket and Torrey, 1969) and auxin (Jacobs, 1952, 1954). While most of these factors...
have been reviewed elsewhere (Torrey et al., 1971; Fukuda, 1996; Turner et al., 2007; Lucas et al., 2013; Milhinhos and Miguel, 2013; Schuetz et al., 2013), the roles of auxin, cytokinins and brassinosteroids are further described below because these hormones have been instrumental in establishing experimental systems where they are used to trigger the differentiation of TEs (Halperin, 1969; Fosket, 1970; Basile et al., 1973; Kohlenbach and Schmidt, 1975; Fukuda and Komamine, 1980; Kubo et al., 2005; Oda et al., 2005; Kwon et al., 2010; Pesquet et al., 2010; Kondo et al., 2015).

I.2.2.b Hormonal induction of TE differentiation in experimental systems

Experimental systems and the discovery of a xylogenic role for auxin and cytokinins

TE formation induced by wounding of Coleus stems constituted the experimental system used to demonstrate for the first time that auxin is a necessary factor for TE differentiation (Jacobs, 1952). Indeed, wounding-induced TE differentiation was inhibited when the auxin-producing leaf above the wound was removed, while applying auxin through the petiole stump restored TE differentiation (Jacobs, 1952). TE differentiation was also promoted by addition of cytokinins to cultured cytokinin-deficient soybean calli, thus demonstrating a xylogenic role for cytokinins (Fosket and Torrey, 1969).

Experimental systems using auxin and cytokinins

A combination of cytokinin and auxin was later found to induce TE differentiation in various experimental conditions such as Coleus stem explants (Fosket, 1970), lettuce leaf disks (Basile et al., 1973) and Zinnia elegans (hereafter Zinnia) cell suspensions (Kohlenbach and Schmidt, 1975). Fukuda and Komamine (1980) upgraded the Zinnia cell suspensions into an optimized hormone-inducible in vitro TE differentiation system. This Zinnia system enabled for better characterizing the cell biology of TEs by getting rid of the influence (and experimental hinders) from the surrounding tissues (Turner et al., 2007).

The discovery of a xylogenic role for brassinosteroids

In particular, the Zinnia system allowed uncovering a potential role for brassinosteroids in TE differentiation as no TEs formed in Zinnia cells treated with uniconazole (Iwasaki and Shibaoka, 1991;
Yamamoto et al., 1997) – an inhibitor of brassinosteroid and gibberellin biosynthesis (Iwasaki and Shibaoka, 1991; Yokota et al., 1991) – unless the cells simultaneously received exogenous brassinosteroids (Iwasaki and Shibaoka, 1991; Yamamoto et al., 1997).

**Experimental systems using brassinosteroids**

Exogenous brassinosteroids alone or in combination with auxin and cytokinin have since been used to induce TE differentiation in experimental systems consisting of *Arabidopsis thaliana* (hereafter Arabidopsis) tissue cultures (Kwon et al., 2010) or cell suspensions (Kubo et al., 2005; Oda et al., 2005; Pesquet et al., 2010). Furthermore, parenchyma cells of Arabidopsis leaf disks can also efficiently and synchronously differentiate into TEs when treated with a mix of auxin, cytokinin and bikinin (Kondo et al., 2015), which is thought to mimic the brassinosteroid signalling cascade (De Rybel et al., 2009). Noteworthy, bikinin functions by inhibiting the glycogen synthase kinase 3 (GSK3), a family of enzymes which blocks the brassinosteroid signalling cascade in absence of brassinosteroids (De Rybel et al., 2009), and/or in presence of the tracheary element differentiation inhibitory factor (TDIF; Ito et al., 2006) signal peptide (Kondo et al., 2014).

**The xylogenic mode of action of auxin, cytokinins and brassinosteroids**

The apparent overlap between the brassinosteroid and TDIF signalling cascades raises the question of whether brassinosteroids are essential for xylem cell specification in normal physiological conditions, or if brassinosteroids are merely inessential, however able to promote xylem differentiation. Auxin and cytokinin are essential for xylogenesis and a mechanism for their action has recently been proposed: a comprehensive approach combining experimental biology and mathematical modelling focusing on the early development of the vasculature of Arabidopsis suggested that auxin and cytokinin interact to define a cytokinin-responsive domain of cell divisions and an auxin-accumulating domain of TE differentiation (De Rybel et al., 2014).
I.2.3. The four modules of the xylem differentiation programmes

I.2.3.a. The differentiation programmes of TEs and fibres consist of four modules

The four major processes of TE and fibre differentiation represent modules that are molecularly and evolutionarily partially independent

After TEs and fibres have elongated and enlarged, their core differentiation programmes seem similar because they consist of the same four modules: SCW deposition, lignification, PCD and autolysis (for recent reviews see Schuetz et al., 2012; Lucas et al., 2013; Escamez and Tuominen, 2014). The need to distinguish between these four modules is dual: firstly, they are anatomically distinct and their respective ontogenies do not fully overlap in time (for review see Escamez and Tuominen, 2014). Secondly, despite some degree of regulatory and molecular interconnection, each of these processes has been successfully decoupled from the others in some Arabidopsis mutants (Turner and Hall, 2000; Avcı et al., 2008; Muñiz et al., 2008; Bollhöner et al., 2013; Smith et al., 2013) or by pharmacological treatment in Zinnia TE cell suspensions (Woffenden et al., 1998; Pesquet et al., 2013). The distinction between the four modules is further justified if considered in an evolutionary perspective (Figure 2), with the appearance of PCD and autolysis predating that of SCW deposition and lignification in the water conducting cells of land plants (Friedman and Cook, 2000; Ligrone et al., 2000, 2012; Escamez and Tuominen, 2014).

Similarities and differences between the differentiation of TEs and fibres

Interestingly, the fibres of some species such as Arabidopsis do not undergo PCD or autolysis before monocarpic senescence (Bollhöner et al., 2012), and observations in poplar trees suggest different molecular mechanisms for PCD and autolysis between TEs and fibres (Courtois-Moreau et al., 2009). Furthermore, fibres have SCWs covering nearly all of the cell surface, while TEs display SCW thickenings with annular or spiral patterns for the first formed primary xylem vessels (protoxylem), reticulate, scalariform or pitted patterns for the metaxylem, and in the secondary xylem,
the vessels harbour pitted SCWs (Bailey and Tupper, 1918; Wooding and Northcote, 1964; Turner et al., 2007; Muñiz et al., 2008; Déjeardin et al., 2010; Pesquet et al., 2010; Oda and Fukuda, 2012a).

Figure 2: Evolution of the water conducting cells in land plants and their differentiation programme in angiosperms.
(A) Simplified phylogenetic tree of land plants (after Kenrick and Crane, 1997) with a description of the main features of their water conducting cells, including the TEs of vascular plants (after data from Friedman and Cook, 2000). The chronological data (Ma: age in million years) was included for the apperition of land plants and for the apperition of vascular plants (after Wellman, 2010; Edwards and Kenrick, 2015; Silvestro et al., 2015).
(B) Scheme representing a simplified description of the differentiation programme of TEs in Angiosperms (after Escarenez and Tuominen, 2014).
The cell walls of TEs and fibres often differ in their lignin contents, as well as in the abundance of different monomers within the lignin heteropolymer (Eriksson et al., 1988; Saka and Goring, 1988; Donaldson, 2001; Gorzsás et al., 2011; paper III). Hence, the four modules of TE and fibre differentiation programmes are grossly similar in their purposes and in their outcomes, but the underlying molecular mechanisms must differ at least in part between the two cell types. Most of the current knowledge on the cell biology and the molecular regulation of xylem differentiation accumulated over the study of TEs, and only to a limited extent over the study of fibres. Therefore, most of the descriptions of the molecular and cellular events of xylem differentiation presented below (sometimes implicitly) refer to TEs, while specific descriptions of fibre differentiation are explicitly exposed when the corresponding knowledge exists.

I.2.3.b. The molecular master switches controlling the xylem differentiation programmes

**TE differentiation is triggered by NAC transcription factors**

The differentiation of TEs can be activated by transcriptional master switches from the NAC (NAM/ATAF/CUC; Aida et al., 1997) transcription factor family, which function downstream of the hormonal signals that drive xylem specification (Kubo et al., 2005). In Arabidopsis TE differentiation is controlled by VASCULAR-RELATED NAC DOMAIN 6 (VND6) and VND7, which were first identified based on their expression profile in TE-differentiating Arabidopsis cell suspensions (Kubo et al., 2005). Arabidopsis plants, tobacco and Arabidopsis cell suspensions as well as poplar leaves overexpressing VND7 or VND6 displayed ectopic differentiation of TEs, including SCW deposition, lignification and PCD (Kubo et al., 2005; Oda et al., 2010; Ohashi-Ito et al., 2010; Yamaguchi et al., 2010b; Schuetz et al., 2014). The trans-differentiating TEs induced by overexpression of Arabidopsis VND7 or VND6 in Arabidopsis plants and poplar leaves harboured SCW patterns reminiscent of protoxylem or metaxylem, respectively (Kubo et al., 2005). Conversely, differentiation of metaxylem or protoxylem was inhibited in Arabidopsis seedlings expressing a dominant repressor version of VND6 or VND7, respectively (Kubo et al., 2005). These observations lead to the suggestion that VND7 specifically triggers the differentiation of protoxylem while VND6 triggers the differentiation of metaxylem (Kubo et al., 2005), although VND7 was later shown to be able to
induce the differentiation of all types of TEs in Arabidopsis (Yamaguchi et al., 2008). In addition, infection of Arabidopsis xylem vessels by the pathogen *Verticillium longisporum* induces the surrounding parenchyma cells to trans-differentiate into TEs in a VND7-dependent manner (Reusche et al., 2012). VND7 may interact with the Arabidopsis VND2 to VND5 (Yamaguchi et al., 2008), which also have the ability to trigger TE differentiation when overexpressed, as do their Arabidopsis homologue VND1 and VND homologues in other species such as poplar or banana (Ohtani et al., 2011; Zhou et al., 2014; Negi et al., 2015). However, the importance of VND1-5 compared to VND6 and VND7 during normal xylem development remains unclear.

**VND6 and VND7 appear as the transcriptional master switches of TE differentiation**

Xylem development can be hindered by overexpressing the Arabidopsis XYLEM NAC DOMAIN1 (XND1) or its poplar or cotton homologues (Zhao et al., 2008; Grant et al., 2010; Li et al., 2014), suggesting that XND1 is a negative regulator of TE formation. On the other hand, only the size of the TEs, but not the overall xylem formation, was reduced in the *xnd1* loss of function mutants, suggesting that physiological levels of XND1 normally retard the onset of SCW deposition during TE cell expansion, possibly by counteracting VND6 and VND7 (Zhao et al., 2008). Similarly, the NAC VND-INTERACTING2 (VNI2) acts as a transcriptional repressor of xylem differentiation, as illustrated by the similar vascular defects in Arabidopsis plants expressing a dominant repressor version of VND7 or VNI2, or overexpressing VNI2 (Yamaguchi et al., 2010a). In summary, several NACs can influence xylem differentiation but only VND6 and VND7 seem to represent the molecular master switches that are sufficient to integrate a diversity of upstream signals into a trigger for the TE differentiation programme.

