Molecular dissection of the role of plant hormones in perennial shoot branching

Molekylær disseksjon av plantehormoners rolle i forgreining hos flerårige planter

Philosophiae Doctor (PhD) Thesis

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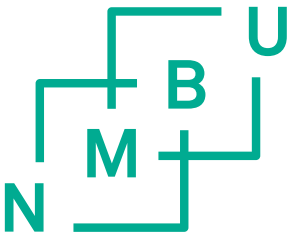

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Vinterbro, 2023
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Summary

Shoot architecture in deciduous trees is determined by the apical meristems and the lateral branching. The formation of above-ground architecture perennials is regulated by a complex spatial-temporal regulation. For decades, intensive research has focused on the identification and characterization of phytohormones that are involved in controlling the complex shoot branching mechanism. This process is governed by a complex hormonal network that forms a strong foundation for understanding the molecular basis for shoot branching. Although, enormous amount of work has been conducted in understanding the mechanism of shoot branching in annuals, very little is known about the molecular process regulating that control branching in woody perennials.

The thesis includes three separate studies. The overall aim has been to provide insights on the knowledge gap related to the role of strigolactone (SL) and gibberellin (GA) along with other interacting hormone pathways in regulating shoot branching in the model perennial woody species (Hybrid aspen). In paper I, the presence and the role strigolactone pathway genes in hybrid aspen was investigated. Previous studies have shown the presence of SL pathway genes in annuals but very little was known about the involvement of SL pathway in perennials. For the first time in perennials, we detected the presence of *DWARF27* (*D27*), *LATERAL BRANCHING OXIDOREDUCTASE* (*LBO*) and *DWARD53-like* (*D53-like*) along with the in-depth understanding of the presence of all the SL pathway gene in above and below ground tissues. The most important finding of this paper was that SL biosynthesis genes *MORE AXILLARY GROWTH3* (*MAX3*) and *MAX4* expressed in high levels in nodal bark rather than AXBs and AXBs expressed high level of SL perception and signaling genes (*MAX2*, *D14* and *D53*) indicating that SL and its precursors are transported from node to AXBs in perennials instead of long-distance transport from roots to axillary buds (AXBs). AXB activation induced by decapitation downregulated most of the genes downstream of *MAX4* apart from *LBO*. In order to specifically understand the role of SL in AXB inhibition, GR24-feeding inhibited the AXB outgrowth once the activation has started, and SL helps to preserve the AXBs in quiescent state until the next growing season by restraining embryonic shoot elongation during the formation of AXBs. Along with SL, both nodes and AXBs also produced GA which may work mutually in promoting AXB activation by overtaking the effects of SL (paper II).

Contrary to the view that GA are branch-inhibitors, our data show that they promote shoot branching. Comprehensive transcript and metabolite studies decoded the role of GA in AXB

activation and outgrowth by rapidly reducing deactivation gene *GA2ox*. This suggests an increase in the GA pool through downregulation of GID1 signaling gene. On the other hand, *GA3ox2*-mediated *de novo* biosynthesis supports AXB elongation and branch formation. Like SL, nodes support the supply of GA precursors from nodes to AXBs. We could show through the combination of metabolite and transcript analysis that $GA_{3/6}$ produced in the quiescent AXBs targets $GA_{1/4}$ through GA2ox mediated deactivation. The study on SL and GA interaction reveal that both the pathways are entangled and confirmed that GA-deactivation is the effective way to regulate GA levels. In paper III, by employing the first ever de novo transcriptome analysis in hybrid aspen, we identified the early molecular responders to AXB activation. We analyzed the global overview of Auxin, Cytokinin, Abscisic acid, Jasmonic acid, Salicylic acid, Brassinosteroids pathway genes at different time points post-decapitation along with validating the SL and GA genes studies in Paper I and II. Our data provided a comprehensive understanding as well as a starting point to understand the previous untouched hormonal pathways in studying shoot branching in perennial woody species.

In conclusion, PhD work has shown the molecular dissection of the role of various plant hormonal pathways involved in shoot branching in perennial woody species, hybrid aspen.

Sammendrag

Skuddarkitektur hos løvtrær bestemmes av det apikale skuddmeristemet og forgreining av sideskudd. Reguleringen av forgreining er kompleks i tid og rom. I mange tiår har forskning satt søkelyset på å identifisere plantehormoner som kontrollerer denne komplekse mekanismen. Det er i dag kjent at prosessen styres av et nettverk av plantehormoner som igjen danner grunnlaget for den molekylære reguleringen. Forskning på mekanismer som styrer forgreining har imidlertid frem til nå hovedsakelig omfattet ettårige planter. Forståelsen av mekanismene og den molekylære prosessen som regulerer forgrening hos flerårige, treaktige planter er derfor lite er kjent.

Det overordnede målet i denne avhandlingen var å gi innsikt i rollen til plantehormoner, spesielt med fokus på strigolakton (SL) og gibberellin (GA), hvordan de samspiller med andre hormoner, og regulerer forgreining hos treaktige planter. Avhandlingen består av tre separate artikler der den flerårige treaktige arten hybridosp (*Populus tremula* × *Populus tremuloides*) er benyttet som modellplante. I artikkel I ble uttrykket og rollen til gener involvert i «SL-veien» undersøkt. RNA ble sekvensert fra avkuttede sideknopper for å studere de molekylære endringene som skjer ved slik avkutting. Tidligere studier har vist uttrykk av SL-biosyntesegener hos ettårige planter, men svært lite er kjent hos flerårige planter. For første gang ble det påviste uttrykk av gener i «SL-veien» i hybridosp. Resultatene viste uttrykk av genet *DWARF27* (*D27*), *LATERAL BRANCHING OXIDOREDUCTASE* (*LBO*) og *DWARD53-lignende* (*D53-lignende*) i tillegg til å gi innsikt i tilstedeværelsen av alle genuttrykk i SL-veien, både i overjordisk og underjordisk vev. Det viktigste funnet i denne artikkelen var at SL-biosyntesegenene *MORE AXILLARY GROWTH3* (*MAX3*) og *MAX4* var høyt uttrykt i nodiebark i stedet for sideknopper. Sideknopper viste høyt uttrykk av SL-persepsjons- og signalgener (*MAX2*, *D14* og *D53*) som indikerer at SL og SL-forløpere transporteres fra nodier til sideknopper i stedet for at det skjer langdistansetransport fra røtter til sideknopper. Aktivering av sideknopper, indusert ved avkutting av toppskudd, nedregulerte de fleste gener nedstrøms for *MAX4* bortsett fra *LBO*. For å forstå rollen til SL i sideskuddhemming ble sideknopper behandlet med GR24, en syntetisk SL analog. Veksten til sideknoppene, etter avkutting av toppskuddet, ble hemmet av GR24. Dette viser av SL bidrar til å holde sideknoppene i en «hvilende tilstand» til neste vekstsesong ved å hemme strekningsveksten hos embryonale skudd. Sammen med SL, produserer både nodier og sideknopper GA, som kan fremme aktivering av sideknopper ved å overta for SL (artikkel II). I motsetning til tidligere

teorier om at GA hemmer sideskuddforgreining, viste våre resultater at GA fremmer forgreining (artikkel II).

Omfattende transkripsjons- og metabolittstudier tydeliggjorde rollen til GA i aktivering og vekst av sideskudd ved raskt å redusere transkripsjonen av GA-de-aktiveringsgenet GA2ox. Dette antyder en økning i «GA-poolen» ved nedregulering av GA-signaleringsgenet GID1. På den annen side, *GA3ox2*-mediert *de novo* biosyntese er involvert i bryting og vekst av sideknopper. I likhet med SL, tilføres GA-forløpere fra nodiene til sideknoppene. Metabolitt- og transkripsjonsanalyser viste at $GA_{3/6}$ produsert i de hvilende sideknoppene påvirker $GA_{1/4}$ -deaktivering ved å stimulere GA2ox. Studien av samspillet mellom SL og GA viser at begge «veier» er koblet og studien bekrefter at deaktivering av GA er en effektiv måte å regulere GA-nivået i sideknopper. I artikkel III ble det, for første gang med hybridosp som modell, gjennomført en *de novo* transkriptomanalyse. Vi identifiserte tidlige molekylære endringene i respons på aktivering av sideknopper. Resultatene ga en oversikt over plantehormonene auxin, cytokinin, abscisinsyre, jasmonsyre, salisylsyre, samt gener i «brassinosteroid-veien» på forskjellige tidspunkt etter avkutting i tillegg til å validere SL- og GA-gen-studiene i artikkel I og II. Resultatene bidro til en større forståelse for de hormonelle veiene som hittil ikke har vært undersøkt men som regulerer skuddforgrening i flerårige trearter. Denne avhandlingen har gitt ny kunnskap, og klargjort forskjellige hormonelle veier involvert i skuddforgrening hos flerårige trearter.

List of publications

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Strigolactone-based node-to-bud signaling may restrain shoot branching in hybrid aspen.**

Niveditha Umesh Katyayini, Päivi L H Rinne, Christiaan van der Schoot (2019).
Plant and Cell Physiology, 2019, Volume 60, Issue 12, Pages 2797–2811
DOI: 10.1093/pcp/pcz17060

II. **Dual Role of Gibberellin in Perennial Shoot Branching: Inhibition and Activation.**

Niveditha Umesh Katyayini, Päivi L. H. Rinne, Danuše Tarkowská, Miroslav Strnad and Christiaan van der Schoot (2020).
Frontiers in Plant Science, 2020, Volume 11, Article 736
DOI: 10.3389/fpls.2020.00736

III. ***De novo*** **transcriptomic analysis of axillary buds in response to decapitation reveals the key hormone pathways in Hybrid aspen**

Niveditha Umesh Katyayini, Mallikarjuna Rao Kovi

(Manuscript)

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1. Introduction

1.1 Plant architecture

Plants as sessile organisms are unable to escape from adverse conditions. Rather, they need to get adapted to prevailing environmental conditions by altering their architecture. The root architecture is altered in response to the availability of nutrients and inherent genetic factors. The shoot system must sustain a range of biotic and abiotic factors such as, herbivory and nutrient supply.

The degree of adaptability of plants to a different architectural plan lies in their indeterminate developmental nature. Their general body structure including the apical-basal axis, is determined at an early stage of the plant's life cycle during embryogenesis, resulting in the formation of a main body axis called shoot apical meristem (SAM) at the apex and basally formed root apical meristems (RAM). The meristem activity during post-embryonic development leads to the formation of root- and shoot- specific organs modifying the body plan throughout the plant's lifecycle to adjust to existing environmental conditions (De Smet and Jürgens, 2007). Lateral shoots or branches are produced from the so-called secondary SAM called axillary meristems (AXMs), that are primarily formed in the axil of the leaves where they join the stem. SAM mainly determines the growth of above ground parts of the plants by forming phytomers (Hollender and Dardick, 2015), the repetitive structural unit consisting of a node (a node is defined as the joint in the stem where a leaf develops), an internode (an internode is the stem section between two nodes) where the stem vascular development occurs, and an AXM or axillary bud (Fig. 1). Both, AXM and SAM have the same growth potential which can give rise to different plant organs. Each AXM functions as a new SAM that forms a secondary growth axis from the lateral bud situated at the leaf axils. Thus, the plant architecture is largely determined by AXM and SAM (Pautler *et al.*, 2013), the development and maintenance of which are crucial for building the plant architecture. Although in *Arabidopsis*, the formation of AXMs is delayed, perennials AXMs arise initially in continuity with the SAM to form axillary buds (AXBs) (Garrison, 1955; Esau, 1977). Thus, the regulation of AXB formation plays an important role in determining shoot architecture predominantly in the development of lateral branches.

The generic architecture of a tree species is determined by the successive architectural phases, called the "architectural model" (Halle and Oldeman, 1970), determining its growth strategy. The tree crown architecture is phyllotactically determined where the AXBs may remain dormant or can form branches post initiation (Barthélémy and Caraglio, 2007). It is uncertain if the herbaceous branching models can be transferred directly to woody perennials, considering their different shoot size,

lifespan, and AXB composition. The transport paths between roots and AXBs might be too long to be effective in branch regulation. AXBs of perennials, unlike those of herbaceous species, are elaborate structures with sturdy scales that enclose a dwarfed, rosette-like embryonic shoot (ES) (Rinne *et al.*, 2015). The branching mechanism and AXB formation in perennial woody species is not well understood yet. The timing of the AXB outgrowth follows two major branching patterns, namely, sylleptic (immediate) and proleptic (delayed) (Hallé, Oldeman and Tomlinson, 1978). Sylleptic branching refers to the formation of branches from AXBs in the same growing season without rest (Wu and Stettler, 1998). In contrast, proleptic branches are formed after the AXBs are formed after a period of dormancy/ rest (Hallé, Oldeman and Tomlinson, 1978; Barthélémy and Caraglio, 2007). The occurrence of sylleptic branching is observed in both temperate zone (Ceulemans *et al.*, 1990; Wu and Hinckley, 2001) as well as tropical woody plants (Hallé, Oldeman and Tomlinson, 1978; Cline and Dong-IL, 2002) usually affected by environmental factors. Proleptic branching determines the outgrowth of the resulting shoots from overwintered buds formed during the preceding growing season by maintain strong apical dominance (Hallé, Oldeman and Tomlinson, 1978; Cline, 1997).

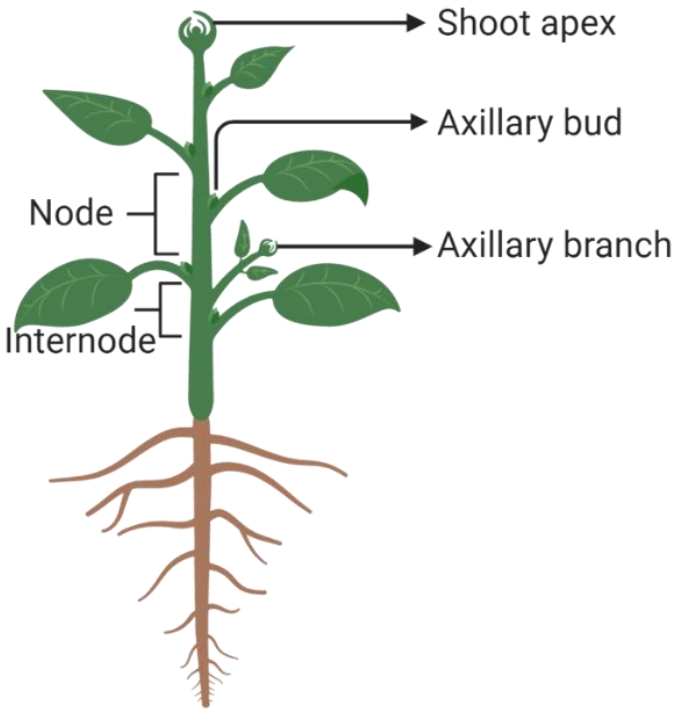


Figure 1. An illustration of plant shoot architecture.

1.2 Apical dominance and Shoot branching

Apical dominance is a central factor that determines branching patterns and shoot branching (Sterck, 2009). AXBs are subjected to apical dominance, a phenomenon where the proliferating shoot apex controls the quiescence of AXBs (Cline, 1997), a classic example where one organ affects the other through "correlative inhibition" (Sachs, 1991; Hillman, 1984). The growth of the AXB is under the control of the shoot apex. AXBs remain inhibited until the apical dominance is removed. However, after bud formation is complete, buds may become dormant or grow into shoots without entering a period of dormancy. In perennial research, dormancy, referred to as a temporary suspension of visible growth in any plant structure containing a meristem (Rohde and Bhalerao, 2007) can be classified into three types, namely: paradormancy (inhibition of growth is caused by other plant parts), ecodormancy (controlled by external environmental conditions) and endodormancy (triggered by internal factors) (Considine and Considine, 2016; Lang *et al.*, 1987).

The inactive state of the AXBs is controlled by auxins produced at the apex, which is referred to as "para-dormancy" (Thimann and Skoog, 1934; Phillips, 1975; Cline, 1991; Cline, 1997; Lang *et al.*, 1987). In plants with strong apical dominance, AXB inhibition is removed by the process called "decapitation" (Rinne, Tuominen and Sundberg, 1993; Cline, 1997), causing relatively any unbranched shoot to change its morphology. (Cline, 1997) categorized branching into 4 stages based on the outgrowth of lateral buds by shoot apex removal: (I) formation of lateral buds; (II) imposition of apical dominance; (III) release of apical dominance by initiation of lateral bud outgrowth, and (IV) elongation and development of shoot branch. In some circumstances, AXBs can re-enter dormancy indicating that the bud release to be a temporary stage(Shimizu-Sato and Mori, 2001).

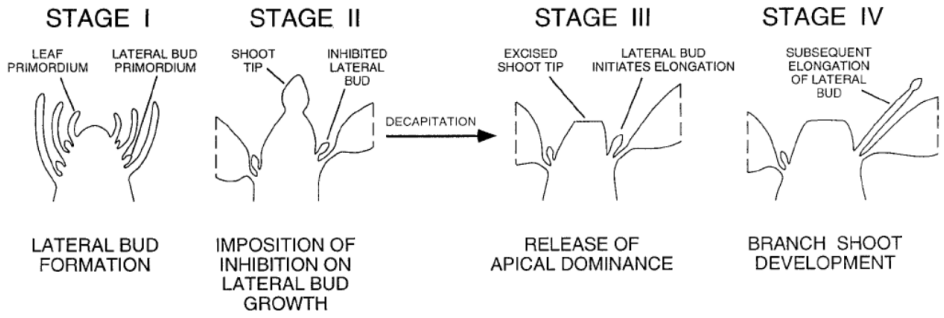


Figure 2. Four stages of apical dominance describing bud release from shoot apex before and after decapitation (adapted from (Cline, 1997).

Shoot branching is an excellent example of a highly plastic developmental process, obtained as a result of the spacio-temporal regulation of axillary bud outgrowth (Rameau *et al.*, 2015). Shoot branching is predominantly dependent on the axillary meristem formation and their subsequent outgrowth regulated by genetic, environmental and hormonal signals (Schmitz and Theres, 2005; Dun, Ferguson and Beveridge, 2006; Ongaro and Leyser, 2008). Branching is highly regulated by plant hormones, which control the developmental activity and distribution according to distinct growth conditions. Release of AXBs from inhibition via decapitation has been used widely in shoot branching studies, a process that can be reverted by exogenous application of auxin to the stump of the decapitated shoot (Thimann and Skoog, 1934; Phillips, 1975; Cline, 1991; Cline, 1997). Recent studies have shown the shoot branching mechanism in annuals (reviewed in (Ongaro and Leyser, 2008; Barbier *et al.*, 2019) which serves as a backbone in understanding branching mechanism in perennials. The complex interplay of the phytohormones during AXB outgrowth is associated with branch inhibitors such as auxin and strigolactone (Thimann and Skoog, 1933; Thimann and Skoog, 1934; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008) as well as gibberellins (Ni *et al.*, 2015; Katyayini *et al.*, 2020).

The first part introduction gives an overview of the nature, occurrence, biochemical features and pathways of strigolactones and gibberellins in perennials. The last part is an outline of the role of these two hormones in controlling shoot branching, also focusing on the interaction between the two.

1.3 Hybrid aspen as a model system in perennials

During the last few decades, much of the focus was given to study shoot branching mechanism in annuals like *Arabidopsis thaliana*. Although annuals like *A. thaliana* were chosen as model plant species in several studies for obvious reasons (small plant size, small genome size, short growing time, high fecundity) (Jansson and Douglas, 2007), a single model system cannot be used to explain all the biological questions. In several aspects, trees represent the opposite extreme of *Arabidopsis* with respect to its long-life spans, seasonality of growth and growth habit (Jansson and Douglas, 2007). The *Populus* genus includes many important woody species, one such important species, *Populus trichocarpa* (Tuskan *et al.*, 2006), has been selected as model tree species for its small genome size (422.9 Mb) and rapid growth.

Branching in woody perennials is generally influenced by both apical dominance and apical control (Cline, 2000; Wilson, 2000). The genus *Populus* shows variation in sylleptic and proleptic branching patterns even within their species genotypes, making it a useful model system to study

shoot branching (Wu and Hinckley, 2001; Wu and Stettler, 1998). In my PhD study, a deciduous proleptic woody perennial, hybrid aspen (*Populus tremula* x *P. tremuloides*- T89 clone) was used (Fig. 3), in which the apical dominance inhibits AXBs branching in the current year, but not the embryonic shoot (ES) development (Rinne *et al.*, 2016). The hybrid aspen AXBs at BMP contains five primordia that results in the formation of scales and protect the next 10 primordia by forming embryonic leaves (Rinne *et al.*, 2015). The development of the AXB is completed at this point and is referred as the bud maturation point (BMP) (Rinne *et al.*, 2015). AXBs remain para-dormant until the following growing season which are activated by decapitation to study the effect of apical dominance on branching. This makes it easier to study the process involving AXB activation rather than the formation of AXB. In trees with sylleptic branching the AXBs are formed in the same growing season thus the BMP is lacking.

Figure 3. Hybrid aspen (T89 clone) grown under controlled conditions in the green house

2. Strigolactones

2.1 History

Strigolactones (SL) were initially discovered as the metabolites that are exuded from the roots of host plants to stimulate seed germination of root parasitic plants (Yoneyama *et al.*, 2010). The first identified SL, Strigol, was isolated from the root exudates of Cotton (*Gossypium hirsutum* L.) as a germination stimulant for witchweed (*Striga lutea* Lour.) (Cook *et al.*, 1966; Cook *et al.*, 1972). But cotton is a non-host for *Striga* species, indicating that, not only host plants (Sorghum, Maize and Proso millet) but also non-host plants can exude SL-like compounds to the rhizosphere (Wang and Bouwmeester, 2018). This led to the proposal that SLs are ubiquitously present in higher plants, possessing different functions. Since then, many compounds that are structurally similar to strigol have been identified in several species of the plant kingdom, and (Butler, 1995) collectively termed them as SLs. Later, another germination stimulant, orobanchol was isolated from the root exudates of red clover (*Trifolium pretense* L.) suggesting that distinct parasitic plants *Orobanche* and *Striga* promote the germination of parasitic plant seeds by utilizing SLs produced from the host plant roots. This has led to serious parasitic infestation by *Striga* species and loss of yield in crop plants (mostly monocotyledonous) in agriculture (Tsuchiya and McCourt, 2009).

In addition to the detrimental role of SL as a germination stimulant of root parasitic plants, recently SLs were found to play a crucial factor in establishing a symbiotic relationship between host plant roots and arbuscular mycorrhizal (AM) fungi to induce hyphal branching which is beneficial for the host plant (Akiyama, Matsuzaki and Hayashi, 2005; Besserer *et al.*, 2006) (Fig. 4). The SL mediated branching plays a crucial role in the interchange of sugars and nitrogen between plants and fungi, respectively (Govindarajulu *et al.*, 2005; Gutjahr, 2014). This mutualistic relationship play an important role to complement the fact that plants release elevated levels of SLs into the soil under phosphate or nitrogen limiting conditions by triggering the developmental response of AM fungi (Gutjahr, 2014).

Few years later, the hidden role of SLs as a crucial regulator of shoot branching inhibitor was elucidated, indicating the role of SL as a new plant hormone class in regulating above-ground plant architecture (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). The biosynthetic mutant *carotenoid cleavage dioxygenase 8* (*ccd8*) are SL deficient in pea i.e., SL inhibits the branching and supply of SL analog GR24 restores the high-branching (Gomez-Roldan *et al.*, 2008). The mutants affected by perception signals can be rescued by exogenous application of GR24 indicating that SLs are long-sought signaling molecules that inhibit shoot branching (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Further studies have shown that, SLs are not only involved in shoot

branching, but also in controlling wide range of plant developmental processes like, root architecture, leaf senescence, cambial growth, secondary growth of shoot and photomorphogenesis (Seto *et al.*, 2012).

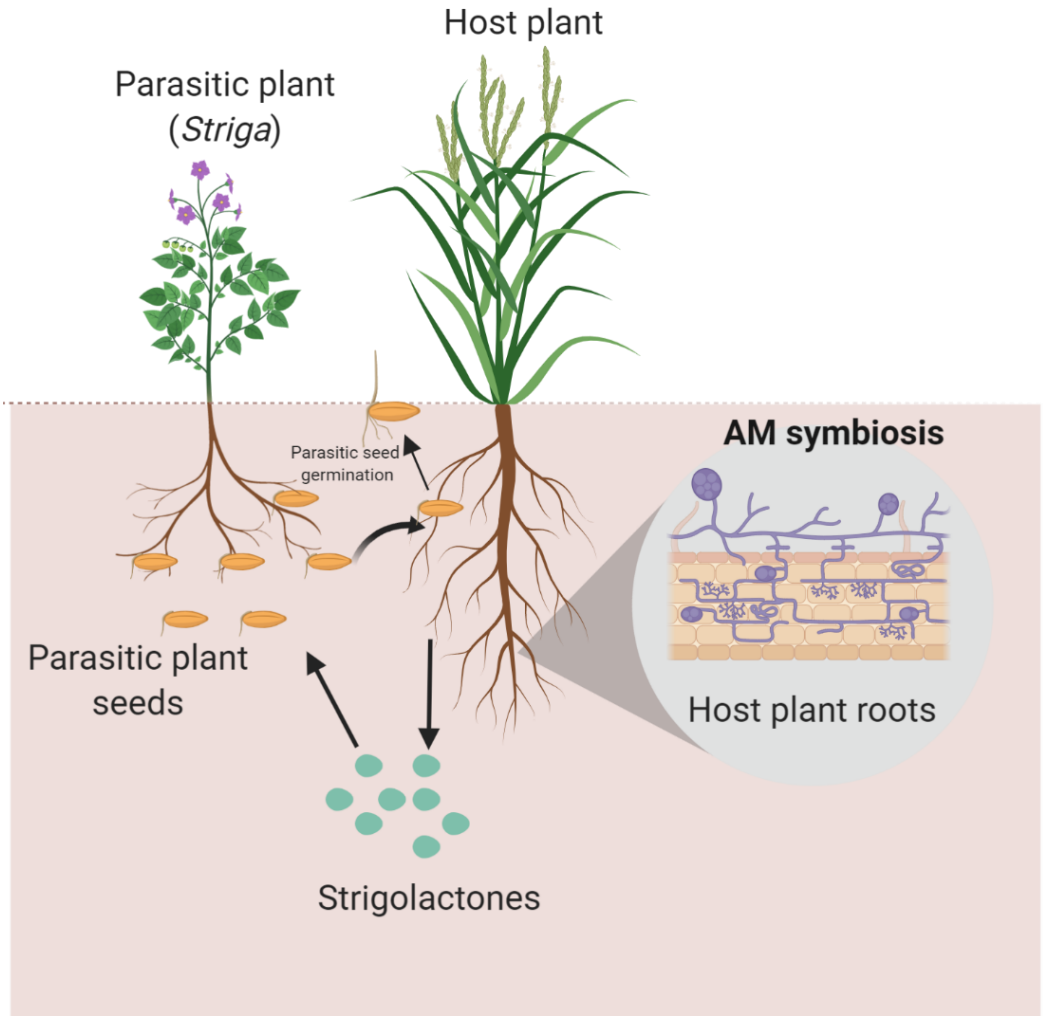


Figure 4. Role of SLs in rhizosphere and AM symbiosis (Redrawn and adapted from (Tsuchiya and McCourt, 2009). SLs are exuded by the host plant roots into the rhizosphere where SLs act as a branching factor for AM fungi promoting hyphal branching. When the seeds reach the parasitic host plant, SL acts as a germination stimulant for parasitic plant (Striga) increasing the risk of parasitization.

2.2 Structure and types of strigolactones

To date,~25 natural SLs have been identified in root exudates of several plant species (Xie, 2016). Strigolactones comprise a small class of carotenoid-derived compounds consisting of a butanolide lactone ring (D-ring) which is linked to a tricyclic lactone (ABC-ring) (Fig. 5). The highly conserved enol-ether C-D ring moiety plays a role in the biological activity and A-B can present variation due to of the attachment of different side groups (Ruyter-Spira *et al.*, 2013; Yoneyama *et al.*, 2010). SLs are divided into two groups based on their second moiety. Canonical SLs comprises of ABCD-ring system, where strigol and related compounds contain ABC-ring system connected to methylbutenolide D-ring via an enol-ether bridge (Al-Babili and Bouwmeester, 2015; Xie *et al.*, 2013; Yoneyama *et al.*, 2018). The modification of AB-rings in canonical SLs can happen through epoxidation, methylation, hydroxylation, or ketolation, representing its structural diversity (Bhattacharya *et al.*, 2009; Al-Babili and Bouwmeester, 2015). On the other hand, the non-canonical SLs lack the A-, B-, or C- rings but rely solely on enol-ether-D-ring moiety. Carlactone (CL) is considered to be the least complex and first reported non-canonical SLs, which is oxidized to Carlactonoic acid (CLA) (Alder *et al.*, 2012; Abe *et al.*, 2014). Plants like maize (Yoneyama *et al.*, 2015; Charnikhova *et al.*, 2018), *Arabidopsis* (Kohlen *et al.*, 2011; Abe *et al.*, 2014), and *Populus* (Xie, 2016) Produce both canonical and non-canonical SLs. Among the several synthetic analogues of SL (including GR5 and GR7), GR24 is often widely used as a model compound for SL and a very active analogue used in several standard bioassays (Zwanenburg and Blanco-Ania, 2018; Xie, Yoneyama and Yoneyama, 2010).

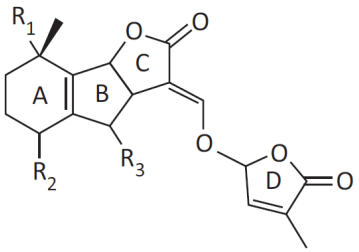


Figure 5. General structure of strigolactone (adapted from (Ruyter-Spira *et al.*, 2013).

2.3 Biosynthesis of strigolactones

Although SLs were found long time ago, not much was known about their biosynthetic pathway. The discovery of SLs as a carotenoid derived compounds was first reported in 2005 (Matusova *et al.*, 2005; Al-Babili and Bouwmeester, 2015). The biosynthetic pathway of SL was elucidated by treating maize carotenoid deficient mutants with a carotenoid biosynthesis inhibitor, fluridone, which blocks the carotenoid formation, resulting in the decreased secretion of germination stimulant rather than

production in *Striga hermonthica* (Matusova *et al.*, 2005). This data suggested a foundation for carotenoid-derived biosynthesis of SL. Genetic evidence has shown the involvement/ identification of several SL biosynthetic genes in the core pathway. In several plant species, mutants of *CAROTENOID CLEAVAGE DIOXYGENASES7* and *8* (*CCD7* and *CCD8*) and *Cytochrome P450* (*Cyt P450*) are SL deficient and exhibit a excess branching phenotype that can be restored by GR24 application (Sorefan *et al.*, 2003; Booker *et al.*, 2004; Umehara *et al.*, 2008; Gomez-Roldan *et al.*, 2008). This provides evidence supporting the involvement of *CCD7*, *CCD8* and Cyt P450 in the biosynthesis of SLs. Later, *d27* (*dwarf27*), another SL-deficient mutant was characterized in rice (Lin *et al.*, 2009) and was further identified and characterized in the *Arabidopsis* ortholog of *OsD27* where the mutant displayed tillering/branching phenotype (Waters *et al.*, 2012a). The plastid-localized D27 is known to encode a novel iron-binding protein (Lin *et al.*, 2009).

A generalized SL pathway occurs in three different cellular compartments i.e., the plastid, the cytoplasm/the symplasm and the nuclei. SL biosynthesis occurs in sequential steps catalyzed by the three canonical biosynthetic enzymes, DWARF27 (D27) and Carotenoid cleavage dioxygenases, CCD7 and CCD8 which encode for the genes *MORE AXILLARY GROWTH3* (*MAX3*) and *MORE AXILLARY GROWTH4* (*MAX4*), respectively, which are localized in plastids (Fig. 6) (Alder *et al.*, 2012; Flematti *et al.*, 2016). CCDs are a family of nonheme iron enzymes that catalyze the cleavage of carotenoids at specific C-C double bonds forming apocarotenoids (Auldridge *et al.*, 2006). Earlier studies showed that in *Arabidopsis* CCD7 uses all-*trans*-β-carotene as a substrate to catalyze the cleavage of C9-10 of β-carotene to yield all-*trans*-β-10'- -carotenal and CCD8 catalyzes the cleavage of 10'-apo-β-carotenal at C13-14 from CCD7 cleavage to produce 13-apo-β-carotenenone (Schwartz, Qin and Loewen, 2004). This suggests that CCD7 and CCD8 acts sequentially in the biosynthetic pathway. Further studies in *Arabidopsis*, pea and rice showed that 9-*cis*-β-carotene is the better substrate for CCDs than all-trans-β-carotene for the formation of an apocartenoid (Alder *et al.*, 2012). Further studies demonstrated that D27 is a β-carotene isomerase that converts all-*trans*-β-carotene to 9-*cis*-β-carotene which becomes a substrate for CCD7 that converts to 9-*cis*-β-apo-10'-carotenal, and in turn oxidized by CCD8 to form a key intermediate for SL biosynthesis and a novel SL-like compound called carlactone (CL), an endogenous SL biosynthetic precursor (Alder *et al.*, 2012; Seto and Yamaguchi, 2014). Moreover, exogenous application of carlactone rescued the shoot branching SL biosynthetic mutants *d27* and *d10* indicating that CL is an intermediate step during the biosynthesis pathway of SLs. The detection of CL in plant tissues using LC-MS/MS lead to a better understanding of the SL biosynthetic pathway (Seto and Yamaguchi, 2014). Since, CL, a canonical SL that contains only A-B rings with enol-ether bridge but lacks B-C rings, requires an extra step for CL to be further oxidized to 4-deoxyorobanchol (4-DO) or 4-deoxystrigol (4-DS).

CL is later exported to the cytoplasm which is catalyzed to CLA by ER localized MORE AXILLARY GROWTH1 (MAX1) (Kameoka and Kyozuka, 2017) a member of the cytochrome P450 family, a heme-containing monooxygenases constituting a large family of proteins in plants (Abe *et al.*, 2014). Grafting experiments demonstrated that MAX1 is a downstream component that catalyzes MAX3 (CCD7) and MAX4 (CCD8) to produce SLs (Booker *et al.*, 2005). It was reported that *Arabidopsis max1* mutant produced 700-fold higher CL than wild-type plants indicating that MAX1 is a direct catalyst for CL (Seto and Yamaguchi, 2014). MAX1 catalyzes the oxidation of the C-19 methyl group of CL to produce Carlactonoic acid *in vitro* using yeast microsomes (CLA; (Abe *et al.*, 2014) or 4-DO (Al-Babili and Bouwmeester, 2015)/ 5DS (Zhang *et al.*, 2014). It was shown that CLA, a non-canonical SL was further methylated by an unknown methyltransferase to form a SL-like compound called methyl carlactonoate (MeCLA). MeCLA was first identified in *Arabidopsis* roots tissues and acts downstream of MAX1 in the biosynthesis of SLs (Abe *et al.*, 2014; Seto and Yamaguchi, 2014).

In *Arabidopsis*, reverse genetics combined with mutational studies revealed a shoot branching enzyme LATERAL BRANCHING OXIDOREDUCTASE (LBO), an oxidoreductase-like enzyme of the 2-oxoglutarate and Fe(II)-dependent dioxygenase family and *lbo*-mutant alleles showed increased shoot branching phenotype (Brewer *et al.*, 2016). LBO oxidizes MeCLA to an active and highly unstable unknown SL-like metabolite called MeCLA +16 Da, an addition of an oxygen atom to MeCLA (Brewer *et al.*, 2016). A recent study determined the structure of MeCLA +16 Da and that LBO converts MeCLA into hydroxymethyl carlactonoate (1'-HO-MeCLA), which is vital for shoot branching regulation (Yoneyama *et al.*, 2020). The conversion of MeCLA to 1'-HO-MeCLA is highly conserved among various plant species including, *Arabidopsis*, tomato, maize, and sorghum (Yoneyama *et al.*, 2020). Figure 6 illustrates a general pathway of SL biosynthesis in plant species. Although extensive studies have been conducted to understand SL biosynthetic pathway in herbaceous species, very little was known about the biosynthesis of SL in perennial woody species like, *Populus* (Wang and Li, 2006; Czarnecki *et al.*, 2014; Zheng *et al.*, 2016).