**NAC transcription factors control the fibre SCW biosynthesis and possibly the entire fibre differentiation**

Regarding the fibres, it remains unclear whether their differentiation is also controlled by molecular master switches. The transcription factors NAC SECONDARY WALL THICKENING PROMOTING FACTOR (NST3)/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN (SND1) and NST1, which are phylogenetically related to VNDs (Zhong et al., 2006; Mitsuda et al., 2007; Zhong et al., 2010a), are expressed in the xylem fibres
and in the interfascicular fibres of Arabidopsis, and can therefore be regarded as candidate regulators of fibre differentiation. Ectopic overexpression of NST1 and NST3/SND1 activates the transcriptional machinery, and subsequent formation, of TE-like SCW thickenings, but apparently not PCD, in non-sclerenchymatous cell types (Mitsuda et al., 2005; Zhong et al., 2006). Furthermore, double mutants for NST1 and NST3/SND1 or expression of a dominant repressor version of NST3/SND1 triggered the loss of functional fibres in Arabidopsis stems (Zhong et al., 2006; Mitsuda et al., 2007; Zhong et al., 2010a, b). This loss of functional fibres could correspond to a loss of the fibre cell type, or alternatively to a failure of existing fibre cells to fulfil their function due to insufficient SCW biosynthesis. Hence, NST1 and SND1/NST3 can clearly be identified as the master regulators of fibre SCW biosynthesis (Mitsuda et al., 2005; Zhong et al., 2006; Mitsuda et al., 2007; Zhong et al., 2008; Zhong et al., 2010a, b), but the current data does not clarify whether NST1 and SND1/NST3 are more generally master switches of the entire fibre differentiation programme in Arabidopsis. This ambiguity is due to the fact that the differentiation of Arabidopsis fibres involves SCW biosynthesis but not PCD. Hence, the observation of PCD cannot be used to distinguish between the biosynthesis of the fibre SCWs and the entire fibre differentiation programme in Arabidopsis. On the other hand it is possible that the fibre differentiation and the SCW biosynthesis programmes are one and the same in Arabidopsis, rendering NST1 and SND1/NST3 potential master switches of fibre differentiation.

I.2.3.c. Polysaccharidic Secondary Cell Wall deposition

Cellulose and hemicelluloses

The secondary walls of the xylem cells are mainly composed of a cellulose framework and a matrix of hemicelluloses, two different types of polysaccharidic polymers which are deposited before the cell wall reinforcement by lignin (Harlow and Wise, 1928; Timell, 1967; Northcote, 1972; Taylor et al., 2003; Somerville, 2006; Scheller and Ulvskov, 2010). More precisely, hemicelluloses consist of ramified polysaccharidic β-(1→4)-linked backbones most often composed of combinations of pentoses such as xylose and arabinose, and hexoses such as mannose, glucuronic acid and glucose (for review see Timell, 1967; Scheller and Ulvskov, 2010). Cellulose, in contrast, is solely composed of β-(1→4)-linked glucose chains which further organize through hydrogen bonds and
Van der Waals interactions in order to form crystalline microfibrils (for review see Brett, 2000; Somerville, 2006). In contrast with primary cell walls, the cellulose microfibrils of the secondary walls are organized into parallel strata, whose orientation allows distinguishing three SCW layers (S1, S2 and S3), which may also differ in their hemicellulose contents and compositions (Wardrop and Bland, 1959; Timell, 1967; Turner et al., 2007; Kaneda et al., 2010).

Biosynthesis and deposition of SCW polysaccharides
The locations and processes associated with the biosynthesis of cellulose and hemicelluloses differ. How the various hemicelluloses are produced is not fully understood but the current knowledge indicates that they are synthesized by membrane-bound cellulose synthase-like (CSL) glycosyl transferases in the Golgi apparatus, and subsequently exported to the cell walls (reviewed by Scheller and Ulvskov, 2010; Rennie and Scheller, 2014). Both the primary wall cellulose and the cellulose of the secondary cell walls are synthesized and deposited at the plasma membrane by rosette-shaped cellulose synthesizing complexes (CSCs) containing cellulose synthase (CESA) enzymes (Mueller and Brown, 1980; Herth, 1983; Kimura et al., 1999; Taylor et al., 2003; Somerville, 2006; Turner et al., 2007; Watanabe et al., 2015). The study of TEs in cell suspensions enabled observing that TE SCW patterning requires the CSCs to be restricted to specific domains of the plasma membrane by the cortical microtubules associated with specific interactors (Oda et al., 2010; Pesquet et al., 2010; Oda and Fukuda, 2012b; Watanabe et al., 2015). The CSCs depositing SCW cellulose in spermatophytes comprise a specific subset of three CESAs (Taylor et al., 2003; Harholt et al., 2012) which were first identified by a mutagenesis approach in Arabidopsis, and named based on their collapsed xylem phenotype: IRREGULAR XYLEM1 (IRX1)/CESA8, IRX3/CESA7 and IRX5/CESA4 (Turner and Somerville, 1997; Taylor et al., 1999; Taylor et al., 2000; Taylor et al., 2003; Somerville, 2006; Turner et al., 2007; Harholt et al., 2012).

The polysaccharides of xylem SCWs as a source of biofuels
Cellulose and to a lesser extent hemicelluloses represent a source of fermentable sugars for the production of biofuels (Pauly and Keegstra, 2010; Rennie and Scheller, 2014). Therefore, the thick cell walls of xylem TEs and fibres can be seen as a major source of biofuels, but their deconstruction and conversion into biofuels is
hindered by the SCW cross-linked hemicelluloses which have a high content of low fermentable pentoses (Petersen et al., 2012; Rennie and Scheller, 2014). Interestingly, the amount of the hemicellulose xylan could be specifically decreased in the fibres of Arabidopsis while maintained in the TEs by expressing a xylan biosynthetic gene downstream of the promoter of VND6 or VND7 in knock-out plants for this xylan biosynthetic gene (Petersen et al., 2012). Some of these Arabidopsis plants with normal TE cell walls and xylan-deficient fibre cell walls had no defects in growth or stem strength, while their release of fermentable sugar was significantly increased (Petersen et al., 2012). This increased saccharification was attributed to a decrease in hemicellulose-mediated tethering of cellulose microfibrils, to an increase in the amount of easy fermentable hexoses in the walls and to a decrease in lignin contents (Petersen et al., 2012). Indeed, lignin and hemicelluloses are considered to bind together in order to increase the strength of the xylem cell walls (Meshitsuka et al., 1982; Boerjan et al., 2003; Zhou et al., 2010; Balakshin et al., 2011). In this accepted model of secondary cell wall architecture, the lignin plays a major biological role in cell wall reinforcement (Stewart et al., 1953; Timell, 1967; Amrhein et al., 1983; Smart and Amrhein, 1985; Boerjan et al., 2003; Bonawitz and Chapple, 2013) which also represents a major hinder for wall deconstruction (Boerjan et al., 2003; Yang and Wyman, 2004; Bonawitz and Chapple, 2013; Wilkerson et al., 2014; Anderson et al., 2015).

I.2.3.d. Lignification

Lignin deposition in xylem cell walls

Lignin is a complex heteropolymer composed of different monomers from the phenylpropanoid pathway, with a predominance of p-coumaryl, coniferyl, and sinapyl alcohols, which differ in their degree of methoxylation (Boerjan et al., 2003; Bonawitz and Chapple, 2010; Ralph, 2010; Vanholme et al., 2010; Hao and Mohnen, 2014). The biosynthetic pathway leading to the production of these monomers, referred to as monolignols, is well understood (Boerjan et al., 2003; Bonawitz and Chapple, 2010; Ralph, 2010; Vanholme et al., 2010; Vanholme et al., 2013; Hao and Mohnen, 2014; Wang et al., 2014) and the enzymatic kinetics of the lignin biosynthetic enzymes have been thoroughly investigated and modelled in the tree species Populus trichocarpa (Wang et al., 2014). These monolignols are thought to be exported by ATP-dependent transporters (Kaneda et al., 2008; Miao and
Liu, 2010; Alejandro et al., 2012; Schuetz et al., 2013; Schuetz et al., 2014) to the cell walls where they undergo oxidative coupling reactions collectively named lignification (Boerjan et al., 2003 and references therein). Whether lignification requires dirigent proteins to guide the polymerization or not remains a point of debate (Davin and Lewis, 2000; Boerjan et al., 2003; Ralph, 2010) in which the former mechanism is considered unlikely (see for review Boerjan et al., 2003; Ralph, 2010). In any case, the supply of reactive oxygen species (ROS) by enzymes such as laccases (Liu et al., 1994; Brown et al., 2005; Berthet et al., 2011; Zhao et al., 2013; Schuetz et al., 2014) and peroxidases (Lee et al., 2013; Shigeto et al., 2015) is required for the oxidative coupling of monolignols. Part of the monolignols and of the ROS required for their polymerization are provided to the xylem fibres and to the TEs by the surrounding cells, meaning that lignification is a partially non-cell autonomous and sometimes occurs post-mortem (Stewart et al., 1953; Stewart, 1966; Pesquet et al., 2013; Smith et al., 2013).