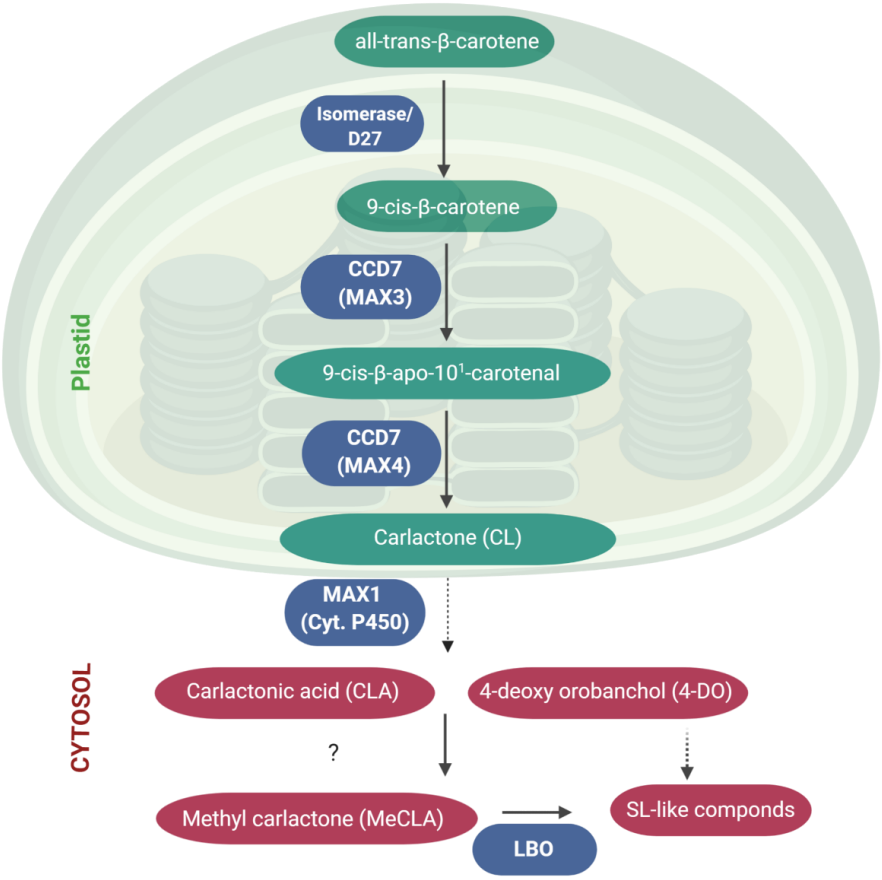


Figure 6. A model of SL biosynthesis pathway (redrawn and adapted from (Katyayini, Rinne and van der Schoot, 2019)

2.4 Strigolactone perception and signal transduction

The molecular mechanisms of SL perception and signaling in plant development have been widely studied in both monocots and dicots. The specificities of different SLs in various biological processes (such as, hyphal branching, parasitic plant germination, development of plant) determines that the receptors involved in the SL recognition is highly specific (Wang and Bouwmeester, 2018). Perception and signal transduction of SLs, similar to other hormones, is mediated via the ubiquitin-proteasome system (Kelley and Estelle, 2012; Morffy, Faure and Nelson, 2016). SL transduction and perception in angiosperms are mediated by two proteins- MORE AXILLARY GROWTH2 (MAX2; in *Arabidopsis* and D3 in rice) and D14 (in rice). The loss of *max2* and SL-biosynthetic mutants display an increase in the AXB outgrowth but cannot be rescued by GR24 application suggesting the importance of the signal transduction pathway (Booker *et al.*, 2005; Gomez-Roldan *et al.*, 2008; Ishikawa *et al.*, 2005; Stirnberg,

van de Sande and Leyser, 2002; Umehara *et al.*, 2008). SL responses require the F-box protein MAX2/D3 which acts as a recognition unit in the SCF^{E3} ligase complex, which consists of conserved SKP1, a CULLIN protein and a specific F-BOX protein that play a key role in SL-triggered proteasomal degradation (Stirnberg, van de Sande and Leyser, 2002; Ishikawa *et al.*, 2005; Stirnberg, Furner and Ottoline Leyser, 2007; Zhao *et al.*, 2014). However, SL perception requires the involvement of an α/β-hydrolase super family protein receptor, DWARF14 (D14), identified in several vascular plants such as petunia (named as DAD2), rice (D14), pea (RMS3), *Arabidopsis* (AtD14) and *Populus* (PtD14) (Fig. 7) (Arite *et al.*, 2009; Hamiaux *et al.*, 2012; Waters *et al.*, 2012b; Zheng *et al.*, 2016). D14 catalyzes the hydrolysis of SLs (Yao *et al.*, 2016). It was recently shown that the SL receptor D14, a non-classical receptor that both hydrolyze SLs as well as serve as a receptor for SLs to bind the active SL isoform (Yao *et al.*, 2016; de Saint Germain *et al.*, 2016; Snowden and Janssen, 2016). The binding and/or hydrolysis of SL that leads to a conformational change of D14 into an active state is an important step in SL signal transduction. Enzymatic studies revealed that D14 proteins, after binding to the SL analog GR24, can be hydrolyzed into an inactive ABC- and D-rings (Lopez-Obando *et al.*, 2015; Hamiaux *et al.*, 2012; Seto and Yamaguchi, 2014). D14 contains the highly conserved Ser-His-Asp catalytic triad that mediate SL hydrolysis and promotes signaling transduction by originating 5-hydroxy-3-methylbutenolide and tricyclic lactone (Waters *et al.*, 2017).

Like other SL signaling components, MAX2/D3 is nuclear localized (Zhao *et al.*, 2014). As proposed, SL triggers the conformational changes of D14 during binding or hydrolysis mediating the physical interaction between D14 and MAX2/D3, which enhances the destabilization of D14 by MAX2, leading to proteasomal degradation (Zhao *et al.*, 2014; Hamiaux *et al.*, 2012; Waters *et al.*, 2017). As mentioned above, degradation of targeted proteins via ubiquitination by the SCF^{E3} ligase- complex is a deciding step during the SL signal transduction pathway.

The first SL repressor identified was DWARF53 (D53) in rice, which was localized specifically in the nucleus and was primarily identified as proteolytic targets of SL signaling (Jiang *et al.*, 2013; Zhou *et al.*, 2013). D53 was discovered in a SL-deficient mutant displaying a semi-dwarf and high tillering phenotypes in comparison to the wild-type phenotype. In *d53* mutant plants present a low tiller number and reduced expression level of D53 (Zhou *et al.*, 2013) suggesting that D53 acts as a negative regulator and repressor of SL downstream signaling in mediating shoot branching (Zhou *et al.*, 2013; Jiang *et al.*, 2013). D53, belonging to a class I ATPase enzyme family protein in rice, and SUPRESSOR OF MAX2 1-like (SMXL) in *Arabidopsis* interacts with AtD14 after SL perception and is targeted for degradation by MAX2 through negative regulation of shoot branching (Stanga *et al.*, 2013; Wang *et al.*, 2015; Soundappan *et al.*, 2015). This leads to the ubiquitination of transcriptional repressors D53/SMXL6/7/8 via 26S proteasome and promote SL responses and signaling. It has been shown that MAX2 interaction with SMXL6/7/8 and D53 is much weaker than those of D14 (Liang *et al.*, 2016; Wang *et al.*, 2015).

In addition, a downstream target of SL signaling, is a transcription factor (TF), *TEOSINTE BRANCHED1* (*TB1*)/*CYCLOPEDIA*/*PROLIFERATING CELL FACTOR1* (*TCP*) family, known to act downstream of D53/SMXLs in signaling pathway necessary for nuclear localization (Aguilar-Martínez, Poza-Carrión and Cubas, 2007). The TFs are well characterized in different species: *BRANCHED1* (*BRC1*) and *BRANCHED2* (*BRC2*) in *Arabidopsis* (Aguilar-Martínez, Poza-Carrión and Cubas, 2007), *TB1* in maize (Doebley, Stec and Hubbard, 1997), *PsBRC1* in *Pisum sativum* L. (Braun *et al.*, 2012), *Fine Culm1*/*OsTB1* in rice (Takeda *et al.*, 2003), and *PtBRC1* and *PtBRC2* in *Populus* (Rinne *et al.*, 2015). These genes show predominant expression in AXBs, and the corresponding mutants resulted in increased secondary shoot growth/ tillering phenotype compared to wild-type that is not GR24 responsive. SL treatment induced expression in *AtBRC1* and *PsBRC1* (Braun *et al.*, 2012) but the branching mutant *brc1* showed insensitivity to SL treatment (Brewer *et al.*, 2009; Minakuchi *et al.*, 2010; Braun *et al.*, 2012). In *Arabidopsis*, *AtBRC1* has a major effect in AXB development and acts as a regulatory hub with *AtBRC2* and both negatively regulate the shoot branching process (Aguilar-Martínez, Poza-Carrión and Cubas, 2007).

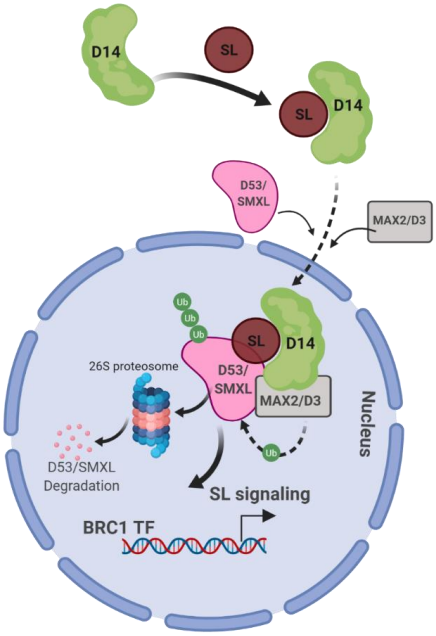


Figure 7. Schematic representation of SL downstream signaling pathway (redrawn and adapted from (Wang *et al.*, 2020)

3. Gibberellins

3.1 Chemical nature and types of gibberellins

Gibberellin (GA) was initially identified in the fungus *Gibberella fujikuroi* (now reclassified as *Fusarium fujikuroi*), which caused a disease in rice known as 'foolish-seedling' (Hedden and Sponsel, 2015). Later, GA was confirmed to be present as a naturally occurring hormone in plants (MacMillan and Suter, 1958). GAs constitutes a type of 6-5 6-5 tetracyclic diterpenoid, with an ent-gibberellane ring structure (Fig. 8). GAs have been classified into two groups based on the number of carbon atoms, containing 19 (with one carboxylic group at the C-7 position, e.g., GA_{20} and GA_9) or 20 carbon atoms (with two carboxylic groups at the C-7 and C-19 positions, e.g., GA_{12} and GA_{53}) (He *et al.*, 2020). In general GAs were named as gibberellin A_1, A_2, etc. based on their order of discovery and structural characterization (MacMillan and Takahashi, 1968).

Among the 136 GAs identified in plants, fungi, and bacteria, only a few GAs have been identified to act as a bioactive hormones namely, GA_1, GA_3, GA_4, and GA_7 (Hedden and Sponsel, 2015), of which GA_3 (known as gibberellic acid) is the most abundant form which is also produced at an industrial scale (Curtis and Cross, 1954). The other existing forms of GAs are either bioactive forms or the deactivated forms (MacMillan, 2001). In *Arabidopsis*, GA_4 is the most active form compared to GA_1 (Eriksson *et al.*, 2006). GA is essential for various aspects of plant growth and development including, stem elongation, leaf expansion, seed germination, seed and flower development, and fruit ripening (Fleet and Sun, 2005; Yamaguchi, 2008; Olszewski, Sun and Gubler, 2002). Although their role in shoot branching is not well characterized yet. The abundance of bioactive GAs is predominantly controlled by several enzymes in both the biosynthesis and catabolism pathways. The plants displaying altered GA-biosynthesis show common GA-deficient phenotypes such as, dwarfism, dark and small leaves, reduced fertility, defect in seed germination whereas high GA content results in increased plant growth and high fertility (Fleet and Sun, 2005; Richards *et al.*, 2001; Olszewski, Sun and Gubler, 2002). Thus, it is crucial to maintain an optimal level of bioactive GAs for normal plant growth and development.

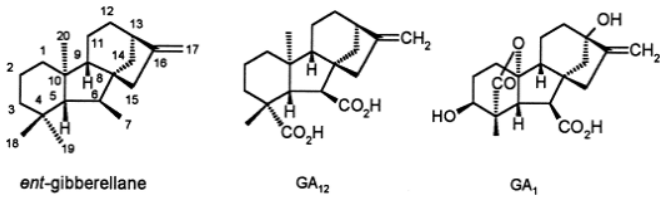


Figure 8. Schematic representation of a general ent-gibberellane skeleton; C_{20} GA form (GA_{12}) and C_{19} GA form (GA_1) (Adapted from (Sponsel, 2003).

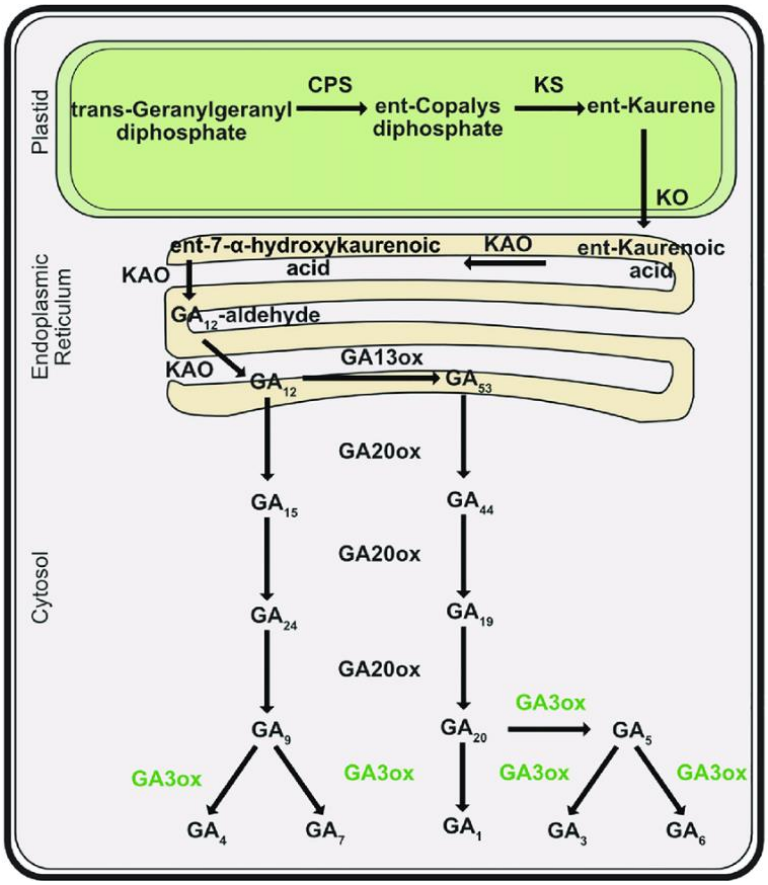
3.2 Gibberellin biosynthesis and signaling

The bioactive GAs are synthesized from trans-geranylgeranyl diphosphate (GGDP), a C_{20} precursor for diterpenoids, in a three-step process which occurs in multiple locations within the cell, including the plastid, endoplasmic reticulum (ER) and cytoplasm (Fig. 9) (Olszewski, Sun and Gubler, 2002; Hedden and Phillips, 2000; Yamaguchi, 2008). The biosynthetic pathway is initiated in the plastid, through the conversion of GGDP to ent-kaurene by a two-step process catalyzed by ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS). In the second step, ent-kaurene is transported to the ER and oxidized to GA_{12}- aldehyde and then to GA_{12} by the action of cytochrome P450-dependent monooxygenases ent-kaurene oxidase (KO) and ent-kaurenoic acid oxidase (KAO). In the third step of the GA biosynthesis, GA_{12} is hydrolyzed to GA_{53} by GA_{13}-oxidase. This step is followed by the conversion of GA_{12} and GA_{53} to distinct precursors and bioactive forms (GA_1, GA_3, GA_4, GA_7) in the cytosol by two parallel pathways: 13-non-hydroxylation and 13-hydroxylation, catalyzed by 2-oxoglutarate-dependent dioxygenases, GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox) (Hedden and Phillips, 2000). GA20oxs modify GA_{12}/GA_{53} to GA_{20}/GA_9 via intermediate precursors GA_{15}/GA_{24} and GA_{44}/GA_{19} in the 13-non-hydroxylation and 13-hydroxylation pathways, respectively (Yamaguchi and Kamiya, 2000). However, the final interconversion requires the enzyme GA3ox to generate GA_4 and GA_1. The concentration of bioactive GAs depends on the rate of their synthesis and deactivation. The deactivation mechanism involves GA2-oxidases (GA2ox) that catalyzes the conversion of bioactive GA_1 and GA_4 to inactive GA_{34} and GA_8 facilitating the catabolic mechanisms such as epoxidation (Zhu *et al.*, 2006) and methylation (Varbanova *et al.*, 2007).

The *Populus* genome consists of multiple genes and enzymes involved in the GA metabolism pathway (Rinne *et al.*, 2011; Rinne *et al.*, 2016). In *Populus*, GA20ox enzymes are encoded by five genes, *GA20ox3*, *GA20ox4*, *GA20ox6*, *GA20ox7*, and *GA20ox8*; the GA3ox enzymes are encoded by two genes, *GA3ox1* and *GA3ox2*; and six GA2ox (*GA2ox1* to *GA2ox6*) (Rinne *et al.*, 2016). Elevated bioactive GA content results in the repression of *GA20ox* and *GA3ox* transcripts and to increased levels of *GA2ox* expression (Hedden and Phillips, 2000). It has been shown that although GA_1 is a widespread bioactive GA, GA_4 is the major bioactive GA with higher affinity for the GA receptor GIBBERELLIN-INSENSITIVE DWARF1 (GID1) than GA_1. Until recently, the mechanism of GA perception by plants was not clear as well as how the GA signals are transduced to produce GA regulated responses. It is known that GA signals are perceived by GID1, which localizes in the nucleus as well as in the cytoplasm (Sun, 2010; Hirano, Ueguchi-Tanaka and Matsuoka, 2008; Ueguchi-Tanaka *et al.*, 2005). GID1 was originally discovered in rice during a

genetic screen for GA signaling mutants (Ueguchi-Tanaka *et al.*, 2005) and studied in *Arabidopsis* as GA receptors (Griffiths *et al.*, 2006; Nakajima *et al.*, 2006). GA signaling involves key nuclear repressors, DELLAs, that belong to a subfamily of GRAS family of transcription regulators (Peng *et al.*, 1997; Silverstone, Ciampaglio and Sun, 1998; Ueguchi-Tanaka *et al.*, 2007; Bolle, 2004). In response to GA, DELLA forms a complex with GID1 receptor (GA-GID1-DELLA) by stimulating the interaction of DELLA with SCF^{E3} complex and subsequently degraded through the 26S proteasome pathway, resulting in the activation of GA responses (Davière and Achard, 2013; Davière and Achard, 2016; McGinnis *et al.*, 2003). In *Arabidopsis*, the soluble receptor GID1 exhibits higher affinity to bioactive GAs than inactive GAs (Nakajima *et al.*, 2006).