**Heterogeneity of xylem lignin**

Once incorporated into the lignin polymer, the aforementioned monolignols constitute β-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, the two latter being the most common units in the xylem of angiosperms (For review see Donaldson, 2001; Boerjan et al., 2003; Bonawitz and Chapple, 2010; Ralph, 2010; Vanholme et al., 2010; Hao and Mohnen, 2014). The proportion of each unit varies between species (see for review Vanholme et al., 2010), between cell types (Saka and Goring, 1985; Eriksson, 1988; Donaldson, 2001; Gorzsás et al., 2011) as well as between different parts of the same cell wall such as the middle lamella, the primary wall and the different layers of the SCWs (Terashima and Fukushima, 1988; Donaldson, 1994). Especially, the xylem vessels generally deposit more lignin than the fibres do, and the lignin of vessels is enriched in G units while that of fibres contains more S units (Saka and Goring, 1985; Donaldson, 2001 and references therein). Furthermore, S lignin is thought to be deposited later than G lignin (Terashima and Fukushima, 1988; Terashima et al., 1993; Donaldson, 2001), which, together with the former observations, suggests a complex regulation of lignin biosynthesis (Terashima and Fukushima, 1988).
Transcriptional regulation of lignification by transcription factors

Campbell and Sederoff (1996) proposed that the regulation of lignin biosynthetic genes’ transcription could represent an additional important regulatory step in the spatial, temporal and compositional control of lignification. For example, the characterization of the promoter activity of the parsley gene encoding for 4-coumarate coenzyme A ligase (4CL)-1, and of the bean gene encoding for phenylalanine ammonia-lyase (PAL)-2 in transgenic tobacco revealed the existence of cis-elements involved in controlling the spatial expression of the corresponding genes (Leyva et al., 1992; Hauffe et al., 1993; Hatton et al., 1995). The sequence of some AC-rich cis-elements (AC elements) controlling the spatial and developmental pattern of the bean PAL-2 promoter activity suggested that the expression of this gene, and by extension the expression of lignin biosynthetic genes in general, could be specifically regulated by MYB-family transcription factors (Hatton et al., 1995). Later studies identified several MYB transcription factors able to directly and specifically regulate the transcription of monolignol biosynthetic genes in Arabidopsis (Zhong et al., 2008; Zhou et al., 2009; Öhman et al., 2013), in maize (Fornalé et al., 2010) and in pine (Patzlaff et al., 2003b; Patzlaff et al., 2003a). In addition to MYBs, the tobacco Pal-box binding protein LIM1 was identified as a specific transcriptional activator for three key monolignol biosynthetic genes (Kawaoka et al., 2000). Recently, a system biology approach aiming to unravel the gene regulatory networks for SCW biosynthesis in Arabidopsis suggested the existence of other specific regulators of lignin biosynthetic gene expression, most of which are MYBs (Taylor-Teeples et al., 2015). This over-representation of MYBs in the specific regulation of monolignol biosynthesis is interesting, especially considering that MYBs are not part of the transcription factors over-represented in the overall SCW-related regulatory network (Taylor-Teeples et al., 2015). It is thus possible that “regulatory sub-networks” specifically control the expression of lignin-related genes, consistent with the fact that lignin represents a “late” evolutionary innovation in the design of water conducting cells in land plants (Friedman and Cook, 2000; Escamez and Tuominen, 2014). Furthermore, the expression of lignin biosynthetic genes is also regulated by more general transcription factors such as the Arabidopsis VND7 (Zhong et al., 2010b; Yamaguchi et al., 2011), NST3/SND1 (Zhong et al., 2010a) and their downstream direct targets MYB46 and MYB83, which are
considered to orchestrate the polysaccharidic SCW biosynthesis and the cell wall lignification (Zhong et al., 2007; Zhong et al., 2008; Ko et al., 2009; McCarthy et al., 2009; Zhong and Ye, 2012). These examples demonstrate that the (direct) transcriptional regulation of monolignol biosynthetic genes is multilayered, including feed-forward loops (Taylor-Teeples et al., 2015) starting with factors that promote the entire differentiation of the lignifying xylem cells through factors that orchestrate the coordinated biosynthesis of complex SCWs down to lignin-specific transcription factors.

**Transcriptional regulation of lignification by chromatin modifications**

Recently, a genome-wide mapping of the transcriptional activation chromatin mark lysine 4 trimethylation of histone H3 (H3K4me3) in the xylem tissue of eucalyptus suggested a potential transcriptional regulation of monolignol biosynthetic genes at the chromatin level (Hussey et al., 2015). In plants, chromatin regions associated with active transcription bear a combination of the activation marks H3K4me3, lysine 36 trimethylation of histone H3 (H3K36me3) and Histone H2B monoubiquitination (H2Bub1) (Roudier et al., 2011; Sequeira-Mendes et al., 2014). While mammals and yeasts require the presence of H2Bub1 marks for the establishment of the H3 methylations associated with active transcription (for review see Cao and Ma, 2011), the situation is different in plants: The genome-wide abundance of H3K4me3 remains roughly unchanged in Arabidopsis loss-of-function mutants for the two E2 enzymes UBIQUITIN CARRIER PROTEIN1 (UBC1) and UBC2, or either of the E3 ligases HISTONE MONOUBIQUITINATION1 (HUB1) and HUB2, which associate into a complex to perform H2B monoubiquitination (Fleury et al., 2007; Cao et al., 2008; Dhawan et al., 2009; Schmitz et al., 2009; Himanen et al., 2012). Interestingly, loss of function in the machinery writing H2Bub1 marks was found to affect H3 methylations and subsequent gene expression of a subset of genes related to flowering (Cao et al., 2008; Dhawan et al., 2009; Schmitz et al., 2009) and to the circadian clock (Himanen et al., 2012). The gene specific effect of H2Bub1 (and/or its writing machinery) on the level of H3K4me3, and potentially of H3K36me3, implies the existence of additional factors that drive this specificity. Specific regulation of the levels of H3K4me3 marks in lignin biosynthetic genes in relation to H2Bub1 marks (and/or
Biotechnological modifications of lignin to reduce lignocellulosic biomass recalcitrance

The transcriptional and enzymatic regulations of monolignol biosynthesis are better characterized than the export and the polymerization of monolignols, which has led to numerous attempts to target these former regulatory steps for the improvement of sugar release from lignocellulosic biomass (Bonawitz and Chapple, 2013). However, many of the efforts to reduce monolignol biosynthesis and/or alter lignin composition by knocking-down monolignol biosynthetic genes have resulted in plants with growth defects that outweigh the gains from improved sugar release (for review see Bonawitz and Chapple, 2013). One apparent exception was reported in greenhouse grown poplars with reduced expression of 4CL-1 and therefore reduced lignin content which was associated with increased growth and higher cellulose content compared with wild-type trees (Hu et al., 1998). However, longer-term experiments on 4CL-1 down-regulated trees suggested that their decreased lignin content was associated with increased susceptibility to vascular embolism and with a negative impact on xylem hydraulics (Voelker et al., 2011). Recently, elaborate metabolic engineering based on “gain-of-function” strategies successfully reduced xylem lignin content and/or recalcitrance to degradation in Arabidopsis (Zhang et al., 2012; Eudes et al., 2014) and poplar (Wilkerson et al., 2014) without affecting the amount of plant biomass. Using pine xylogenic cell suspensions, Wagner et al. (2015) also provided a proof of concept that the less recalcitrant S-lignin (Li et al., 2010) could be introduced in gymnosperms which otherwise mainly contain G-lignin. In Arabidopsis the knowledge of the transcriptional regulation of TE and fibre differentiation allowed to perform “gene rewiring” to increase the amount of polysaccharides and drastically reduce the amount of lignin in the SCWs of fibres, without affecting the TEs (Yang et al., 2013). If applied to woody species, this approach is thus expected to maintain xylem hydraulics and normal plant growth while simultaneously increasing the amount of extractable sugars and decreasing the recalcitrance of fibre cell walls. However, the use of a master switch transcription factor such as NST1 or its orthologues to create a fibre-specific artificial feed forward loop (Yang et al., 2013) in another species than Arabidopsis may result in the premature induction of fibre
programmed cell death. In the TEs of Arabidopsis, the occurrence of premature cell death has been shown to prevent SCW deposition (Muñiz et al., 2008) and a similar output could be expected in the fibres of other species. Hence, it is important to identify and use specific regulators of lignin biosynthesis and of polysaccharidic SCW deposition in order to implement such an elegant “gene rewiring” strategy (Yang et al., 2013) in wood, without taking the risk of inducing premature wood fibre PCD.

I.2.3.e. Programmed Cell Death

Definition of programmed cell death
Programmed cell death is an important mechanism for the development and the health of multicellular organisms, as well as for the fitness of populations of unicellular organisms (Lockshin and Zakeri, 2001; Ameisen, 2002; Fendrych et al., 2014; Bidle, 2015). Lockshin and Williams (1964) coined the term “programmed cell death” to describe developmentally predictable, non-accidental sequences of events during cell death that had been observed since the 19th century (for a historical perspective see Clarke and Clarke, 1996; Lockshin and Zakeri, 2001). Studies in the metazoan model organism Caenorhabditis elegans lead to the discovery of a genetic regulation of PCD (Horvitz et al., 1983), which was confirmed by the identification of a caspase (cysteine-dependent aspartate proteinease) as the first characterized positive regulator of developmental cell death (Yuan et al., 1993; Alnemri et al., 1996; Lockshin and Zakeri, 2001, 2002). The developmental cell deaths of TEs and fibres are both considered to be (genetically) programmed because poplar fibres die in a coordinated fashion (Courtois-Moreau et al., 2009) and TE cell death is associated with a conserved sequence of physiological events (for review see Escamez and Tuominen, 2014). Furthermore, in the case of TEs, cell death is a functionalizing event which contributes to the biological, post-mortem function of these cells (Raven, 1977; Friedman and Cook, 2000; Ménard et al., 2015).

Programmed cell death and autolysis are different processes
Fully differentiated xylem TEs and Fibres can be clearly identified as dead because they have undergone complete protoplast autolysis, defined as the degradation a cell’s content by its own enzymes (O'Brien and Thimann, 1967; Torrey et al., 1971;
Wodzicki and Brown, 1973; Shiningger, 1979; Fukuda, 1996; Escamez and Tuominen, 2014). Although fully autolyzed cells ought to be dead, this does not necessarily imply that programmed cell death and autolysis are part of the same cellular process (for review see Escamez and Tuominen, 2014). O’Brien and Thimann (1967) had already proposed that TE protoplast autolysis could involve different mechanisms from TE PCD. More recently, TE PCD and autolysis were proven to be genetically distinct in Arabidopsis mutants in which loss-of-function of the cysteine proteases XYLEM CYSTEINE PROTEASE 1 (XCP1) or both XCP1 and XCP2, or METACASPASE9 (MC9) impaired autolysis without affecting PCD (Avci et al., 2008; Bollhöner et al., 2013). As a consequence, the occurrence of PCD cannot be deduced based on the sole observation of autolytic features, but rather requires the observation of a clearly established sequence of morphological, physiological and molecular events (Escamez and Tuominen, 2014).

Physiological events associated with TE PCD

The instant of death in TEs is marked by the rupture of the tonoplast and the concomitant demise of cyclosis (Groover and Jones, 1999; Bollhöner et al., 2012), because it constitutes a point of no-return in the progression of the TE cell death (Fukuda, 1996). Therefore, any event that is part of the cell death programme must take place before the vacuolar collapse, while later events should be considered as post-mortem (Escamez and Tuominen, 2014). To date, only few pre-mortem physiological events have been associated with TE PCD. During the most studied type of metazoan PCD, apoptosis (Kerr et al., 1972), the integrity of the mitochondrial envelope is compromised, leading to the release of the cytochrome c into the cytoplasm, triggering a PCD signalling cascade (Cain et al., 2002; Garrido et al., 2006). The existence of such mechanism during TE PCD was investigated in differentiating *Zinnia elegans* cell suspensions, in which mitochondria undergo subtle morphological changes and loss of membrane potential before TE cell death (Yu et al., 2002). In this system, TE vacuolar collapse could be induced by pharmacologically triggering the loss of mitochondrial membrane potential, but not by triggering the release of cytochrome c (Yu et al., 2002). Thus, in contrast with metazoan apoptosis, TE PCD does not rely on a cytochrome c-dependent signalling although it is affected by mitochondrial membrane integrity (Yu et al., 2002). Shortly before TE cell death, altered tonoplast permeability was
observed in differentiating Zinnia cell suspensions (Kuriyama, 1999; Obara et al., 2001). In addition, vacuolar collapse could be induced upon treatment with probenecid to affect anion transport across the tonoplast (Kuriyama, 1999). Finally, Zinnia TE PCD could be induced by calcium influx, probably in response to extracellular proteolysis, as demonstrated by exogenous application of pharmacological compounds that promoted or inhibited these processes (Groover and Jones, 1999). From the previous observations, it seems that the molecular regulations of mitochondrial membrane and tonoplast integrity, and of calcium influx, in turn regulate TE PCD.