A



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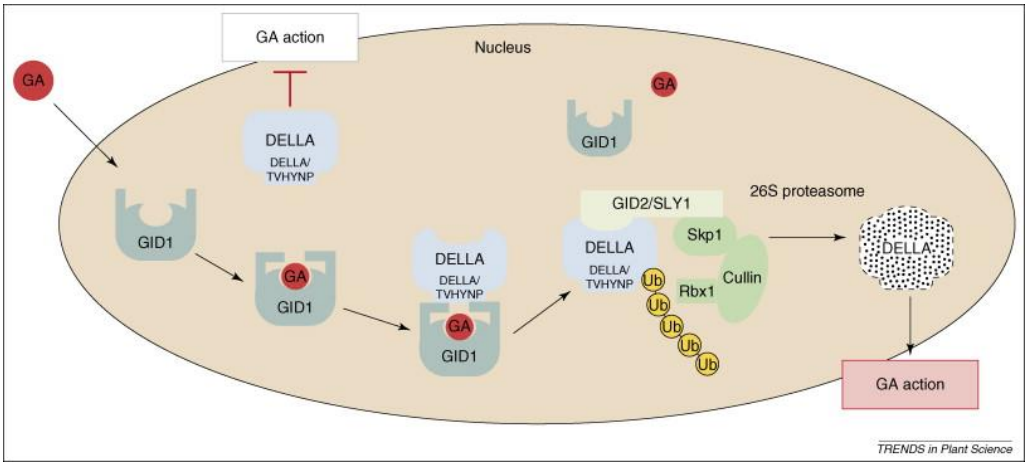

Figure 9. (A) Gibberellic acid biosynthesis and catabolism pathway and (B) GID1 mediated signaling pathway in higher plants (Hirano, Ueguchi-Tanaka and Matsuoka, 2008; Igielski and Kępczyńska, 2017)

3.3 Role of strigolactones and gibberellins in controlling shoot branching

Shoot branching is a process regulated by an intricate interaction between hormones, nutrients and environmental factors (Roman *et al.*, 2016). Among hormones, auxin plays a crucial role. Auxin is predominantly synthesized in young leaves of the shoot apex (Ljung, Bhalerao and Sandberg, 2001). It is transported basipetally through a specific polar auxin transport stream (PATS) in the main stem showing that the apical dominance depends on PATS. Auxin does not enter the buds thereby acting indirectly on the outgrowth of buds (Hall and Hillman, 1975; Prasad *et al.*, 1993). The increased auxin transport in the main stem inhibits the outgrowth of AXB through its ability to establish its own PAT stream from AXB into the stem (Domagalska and Leyser, 2011; Bennett *et al.*, 2016). This co-relation between AXB outgrowth and auxin level is generally stated as the classical theory of apical dominance (Dun, Ferguson and Beveridge, 2006). Although auxin usually suppress branching, after decapitation, it is unable to fully inhibit the outgrowth of lateral bud as the auxin already in the PATS did not show any reduction after decapitation (Morris *et al.*, 2005; Beveridge, Symons and Turnbull, 2000; Li *et al.*, 1995). It has also been shown that decrease in the auxin level post-decapitation is not essential to trigger bud outgrowth initiation. (Morris *et al.*, 2005) shown that auxin depletion in pea plants (*Pisum sativum L.*) after decapitation was relatively to slow along the stem to cause the AXB outgrowth.

Unlike auxin, cytokinin (CK) is known to be transported acropetally from root to shoot in the xylem and are synthesized in both roots and shoots (Chen *et al.*, 1985; Nordström *et al.*, 2004). The long-distance transport of CK induce shoot branching by activation of AXBs (Ongaro and Leyser, 2008; Müller and Leyser, 2011) and act directly to AXB release from dormancy through direct application of exogenous CK to the AXBs (Sachs and Thimann, 1967; Miguel *et al.*, 1998). CK is known to act antagonistically to auxin in the control of bud outgrowth (Teichmann and Muhr, 2015) as the level of CK increases during bud growth activation (Emery, Longnecker and Atkins, 1998). Recently, it has been shown that CK targets transportation of auxin in order to regulate AXB outgrowth (Waldie and Leyser, 2018). Additionally, CK acts as a secondary messenger by for stem auxin by regulating the level of CK oxidases to control shoot branching (Nordström *et al.*, 2004; Tanaka *et al.*, 2006; Shimizu-Sato, Tanaka and Mori, 2008). Along with CK, SL also acts as a crucial regulator of bud outgrowth by repressing shoot branching. Apically produced auxin is transported down the stem to upregulate SL synthesis genes thus repressing the bud outgrowth as well as downregulate the CK levels to promote AXBs outgrowth (Barbier *et al.*, 2019). Unlike Auxin, CK and SL may be transported to the AXBs from the main stem (Teichmann and Muhr, 2015). Additionally, the expression of CK and SL signaling genes during bud outgrowth denotes that the AXBs may be the site of action for these two hormones (Roman *et al.*, 2016; Dierck *et al.*, 2016; Mason *et al.*, 2014). Although the function of CK and SL in AXB outgrowth is not completely defined, they act to control the early bud outgrowth at different stages. BRC1/TB1 is a key transcriptional factor that acts as a common target point in SL and CK branch regulatory pathways determining the activation potential of the buds. *BRC1* acts as a negative regulator of shoot branching and as an integrator of multiple pathways. In *Arabidopsis*, *BRC1* acts as a branch repressor but in certain cases is unable to prevent bud outgrowth due to environmental and developmental stimuli which can in turn modulate the transcription of *BRC1* (Aguilar-Martínez, Poza-Carrión and Cubas, 2007; Dun *et al.*, 2012; Leyser, 2009; Seale, Bennett and Leyser, 2017). BRC1 act locally in the buds and mainly functions as a hub for shoot branching mechanisms (Wang *et al.*, 2019). In Pea, BRC1 positively regulates bud activators, CK and negatively regulates SL (Braun *et al.*, 2012; Dun *et al.*, 2012). In this system, apical dominance is caused by the increase and decrease of SL and CK biosynthesis genes in the stem, respectively, thereby regulating the transport of CK and SL into the AXBs (Dun *et al.*, 2012; Dun *et al.*, 2013; Seale, Bennett and Leyser, 2017). Thus, auxin and CK regulate decapitation-induced branching whereas, SLs role is retained in intact unbranched plants (Young *et al.*, 2014). The role of GAs in the aforementioned shoot branching hormonal network is still not well characterized.

GAs is generally involved in stem elongation, germination, dormancy, flowering, leaf, and fruit development (Hedden and Sponsel, 2015). GA-deficient mutants show higher shoot branching phenotype than the wild types especially in *Arabidopsis* (Silverstone, Ciampaglio and Sun, 1998), rice (Lo *et al.*, 2008), and pea (Weller, Ross and Reid, 1994). Additionally, GAs regulate shoot branching in several perennials such as, *Jatropha curcas* (Ni *et al.*, 2015; Ni *et al.*, 2017), sweet cherry (*Prunus avium*) (Elfving, Visser and Henry, 2011) and hybrid aspen (Rinne *et al.*, 2016). However, the mechanistic way GA controls apical dominance and shoot branching has not been characterized yet. A study in pea shows that GA plays a key role in inhibiting lateral bud outgrowth (Scott, Case and Jacobs, 1967). The dwarf mutant of *Arabidopsis gai* (*gibberellin insensitive*) showed reduced apical dominance (Koorneef *et al.*, 1985). It has been shown in *Populus* and rice that mutants in GA biosynthetic genes and overexpressors of GA-deactivating genes *GA2ox* resulted in increased branching/ tillers suggesting the negative role of GA in shoot branching (Agharkar *et al.*, 2007; Mauriat, Sandberg and Moritz, 2011; Zawaski and Busov, 2014; Lo *et al.*, 2008; Olszewski, Sun and Gubler, 2002). It was recently reported that GA acts as a positive regulator in mediating lateral bud outgrowth in the perennial woody species *Jatropha curcas* (Ni *et al.*, 2015).

In addition, several studies have shown that biosynthesis of GA is modulated by GA dioxygenases regulation. The mutant studies in *Arabidopsis* revealed an increase transcript abundance of *GA20ox* and *GA3ox* and decreased abundance of *GA2ox* in response to GA biosynthesis inhibitors (Phillips *et al.*, 1995; Thomas, Phillips and Hedden, 1999; Chiang, Hwang and Goodman, 1995). On the contrary, plants treated with bioactive GAs repress the GA biosynthesis and activate the GA catabolism genes (Phillips *et al.*, 1995; Thomas, Phillips and Hedden, 1999). Additionally, the interaction between GA biosynthesis and signaling genes was studied by investigating the signaling mutant *gai* with the presence of high level of bioactive GAs and increased biosynthetic activity by *GA20ox* (Thomas, Phillips and Hedden, 1999; Phillips *et al.*, 1995; Yamaguchi, 2008). In support to this, GA has been found to induce AXB formation and activation in *Populus* (Rinne *et al.*, 2011; Rinne *et al.*, 2016). These data suggest that a feedback and feed forward regulation governed by GAs are required to maintain the equilibrium between the GA metabolism genes in controlling shoot branching (Ueguchi-Tanaka *et al.*, 2005; Rinne *et al.*, 2016; Yamaguchi, 2008).

4. Other plant hormones involved in shoot branching

4.1 Auxin

Auxin was the first hormone linked to the study of regulation of shoot branching and has been in the limelight for over 100 years. The pioneering experiments of (Thimann and Skoog, 1933; Thimann and Skoog, 1934) demonstrated that the shoot apex removal mainly stimulated the outgrowth of AXB and the application of auxin to the stump of a decapitated plant could be suppressed by the application of auxin. An enormous number of contributions have been given to build auxin story on the understanding of how auxin repress the bud growth and regulate the shoot branching mechanism (Kepinski and Leyser, 2005; Bennett *et al.*, 2016). The most abundant type of auxin is indole-3-acetic acid (IAA) synthesized in shoot apex and young growing leaves (Ljung, Bhalerao and Sandberg, 2001) and its removal eliminates the major auxin source from the shoot apex. Auxin is transported rootward through directional cell-to-cell transport through the polar auxin transport (PATS), although the hormone does not enter the bud (Hall and Hillman, 1975; Prasad *et al.*, 1993) and acts indirectly on the axillary bud outgrowth (Blakeslee, Peer and Murphy, 2005). Removal of shoot tip through decapitation results in the AXB activation and application of auxin back on the decapitated stem restores its inhibition. Although the mechanism of auxin has been studied for decades, the mechanism of inhibition of AXMs through auxin still remains unclear. It was very clear through the secondary messenger model, that auxin moving basipetally through the stem acts indirectly through competitive inhibition of auxin export from AXBs thereby regulating the bud activity (Balla *et al.*, 2011; Balla *et al.*, 2016); reviewed in (Müller and Leyser, 2011). One such auxin efflux carrier proteins PIN-FORMED1 (PIN1) is known to play a critical role in auxin canalization and transport in the shoot which is a highly polar and high-conductance PATS (Paponov *et al.*, 2005; Petrášek and Friml, 2009; Barbier *et al.*, 2019; Adamowski and Friml, 2015). It was showed that the export of auxin from the bud can travel across the stem in order to provide bud-bud competition via exchange of auxin between connective auxin transport (CAT) and PATS (Bennett *et al.*, 2016). The auxin exchange through PATS cannot fully illustrate the competition between the buds (Bennett *et al.*, 2016; Bennett *et al.*, 2014). The mediation of bud-bud-communication proposed through CAT, showed decreased branching in the mutants due to impaired communication in local transport of auxin in *Arabidopsis* (Bennett *et al.*, 2016). CAT enables the movement of auxin outside PATS through the surrounding tissues mediated by other major contributors PIN3, PIN4, PIN7 (Bennett *et al.*, 2016). Whereas, in Pea, the auxin export from AXBs did not prevent the early bud outgrowth via decapitation instead, rather the inhibitory effect was observed only after two days (Chabikwa, Brewer and Beveridge, 2018). These data indicate that initiation of AXB outgrowth via decapitation does not depend on auxin canalization and export

from the bud rather is important for sustained bud outgrowth (Barbier *et al.*, 2019). For many years, indirect effect of auxin was described by presuming that the auxin was transported to the axillary buds by the secondary messenger, like cytokinin (CK) (Cline, 1991).

4.2 Cytokinin

Cytokinin (CK) was postulated to function as a secondary messenger of auxin during bud activity regulation. CK acts antagonistically to auxin in bud outgrowth control (Shimizu-Sato, Tanaka and Mori, 2008). CK has long been implied as the promoter of AXB outgrowth (Müller and Leyser, 2011; Wickson and Thimann, 1958; Maurya *et al.*, 2020a) and is synthesized throughout the plant shoots and roots (Nordström *et al.*, 2004). CKs promote bud outgrowth by promoting cell division. CKs are transported acropetally in the xylem sap and enter the AXB to promote growth. Several experimental evidence have shown that CK application to the buds trigger outgrowth even with apically applied auxin or in the presence of shoot apex (Wickson and Thimann, 1958; Faiss *et al.*, 1997; Chatfield *et al.*, 2000; Dun *et al.*, 2012; Roman *et al.*, 2016). The initial CK synthesis is mediated by *isopentyl transferase* (*IPT*) that helps in maintaining CK levels and are downregulated by auxin in the main stem (Tanaka *et al.*, 2006). Auxin inhibits CK biosynthesis directly through the *AXR1*-dependent CK signaling pathway (Nordström *et al.*, 2004) and regulate CK levels by regulating cytokinin oxidase (CKX) expression to control shoot branching. Decapitation increased the expression of *IPT* in the nodal stem in pea and chickpea (Tanaka *et al.*, 2006; Turnbull *et al.*, 1997) and the outgrowth of lateral bud is due to the increased accumulation of CK locally. It was also shown that decapitation induces the expression of *IPT3* in nodal stem in *Arabidopsis* (Müller *et al.*, 2015). These data signify that CK is required to overcome the bud inhibition (apical dominance) rather than bud growth induced by decapitation and shoot branching regulation differ between intact and decapitation induced outgrowth of AXBs (Müller *et al.*, 2015; Barbier *et al.*, 2019). In *Arabidopsis*, CK is recognized at the ER by members of the HISTIDINE KINASE (AHK) kinase receptor family (Inoue *et al.*, 2001; Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2005) which targets ARABIDOPSIS RESPONSE REGULATORs (ARRs) in the nucleus, which are CK signaling regulators playing a pivotal role in shoot branching (Schaller, Bishopp and Kieber, 2015).

4.3 Abscisic acid

Abscisic acid (ABA) is known to play important roles in several phases during the plant life cycle (Seo and Koshiba, 2002; Hayes, 2018). It has long been shown that ABA acts as an inhibitor in the upstream control of AXB outgrowth (Nguyen and Emery, 2017). In perennial species, ABA has been known to be an important phytohormone which is essential for survival in suboptimal

conditions (Pan *et al.*, 2021) and to transition from paradormancy to endodormancy (reviewed in (Barbier *et al.*, 2019). Recent studies indicate that ABA is involved in shoot branching regulation in response to light quality and intensity. Studies in several species showed that ABA negatively correlates with bud activity and application of ABA exogenously inhibited the bud outgrowth in *Arabidopsis*, pea, and tomato (*Solanum lycopersicum*) and hybrid aspen (Yao and Finlayson, 2015; Singh *et al.*, 2018; Maurya *et al.*, 2020b). While Fluoridone, an ABA biosynthetic inhibitor promoted AXB outgrowth in *Rosa hybrida* (Le Bris *et al.,* 1999). In poplar (*Populus x canescens*) the reduction in ABA level in explants led to increased AXB outgrowth (Arend *et al.*, 2009). Several ABA biosynthetic mutants displayed an increased branching phenotype (González-Grandío *et al.*, 2017b; Reddy *et al.*, 2013) and ABA accumulation in buds negatively correlates to R:FR ratio (Holalu and Finlayson, 2017). ABA was shown to act downstream of BRC1 in *Arabidopsis* suggesting that it may not be the early responders to decapitation (Barbier *et al.*, 2019) but *BRC1* did not respond to exogenous application of ABA (Yao and Finlayson, 2015). Together, these data suggests that ABA modulates bud dormancy upon change in light intensity, shade and transition from paradormancy to endodormancy (reviewed in (Barbier *et al.*, 2019). The role of ABA in shoot branching in perennial species needs to be explored.

4.4 Jasmonic acid

Jasmonic acid (JA) is a critical hormone for plant development and defense regulation (Yang *et al.*, 2019). JA has largely been known in regulating plant stress responses (mechanical, herbivore and insect damage) and plant resistance-related pathway (Kazan, 2015; Ahmad *et al.*, 2016; Wasternack and Strnad, 2016; Ruan *et al.*, 2019). Jasmonates consists of JA, methyl jasmonate (MeJA) and JA-Ile (an isoleucine conjugate) are the derivatives of a class of fatty acids (Sun *et al.*, 2021). Apart from its involvement in biotic and abiotic stress, JA has been recently known to be also involved in regulating growth and development (Wasternack and Hause, 2013; Campos, Kang and Howe, 2014). There are also evidences which shows the role of JA in response environmental stress by regulating gene expression (Gupta *et al.*, 2017). Over the past few decades, several studies in *Arabidopsis* have progressed the understanding of JA signaling pathway where JA levels become relatively low in the absence of invaders thereby increasing the abundance of the repressor JASMONATE ZIM domain (JAZ) proteins that interact with a specific JA-regulated transcription factor such as, MYC (Hickman *et al.*, 2017). The crosstalk between JA and other plant hormones in response to biotic and abiotic stresses have been widely studied (Yang *et al.*, 2019) but the role of JA pathway genes in shoot branching has not been elucidated yet.

4.5 Salicylic acid

Like JA, Salicylic acid (SA) is a major player in regulating plant innate immunity/defense responses. It was shown that SA triggers synthesis of JA and plants with higher level of SA also show higher levels of JA. The exogenous application of SA by spraying displayed a significant increase in shoot growth after 7 days of treatment in Soya bean (*Glycine max* (L.) Merr. cv. Cajeme) (Gutiérrez-Coronado, Trejo-López and Larqué-Saavedra, 1998). It was also shown the presence of highest SA in the barks of willow which could in turn help to measure the changes in the content of SA upon stress responses (Petrek *et al.*, 2007). Recent studies showed the interplay between JA and SA signaling pathway in *Populus* and observed the striking differences between SA signaling in perennials and annuals with response to plant defense against pathogens (Ullah *et al.*, 2022). The first study on the mechanism of SA-mediated disease resistance by SL in *Arabidopsis* was studied to better understand the role of SL in defense responses (Kusajima *et al.*, 2022). However, no studies have shown the involvement of SA pathway genes in AXB outgrowth.

4.6 Brassinosteroids

Brassinosteroid (BR) are the growth promoting hormones that regulate diverse aspects of physiological processes in plants. Until date, only fewer research works have been conducted that show the role of BR in shoot branching. BR synthesis genes *CYP724B* and *CYP90B* promote shoot branching in rice by producing increased tillers and mutants significantly decreased the tiller number (Wu *et al.*, 2008; Tong *et al.*, 2009). Several transcriptome and biochemical analysis revealed the key role of a BR transcription factor BRI1 EMS SUPPRESSOR1 (BES1) in the SL-regulated shoot branching where BES1 interacts with D53-like SMXLs and D14-MAX2 in *Arabidopsis* to inhibit *BRC1* expression (Hu *et al.*, 2020). In tomato, BR synthesis and signaling genes promote branching and are involved in the release of apical dominance (Xia *et al.*, 2021). However, more detailed studies need to be conducted to define the role of BR in shoot branching and its interactions with other plant hormones.

2. Objectives of this study

The main objective of the present study was to improve the understanding of the molecular mechanism plant hormones use to control shoot branching in model woody species, *Populus*.

More specifically, the following objectives were formulated:

- To investigate the presence of SL pathway genes in the *Populus trichocarpa* genome and its expression in hybrid aspen (Paper I). Our main goal was also to find out the major hubs for SL biosynthesis and perception which in turn would help to better understand the role of SL in inhibiting AXB outgrowth in intact plants

- To investigate the role of GA in shoot branching by mapping the expression of all the GA pathway genes in intact plants as well as in plants with decapitated AXBs and nodes through comprehensive transcriptomic and metabolite analyses in perennial hybrid aspen. This work aimed to test the hypothesis to understand the dual role of GA in AXB outgrowth as well as to investigate the interference of GA_3, GA_4 and GR24 on the expression of SL- and GA-pathway genes (Paper II)

- To investigate the molecular mechanisms and genome-wide identification of DEGs in different hormonal pathways responsible for AXB activation and outgrowth, using hybrid aspen as a model species (Paper III). This work aimed at testing the hypothesis whether the data from SL and GA pathway genes in hybrid aspen (from paper I and II) is validated using RNA-seq. This work also aimed at investigating transcriptome changes in response to decapitation in selected AUX, CK, ABA, JA, SA, and BR pathway genes.

3. Materials and methods

3.1 Plant material and growing conditions

The studies in this thesis were conducted with the model woody perennial, Hybrid aspen (*Populus tremula* x *Populus tremuloides*) clone T89 (Fig 3), that does not branch under controlled conditions in the climatic chambers or in the greenhouse under long days (LD), was used as a source of material in all the experiments (Paper I, II, III). T89 clone was initially micro-propagated *in vitro* in the growth chambers at 20°C for a period of 5 weeks and thereafter transferred to a greenhouse at 20 °C, 18 h photoperiod, 200-250 µmol m^{-2} s^{-1} (osram) and 60% relative humidity (RH). A detailed description of the growth conditions can be found in paper I. Only plants that reached the height of 80-100 cm and had stable leaf production and elongation rates were chosen to conduct the experiments. Tissue and organ samples from intact plants were collected for studying the expression of SL and GA pathway genes (Paper I and II).

3.2 Decapitation assay

T89 plants were decapitated at the BMP approximately 40 cm below the apex as stated in Fig 10. The mature AXBs (sampled at 0, 2, 6, 12, 24 and 48 h) and nodal bark samples (sampled at 0, 2, 6 and 12 h) were harvested just below the BMP for transcript analysis of SL and GA pathway genes at various time points post-decapitation (Paper I and II). Quantification of GAs was also conducted using the AXBs below BMP post-decapitation (Paper II). In paper III, the points selected for the study included 0, 6, 12, 24, 48 and 72 h post-decapitation. The samples from 24 h, 48 h and 72h were included in the analysis to mitigate the circadian effects. This was done by requiring that candidate genes should be between time point 0 and 24 h, 48 h and 72 h.

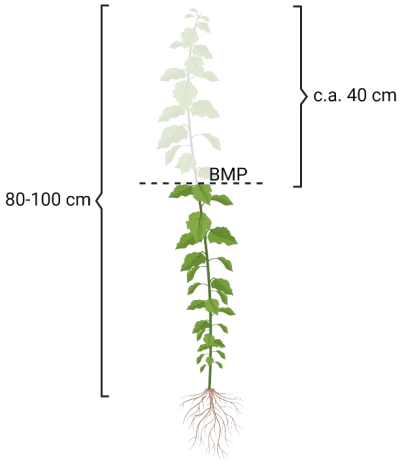

Figure 10. Hybrid aspen decapitated at BMP

3.3 Xylem-feeding experiments

We used the bud-internode units (single-node cuttings) with the AXB at the higher end of approx. 2 cm long segments without leaves (paper I, II; Fig. 11). Xylem-feeding of 10 µM GR24 (rac-GR24; Chiralix BV, The Netherlands), 10 µM GA_3 and 10 µM GA_4 (Sigma-Aldrich) was supplied to the bud via stem vasculature (Paper 1 & II). The stem was placed in the water while cutting to avoid any air cavity formation in the xylem channel. The base of the stem of the single-node cutting was punctured through the pores into the Styrofoam sheet. The cutting was immediately placed in the water medium/hormone solution to float with the lower end of the stem immersed in the water/ hormone solution. All the treatments were monitored in growth chambers under the controlled condition (18 h light, PPFD 160-200 µmol m^{-2} s^{-1}, 20 °C, 60% RH).

Figure 11. A general experimental setup of xylem-feeding of hormones in single-node systems of hybrid aspen used in the study (Paper I & II)

3.4 Bud burst evaluation

AXB burst was monitored for 14 days in xylem-fed water control, 10 µM GR24, GA_3 and GA_4 treated cuttings (Paper II). The bud bursting was expressed as Σ_{14}-values, which determines the speed of burst in days and the number of bud bursts (Rinne *et al.*, 2011). The Σ-score 14 represents the bud burst at day 1 and Σ-score 1 represents the bud burst at day 14, and so on.

3.5 GA measurements

GA content of the AXBs and apices harvested from zone 1-6 was measured using LC-MS/MS with $^2H_2$-labeled GA as an internal standard (Paper II) (Urbanová *et al.*, 2013).

3.6 Analysis of gene expression by qRT-PCR

AXB and nodal bark samples were extracted from different experiments (as described in paper I, II) for RNA isolation. Samples were frozen in liquid nitrogen and stored at − 80 °C for total RNA extraction. Frozen buds and bark tissues were ground with a mortar in 500 µl extraction buffer and processed further as described in paper I. Quantitative reverse transcription-PCR (qRT-PCR) analyses were performed with the Applied Biosystems 7500 Fast Real-Time PCR system, using SYBR® select PCR master mix (Applied Biosystems) (Paper I, II). Transcript levels were normalized with *Populus* actin as a reference gene.

3.7 Statistical analyses

For the decapitation induced gene expression changes, effect of hormones on AXB burst using feeding experiment, to assess the AXB length, ES length, embryonic leaf numbers were assessed by using analyses of variance (ANOVA; one-way for decapitation experiments (Paper I and II) and two-way for xylem-feeding experiments (Paper I and II) in combination with Fischer's LSD test in the general linear model mode and by regressive analysis using the Minitab statistical software (Minitab 18.1, Minitab Inc., PA, USA) ($p \leq 0.005$). Data analysis was also performed using Microsoft Excel.

3.8 Bioinformatic tools

Bioinformatic tools used in the research work is mentioned below:

Purpose	**Web links**
BLAST	http://www.ncbi.nlm.nih.gov/BLAST http://www.phytozome.net http://popgenie.org/
Primer design	http://bioinfo.ut.ee/primer3-0.4.0/
Phylogenetic analysis	www.megasoftware.net
Sickle	https://github.com/najoshi/sickle/blob/master/README.md
FASTQC	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
CEGMA	http://korflab.ucdavis.edu/Datasets
RSEM version 1.1.11	http://deweylab.biostat.wisc.edu/rsem
EdgeR	https://www.r-project.org/
WEGO 2.0	https://wego.genomics.cn/
eggNOG (v5.0)	http://eggnog5.embl.de/#/app/home
REVIGO	http://revigo.irb.hr/
KEGG	https://www.genome.jp/kegg/
Blast2GO	https://www.blast2go.com/
Clustvis	https://biit.cs.ut.ee/clustvis/

3.9 Analysis of gene expression by RNA sequencing

To investigate the effect of decapitation of transcript levels of genes related to hormonal regulatory networks in hybrid aspen, we harvested the AXBs below the BMP in intact as well as in decapitated plants (6, 12, 24, 48, 72 h). RNA was extracted from each sample as described in paper I and II. After DNA removal and purification of RNA, the samples were shipped to BGI Tech, Hong Kong for cDNA library preparation and sequencing using an Illumina sequencing platform as described in paper III. *De novo* transcriptomic assembly was constructed using the pipeline mentioned in Fig. 12 as the hybrid aspen genome is poorly characterized. A false discovery rate (FDR) of ≤ 0.005 was used as a cut off to classify differentially expressed genes. The analysis included gene ontology (GO) enrichment analysis, KEGG pathway analysis, COG analysis as described in paper III.

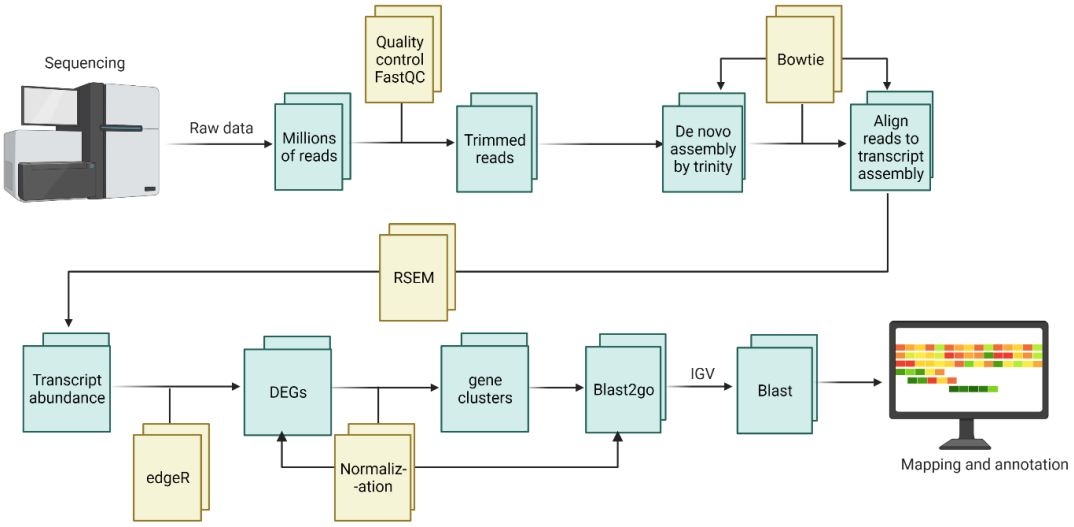

Figure 12. Pipeline for RNA-sequencing (adapted from (Kovi *et al.*, 2016; Kovi *et al.*, 2017); described in paper III). Created with BioRender.com

4. Main results and discussion

4.1 Paper I:

4.1.1 Identification of SL biosynthesis and signaling pathway in hybrid aspen

SLs, a new class of plant hormones are involved in shoot branching. The spatio-temporal regulation of bud outgrowth is usually managed by shoot branching patterns. Multiple pathways regulate the process of AXB outgrowth in perennials. Biosynthetic and signaling pathways are tightly regulated by the involvement of several gene families and enzymatic steps to produce SLs and its derivatives. In contrast with annuals such as *Arabidopsis*, petunia, rice and pea, much less is known about the role of SL during branching in perennials. Over the years, genetic, physiological and biochemical analysis have led to the following two hypotheses of bud activation. The first referred to as a direct-action or second-messenger hypothesis, refers to the role of auxin produced in the main stem that upregulates the production of SL that by moves to AXBs and inhibits the bud outgrowth via upregulation of *BRC1* (Brewer *et al.*, 2009; Aguilar-Martínez, Poza-Carrión and Cubas, 2007; Shinohara, Taylor and Leyser, 2013). The other hypothesis known as the auxin-transport canalization- based mechanism, explain the indirect action of auxin in bud outgrowth inhibition, where buds act as the auxin source enabling its need to efficiently export auxin to the stem (auxin sink) owing to the dormant buds must establish the export of auxin flow to the main stem for its activation. This in turn reduces the sink strength of the stem preventing the other buds from auxin export (Li and Bangerth, 1999; Waldie, McCulloch and Leyser, 2014; Prusinkiewicz *et al.*, 2009; Domagalska and Leyser, 2011). Despite the distinct differences between different model species, the structural diversity of identified SL-like compounds (Wang and Bouwmeester, 2018), and the difference in explanatory paradigms, SL biosynthesis and perception are shown to be highly conserved across the plant kingdom (Waters *et al.*, 2017). However, not much is known about the molecular mechanism of SL pathway genes in woody perennials (Wang and Li, 2006; Czarnecki *et al.*, 2014; Zheng *et al.*, 2016). This is surprising, considering that many trees have AM fungi associations, and that forests cover vast stretches of the earth surface (Crowther *et al.*, 2015), constituting critically important ecosystems (Watson *et al.*, 2018). Due to the importance of shoot branching in the determination of photosynthetic light use efficiency and biomass yield in woody perennial trees, firstly we investigated the if all the SL pathway genes are present in the woody model tree species, *Populus*.

Primarily, we report previously unidentified sequence homologues of three members of the SL pathway genes, namely *D27* (3 homologs), *LBO* (1 homolog), and *D53-like* (3 homologs). Further, we also provide evidence that the entire SL pathway is conserved in the *Populus* genome. The

presence of the complete SL pathway may suggest that suppression of branching might be similar to what has been observed in herbaceous species (Lopez-Obando *et al.*, 2015). Despite the conserved nature of the SL pathway and signaling genes, there are reasons to believe that the perennial lifestyle and growth habits of deciduous trees (Barthélémy and Caraglio, 2007; Tomlinson, 1983; Millet, Bouchard and Édelin, 1999) require adaptations in both the local mechanisms and in the layout of the hierarchical control networks that govern shoot branching. AXBs of hybrid aspen contain sturdy scales which acts as a protective barrier (Rinne *et al.*, 2015) whereas herbaceous species consists of buds without scales (Grbić and Bleecker, 2000; Long and Barton, 2000). In addition, in the extending shoot system of trees new AXBs arise at locations that are increasingly remote from the SL-producing root tips. Together, these physical constraints indicate that the two branching models developed for herbaceous plants can not directly transfer to woody perennials.

4.1.2 Is root-to-shoot signaling inhibiting branching?

SLs regulate several developmental events, including root development, stem growth and senescence (Seto *et al.*, 2012; Al-Babili and Bouwmeester, 2015), but little is known about the spatial and temporal control of SL biosynthesis and signaling (Kameoka and Kyozuka, 2017). In fact, SLs appears to be almost absent from the shoot of most plant species (Zhang *et al.*, 2018). The direct-action model (Dun *et al.*, 2012; Brewer *et al.*, 2015) depicts the roots as the primary source of branch-inhibiting SLs, although shoots can also produce SL themselves (Beveridge, Symons and Turnbull, 2000; Turnbull, Booker and Leyser, 2002; Simons *et al.*, 2006). In this model, root-produced SL is transported upward through the xylem to suppress AXBs. Despite the initial confirmation of this model (Kohlen *et al.*, 2011; Kohlen *et al.*, 2012), the evidence for xylem-transport is limited and so far, unconfirmed (Xie *et al.*, 2015; Yoneyama *et al.*, 2018). Instead, as a rather lipophilic compound (Yoneyama *et al.*, 2018) CL might move via phloem strands through the graft interface. Notably, sieve tubes do contain lipophilic compounds, among which many are hormones (Guelette, Benning and Hoffmann-Benning, 2012; Benning *et al.*, 2012). On the other hand, it also seems unlikely that roots would transport SL via the phloem to AXBs as phloem transport tends to be from source leaves to roots rather than the other way around.