**Molecular factors associated with the positive regulation of TE PCD**
The induction and execution of plant PCD have been proposed to rely on the action of proteases in the same way as caspases, which mediate proteolytic signalling cascades during apoptosis (Lam, 2005; Woltering, 2010). Caspase-like activities have been reported in differentiating xylem in poplar trees (Han et al., 2012) and in xylogenic Zinnia cell suspensions (Twumasi et al., 2010), in which treatment with caspase inhibitors decreased TE differentiation (Lakimova and Woltering, 2009; Twumasi et al., 2010). However, caspase homologues cannot be found in plants and the caspase-like activity detected in differentiating xylem (Twumasi et al., 2010; Han et al., 2012) could not be unambiguously linked to TE PCD. Plants possess structural analogues of caspases called metacaspases (MCs) which display a different substrate specificity (Uren et al., 2000), but which were anyway proposed to function in promoting PCD. Consequently, MC9, the only metacaspase whose expression is induced during Arabidopsis in vitro TE differentiation was proposed as a positive regulator of TE PCD (Turner et al., 2007). Similarly, numerous other TE expressed cysteine-dependent proteases (Ye and Varner, 1996; Beers and Freeman, 1997; Zhao et al., 2000; Bozhkov and Jansson, 2007; Turner et al., 2007), and a serine-dependent protease (Groover and Jones, 1999), were proposed to be positive effectors of TE PCD. However, only three of these proteases were genetically characterized, revealing that they function in *post-mortem* autolysis (Avcı et al., 2008; Bollhöner et al., 2013), while none of the above proteases has so far been demonstrated to be a positive regulator of PCD *per se*. Even though a function for MC9 could not clearly be established in TE PCD (Bollhöner et al., 2013), MC9 has been shown to control the ROS-induced spreading of cell death in
Arabidopsis leaves by activating the pro-death signalling GRIM REAPER (GRI) peptide (Wrzaczek et al., 2015). It is therefore possible that MC9 controls a yet unknown form of cell death signalling. Another reason for MC9 to remain a candidate positive regulator of TE PCD is the fact that a MC9 homologue has been shown to regulate the mode of cell death during spruce embryo suspensor PCD, via regulating autophagy in these cells (Minina et al., 2013).

**Potential participation of autophagy in TE PCD**

The cellular process of autophagy (De Duve and Wattiaux, 1966) has been proposed as a mechanism that promotes plant PCD in general (van Doorn et al., 2011) and fibre PCD (Courtois-Moreau et al., 2009) and TE PCD (Kwon et al., 2010) in particular. Autophagy was originally discovered by the observation of vesicles named autophagosomes that could engulf some cytoplasmic content in a cell in order to target it for degradation in lytic compartment such as lysosomes or vacuoles (De Duve and Wattiaux, 1966). This vesicular pathway for degradation has later been renamed macroautophagy on account of the discovery of morphologically distinct but mechanistically overlapping autophagic (“self-digestion”) pathways such as microautophagy, chaperone-mediated autophagy and organelle-specific autophagy (For review see Liu and Bassham, 2012). Macroautophagy is the best characterized autophagic pathway owing to the discovery in yeasts of specific genes involved in its regulation (ATG genes; Klionsky et al., 2003), most of which are conserved in animals and plants (Yang and Klionsky, 2010; Liu and Bassham, 2012). Due to its predominance in the literature, the process of macroautophagy is usually referred to (including below) as autophagy (Figure 3). Autophagy was originally recognized to function in nutrient recycling during starvation (Mortimore and Pösö, 1987; Mizushima et al., 2004; Thompson et al., 2005; Yang and Klionsky, 2010) through bulk degradation of cytoplasmic content. Autophagy is now known to also operate in favourable trophic conditions in Arabidopsis (Guiboileau et al., 2012). Furthermore, some instances of selective autophagic degradation of specific targets have been reported (Yang and Klionsky, 2010; Derrien et al., 2012; Michaeli et al., 2015; Wurzer et al., 2015). Specific target degradation by autophagy has been linked to the existence of adaptor proteins which bind to both the autophagosome membrane-bound ATG8 protein (considered a marker for autophagosomes; Klionsky et al., 2012) and the targeted cargo (Yang and Klionsky, 2010; Svenning...
et al., 2011; Wurzer et al., 2015). The fact that autophagy does not solely function in bulk cytoplasmic degradation but that it also targets specific proteins for degradation implies a potential regulatory role for autophagy in cellular processes, including PCD. However, there is still a debate in both animal (Clarke and Puyal, 2012; Denton et al., 2012; Shen et al., 2012; Gump et al., 2014; Liu and Levine, 2014) and plant research (van Doorn et al., 2011; Hackenberg et al., 2013; Minina et al., 2013; Lv et al., 2014; Minina et al., 2014) regarding whether autophagic cell death exists, whether increased autophagy is merely present in some cells undergoing PCD without contributing to the cell death processes, or whether autophagy indirectly contributes to PCD by maintaining the organization of the dying cells which therefore die in a controlled rather than necrotic fashion.

Figure 3: Simplified scheme of the cellular process of autophagy (macroautophagy).
Before this thesis (Paper II), only one published study addressed the role of autophagy in the TEs, claiming the occurrence of autophagic cell death during Arabidopsis TE differentiation (Kwon et al., 2010). However, this study relied on constitutive down-regulation or constitutive, ectopic induction of autophagy which both affected xylem differentiation rates (Kwon et al., 2010), rendering impossible the interpretation of the results in terms of TE PCD. In the case of fibre PCD, the contribution of autophagy to the cell death processes was hypothesized based on induction of ATG genes and based on morphological features of cytoplasmic degradation, but autophagic cell death was not demonstrated (Courtois-Moreau et al., 2009). Indeed, autophagic cell death is difficult to demonstrate. In theory, demonstrating autophagic cell death requires at least the observation of an increased level of autophagy in the dying cells, as well as an effect of autophagy inhibition on the observed PCD process (as can be derived from the recommendations from the Nomenclature Committee on Cell Death; Galluzzi et al., 2012). Therefore, to date there is not a single cellular process or molecular effector whose role is unambiguously characterized as to specifically trigger or execute PCD in xylem.

**Negative regulation of TE PCD**

The absence of any known positive regulator of PCD in xylem could come from the fact that evolution co-opted existing genes with a non-death related function to regulate PCD (Ameisen, 2002; Galluzzi et al., 2008); genes which likely also retained all or part of their ancestral pro-life function (Ameisen, 2002; Gallusi et al., 2008), hindering the unambiguous identification of their role in PCD. In addition, the potential functional redundancy of positive PCD regulators or the probable deleterious effect of impaired PCD on health and development likely hinder the discovery of factors that promote PCD. Alternatively, it could be that in at least some types of PCD, no positive regulator exists: In theory, PCD need not be mediated by specific positive regulators, but may solely require the presence of cell death repressors whose action is stopped in a genetically controlled fashion. Such a negative regulator of TE PCD could be the tetra-amine synthase ACAULIS5 (ACL5), or its product thermospermine, based on the observation that TEs undergo premature cell death in Arabidopsis acl5 mutants (Muñiz et al., 2008). On the other hand, protoxylem TEs are not affected by the loss of ACL5 function in Arabidopsis (Muñiz et al., 2008), suggesting that either there exist a different type of PCD in
protoxylem TEs from other TEs or that ACL5 is involved in a more complex (PCD-related) regulatory mechanism. It is also unclear whether ACL5 functions in TEs via its product because exogenous application of thermospermine in TE-differentiating Zinnia cell suspensions was shown to inhibit the entire differentiation process (Kakehi et al., 2008). Hence, despite a great amount of available data and clever hypotheses, the regulation of TE PCD remains elusive, possibly because it has been investigated too often within the conceptual framework of animal PCD, or without a careful distinction between PCD and cellular autolysis.

I.2.3.f. Programmed autolysis

Protoplast autolysis and cell wall autolysis
The occurrence of protoplast autolysis in TEs and fibres has been deduced from the observation that their protoplasts become fully degraded while their thick cell walls prevent protoplast elimination by neighbouring cells (Esau, 1943; O'Brien and Thimann, 1967; O'Brien, 1970; Wodzicki and Brown, 1973; Groover et al., 1997; Avci et al., 2008; Courtois-Moreau et al., 2009; Bollhöner et al., 2012; Bollhöner et al., 2013). Another dimension of TE autolysis is the partial degradation of the side walls and of the end walls, the latter leading to the creation of the perforation plate whose structure greatly influences the xylem hydraulics (O'Brien and Thimann, 1967; O'Brien, 1970; Carlquist, 2012; Feild and Brodribb, 2013). Despite the long standing observation of this partial wall degradation and of its impact on xylem hydraulics, its regulation and molecular effectors remain unknown. The underlying mechanism has been proposed, but not proven, to rely on the release of cell wall polysaccharide-degrading enzymes by the dead TEs during the bulk autolysis of their protoplast (O'Brien and Thimann, 1967; O'Brien, 1970).

A genetic programme for autolysis
The complete autolysis of protoplasts during the development of TEs and fibres has three implications: Firstly, the completion of full protoplast autolysis can only be a post-mortem event, although it cannot be excluded that autolytic processes start before the instant of death. Secondly, the preparation and execution of the protoplast autolysis must be coordinated with the other modules of the differentiation programme for the cells to become functional. Thirdly, there must exist a genetic programme for autolysis, for these cells to build up their autolytic machinery (Escamez and
Tuominen, 2014). Consistently, the expression or activity of proteases, endonucleases and ribonucleases have been detected in differentiating xylem in Zinnia, Arabidopsis and poplar plants as well as in TE-differentiating Arabidopsis and Zinnia cell suspensions (Thelen and Northcote, 1989; Minami and Fukuda, 1995; Ye and Droste, 1996; Ye and Varner, 1996; Beers and Freeman, 1997; Aoyagi et al., 1998; Woffenden et al., 1998; Zhao et al., 2000; Demura et al., 2002; Funk et al., 2002; Ito and Fukuda, 2002; Pesquet et al., 2004; Ehlting et al., 2005; Kubo et al., 2005; Pesquet et al., 2005; Turner et al., 2007; Avci et al., 2008; Farage-Barhom et al., 2008; Courtois-Moreau et al., 2009; Yamaguchi et al., 2011; Han et al., 2012; Bollhöner et al., 2013 Paper II). In order to protect the cells from a premature demise by their own hydrolytic machinery, it was proposed that these enzymes could be stored in the vacuole from which they would be released upon death by the vacuolar rupture (Wodzicki and Brown, 1973; Fukuda, 1997; Groover et al., 1997; Obara et al., 2001). This vacuolar localization of autolytic hydrolases is supported by the fact that several of them harbour an endomembrane-targeting signal peptide (Aoyagi et al., 1998; Avci et al., 2008). Furthermore, several of these hydrolases have an acidic optimal pH for their activity (Thelen and Northcote, 1989; Ye and Varner, 1996; Vercammen et al., 2004), supporting their localization to the vacuole (Fukuda, 1997) and the proposed role of the vacuole rupture in activating the bulk autolysis of the protoplast (Wodzicki and Brown, 1973; Fukuda, 1997; Groover et al., 1997; Obara et al., 2001; Avci et al. 2008; Bollhöner et al., 2013). On the other hand, plant cells contain other acidic endomembrane compartments which could also be responsible for cytoplasmic acidification at the end of PCD (Fendrych et al., 2014; Luo et al., 2015) and to which some of these proteases could also localize before the onset of the bulk autolysis (Ménard et al., 2015).

**Molecular executors of the TE autolysis**

Among the different hydrolases thought to be associated with TE protoplast autolysis, only a few have been further characterized. The Zn\(^{2+}\)-dependent S1-type Zinnia endonuclease 1 (ZEN1) is the only nuclease that has been shown to have the ability to degrade nuclear DNA *in vitro* and to contribute to nuclear degradation during TE autolysis in Zinnia cell suspensions (Ito and Fukuda, 2002). TE autolysis was also impaired by pharmacological treatment of Zinnia cells with a cysteine protease and proteasome inhibitor, but not with a specific proteasome inhibitor, providing
the first piece of evidence for the involvement of cysteine-dependent proteases in TE autolysis (Woffenden et al. 1998). TE autolysis was indeed found incomplete when observed by electron microscopy in Arabidopsis loss-of-function mutants for the papain-like cysteine protease (PLCP) XCP1, for both XCP1 and XCP2 (Avci et al., 2008), as well as for the metacaspase MC9 (Bollhöner et al., 2013). Interestingly, MC9 was detected in the cytoplasm of TEs and was therefore assumed to remain inactive until the acidification of the cytoplasm by the vacuolar rupture (Bollhöner et al., 2013); although this thesis identifies a pre-mortem role for MC9 as well as an additional vacuolar localization (Paper II). XCP1 and XCP2 were found to localize mainly inside the vacuole of the TEs, where they are thought to contribute to intra-vacuolar hydrolytic processes before the onset of the bulk protoplast autolysis (Avci et al., 2008). The apparent overlap between the functions of XCP1, XCP2 and MC9 prompted Bollhöner et al. (2013) to investigate their potential relationship, especially because proteolytic cascades can lead to the activation of such papain-like cysteine proteases (van der Hoorn et al., 2004; Richau et al., 2012). However XCP2 behaved as a target for degradation by MC9 in vitro, and the analysis of papain-like cysteine protease (PLCP) activity in mc9 xcp1 xcp2 triple mutants clearly indicated that MC9 rather activates at least one other unidentified PLCP (Bollhöner et al., 2013). The identification of PLCPs as executioners of bulk TE autolysis is not surprising because this type of hydrolases is known to be remarkably stable in proteolitically harsh conditions (Richau et al., 2012). In contrast no serine-dependent protease has been shown to contribute to autolysis, but some of them instead seem to act in the early signalling of TE differentiation (for review see Petzold et al., 2012) or probably in the signalling of TE PCD (Groover and Jones, 1999).

**The programme for autolysis includes safeguards**

The process of TE autolysis itself can be expected to have consequences beyond the autolyzing TE itself because it likely triggers the release of a number of molecules. Observations of xylem differentiation within *in vitro* tobacco explants lead to the hypothesis that autolyzing TEs could release auxin and cytokinins, thus providing positional information for TEs to differentiate end-to-end and for the cambium to produce more xylem cells (Sheldrake and Northcote, 1968). Furthermore, anatomical characterization of xylem differentiation in oat coleoptiles suggested that autolyzing TEs release some hydrolases (O’Brien...
and Thimann, 1967; O’Brien 1970). However, the protoplasts and the cell walls of xylem parenchyma cells adjacent to autolyzing TEs remain apparently undamaged in oat and in maize, suggesting that mechanisms must exist to protect the neighbouring cells during TE autolysis (O’Brien and Thimann, 1967; O’Brien, 1970; Srivasta and Singh, 1972). One such mechanism of “programmed safeguard” relies on the protein TED4 which was identified based on its expression during TE differentiation of Zinnia cell suspensions and in the xylem of Zinnia plants (Demura and Fukuda, 1993; Demura and Fukuda, 1994). Both TEs and non-TE cells secrete the TED4 protein during xylem differentiation in order to inhibit the otherwise harmful activity of the proteasome 20S subunit released by autolyzing TEs (Endo et al., 2001). The secretion of TED4 by immature xylem cells in anticipation of TE autolysis (Endo et al., 2001) further exemplifies the programmed nature of TE autolysis. In addition, the fact that both TEs and non-TEs seem to implement safeguards against potential adverse effects of TE autolysis suggests that xylem cells cooperate in the spatial restriction of autolysis, in addition to their known cooperation during lignfication.
I.3. Aims of the research

The general aim of the work presented in this thesis was to discover new regulations of lignification and TE programmed cell death during xylem development. Such new regulations were indeed expected to provide novel knowledge of xylem development as well as trails for future (bio)technological applications.

To discover new regulations of lignification, my colleagues and I first aimed to identify new regulators of TE lignification by performing a screen in Zinnia elegans xylogenic cell suspensions as an experimental system (Paper I). The next aim consisted in testing the potential involvement of candidate genes in controlling lignification by using a reverse genetic approach in the model plant Arabidopsis thaliana (Paper I, Paper III). Finally, this work aimed to unravel the function of at least one promising candidate gene specifically involved in non-cell autonomous lignification in order to unravel new regulations of lignification (Paper III, Paper IV).

To discover new regulations of TE PCD, the role of the candidate PCD regulator Arabidopsis METACASPASE9 was investigated at the cellular level using Arabidopsis xylogenic cell suspensions as an experimental system (Paper II).
II. Results and discussion

II.1. New regulations of xylem lignification

II.1.1. Identification of new regulators of non-cell autonomous TE lignification

II.1.1.a. Screening for candidate genes potentially regulating lignification

The existence of a post-mortem lignification demonstrates the partial non-cell autonomy of lignification

Observations of the xylem chemistry along stem cross sections in trees revealed that the amount of lignin in the cell walls of xylem TEs and fibres increased after the death of these lignifying cells (Stewart et al., 1953; Stewart, 1966). In addition, the xylem parenchyma cells were shown to provide the neighbouring, dead, lignifying cells with ROS in the stems of Zinnia elegans, presumably to allow post-mortem oxidative coupling of monolignols (Ros Barceló, 2005). We confirmed that lignification occurs partially post-mortem by two approaches: First, in the stems of Zinnia plants, our observations of xylem lignification in cross sections of consecutive internodes showed that TE lignification had progressed beyond the TE life span (Paper I, Fig. 2). Second, in xylogenic Zinnia cell suspensions, dead, non-lignified TEs that had been treated with the inhibitor of monolignol biosynthesis piperonylic acid (PA) could lignify post-mortem following the exogenous supply of monolignols (Paper I, Fig. 1). Consequently, we deduced that TE lignification occurs at least partially in a non-cell autonomous manner and we confirmed that the parenchymatic non-TE cells in Zinnia xylogenic cell suspensions provide the dead, lignifying TEs with both ROS and monolignols (Paper I, Fig. 7). Furthermore, these observations established the xylogenic Zinnia elegans cell suspensions as a suitable system to screen for candidate regulators of lignification, including potential regulators of the non-cell autonomous component of TE lignification.

Differentially expressed genes during inhibition of lignification in xylogenic Zinnia cell suspension

We discovered that the ethylene signalling inhibitor silver (Beyer et al., 1976), in the form of thiosulfate (silver thiosulfate: STS), could
arrest both TE lignification and PCD in Zinnia xylogenic cell suspensions (Paper I, Fig. 3). Hence, differentiating Zinnia cell suspensions were treated with 60 µM STS, and screening for differentially expressed genes between STS-treated and untreated conditions was performed by using suppression subtractive hybridization (SSH) (Paper I, Supplementary Data Set 1 online). Several genes known or thought to be involved in lignin biosynthesis were differentially expressed upon STS treatment (Paper I), strongly suggesting the existence of other regulators of lignification within the genes differentially expressed in response to STS.

II.1.1.b. Confirmation of candidate genes as regulators of lignification

Candidate genes identified in Zinnia have orthologues which control lignification in Arabidopsis

The next step in finding new regulators of lignification consisted in identifying homologues for the Zinnia candidates in the genome of Arabidopsis in order to test their potential lignin-related role by reverse genetics. T-DNA insertion lines for fifty-four different Arabidopsis genes, homologous to the Zinnia candidates, were obtained and the cell wall properties of their xylem-rich hypocotyls were analyzed by pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) (Paper I, Fig. 9; Supplemental Data Set 2 online; Paper III, Fig. 2). The relative contents of total lignin, G-type, S-type or H-type lignin, or the ratio of S lignin to G lignin, were significantly different from wild-type levels (Welch-corrected T-test) in the T-DNA lines of sixteen of the candidate genes. Further multivariate data analysis of the cell wall components by orthogonal projections of latent structures-discriminant analysis (OPLS-DA) (Bylesjö et al., 2006) revealed significantly different cell wall properties between the wild-type and the T-DNA lines for twenty of the candidate genes. The T-DNA lines for nine of the candidate genes showed significant differences from wild-type using both Welch-corrected T-test and OPLS-DA analysis (Figure 4), indicating that these genes are putative new regulators of lignification.
**Table:**

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<th>H lignin</th>
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**Figure 4:** Screening for regulators of non-cell autonomous lignification.

Relative lignin contents in Arabidopsis T-DNA insertion mutants for potential regulators of lignification were analyzed by MS-Py ([Paper I](#)) and are displayed as percentages of the corresponding relative lignin contents in wild-types. The displayed candidate regulators were selected based on two criteria: (i) The corresponding T-DNA lines displayed relative lignin contents that were significantly different from wild-type levels according to a Welch-corrected T-test (n=3), as indicated by an asterisk (p<0.05). (ii) Pairwise comparison of pyrolysates by orthogonal projections to latent structures-discriminant analysis (OPLS-DA) indicated a significant difference ("Q2(cum)">0.5) in the cell wall structures of the T-DNA lines compared with wild-type. The SAIL_580_C07 mutant was included because eventhough its relative contents of total, G, S or H lignin did not significantly differ from the wild-type, its ratio of S-type to G-type lignin was significantly different from the wild-type (data not shown). The previously characterized mutants c4h-3 (Ruegger and Chapple, 2001) and ccr1-3 (Derikvand et al., 2008) were included as controls, which displayed the expected changes in lignin properties compared to wild-type.