4.1.*3* Node- to AXB signaling of SL is the dominant mechanism in trees

Our results highlight that the SL biosynthetic genes are not expressed in AXBs in hybrid aspen. On the other hand, both SL biosynthesis and signaling genes show expression in both nodal bark tissues as well as roots by synthesizing SL, indicating that SL produced in the roots in hybrid aspen only

move to the lower AXBs which are in close proximity to the roots. However, we observed that the expression of *MAX3* was extremely high (65 times) in the source node compared to *MAX4,* whereas sink nodes expressed *MAX3* and *MAX4* at 200 and 500 times higher than the root tips indicating that the nodal bark associated with AXBs are almost exclusively reliant on the import of CL and downstream products. The expression so exceptionally high in the nodes of young developing AXBs suggests that *MAX3* and *MAX4* required to constrain the elongation of the developing embryonic shoot. We conclude that in trees, nodal bark is the major hub for signaling to AXBs instead of roots. From our data, along with nodal bark, AXBs also express *MAX1* and *LBO*, suggesting that AXBs import CL, CLA and MeCLA for its conversion locally. Although the role of *LBO* in *Arabidopsis* catalyzes the hydroxylation of MeCLA to an unidentified product (Brewer *et al.*, 2016), their expression in source nodes is highest and the AXBs associated with it shows upregulation in gene expression one day post-decapitation supporting the hypothesis of its involvement in the inhibition of branching. The distinct role of *LBO* in nodes and in the activated and growing AXBs of hybrid aspen, remains to be established. This local node-to-AXB transport usually involves non-canonical SLs suggesting that branch inhibition signals might be governed by non-canonical rather than canonical SLs (Brewer *et al.*, 2016; Yoneyama *et al.*, 2018). This locally orchestrated way of node-to-AXB signaling solves the problem presented by the continuously expanding shoot system of a tree, where distances can become increasingly prohibitive for effective root-to-AXB signaling. It is tempting to speculate that in hybrid aspen root-produced SLs (expressing *MAX3*, *MAX4*, *MAX1*, and *LBO*) mostly serve to attract AM fungi, whereas nodes regulate AXB activation and outgrowth. It remains to be seen if this holds true also under conditions of phosphate starvation, which is known to upregulated SL biosynthesis in roots of e.g. tomato, rice and Petunia (López-Ráez *et al.*, 2008; Umehara *et al.*, 2015; Drummond *et al.*, 2015). Our data also shows the expression of the SL receptor, *D14, the* F-box *MAX2*, and the *D53-like* in both nodes and leaves suggesting that all the SL signaling genes are localized in the vascular bundles (Stirnberg, Furner and Ottoline Leyser, 2007; Shen, Luong and Huq, 2007; Zhou *et al.*, 2013; Soundappan *et al.*, 2015), and matches the regulation of AXB as proposed in the auxin canalization model (Domagalska and Leyser, 2011). In hybrid aspen, the embryonic shoot inside the scales continues with the stem (Rinne *et al.*, 2015) and the stem-AXB interface is likely to be involved in the regulation of AXB activation, burst and outgrowth by involving a canalization process. Both the downstream targets, *BRC1* and *BRC2*, are upregulated during AXB formation and development and maturation (Rinne *et al.*, 2015) and our results showed *BRC1* downregulation 2 h after decapitation. The important implication is that increased production of CLA and downstream SL-like compounds, as well as the downstream targets of SL signaling do not appear to prevent the

development of a side shoot. Rather, the elongation of the dwarfed embryonic shoot is postponed to the next growing season (van der Schoot, Paul and Rinne, 2013). Our observations also demonstrated that all the SL pathway genes are either downregulated or showed the general tendency of downregulation (*D27a*, *D27b*, *MAX2a* and *D53-like3*) upon decapitation within the first 24 h during the activation of AXBs with the exception of *LBO* that shows an upregulation with the reason unknown. We conclude that SL and the downstream products *BRC1* and *BRC2* do not prevent primary morphogenesis at the SAM and shoot formation, but possibly elongation of the embryonic stem which occurs 24 h post decapitation.

4.1.4 Feedback regulation and transport of SL in shoots of hybrid aspen

In single node cuttings AXB activation and outgrowth are not affected by the roots or leaves, and comparison with the decapitation experiments should be done cautiously. However, it enabled us to investigate activation and outgrowth in young developing AXBs. Our data showed that, like in case of the decapitation experiment, mature AXBs and their enclosed embryonic shoot had elongated at day 3. The grossly similar timeframe indicates that the underlying changes in gene expression changes could be comparable. Whereas in the decapitation experiment neo-formed primordia/leaves had not yet started at 96 h, in single node cuttings showed that at day 5 and day 7 several primordia/leaves had emerged. Our results demonstrated that GR24- treatment did not prevent the AXB outgrowth once their activation by ES elongation has started. Our investigation on the effect of concentration dependent GR24 resulted in a consistent and significant response. While the response of GR24- feeding on SL pathway genes in mature and young AXBs showed that both *MAX1* homologs showed positive feedback from GR24 treatment. Our data also proved that GR24 did not have any significant effect on the branch inhibitors, *BRC1* and *BRC2*. In brief, feeding GR24 via the xylem to isolated single node systems significantly affects both CL conversion to CLA, and the production of components of the receptor system, but not the downstream target genes *BRC1* and *BRC2*. Because single node cuttings are not only devoid of roots, but also of leaves, no obvious source of sugars is available for AXB activation through BRC1 repression (Mason *et al.*, 2014; Fichtner *et al.*, 2017; Yang *et al.*, 2018). This more or less rules out the nutrient-diversion theory (Kebrom, 2017) as a general theory for AXB burst regulation. This might indicate that sugars are not strictly required to initiate AXB activation, although they might be needed to fuel outgrowth. Similarly, CKs from the roots do not play a role, but node producing CKs might be important (Ferguson and Beveridge, 2009). The SL working models that describe the results obtained with the three experimental situations, propose that in hybrid aspen node-to-AXB signaling is the ruling principle, and that roots contribute little SL to the lower positioned mature AXBs, if at all. The mature AXB itself can also synthesize SL-like compounds downstream of CL, but not CL itself. In

the first 48h, decapitation downregulates *MAX1* genes as well as all SL signaling genes, except for LBO, which is upregulated. The further changes in genes expression up to the 5d time point, whether they concern up-or downregulation, are neutralized by GR24-feeding, indicating that it affects both CL production as well as formation of the SL receptor complex.

4.2 Paper II:

Although it was shown that SL cannot prevent the bud outgrowth once the AXB activation has started (Paper I), the role of GA in AXB activation and outgrowth in perennial trees needs further investigation (Rinne *et al.*, 2016). In hybrid aspen, AXB development and outgrowth involves local regulation of GA pathway genes (Rinne *et al.*, 2016). According to previous reports, GA pathway plays an important role in shoot branching, most importantly ES dwarfing due to GA-deficiency and the AXB activation induced by the upregulation of GA biosynthesis gene *GA3ox2* upon decapitation indicate that GA biosynthesis forms a crucial part in shoot branching (Rinne *et al.*, 2015; Marzec, 2017). Because GA precursors and bioactive GAs can move short and long-distance to regulate developmental events (Binenbaum, Weinstain and Shani, 2018), nodes may assist development and outgrowth of AXBs by delivering GA and other hormones like SL via the vasculature system (Katyayini, Rinne and van der Schoot, 2019). In this study, we conducted a comprehensive analysis of GA-pathway genes in various plant parts as well as the effect the decapitation would have on the AXBs and its associated nodes- important determinants of GA and SL homeostasis via hormone feeding. It also included the metabolite analysis of all the GAs in different AXBs of intact plants.

4.2.1 Axillary buds activate due to diminished primary responders (*GA2ox*s) in perennial hybrid aspen

We previously reported the identification of few GA biosynthesis genes and signaling involved in AXB activation in hybrid aspen (Rinne *et al.*, 2016). Until this study was made, no reports were available earlier on the identification and profiling of the entire GA pathway genes in hybrid aspen. To obtain further insights on the remaining unidentified GA pathway genes involved in the regulation of AXB outgrowth, we analyzed their expression upon decapitation. Most importantly the effect of GA_3 and GA_4 on AXBs were analyzed to uncover its role and effect on GA pathway genes in hybrid aspen.

All the seven *GA20ox* genes showed expression in AXBs with *GA20ox5* detected with highest transcript level overall and *GA20ox8* was expressed more generally in all the plant parts analyzed. This indicates that all the *GA20ox* genes are present in the AXB which helps in maintaining its

dwarf shoots. Although our metabolite analysis showed that, $GA_{1/4}$ level was much lower in AXBs in comparison to apices because AXBs showed high expression of *GA2ox* family genes that might lead to the deactivation of bioactive GAs (Middleton *et al.*, 2012). These bioactive GAs influence the balance between GA biosynthesis and deactivation via decreased deactivation and signaling followed by biosynthesis (Olszewski, Sun and Gubler, 2002; Yamaguchi, 2008). Thus, the quiescent state of the AXBs is maintained by the high expression level of *GA2ox* genes to protect the proleptic nature of the hybrid aspen Shoots. *GA2ox* genes are downregulated during the activation of AXBs followed by early downregulation of GA signaling genes (GID1s) upon decapitation indicating that increased GA led to the decreased GID1 levels with an increase in the level of GA thereby maintaining the homeostatic level. On the other hand, the overall expression levels were much lower in the apices compared to AXBs and sink leaves produced highest GA precursors. Also, the genes expressed in plant parts other than AXBs might have specific tissue or organ specific expression. Since quiescent AXBs express low levels of $GA_{1/4}$ despite the high expression of GA biosynthesis, signifies that dwarfed ES of the quiescent AXBs are deficient of GA independent of GA biosynthesis. While $GA_{3/6}$ maintain the quiescent state of the AXBs, it acts by deactivating $GA_{1/4}$ through *GA2ox* upregulation (Ito *et al.*, 2017) which in turn can conserve the quiescent AXBs in GA_4- deficient state. GA_4 is known to have an opposite effect to GA_3 in promoting AXB outgrowth via cell division and elongation (Hedden and Sponsel, 2015). Hence, the quiescent state of the AXBs is majorly maintained by the low levels of GA_4 as well as the previously studied SL and BRC-1 mediated ABA signaling (Katyayini, Rinne and van der Schoot, 2019; González-Grandío *et al.*, 2017a; Wang *et al.*, 2019). On the other hand, the two *GA3ox* genes (*GA3ox1* and *GA3ox2*) showed an opposite expression pattern, especially with respect to apices where the presence of expression pattern of *GA3ox1* in apices was studied previously (Israelsson *et al.*, 2004). Although the expression of *GA20ox* genes in apices was lower, *GA3ox2* compensated it with its high transcript levels indicating that both *GA3ox1* and *GA3ox2* are regulated developmentally during tissue maturation, rather than tissue specific. *GA3ox1* is expressed at higher levels in source leaves. Thus, the level of *GA3ox2* was high in actively proliferating tissues like apices and roots whereas, *GA3ox1* was in source nodes and leaves. The GA-signaling receptors *GID*s were expressed at low levels in apices indicating the negative correlation of GA receptors with the high production of bioactive GAs through GA biosynthesis gene, *GA3ox2*. Although our data shows that GA pathway genes are ubiquitously present in AXBs, the outgrowth of AXBs require precursors exported from node to AXBs via the downregulation of *GA20ox* genes in AXBs and subsequent upregulation in nodes post-decapitation. This data was also supported by the metabolite analysis of precursors with an increase in their levels in AXBs upon decapitation. This

data is consistent with the findings from paper I that node-to-AXB signaling mediated by SL maintains the AXB in a quiescent state (Katyayini, Rinne and van der Schoot, 2019).

4.2.2 GA-SL interference modulate shoot branching in hybrid aspen

In order to uncover the role of GA_3, GA_4 and GR24 on the expression of GA- and SL- pathway genes, xylem feeding experiments were conducted in the single-node cuttings. AXBs were fed with GA_3, GA_4 and GR24 followed by monitoring the AXB behavior at the 3- and 5-day time points and gene expression analysis of SL and GA pathways genes. It was shown earlier that GA_3 does not trigger the outgrowth of AXBs contrary to GA_4 (Rinne *et al.*, 2011). Our previous analysis showed that SL inhibits AXB outgrowth and keeps the AXB in a quiescent state (paper I; (Katyayini, Rinne and van der Schoot, 2019). *GA2ox* family genes responded strongly to both GA_3 and GA_4. GR24 feeding did increase expression of both *GA3ox1* and *GA3ox2* genes at day 5. A putative increase in the GA biosynthesis by GR24, might explain why GR24 feeding reduced *GID1* expression levels to a similar level as GA_3 and GA_4. In hybrid aspen, SL pathway and perception genes were highly expressed in mature AXBs, and decapitation downregulated the genes involved in the pathway along with *BRC1* (paper I). Hence, the action of SL and GA2ox is both responsible for the quiescent state of the AXBs in the intact plants (paper I and II) and decapitation lowers the SL signaling by increasing the CK and GA signaling (Ni *et al.*, 2017; Duan *et al.*, 2019; Katyayini, Rinne and van der Schoot, 2019). GA_3 and GA_4 feeding lead to the decrease in expression level of SL biosynthesis gene *MAX1* which is supported by the earlier observations (Ni *et al.*, 2017; Ito *et al.*, 2017). It was also shown that GA and GR24 acts separately on various transcriptional targets (Lantzouni, Klermund and Schwechheimer, 2017) which clarifies the reason GR24 promotes ES elongation (paper I). But how GA and SL align their activities during AXB outgrowth in later stages remains elusive.

4.2.3 Metabolite analysis revealed the involvement of bioactive GAs- GA_3 and GA_6 in the maturation of AXBs and not outgrowth

The main findings from the comprehensive metabolite analysis (Urbanová *et al.*, 2013) correlate gene expression with the actual levels of GA and its precursors in the AXBs from distinct zones along the stem in intact plants as well as in buds from decapitated plants. As GA pathway is under homeostatic control, the expression of GA-pathway genes suggests that AXBs were sensitized to low GA levels, whereas decapitation de-sensitized them to GA, indicating an increased availability of GA. Our analysis involved the bioactive GAs in both 13-hydroxylation and non-13-hybroxylation branches in apices, although GA_1 had dominant levels, GA_4 level was quite low and GA_3 was almost undetectable in the apices. The presence of GA_3 and GA_6 in the AXBs could be

linked to the function of *GA3ox1*. On the other hand, a study from (Israelsson *et al.*, 2004) also supported the fact that *GA3ox2* function in converting the precursor GA_9 and GA_{20} to GA_7, GA_4, GA_1. Although GA_4 is known to promote elongation (Israelsson *et al.*, 2004), GA_1 showed higher expression levels in the apices (paper II). Although GA_3 is used habitually in many studies, it differs with its effect compared to GA_4. It was shown that GA_3 and GA_4 have distinct and opposite function in regulating AXB outgrowth (Rinne *et al.*, 2011). GA_3 induced abscission in AXBs under both long days (quiescent AXBs) and short days (dormant AXBs) (Rinne *et al.*, 2011). GA2ox was found to be localized in the band below SAM by safeguarding it from the high influx of GA_4 (Sakamoto *et al.*, 2001; Jasinski *et al.*, 2005; King *et al.*, 2008; Bolduc and Hake, 2009). In grasses, GA2ox cannot deactivate GA_3 but can induce the floral meristem through entry of GA_3 to SAM but, GA_4 is able to enter the stream after the disappearance of GA2ox expression band (King *et al.*, 2003). As GA_3 is not deactivated, supplied GA_3 is expected to result in deactivation of endogenous GA_4, because GA_3 can significantly upregulate *GA2ox* genes (paper II). Thus, absence of AXB outgrowth by GA_3-feeding might be a consequence of GA_4 deficiency and loss of GA_4-mediated activation and outgrowth. Our findings mainly detect the presence of $GA_{3/6}$ in quiescent AXBs, and the levels decreased upon decapitation. This data is in line with the fact that GA_3 cannot upregulate growth-related α-clade 1,3-β-glucanases promoted outgrowth in genetically modified hybrid aspen (Rinne *et al.*, 2016).

4.3 Paper III:

In the third manuscript, we explored the changes in gene expression in different hormone pathway genes occurring during decapitation induced AXB outgrowth in *Populus*. The experimental layout was the same as in paper I and II except that we did not choose the very early points 2 h and 4 h post decapitation. Six-time points which included control (non-decapitated) were analyzed.

4.3.1 Effect of decapitation on the transcriptome responses in proleptic woody species *Populus*

While several studies examine the role of different plant hormones in shoot branching in annuals, to the best of my knowledge no comprehensive analysis have explored the underlying initial changes in gene expression of different hormonal pathways in decapitation-activated axillary buds in perennial woody species. The finding presented here (paper III) investigated the transcriptional response of decapitation induced early-bud activation changes in the previously studied plant hormones SL and GA (paper I and II) along with the other plant hormones such as, auxin, CK, ABA, JA, BR and SA (paper III). Of the 44,000 predictive genes in *Populus trichocarpa* genome, 8997 (20.40%) were differentially expressed genes (DEGs) in the RNA seq analysis (FDR<0.005)

of decapitation induced AXBs at different time points compared to control AXBs. It is also important to note that although the genome is well characterized, the information could still be limited for this *Populus* hybrid genotype. Hence, *de novo* based transcriptomic analysis was conducted over reference-based assembly to map back the reads obtained post-sequencing and identifying DEGs. Our observations showed that gene expression changes occur as early as 6 h post-decapitation and dramatic increase in the number of up- and downregulated genes were observed at 48 h post-decapitation which clearly indicate that these relate to elongation of ES (paper I). These results suggest that the important changes leading to the bud outgrowth in *Populus* occurs early in the process and the results seem to coincide with those obtained from paper I and II. It is also in concurrence with the previous results from (Rinne *et al.*, 2015; Rinne *et al.*, 2016).

The functional gene enrichment analysis (GO) performed in control and decapitated (6, 12, 24, 48 and 72 h) showed that the GO terms were highly represented between 12 h to 48 h post decapitation. Lipid cellular process, transport, localization, cellular metabolism, translation, cellular biosynthesis, response to stress, metabolism, transport, biosynthesis, response to stress were some of the GO terms overrepresented between 12-48 h after decapitation.

4.3.1 Effect of decapitation on other plant hormonal pathway genes in perennial *Populus*

Consistent with the study of changes in SL and GA pathway genes induced by decapitation (Paper I & II), the current paper III is also aimed to decipher the role of various hormone-related genes to auxin, CK, ABA, JA, SA and BR by the RNA sequencing analysis. The main highlights are as follows. CK promotes shoot branching through AXB activation (del Rosario Cárdenas-Aquino, Sarria-Guzmán and Martínez-Antonio, 2022). Among the CK genes, *IPTs* are the most important genes that play a role in shoot branching in *Arabidopsis* (Müller *et al.*, 2015) which showed downregulation in gene expression upon decapitation except *IPT3* (paper III). This is consistent with the results from (Müller *et al.*, 2015). The increase in CK level two days after decapitation (paper III) could be one of the factors for inducing ES elongation followed by AXB activation mainly through the AXB produced CK locally (Nordström *et al.*, 2004; Tanaka *et al.*, 2006; Ferguson and Beveridge, 2009). Most of the signaling genes belonging to *ARR1* family showed an increase in expression after 72 h (paper III). These results are consistent with the previous findings that CK plays a key role in positively regulating shoot branching. On the contrary, ABA is known to act as a negative regulator in bud activity in *Arabidopsis* (Emery, Longnecker and Atkins, 1998; Yao and Finlayson, 2015; Reddy *et al.*, 2013) and many studies have shown that the accumulation of ABA in buds correlates negatively to bud outgrowth (Tucker and Mansfield, 1971; Tucker, 1977; Tamas *et al.*, 1979; Knox and Wareing, 1984; Gocal *et al.*, 1991; Mader, Emery and Turnbull,

2003). The studies related to the effect of R:FR on ABA levels showed that it acts as a general regulator of branching and acts independently of auxin and SL signaling and *BRC1* (Yao and Finlayson, 2015). There has been no evidence till date on the effect of decapitation in regulating bud activity via ABA pathway genes. In our study, a range of ABA biosynthetic genes (*NCEDs*) were upregulated as early as 6-12 h and accumulation of ABA requires *NCED3* (González-Grandío et al., 2017a). It was shown that accumulation of ABA excessively results in branching inhibition (Mader, Emery and Turnbull, 2003; Holalu and Finlayson, 2017). Additionally, the ABA-catabolism gene *CYP707A* was initially upregulated at 6 h and down-regulated at 12 h followed by suppressing AXB outgrowth through downregulation of *ABRE* at 72 h (except the upregulation of *ABRE2* and *5* at 24 h) indicating that *ABRE* acts in the negative feedback mechanism of ABA signaling.

Genes related to plant hormones (JA and SA) involved in stress responses and defense (Yang *et al.*, 2019) were also affected by decapitation. One such critical hormone that regulates stress responses is Jasmonic acid (JA). The genes related to JA biosynthesis showed increased expression level as early as 6 h whereas, JA signaling showed downregulation at a later time point of 72 h suggesting the defense signaling genes mediated by JA pathway showed increased response to decapitation mediated activation (paper III). In the same line, although SA pathway has not been studied in response to shoot branching, we identified several SA related genes which might have a role in shoot branching. Among them, *ICS1/2* and *PAL* genes which are important in SA biosynthesis and synthesis, both showed an upregulation at 12 h after decapitation. SA pathway genes showed downregulation much earlier than JA signaling genes (paper III). Apart from these two hormones, a very well-known hormone BR has been known to act as a growth-promoting steroid hormone. Although BR pathway is well characterized in *Arabidopsis* (Hu et al., 2020), there is not much known about its regulation till now in perennials. Our data showed that the important BR signaling genes *BRI1*, *BES1* and *BZR1* showed similar expression pattern with after decapitation and down regulated at 72 h (paper III). *DWF1* and *DET2* also showed a similar expression pattern. In *Arabidopsis* there was an increased accumulation of BR and the expression of *BZR1* was upregulated upon apical bud removal (Xia *et al.*, 2021). An increase in the BR content was observed in both bud and nodes upon decapitation which coincides with our decapitation data with the downregulating effect of signaling genes (Xia *et al.*, 2021).

5. Conclusions

The first part of this thesis (paper I) aimed to shed the light on the molecular mechanisms associated to the role of SL pathway genes in perennial shoot branching in hybrid aspen. The results showed that nodes rather than roots are the primary supplier of SL and its precursors to AXBs and AXBs themselves can synthesize SL-like compounds downstream of CL. It is also shown that SL might be necessary to inhibit AXB activation in intact plants, but elongation of ES requires SL activity. Even though SL restrain the bud outgrowth in intact plants (para-dormant) and during the formation of AXBs, it cannot overtake the factors that promote the activated AXBs.

The second part of the study (paper II) aimed to understand the role of GA in shoot branching in perennial woody species, hybrid aspen and to investigate the transcriptomic and metabolite analysis of GA pathway components. The results showed that accumulation of $GA_{3/6}$ mediated by SL and *GA3ox1*, helps to keep the AXBs in a paradormant state. This is done through the upregulation of *GA2ox* genes by $GA_{3/6}$ and deactivation of $GA_{1/4}$ by GA2ox. On the other hand, AXB activation by decapitation is caused by the downregulation of GA deactivating genes *GA2oxs*. *GA2ox* genes makes the $GA_{1/4}$ pool available for GA signaling. The increase in *GA3ox2*-mediated GA biosynthesis by $GA_{1/4}$. This opposite roles of $GA_{1/4}$ and $GA_{3/6}$ and dual roles (inhibitory and activation) of GA explains why the GA model in shoot branching remains obscure.

The final aim of the study was to investigate the genome-wide study of the regulatory hormone pathways. Paper III investigated and validated the role of SL and GA associated genes (from Paper I and II) along with other hormone pathways (AUX, CK, ABA, SA, JA, BR), by analyzing the transcriptome responses of AXBs below BMP to decapitation compared to control in the model tree species hybrid aspen. This comprehensive study provided an insight into understanding the complex molecular mechanisms involved in shoot branching upon decapitation. Our study also provides a starting point to unravel the role of shoot branching in previously unstudied plant hormones in perennial woody species.

6. Further perspectives

The results of this study added further knowledge about the hormonal control of shoot branching in proleptic hybrid aspen. The results in this thesis show a clear understanding on the role of SL in AXB inhibition in intact plants. Since SL is produced is a minute quantity in buds, it was difficult to quantify the amount of SL in different zones along the stem. Hence, a more sophisticated method for detecting SL using LC/MS-MS needs to be developed. In order to better understand the role of other plant hormones (auxin, CK, ABA, JA, SA, BR), it is crucial to analyze the content of each hormone in the AXBs as well as the effect of decapitation on the hormonal content for the same. To better understand the interdependence of SL and GA pathway along with the changes in other hormonal pathways, transcriptional studies using RNA sequencing may be performed for the AXBs treated with SL analog GR24, GA_3 and GA_4 in a single node feeding system. It would also be helpful to study physiological and molecular characterization of some interesting mutants in hybrid aspen by CRISPR/Cas9- directed mutagenesis.

7. References

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Paper I

PCP
PLANT & CELL PHYSIOLOGY

Strigolactone-Based Node-to-Bud Signaling May Restrain Shoot Branching in Hybrid Aspen

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Regular Paper

The biosynthesis and roles of strigolactones (SLs) have been investigated in herbaceous plants, but so far, their role in trees has received little attention. In this study, we analyzed the presence, spatial/temporal expression and role of SL pathway genes in *Populus tremula* × *Populus tremuloides*. In this proleptic species, axillary buds (AXBs) become para-dormant at the bud maturation point, providing an unambiguous starting point to study AXB activation. We identified previously undescribed *Populus* homologs of *DWARF27* (*D27*), *LATERAL BRANCHING OXIDOREDUCTASE* (*LBO*) and *DWARF53-like* (*D53-like*) and analyzed the relative expression of all SL pathway genes in root tips and shoot tissues. We found that, although AXBs expressed *MORE AXILLARY GROWTH1* (*MAX1*) and *LBO*, they did not express *MAX3* and *MAX4*, whereas nodal bark expressed high levels of all SL biosynthesis genes. By contrast, expression of the SL perception and signaling genes *MAX2*, *D14* and *D53* was high in AXBs relative to nodal bark and roots. This suggests that AXBs are reliant on the associated nodes for the import of SLs and SL precursors. Activation of AXBs was initiated by decapitation and single-node isolation. This rapidly downregulated SL pathway genes downstream of *MAX4*, although later these genes were upregulated coincidently with primordia formation. GR24-feeding counteracted all activation-related changes in SL gene expression but did not prevent AXB outgrowth showing that SL is ineffective once AXBs are activated. The results indicate that nodes rather than roots supply SLs and its precursors to AXBs, and that SLs may restrain embryonic shoot elongation during AXB formation and para-dormancy in intact plants.

Keywords: Axillary bud • DWARF27 (D27) • DWARF53-like (D53-like) • LATERAL BRANCHING OXIDOREDUCTASE (LBO) • *Populus*.

Introduction

In deciduous trees, crown architecture arises through the coordinated action of terminal and axillary meristems (AXMs). In contrast to annuals, like *Arabidopsis* (Grbić and Bleecker 2000, Long and Barton 2000, Greb et al. 2003), the AXMs of deciduous trees arise in the axils of emerging leaves and produce axillary buds (AXBs) with bud scales (Garrison 1955). The timing and pattern of branch formation reflect different branching styles. In sylleptic branching, newly formed AXBs produce branches in the same season, whereas in proleptic branching they may produce them only in the following seasons (Hallé et al. 1978, Ceulemans et al. 1990, Wu and Stettler 1998, Barthélémy and Caraglio 2007). The sylleptic branching pattern is strongly influenced by the prevailing environmental conditions, revealing considerable plasticity in architectural design. On the other hand, in proleptic species AXB outgrowth is postponed to the next growing season, resulting in a more robust branching pattern (Cline 1997).

Hybrid aspen (*Populus tremula* × *Populus tremuloides*, clone T89) is a typical proleptic species. AXBs develop during the growing season until the dwarfed side shoot, enclosed by five scales, has produced about 10 embryonic leaves. This point is referred to as the bud maturation point (BMP; Rinne et al. 2015). These mature AXBs partially dehydrate and remain para-dormant, at least until the next growing season, but they can be activated expeditiously by decapitation. This allows the investigation of processes that exclusively relate to AXB activation, and not to AXB formation. In sylleptic tree species, where branches are initiated in the same season, such unambiguous starting point is lacking.

In woody perennials, very little is known about the molecular processes that control branching. By contrast, these processes are under intensive investigation in herbaceous annuals, like *Arabidopsis* and pea, as well as in the monocot rice (Sorefan et al. 2003, Domagalska and Leyser 2011, Wang and Li 2011). They show that the AXB activation is regulated by a network of interacting hormones. Although auxin and cytokinins are the classic branching hormones (King and Van Staden 1988, Müller and Leyser 2011), recent work with woody species shows that gibberellins (GA) also play a role (Ni et al. 2015, Rinne et al. 2016). Crucial newcomers in this network are carotenoid-derived terpenoid lactones, referred to as strigolactones (SLs) that suppress branching (Gomez-Roldan et al. 2008, Umehara et al. 2008, Ferguson and Beveridge 2009).

So far, all natural SLs have been isolated from root exudates and identified based on their capacity to stimulate germination of parasitic plant seeds (Kobae et al. 2018). The first SL, identified in root exudates of cotton, was named strigol because it stimulated the germination of witchweed (*Striga lutea* Lour) seeds (Cook et al. 1966, Cook et al. 1972). When *Striga* seeds are in close proximity of the roots of a strigol exuding host plant, they will germinate and parasitize the plant. The finding

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that not only host plants but also non-hosts like cotton exuded SL-like compounds to the rhizosphere, indicated that SLs had some distinct function unrelated to parasitic seed germination (Wang and Bouwmeester 2018). Such non-host SL exudation was found to attract arbuscular mycorrhizal (AM) fungi to colonize plant roots, particularly under conditions of phosphate starvation (Yoneyama et al. 2007, López-Ráez et al. 2008, Carbonnel and Gutjahr 2014). In an established symbiotic relationship, the AM fungi deliver phosphate to the plant, while in return the plant provides sugars (Akiyama et al. 2005, Besserer et al. 2006).

In addition to inhibiting shoot branching and attracting AM fungi, SLs have crucial roles in secondary growth, root development and leaf senescence (Gomez-Roldan et al. 2008, Umehara et al. 2008, Kapulnik et al. 2011, Rasmussen et al. 2012, Yamada et al. 2014). The role of SL in shoot branching has been analyzed in branching mutants of *Arabidopsis* (Sorefan et al. 2003, Booker et al. 2004, Booker et al. 2005, Domagalska and Leyser 2011, Seto and Yamaguchi 2014), pea (Beveridge et al. 1997, Hamiaux et al. 2012), petunia (Drummond et al. 2009, Hamiaux et al. 2012) and rice (Wang and Li 2011, Zhang et al. 2014).

A generalized SL pathway can be subdivided into three distinct parts, which are spatially separate: the plastid, the cytoplasm/symplasm and the nuclei of cells in the target areas (**Fig. 1**). In the plastid carotenoid pathway (Matusova et al. 2005), all-*trans*-β-carotene is converted to the biosynthetic intermediate carlactone (CL), which is exported to the cytoplasm (Alder et al. 2012, Kobae et al. 2018, Yoneyama et al. 2018). CL biosynthesis involves three important classes of plastid enzymes. In *Arabidopsis*, these include the isomerase DWARF27 (D27), and two carotenoid cleavage dioxygenases (CCD7 and CCD8), encoded by *MORE AXILLARY GROWTH3* (*MAX3*) and *MORE AXILLARY GROWTH4* (*MAX4*), respectively.

CL is a chemically stable and graft-transmissible intermediate that must be converted by the ER-anchored enzyme MORE AXILLARY GROWTH1 (MAX1; Cytochrome P450) to carlactonoic acid (CLA; Abe et al. 2014) or 4-deoxyorobanchol (4DO; Alder et al. 2012). CL and CLA are non-canonical SLs that possess the essential enol ether-D-ring moiety required for biological activity (Zwanenburg et al. 2009) but not the complete ABCD ring system found in canonical SLs (Yoneyama et al. 2018). CLA, the universal precursor of a variety of species-dependent SLs (Iseki et al. 2018), is methylated to methyl carlactonoate (MeCLA) in roots and shoots by an unidentified enzyme (Abe et al. 2014, Iseki et al. 2018, Yoneyama et al. 2018). In *Arabidopsis*, MeCLA is a substrate of the 2-oxoglutarate-dependent dioxygenase LATERAL BRANCHING OXIDOREDUCTASE (LBO), which oxidizes MeCLA to a compound referred to as MeCLA+16D (Brewer et al. 2016).

SL perception requires the F-box protein MAX2 and the unconventional hormone receptor DWARF14 (D14), a protein of the α/β-fold hydrolase superfamily. In *Arabidopsis*, as well as rice, SL triggers interactions among D14, MAX2 and SMXL/D53 in the nuclei of target cells (Zhou et al. 2013, Zhao et al. 2015, Liang et al. 2016, Yao et al. 2016). In rice, the D53 protein was identified as a repressor of the SL signaling pathway, which is targeted for degradation after SL treatment (Jiang et al. 2013).

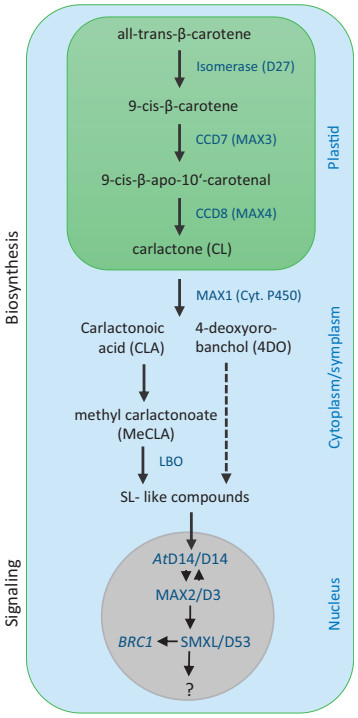

Fig. 1 Generalized scheme of SL biosynthesis and signaling. The schema envisions three compartments: the biosynthetic compartment of the plastid (green) where CL is produced, the cytoplasmic and the symplasmic compartment (light blue) where excreted CL is converted to CLA/4DO by MAX1, and the nucleus (grey) where perception occurs. CLA is converted to MeCLA, and further by LBO. The SL-like compounds downstream of MeCLA and 4DO (stippled line) are imported into the nuclei of target cells, where *At*D14/D14 interacts with the F-box protein MAX2/D3 in an SL-dependent manner to ubiquitinate and degrade the transcription repressor SMXL/D53, resulting in expression of *BRC1*.

The rice F-box protein DWARF3 (D3, ortholog of *Arabidopsis* MAX2) plays a crucial role in mediating this degradation. It requires D14 to ubiquitinate D53 for degradation by the D14-SCF^{D3} ubiquitin ligase, to promote SL signaling and responses (Jiang et al. 2013, Zhou et al. 2013). A downstream target of SL signaling is the gene *BRANCHED1* (*BRC1*)/*TEOSINTE BRANCHED1* (*TB1*), which encodes a transcription factor that suppresses shoot branching (Doebley et al. 1997, Aguilar-Martínez et al. 2007, Finlayson 2007, Finlayson et al. 2010, Seale et al. 2017).

Although in annuals SL biosynthesis and signaling genes are largely conserved (Yao et al. 2018), in woody perennials their presence and function remain mostly unexplored. Given the distinct initiation, development and composition of AXBs in hybrid aspen (Rinne et al. 2015), it is uncertain if the complete pathway is present and functionally conserved in *Populus*. So far, few SL pathway genes have been identified in perennials

(Wang and Li 2006, Czarnecki et al. 2014, Zheng et al. 2016). However, it has been reported that *Populus* root exudate contains 4DO, a canonical SL, and the non-canonical SLs CLA and MeCLA (Xie 2016). Tentative evidence indicates that the inhibition of shoot branching is mediated by non-canonical SLs (Yoneyama et al. 2018). Indeed, grafting experiments with pea, *Arabidopsis* and petunia showed that root-produced CL can be imported by the shoot (Beveridge et al. 2000, Morris et al. 2001, Turnbull et al. 2002). However, to inhibit branching in *Arabidopsis* it must be converted by MAX1 to CLA, as CL is ineffective in *max1* mutants (Scaffidi et al. 2013). In addition, these studies showed that SL biosynthesis genes can also be expressed in shoots, as a wild type scion on an SL-deficient mutant stock does not display a branching phenotype. However, so far SL-like compounds have not been isolated from shoots, indicating that their levels may be very low (Kobae et al. 2018).

In AXBs of hybrid aspen, two *MAX1* orthologs and two orthologs of the SL target gene *BRC1* are expressed (Rinne et al. 2015). All four genes were upregulated during AXB development, reaching their highest levels in mature AXBs, whereas decapitation at the BMP downregulated them in the proximal AXBs (Rinne et al. 2015). In agreement with this, in *Populus* × *canescens*, knockdown of SL biosynthesis genes reduced *BRC1* expression and induced branching, like knockdown of *BRC1* and *BRC2* (Muhr et al. 2016, Muhr et al. 2018). Together, these findings suggest that at least part of the SL biosynthesis and signaling genes as well as downstream targets are functional in *Populus*.