For each analyzed T-DNA line, the expression of the corresponding wild-type gene was recovered from cell-type specific micro-array analysis from Arabidopsis root cells (Brady et al., 2007) that were later analyzed and organized to represent spatio-temporal expression data (Cartwright et al., 2009). The average expression of each gene in "old" xylem vessels (sections 7-12 from Brady et al., 2007) was displayed as "TEs" while "non-TEs" represent the average expression in stele cells (including procambial and parenchymatic cells) from the same sections. The expression of At2g43120 was not measured due to the absence of a corresponding probe on the micro-array chip used by Brady et al. (2007). Ɨ marks indicate genes whose expression in Arabidopsis xylogenic cell suspensions remained at least 75% of their maximum expression after TE cell death (after data from Kubo et al., 2005), indicating a possible involvement in *post-mortem* lignification ([Paper I, Supplemental Data Set 2 online](#)).
II.1.1c. Identifying genes regulating non-cell autonomous lignification

Several regulators of lignification are expressed in lignifying and non-lignifying xylem cells

In order to identify new regulations of lignification, the sole identification of new regulators is not sufficient because these regulators may simply represent new intermediates in already known regulatory pathways. The non-cell autonomous component of lignification had remained poorly characterized before the work presented in this thesis. We therefore reasoned that the functional characterization of candidate regulators of the non-cell autonomous lignification would shed light on new regulations of lignification. To determine if a gene could function in non-cell autonomous lignification, we looked for its potential expression in non-lignifying cells using publicly available expression data. We found that five out of the nine aforementioned regulators of lignification maintained a high level of expression after TE PCD in Arabidopsis xylogenic cell cultures (Kubo et al., 2005), indicating that these genes were (also) expressed in parenchymatic non-TE cells (Figure 4; Paper I, Supplemental Data Set 2 online). Furthermore, three out of the nine genes had previously been found to be expressed in both the TEs and their neighbouring non-lignifying non-TE cells in the main root of Arabidopsis seedlings (Figure 4) (Brady et al., 2007; Cartwright et al., 2009). These three genes (namely At5g61750, At3g25640 and At2g38870) were clearly more expressed in non-TEs than in TEs, suggesting that they could mainly function in non-cell autonomous lignification. However, the transcripts of some of the genes expressed beyond TE cell death in xylogenic Arabidopsis cell suspensions (Kubo et al., 2005) had not been detected in non-TEs in Arabidopsis roots (Brady et al., 2007; Cartwright et al., 2009), and conversely. Hence, the available expression data (Kubo et al., 2005; Brady et al., 2007; Cartwright et al., 2009), however useful, seems incomplete and it cannot be used alone to infer a role in non-cell autonomous lignification. Based on this available expression data and on the histochemical β-glucuronidase promoter-reporter assay for one of the candidate genes (At1g32230; Paper I, Fig. 8), we could therefore identify promising candidates probably involved in non-cell autonomous part of lignification. On the other hand, it remained unclear whether they could be specific regulators of the non-cell autonomous part of lignification because none of these genes could be shown to be specifically expressed in the non-lignifying
xylem cells. Nevertheless, the available expression data had been obtained by micro-array using a chip that did not allow for detection of the transcripts from one of the nine genes of interest; the PIRIN2 (At2g43120) gene, which could therefore still represent a specific regulator of non-cell autonomous lignification.

**PIRIN2 is a regulator of lignification which is not expressed in lignifying TEs**

The Arabidopsis PIRIN2 (PRN2) was the only Arabidopsis PRN, out of the four orthologues (Paper III, Supplementary Fig. 1) of the candidate lignin regulator Zinnia PIRIN (Paper I), whose knock-out lines showed altered lignin properties compared with wild-type in Py-GC/MS analyses (Figure 4; Paper III, Fig.2). The spatio-temporal expression pattern of PIRIN2 was studied by monitoring the activity of its promoter, using the histochemical β-glucuronidase promoter-reporter assay (Paper III, Fig. 1). We found the promoter of PIRIN2 to be active in xylem parenchyma cells but not in the lignifying xylem tracheary elements (Paper III, Fig. 1), indicating that PRN2 regulates specifically the non-cell autonomous component of TE lignification. Therefore we undertook characterizing the function of PRN2 during non-cell autonomous lignification as a mean to uncover new regulations of lignification.

**II.1.2. Transcriptional regulation of non-cell autonomous lignification**

**II.1.2.a. Transcriptional regulation of lignin biosynthetic genes at the chromatin level**

**PIRIN2 works as a transcriptional regulator of lignin biosynthetic genes**

Detailed analyses of the cell wall composition in the hypocotyls of prn2 knock-out mutants and in PRN2 overexpressing plants (Zhang et al., 2014) by Py-GC/MS and by Fourier transform infrared (FTIR) microspectroscopy revealed that PRN2 functions as a negative regulator of S lignin biosynthesis, and to a lesser extent as a negative regulator of G lignin biosynthesis (Paper III, Figs. 2,3,5). Earlier reports had determined that the human PIRIN orthologue could function as a transcriptional co-regulator (Wendler et al., 1997; Dechend et al., 1999). We therefore hypothesized that the Arabidopsis PIRIN2 could control lignin biosynthesis by regulating the expression of lignin biosynthetic
genes. Indeed, real-time quantitative PCR (qPCR) analyses showed increased expression of several lignin biosynthetic genes in the hypocotyls and the stems of prn2 mutants compared with wild-type levels, while PRN2 overexpressors consistently displayed opposite expression patterns (Paper III, Figs. 3, 6). Hence, PRN2 functions as a transcriptional regulator of lignification. However, PRN proteins have never been shown to directly bind to chromatin, which suggested that the Arabidopsis PRN2 is a transcriptional co-regulator like its orthologue in human.

**PIRIN2 interacts with the transcriptional regulator HUB2**
To determine whether PIRIN2 could function as a transcriptional co-regulator, we identified potential interactors of the PIRIN2 protein by yeast-two-hybrid screening (Paper III, Supplementary Table 1). Next, the cell wall properties were analyzed by Py-GC/MS in the hypocotyls of T-DNA insertion lines for the eighteen potential candidate interactors (Paper III, Supplementary Fig. 6), revealing a decrease in the proportion of S lignin in the T-DNA lines for the HISTONE H2B MONOUBIQUITINATION2 (HUB2) (Fleury et al., 2007; Cao et al., 2008). The Arabidopsis HUB2 had earlier been shown to function in a protein complex that binds to chromatin and performs monoubiquitination of histone H2B (H2Bub1) (Fleury et al., 2007; Cao et al., 2008). The H2Bub1 chromatin marks have been associated with increased trimethylation of lysine 4 and possibly of lysine 36 of histone 3 (H3K4me3 and H3K36me3, respectively) at specific loci, triggering the subsequent up-regulation of the corresponding target genes (Cao et al., 2008; Schmitz et al., 2009; Himanen et al., 2012). Hence, we hypothesized that HUB2 could function as a positive transcriptional regulator of S lignin biosynthesis, and that PRN2 could interact with and antagonize the function of HUB2. The ability of HUB2 and PRN2 to interact together was confirmed by yeast-two-hybrid assay and by co-immunoprecipitation (Co-IP) (Paper III, Fig. 4). The possible physiological interaction between HUB2 and PRN2 was also supported by histochemical β-glucuronidase promoter-reporter assay, which revealed HUB2 promoter activity in xylem, including the non-TE cells (Paper III, Fig. 5). In addition, we found that lignin biosynthetic genes were down-regulated in the hypocotyls of hub2 T-DNA knock-out mutants (Paper III, Fig. 5), consistent with our hypothesis that HUB2 is a positive transcriptional regulator of S-type lignin biosynthesis.
PIRIN2 tunes down the HUB2-dependent transcriptional activation of lignin biosynthetic genes

To verify if PRN2 could antagonize the positive regulation of S lignin biosynthesis by HUB2, we performed Py-GC/MS analysis to compare the proportion of S lignin between the hypocotyls of wild-type, prn2 and hub2 single mutants, and prn2 hub2 double mutants. While the hub2 mutants displayed a trend towards a lower proportion of S-type lignin than wild-type, both the prn2 and prn2 hub2 plants showed increases in S lignin compared to wild-type (Paper III, Fig. 5), suggesting that PRN2 and HUB2 function within the same pathway. Furthermore, Py-GC/MS analysis of stem samples revealed that the proportion of S lignin was further decreased in PRN2 overexpressing plants than in hub2 knock-outs, while hub2 mutants overexpressing PRN2 contained the same proportion of S lignin than hub2 mutants alone (Paper III, Fig. 5). Therefore, the negative regulation of lignin biosynthesis by PRN2 requires the presence of HUB2. Together, our data indicates that PRN2 functions as an antagonist of the up-regulation of lignin genes by HUB2. HUB2 has never been shown to function as a transcription factor, but rather to up-regulate specific target genes by promoting chromatin modifications associated with active transcription such as H3K4me3 marks (Cao et al., 2008; Himanen et al., 2012). H3K4me3 marks have recently been proposed to play a role in the regulation of lignin biosynthetic genes in the developing wood of eucalyptus (Hussey et al., 2015). Hence, we hypothesized that PRN2 could interact with HUB2 in order to repress the downstream establishment of the H3K4me3 marks at the loci of lignin biosynthetic genes.

PIRIN2 modulates chromatin marks

To test if PRN2 could affect the amount of H3K4me3 marks in the chromatin of lignin biosynthetic genes, we undertook an approach that relies on chromatin immunoprecipitation (ChIP) using an anti-H3K4me3 antibody followed by qPCR for quantification of the immunoprecipitated chromatin. Due to the amount of material required for these ChIP-qPCR analyses, hypocotyls could not be used. Instead, the lower parts of the main stem from fifteen plants had to be pooled to create each biological replicate. We first verified that the expression of lignin biosynthetic genes was up-regulated in the stems of prn2 plants compared with wild-type (Paper III, Fig. 6), as it was the case in the hypocotyls. Next, ChIP-qPCR analysis of five lignin biosynthetic genes indicated a clear trend towards an increased amount of H3K4me3 in prn2
plants compared with wild-type (Paper III, Fig. 6). Refined ChIP-qPCR analysis of four loci within the gene encoding for the S lignin-related caffeic acid O-methyltransferase (COMT) confirmed that H3K4me3 marks were more abundant in prn2 compared with wild-type (Paper III, Fig. 6). Furthermore, none of the four loci within the COMT gene displayed changes in H2Bub1 (Paper III, Fig. 6), demonstrating that PRN2 modulates the abundance of H3K4me3 downstream of HUB2 rather than the abundance of H2Bub1 controlled by HUB2. Collectively, these observations indicate that there exists a transcriptional regulation of the non-cell autonomous component of lignification. This transcriptional regulation takes place at least in part at the chromatin level and involves PRN2 and HUB2. HUB2 is involved in establishing all H2Bub1 marks genome-wide in Arabidopsis (Cao et al., 2008) but loss of function of the protein complex that establishes H2Bub1 only affects the abundance of H3K4me3 in specific subsets of genes (Cao et al., 2008; Himanen et al., 2012). Consequently, factors must exist that drive this specificity and our data strongly suggest that PRN2 is one such co-regulator involved in the specific regulation of lignin biosynthetic genes by a HUB2-containing protein complex. PRN2 works as a negative regulator of H3K4me3 establishment downstream of HUB2, which implies the existence of yet unidentified factors that establish HUB2-dependent H3K4me3 marks in specific target genes. Interestingly, H2Bub1 and the downstream H3K4me3 marks regulate the expression of circadian clock genes in Arabidopsis (Himanen et al., 2012) and H2Bub1 marks are involved in recruiting transcription factors that regulate circadian clock genes in mammalian cells (Tamayo et al., 2015). By extension, it is possible that the HUB2-containing protein complex and/or the H2Bub1 marks recruit proteins that establish H3K4me3 marks as well as transcription factors that regulate the expression of lignin biosynthetic genes in Arabidopsis.

II.1.2.b. Transcriptional regulation of lignin biosynthesis by protein complexes

Identification of a PIRIN2-interacting transcription factor

By analogy with the human PIRIN which interacts with several different transcriptional regulators (Wendler et al., 1997; Dechend et al., 1999), we hypothesized that the Arabidopsis PIRIN2 could also modulate the activity of other transcriptional regulators of lignin biosynthetic genes than the sole HUB2. Among the eighteen potential PRN2 interactors that we had identified by yeast-two-
hybrid screening (Paper III), one was a putative transcription factor: the basic-helix-loop-helix (bHLH) family protein bHLH62 (At3g07340). The ability of bHLH62 to interact with PRN2 was confirmed by yeast-two-hybrid assay and by Co-IP (Paper IV, Fig. 1). Furthermore, gene expression analysis from individual cell types in the main root of Arabidopsis seedlings (Brady et al., 2007; Cartwright et al., 2009) indicated the presence of bHLH62 transcripts in both the lignifying TEs and in the non-TEs in which PRN2 is also expressed. Hence, bHLH62 and PRN2 can interact in a physiological context.

bHLH62 is a regulator of polysaccharidic SCW biosynthesis and lignification

For PRN2 to function as a co-regulator of bHLH62 in the transcriptional control of lignin biosynthesis, bHLH62 must itself be a regulator of lignin biosynthesis. At first, this did not seem to be the case because analysis of the cell wall composition by Py-GC/MS in the hypocotyls of a T-DNA insertion line for bHLH62 did not reveal any difference in lignin composition compared with wild-type (Paper III, Supplementary Fig. 6). However, careful characterization of the used T-DNA insertion line (SALK_091124) revealed that despite the absence of full-length bHLH62 transcript, this line could produce a partial bHLH62 transcript including the bHLH-domain (data not shown). To better investigate the potential role of bHLH62 in regulating lignification, we obtained another T-DNA insertion line which we confirmed to be a knock-out mutant (hereafter bhlh62-1) suitable for reverse genetic analysis (Paper IV, Fig. 2). Analysis of cell wall contents by Py-GC/MS of the lower main stem of bhlh62-1 showed a significant decrease in S lignin, and to a lesser extent in G lignin, compared with wild-type (Paper IV, Fig. 3). Similar trends were observed in hypocotyls although these differences were not significant, thus justifying to conduct further analyses in stems rather than hypocotyls. Stem cross-sections stained for lignin with phloroglucinol indicated that the differences in lignin contents between wild-type and bhlh62-1 did not arise from a difference in xylem anatomy and/or in the rate of xylem differentiation, but rather from differences in SCW lignification (Paper IV, Fig. 3). Consistently, additional Klason analysis of cell wall residues from the lower main stem of bhlh62-1 showed decreased lignin content compared with wild-type (Paper IV, Fig. 4). In addition, the amounts of cell wall polysaccharides seemed lower in bhlh62-1 than in wild-type (Paper IV, Fig. 4). Together, our results attribute a role for bHLH62 in the positive
regulation of polysaccharidic SCW edification and of SCW lignification.

**PIRIN2 is an antagonistic interactor of bHLH62 in the regulation of lignification**

To determine the potential relation between PRN2 and bHLH62, we compared the cell wall contents of the lower main stems of wild-type, bhlh62-1, prn2-2, and bhlh62-1 prn2-2 double mutants. The total lignin content of the prn2-2 mutant did not seem to differ from that of the wild-type, while both bhlh62-1 and bhlh62-1 prn2-2 plants displayed a similar decrease in lignin content compared with wild-type (**Paper IV, Fig. 4**). Hence, bHLH62 and PRN2 seem to function together in the regulation of lignification. This regulation of lignification was hypothesized to take place at the transcriptional level, based on the suspected function of PRN2 as a transcriptional co-regulator and based on the predicted function of bHLH62 as a transcription factor. The expression of lignin biosynthetic genes measured by qPCR in the lower main stem of the aforementioned lines showed a great variability, leading to the absence of significant difference between the bhlh62-1 mutant and the wild-type (**Paper IV, Fig. 4**). As could be expected, the expression of most of the monitored lignin biosynthetic genes tended to show an increased expression in the prn2-2 mutant compared with the wild-type (**Paper IV, Fig. 4**). Interestingly, the bhlh62-1 prn2-2 double mutants displayed expression levels similar to those in the bhlh62-1 mutants rather than in the prn2-2 mutants for most of the lignin biosynthetic genes (**Paper IV, Fig. 4**), indicating that PRN2 functions as an antagonistic co-regulator of bHLH62. Hence, while bHLH62 could be involved in regulating polysaccharidic SCW biosynthesis and cell-autonomous lignification without PRN2, PRN2 and bHLH62 seem to function as an antagonistically in the non-TEs, in order to modulate the non-cell autonomous component of lignification (**Paper IV, Fig. 5**). The non-cell autonomous lignification is also transcriptionally regulated by a PRN2- and HUB2-containing protein complex and it remains to be determined whether or not bHLH62 belongs to the same complex in non-TEs. In any case, it is clear that there exists at least one PRN2-containing protein complex (see Figure 5 for a plausible complex) which transcriptionally regulates the non-cell autonomous lignification. Although it is a common feature across the tree of life that most proteins do not function as single units but as multimers, most of these multimers are homodimers and the occurrence of high order
heteromultimers is very rare (Lynch, 2013). Hence, the existence of a potential complex involving at least HUB2 and its obligatory interactors (Cao et al., 2008) as well as PRN2 would represent a somewhat idiosyncratic situation which may be specific to the regulation of non-cell autonomous lignification.

Figure 5: Plausible protein complex regulating lignin gene expression. Information on protein-protein interaction were obtained from the Arabidopsis Interactome Mapping Consortium (Dreze et al., 2011; http://interactome.dfci.harvard.edu) and from the work presented in this thesis to assemble a plausible protein complex that could regulate the expression of lignin biosynthetic genes. According to this model, PRN2, HUB2 and bHLH62 could function within a unique protein complex which links the establishment of H2Bub1 chromatin marks to the downstream H3K4me3 and to the subsequent transcriptional activation of the target genes.
II.1.3. Conclusions and perspectives on new regulations of xylem lignification

The work presented above experimentally confirmed that lignification of xylem TEs is partially non-cell autonomous and occurs partially post-mortem in the Zinnia xylogenic cell suspensions as well as in whole Zinnia and Arabidopsis plants. These systems subsequently enabled identifying the Arabidopsis PIRIN2, which is not expressed in lignifying xylem cells, but which nevertheless regulates lignification, and therefore constitutes a specific regulator of non-cell autonomous lignification. The functional characterization of PRN2 revealed that the non-cell autonomous component of lignification is transcriptionally regulated, including by the modulation of chromatin marks associated with active transcription. Transcriptional regulation of lignification at the chromatin level had previously been hypothesized (Hussey et al., 2015) but the work presented here provides the first demonstration of a direct regulation of lignin biosynthetic genes via chromatin modifications. Furthermore, the non-cell autonomous lignification is regulated by at least one PRN2-containing protein complex (Figure 5), or by two separate complexes in which PRN2 would act as an antagonist of the positive transcriptional regulators HUB2 and bHLH62. Finding and characterizing other potential protein interactors for PRN2, HUB2 or bHLH62 could shed light on whether PRN2 functions as a general negative transcriptional co-regulator in different protein complexes or whether PRN2, HUB2 and bHLH62 operate within one single complex. Either way, the existence of non-cell autonomous lignification and of its specific regulation(s) constitutes one example of cooperation between the different xylem cell types during xylem development. Such cell cooperation likely involves some form of coordination between the cooperating cells. Indeed, mobile factors such as proteins, peptides, micro-RNAs and hormones have already been shown to be involved in the coordination of early xylem differentiation with other tissues, or within the same tissue (Jacobs, 1952; 1954; Fosket and Torrey, 1969; Iwasaki and Shibaoka, 1991; Yamamoto et al., 1997; Matsubayashi et al., 1999; Zhong and Ye, 1999; Eriksson et al., 2000; Emery et al., 2003; Biemelt et al., 2004; Caño-Delgado et al., 2004; Motose et al., 2004; Ohashi-Ito et al., 2005; Ito et al., 2006; Carlsbecker et al., 2010; Kobayashi et al., 2011; Kondo et al., 2011; Pesquet and Tuominen, 2011; De Rybel et al., 2014; Kondo et al., 2014). Investigating the intercellular signalling
between differentiating cells at later stages of xylem formation could reveal factors coordinating the cooperation between the cells involved in non-cell autonomous lignification. Regarding potential applications, the knowledge that parenchyma cells are involved in the lignification of xylem TEs and fibres means that these xylem parenchyma cells represent a new target cell type for biotechnological strategies to overcome lignocellulosic biomass recalcitrance. For example, orthologues of the Arabidopsis PIRIN2 could be targeted to reduce lignification or alter lignin composition in species that are relevant for biomass production, in order to ease the conversion of lignocellulosic biomass into biofuels (see patent by Boerjan, W., Vanholme, R.M.I., Turumtay, H., Tuominen, H. Escamez, S. and Zhang, B.; 2015).

II.2. New regulations of xylem cell death

II.2.1. Restriction of cell death to the target cell type

II.2.1.a. Dying TEs implement safeguards to protect the surrounding cells

Xylogenic cell suspensions as a relevant system to functionally characterize METACASPASE9

The potential involvement of the Arabidopsis METACASPASE9 (MC9) in TE PCD had earlier been hypothesized (Turner et al., 2007; Bollhöner et al., 2013) based on two grounds: (i) Because several metacaspases (MCs) had been linked to the regulation of several types of plant PCD (He et al., 2008; Coll et al., 2010; Watanabe and Lam, 2011) and (ii) because MC9 is the only MC gene whose expression is induced during TE differentiation in Arabidopsis cell suspensions (Turner et al., 2007). Comparison of Arabidopsis mc9 loss-of-function mutants with wild-type seedlings did not reveal any significant change in the distance of protoxylem TE PCD from the root tip which suggested that MC9 did not control PCD (Bollhöner et al., 2013). On the other hand, such analyses showed a great variability between and within experiments, possibly due to the difficulties associated with live imaging of xylem in planta and because very few TEs die at any given time. To overcome these technical hindrances we undertook the functional characterization of MC9 using a reverse genetic approach in Arabidopsis xylogenic cell suspensions (Pesquet et al., 2010) (Paper II). First, we confirmed the expected TE-specific
expression of MC9 by monitoring a MC9:GFP fusion expressed under the transcriptional control of MC9 endogenous promoter in differentiating cell suspensions (Paper II, Fig. 1). Next, we generated cell lines with down-regulated MC9 expression using RNAi (hereafter MC9-RNAi) and we verified that autolysis was impaired in MC9-RNAi TEs (Paper II, Fig. 1), as expected from the known role of MC9 in TE autolysis in whole plants (Bollhörner et al., 2013). Hence, the Arabidopsis xylogenic cell suspension system appeared as a suitable system to further characterize the role of MC9 in TEs, including its potential involvement in programmed cell death.

**METACASPASE9 operates in dying TEs to prevent ectopic death of non-TEs**

To monitor TE PCD, differentiating wild-type and MC9-RNAi cell suspensions were stained with the viability dye fluorescein diacetate (FDA) and observed at different times over the course of their differentiation. The proportion of dead TEs did not significantly differ between wild-type and MC9-RNAi cell suspensions at any time point (Paper II, Fig. 1), suggesting the absence of a role for MC9 in regulating TE cell death. Instead, observations with the FDA staining revealed the occurrence of ectopic cell death of parenchymatic non-TEs in differentiating MC9-RNAi lines (Paper II, Fig. 2). Because MC9 is specifically expressed in the TE cell type, we hypothesized that the MC9-RNAi TEs were responsible for the ectopic cell death of the non-TEs. Indeed, earlier work in Zinnia xylogenic cell suspensions have shown that autolyzing TEs could release potentially harmful cellular material, whose deleterious effect had to be prevented by the secretion of a protective inhibitor (Endo et al., 2001). Consistent with a harmful effect of dying MC9-RNAi TEs on non-TEs, the temporal progression of non-TE cell death correlated with the temporal progression of TE PCD in MC9-RNAi lines (Paper II, Fig. 2). Inducing the differentiation of a mix of wild-type and MC9-RNAi cells (1:1) suppressed the ectopic cell death of the non-TEs, while purposely reducing the differentiation of TEs in MC9-RNAi cell suspensions also reduced the ectopic cell death, further suggesting that MC9-RNAi TEs caused the ectopic non-TE death. Therefore, the collective evidence strongly suggests that MC9 operates within the differentiating TEs where it implements safeguards to prevent the dying TEs from becoming harmful for the non-TEs.
II.2.1.b. The spatial restriction of cell death requires tight regulation of autophagy in TEs

**METACASPASE9 regulates the level of autophagy in TEs**

In a review article Bozhkov and Jansson (2007) had proposed that the restriction of cell death to the target cell type during PCD in plants relies on the cellular process of autophagy. Interestingly, autophagy was associated with the progression of PCD in xylem fibres (Courtois-Moreau et al., 2009) and in TEs (Kwon et al., 2010). Furthermore, during the PCD that occurs in developing spruce somatic embryos, a metacaspase regulates the level of autophagy and thereby enables the correct progression of cell death (Minina et al., 2013). Hence, we hypothesized that MC9 could regulate autophagy in TEs in order to spatially restrict cell death. Cell type-specific quantification of autophagy by differential interference contrast (DIC) microscopy observations after inhibition of autophagic body degradation by Concanamycin A (Yoshimoto et al., 2004) revealed a higher level of autophagy in TEs than in non-TEs (**Paper II, Fig. 3**). Interestingly, we observed a higher level of autophagy in *MC9*-RNAi TEs than in wild-type TEs, while the level of autophagy remained similar in the non-TEs of both genotypes. Hence, MC9 appears to tune-down the level of autophagy in TEs. This implies that MC9 functions in part before TE PCD, and not solely during post-mortem autolysis as previously hypothesized based on the cytoplasmic localization of MC9 in whole plants and because MC9 is active at low pH (Bollhöner et al., 2013). The acidic pH requirement for MC9 activity and our observation that MC9 functions pre-mortem led us to hypothesize that MC9 could also be localized in the vacuoles of TEs, an acidic organelle where part of the autophagic process takes place. To test this hypothesis, we observed a MC9:GFP fusion protein expressed under the control of *MC9* promoter in the TEs of Arabidopsis roots by using confocal laser scanning microscopy (cLSM). GFP signal was mainly detected in the cytoplasm of TEs, although GFP-positive punctuates could also be observed in the vacuolar compartment (**Paper II, Fig. 4**). The number of vacuolar MC9:GFP punctuates was significantly increased after treating the seedlings with Concanamycin A, which inhibits vacuolar degradation of autophagic bodies as well as quenching of the GFP by the acidic pH (**Paper II, Fig. 4**). Simultaneous treatment of the seedlings with Concanamycin A and the inhibitor of autophagosome formation wortmannin (Blommaart et al., 1997) significantly decreased the amount of vacuolar
MC9:GFP punctuates compared with Concanamycin A treatment alone (Paper II, Fig. 4), which suggests that these punctuates could represent autophagic bodies. Importantly, the vacuolar localization of MC9 indicates that MC9 can function before TE PCD, making it possible for MC9 to regulate the level of autophagy in living TEs.

The regulation of TE autophagy by MC9 restricts cell death to the target cell type

To test whether MC9 could restrict cell death to the TEs by tuning down the level of autophagy in this cell type, we undertook repressing autophagy specifically in MC9-RNAi TEs. For this purpose we created an RNAi fragment targeting the autophagy gene ATG2 (ATG2-RNAi), which is known to be essential for autophagy in Arabidopsis (Inoue et al., 2006; Hackenberg et al., 2013). The ATG2-RNAi was placed under the transcriptional control of the promoter of the SCW-specific cellulose synthase synathase gene IRREGULAR XYLEM1 (IRX1; Turner and Somerville, 1997; Taylor et al., 2000), which is active in the TEs but not in the parenchymatic non-TEs. Next, we generated MC9-RNAi proIRX1::ATG2-RNAi double transgenic cell lines in which we could verify that MC9 was still down-regulated (Paper II, Supplementary Fig. 1), and that the level of TE autophagy was lower than in MC9-RNAi cell suspensions (Paper II, Fig. 5). Interestingly, decreasing the level of autophagy in MC9-RNAi TEs by the additional proIRX1::ATG2-RNAi autophagy knock-down suppressed the ectopic death of the non-TE cells (Paper II, Fig. 5). Therefore, tight regulation of the level of autophagy in TEs by MC9 is required to confine cell death to the TE cell type (Figure 6), at least in the Arabidopsis xylogenic cell suspension system. Because of the numerous plant systems in which metacaspase activity and autophagy have been detected during programmed cell death (For review see Minina et al., 2014; Van Durne and Nowack; 2016), it is tempting to hypothesize that MC-dependent regulation of autophagy during PCD could represent a general mechanism for protecting the surrounding cells.
**Figure 6:** Plausible model for the restriction of cell death to TEs by the MC9-mediated control of TE autophagy. In this model, the level of autophagy in TEs is tuned down by MC9 to prevent the excessive release of harmful molecules (e.g. toxins, lytic enzymes or pro-death signal peptides) and/or to foster the release of safeguard molecules (e.g. inhibitors of lytic enzymes or pro-life signal peptides) from the TEs.
II.2.2. Conclusions and perspectives on new regulations of xylem cell death

The work presented above relied on the functional characterization of the candidate xylem PCD regulator METACASPASE9 to discover new regulations of the programmed cell death of tracheary elements. The quantification of TE PCD at the level of the cell population in Arabidopsis xylogenic cell cultures (Paper II, Fig. 5), together with the previous observation of TE PCD in the mc9 loss-of function mutant plants (Bollhöner et al., 2013), did not suggest a direct involvement of MC9 in the execution of TE PCD per se. Instead, MC9 regulates the level of autophagy in TEs to prevent them from becoming harmful for the surrounding cells. Indeed, TEs must accumulate a molecular arsenal which executes the TE post-mortem autolysis and which could contribute to the execution of the cell death programme. It is thus conceivable that while preparing for cell death and autolysis, as well as during the post-mortem autolysis, TEs could release harmful molecules against which safeguards must be implemented during the progression of TE PCD (as exemplified by Endo et al., 2001). The implementation of such safeguards relies at least partially on the regulation of autophagy by MC9 in TEs, which therefore represents a form of cell death regulation in that it spatially restricts cell death. This newly discovered regulation of xylem cell death also represents an example of cell cooperation between the TEs and the non-TEs because the non-TEs are protected at least in part by the TEs themselves. Investigating the molecules released by the TEs with different levels of MC9 expression or different levels of autophagy, for example by analyzing the extracellular medium of xylogenic cell suspensions, could shed light on the intercellular signalling that restricts xylem cell death to the target cell types downstream of autophagy. The results of investigations on the role of autophagy during PCD have often been difficult to interpret, leading to conflicting views on whether autophagy is a “pro-life” or a “pro-death” process, both in plant systems (Lv et al., 2014; Minina et al., 2014) and in animal systems (Denton et al., 2012; Clarke and Puyal, 2012; Shen et al., 2012; Liu and Levine, 2014). Part of the difficulty in interpreting the wealth of data on autophagy during PCD may arise from the fact that most studies relied on the constitutive promotion or constitutive inhibition of autophagy, rather than on alterations of autophagy specifically in the cells undergoing PCD. In contrast, the present study relied on cell-type specific down-regulation of autophagy and provided
evidence that autophagy in certain cells can dramatically affect the surrounding cells, consistent with recent discoveries that autophagy can influence protein secretion (Torisu et al., 2013; Bhattacharya et al., 2014; Cinque et al., 2015; Kang et al., 2015; Kraya et al., 2015; Nowag and Münz, 2015). It is therefore possible that modulation of autophagy could be used in particular target cells to affect or kill the surrounding cells for therapeutic or biotechnological applications.
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