Our first aim was to investigate whether close homologs of the *Arabidopsis* and rice SL pathway genes (**Fig. 1**) were present in the *Populus trichocarpa* genome (Tuskan et al. 2006), and if and where they were expressed in hybrid aspen. In addition, we aimed to assess whether the unique lifestyle of woody perennials and their complicated bud structure would put different demands on the spatial layout of the SL biosynthesis and signaling paths. Here, we, identified all SL pathway genes in the *P. trichocarpa* genome, and analyzed their expression in roots and shoot tissues of the non-branching hybrid aspen seedlings (**Fig. 2**). Nodal bark, rather than root tips, appeared to be major hubs for SL biosynthesis, whereas the AXBs were dominant centers of SL perception. Decapitation-activated AXBs rapidly downregulated SL pathway genes coincident with the start of embryonic shoot (ES) elongation, suggesting that SL inhibits this elongation in intact plants.

Results

Expression of SL biosynthesis genes in hybrid aspen

The first enzyme in the SL biosynthesis pathway is isomerase D27 (**Fig. 1**), but so far it has not been reported for woody perennials. We identified three close homologs of the *Oryza sativa D27* gene (Lin et al. 2009) in the *P. trichocarpa* genome (Supplementary Fig. S1), and named them *D27a*, *D27b* and *D27c*. Transcripts of *D27a* and *D27c* were expressed in most plant parts, whereas *D27b* was undetectable. *D27a* transcript levels were higher than those of *D27c*, in the apex and particularly in the sink and source leaves (**Fig. 3A**). Remarkably, in roots, thought to be the major source of SL, *D27a* transcripts were undetectable, and *D27c* expression was also very low (**Fig. 3A**). However, roots expressed *MAX3* and *MAX4*, the two downstream SL biosynthesis genes that mediate CL production (**Figs. 1**, **3B**). By contrast, the expression of *MAX3* and *MAX4* was virtually absent in developing and mature AXBs, but surprisingly the associated nodes expressed both genes at high levels (**Fig. 3B**). The expression of *MAX3* was higher than that of *MAX4* in both sink and source nodes. The sink nodes, which support the young developing AXBs, expressed both genes at very high levels (**Fig. 3B**, inset). *MAX1* genes were expressed in all plant parts, including the AXBs (**Fig. 3C**). However, because the AXBs themselves did not express *MAX3* and *MAX4*, *MAX1* must serve to convert imported CL. As the expression of *MAX3* in both sink and source nodes, and *MAX4* in sink nodes, were at exceptionally high levels compared to roots (**Fig. 3B**), the AXBs of hybrid aspen are likely to import CL from the nodes rather than from the roots.

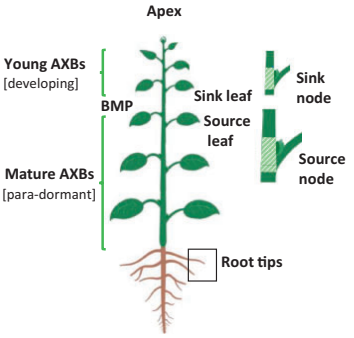

Fig. 2 Cartoon depicting the position of young and mature AXBs. The developing young AXBs become para-dormant at the BMP. Sink node and source node denote bark tissue, isolated from the nodes of young and mature AXBs, respectively (hatch pattern). Root material was isolated exclusively from root tips.

In *Arabidopsis*, a downstream product of CLA is the methyl ester MeCLA (**Fig. 1**), which can directly interact with the SL signaling component D14 (Abe et al. 2014). However, MeCLA is also substrate for LBO (**Fig. 1**), and conversion into other SL-like compounds might be required for at least some of its bioactivity (Brewer et al. 2016). To date, no information is available about its precise role, and whether it is conserved in woody species.

To identify the *LBO* gene, we searched the *P. trichocarpa* genome for a putative ortholog of *AtLBO* (encoded by locus *At3g21420*) and identified a protein encoded by Potri.010G023600 as *PtLBO*. The number of amino acids in *PtLBO* (364 aa) is identical to that in *AtLBO* (Supplementary Fig. S2) and exhibits 84% similarity and 66% identity at the

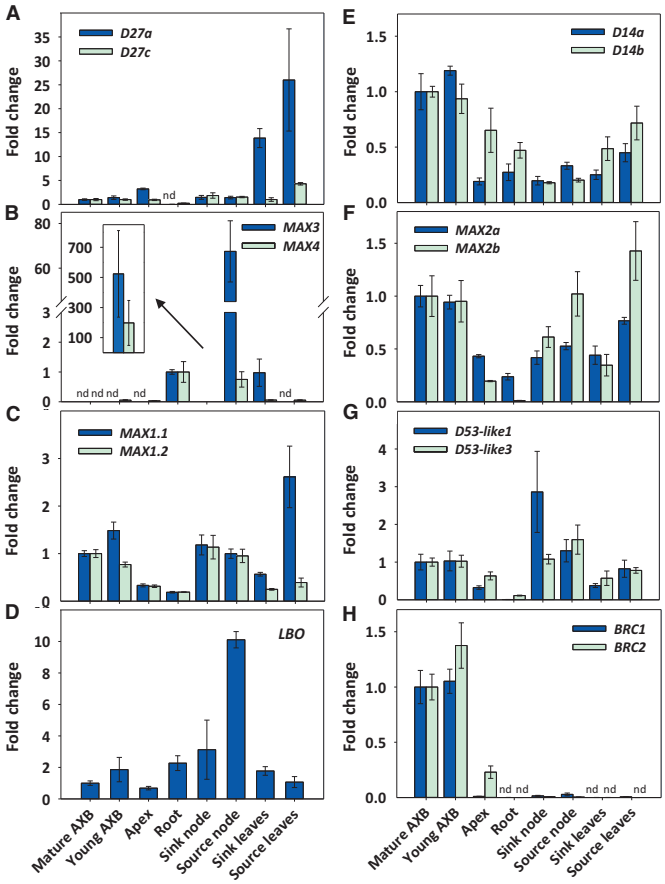

Fig. 3 Expression of SL biosynthesis genes in different plant parts. Expression (fold change) was analyzed in mature AXBs, young AXBs, apex, root, sink node, source node, sink leaves and source leaves. (A) *D27a* and *D27c*. (B) *MAX3* and *MAX4*. (C) *MAX1.1* and *MAX1.2*. (D) *LBO*. (E) *D14a* and *D14b*. (F) *MAX2a* and *MAX2b*. (G) *D53-like1* and *D53-like3*. (H) *BRC1* and *BRC2*. Values represent the means of three biological replicates ±SE ($n = 6$ plants). nd, not detected. The expression value of the mature AXBs or roots was set at 1.

amino acid level. *LBO* was expressed throughout the plant, including AXBs, but the highest relative expression was found in source nodes, followed by sink nodes and roots (**Fig. 3D**).

Expression of SL signaling genes in hybrid aspen

In *Arabidopsis*, the α/β-hydrolase D14 and the F-box protein MAX2 are essential components in the SL-dependent suppression of AXB outgrowth. D14, thereby, functions as an SL receptor with catalytic activity. Although D14 is localized in the cytoplasm and nucleus (Chevalier et al. 2014), the nuclear pool is responsible for D14 function (Liang et al. 2016). SL triggers the physical interaction among nuclear-localized D14, MAX2 and SMXL7/D53 in the nuclei of target cells, resulting in degradation of SMXL7/D53 (Liang et al. 2016). *D14* as well as *MAX2* homologs have been identified previously in a *Populus* species (Czarnecki et al. 2014, Zheng et al. 2016), but their tissue-specific expression and role in AXBs have not been investigated. The present data show that in hybrid aspen all plant parts expressed *D14a* and *D14b*. Transcript levels in AXBs were two to three times higher than in roots, while levels in the corresponding nodes were somewhat lower than in roots (**Fig. 3E**). A similar trend was found for *MAX2a* and *MAX2b* transcripts, although here the lowest expression level was in roots instead of nodes (**Fig. 3F**). The relative expression of both signaling genes, *MAX2* and *D14*, was highest in AXBs. However, *MAX2b* was also well expressed in source tissues (**Fig. 3E, F**). Thus, although the production of SL-like compounds downstream of CL occurs predominantly in both sink and source nodes, SL perception appears particularly dominant in AXBs (**Fig. 3E–G**).

Downstream targets of SL signaling in hybrid aspen

In rice, enhanced SL signaling results in the proteasomal degradation of the *Os*D53 (**Fig. 1**), a suppressor of SL signaling, resulting in inhibition of AXB activation and outgrowth (Jiang et al. 2013, Zhou et al. 2013). Using phylogenetic analysis, we identified three *P. trichocarpa* homologs of *Os*D53, which we named *D53-like1*, *D53-like2* and *D53-like3* (Supplementary Fig. S6). All three genes were expressed throughout the plant, with the possible exception of roots in the case of *D53-like1* and *D53-like2* (**Fig. 3G;** Supplementary Fig. S3A). As *D53-like2* was unresponsive to decapitation, we considered it not relevant for branching (Supplementary Fig. S3B). Although hardly expressed in roots, AXBs and their associated nodes expressed *D53-like1* and *D53-like3* at appreciable levels (**Fig. 3G**). Expression in the apex was about half of that in AXBs and nodes.

Among the downstream targets of SL in *Arabidopsis* is the branch-inhibitor gene *BRC1* (**Fig. 1**), which encodes a class II TB1 CYCLOIDEA PCF (TCP) type transcription factor (Aguilar-Martínez et al. 2007, Finlayson 2007) that represses cell proliferation (Schommer et al. 2014). As we showed previously, hybrid aspen has two *BRC* genes, *BRC1* and *BRC2*, which are upregulated in developing AXBs (Rinne et al. 2015). Here, we confirm that *BRC1* and *BRC2* are highly expressed in AXBs, but that their relative expression elsewhere in the plant was very low or undetectable, except for *BRC2* in the shoot apex (**Fig. 3H**). This suggests that SL signaling targets *BRC1* and *BRC2* in the dwarfed side shoots of the AXBs to inhibit outgrowth.

Decapitation-induced developmental changes in AXBs

To assess the role of SL biosynthesis and signaling in the activation of mature, developmentally inactive AXBs, plants were decapitated at the BMP. Changes in gene expression were analyzed in the AXB proximal to the decapitation point. To provide context to these gene expression changes, we investigated the time-frame of decapitation-induced developmental changes in the proximal AXB (**Fig. 4**). The lengths of the AXBs and ESs were measured, and the number of embryonic leaves counted at regular intervals post decapitation (**Fig. 4B**). The length of the proximal AXB increased gradually after decapitation, and the increase was statistically significant after 48 h. The elongation of the ES shoot followed a similar pattern, albeit a statically significant increase occurred 1 d earlier (**Fig. 4B**). The number of embryonic leaves was constant over the entire 96-h period, showing that no neo-formed leaves were produced (**Fig. 4B**). Together, the data show that decapitation-induced changes in gene expression during the first 48 h clearly relate to elongation of the ES stem, and not to the formation of new leaves at the shoot apical meristem (SAM) of the ES.

Post-decapitation expression of SL biosynthesis and signaling genes

To pinpoint the role of SL biosynthesis and signaling in the early activation events of the proximal, mature AXBs, we restricted our analyses to the genes that were expressed in the AXBs themselves (**Fig. 3**). The expression of *D27*, *MAX1*, *LBO*, *D14*, *MAX2* and *D53-like* genes, as well as the downstream target genes *BRC1* and *BRC2*, was analyzed in AXBs during the critical 0–48 h post-decapitation period (**Fig. 5**).

The expression of *D27a* and *D27c* was somewhat reduced between 6 and 12 h after decapitation, and thereafter gradually recovered (**Fig. 5A**), although these changes were not statistically significant. As *MAX3* and *MAX4* were not expressed in AXBs of intact plants (**Fig. 3B**), the modest decapitation-induced alterations in the two *D27* genes might not relate SL-mediated events in the AXBs. *MAX1.1* and *MAX1.2* expressions, and putative CLA production, were significantly reduced by decapitation between 2 and 6 h (**Fig. 5B**). *LBO* expression showed a statistically significant increase that started between 12 and 24 h (**Fig. 5C**).

Although *D14a* and *D14b* were specifically expressed at high levels in all AXBs of intact plants (**Fig. 3E**), decapitation significantly reduced transcript levels in the AXB proximal to the decapitation point (**Fig. 5D**). The transcript levels of both *D14* genes declined significantly between 2 and 6 h, and onward. Although *D14b* expression diminished more gradually, both *D14* genes had the same low level at the 48 h time point (**Fig. 5D**). The expression of F-box genes *MAX2a* and *MAX2b* also decreased relatively early, between 2 and 6 h post decapitation, although *MAX2a* expression tended to recover (**Fig. 5E**). Of the two *D53-like* genes, *D53-like1* expression was significantly reduced by decapitation between 0 and 2 h. By contrast, the decrease in *D53-like3* expression was only transient, and it increased significantly between 12 and 24 h (**Fig. 5F**).

The downstream target of SL signaling, *BRC1*, which was highly expressed in mature AXBs of intact plants (**Fig. 3H**), was rapidly and strongly downregulated after decapitation between 0 and 2 h in the proximal AXB. *BRC2* expression was more gradually and modestly reduced (**Fig. 5G**).

Taken together, the downregulation of *MAX1.1*, *MAX1.2*, *D14a*, *D14b*, *MAX2b*, *D53-like1*, *BRC1* and *BRC2* as well as the upregulation of *LBO* (**Fig. 5**) preceded the initial phase of ES stem elongation, and the subsequent neo-formation of leaves (**Fig. 4**).

Developmental changes in AXBs of GR24-treated single-node systems

As we found that SL pathway dynamics within the AXB-node complex reflected the transition from inactivity to activation, we hypothesized that an increase in SL content will prevent AXB activation. To investigate the effect of SL application, we used single-node systems to xylem-feed the SL analog GR24 into AXBs (**Fig. 6**). These systems, commonly used to study bud burst (Rinne et al. 2011, Brewer et al. 2015, Rinne et al. 2016, Seale et al. 2017, Xie et al. 2017), are particularly useful in woody perennials where direct application to the buds is ineffective. Xylem-feeding also enables the investigation of AXB activation independent from the constraints of apical dominance and leaf- or root-derived signals.

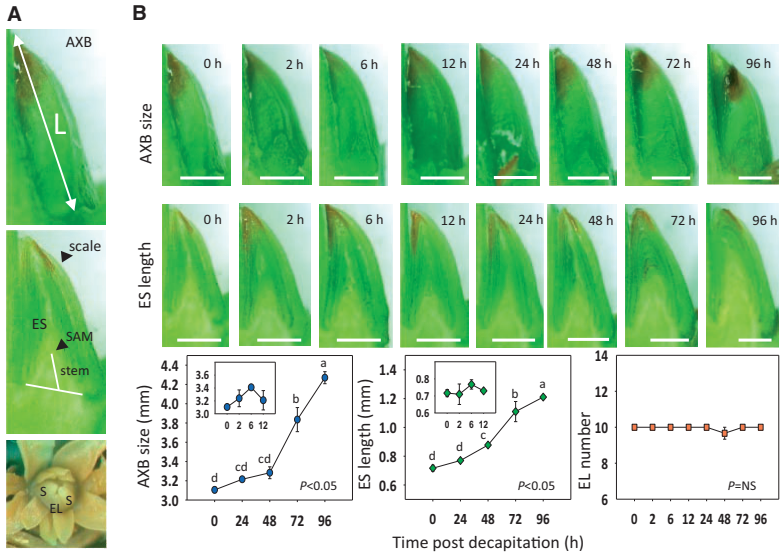

Fig. 4 AXB size, ES length and the number of embryonic leaves (EL) in the AXB proximal to the plant decapitation point. (A) The size of the AXBs was measured as indicated (L, upper panel). ES length was measured from the surface of the SAM to the basal line connecting the outer bud scales (middle panel). EL number, as depicted, was counted in fixed AXBs (lower panel). (B) Upper row photographs show AXB sizes, measured at 0, 2, 6, 12, 24, 48, 72 and 96 h post decapitation. The lower row shows ES lengths at the same time points. The line graphs show AXB size (left), ES length (middle) and EL number (right) at the indicated times after plant decapitation. Different letters indicate statistical differences between the time points (one-way ANOVA with *post hoc* Fisher's LSD test; *P*-value at least <0.05; NS, not significant). Scale bars, 1.0 mm.

GR24 (10 μM) was fed into the internode base of single-node systems for 3, 5 or 7 d. At day 3 (72 h), the young as well as the mature AXBs that were kept on water (controls) were already enlarging, while GR24-treated young and mature AXBs were slightly less elongated. However, at days 5 and 7 the effect was reversed, particularly in the case of mature AXBs (**Fig. 6**). The 7-d time point was repeated in a separate experiment, with a similar result. However, in both experiments, the promoting effect of GR24 on AXB size was not statistically significant (**Fig. 6**, inset). The sturdy outer scale of the mature AXBs did not elongate much, and AXB enlargement at day 7 was mostly due to the protrusion of the inner scales from the tip of the buds (Supplementary Fig. S4). GR24-feeding had a similar but more pronounced effect on the elongation of the ES. At the 7-d time point, the ESs of GR24-fed mature AXBs were significantly longer than the controls. The 7-d time point was repeated in a separate experiment, confirming that GR24 could enhance ES elongation once AXBs were activated (**Fig. 6**, inset).

Notably, in mature AXBs the number of embryonic leaves had increased from 10 to 12 by day 5 (120 h), and at day 7 (168 h) several additional primordia had emerged, with or without GR24. Young AXBs possessed fewer embryonic leaves at the time of single-node isolation, but also here the number rose steadily without any visible interruption. Although the GR24-fed young AXBs appeared to slightly delay leaf initiation, the differences in leaf numbers were not statistically significant (**Fig. 6**, inset).

Effects of GR24 on gene expression in AXBs of single-node systems

As GR24-feeding only affected the elongation of the ES in a statistically significant way, the early changes in gene expression must relate to ES elongation. Here, we investigated, how GR24-feeding would affect the SL pathway genes (**Fig. 1**) in mature and young AXBs. In the decapitation experiments, we probed the early changes in gene expression in the period preceding primordia formation (0–48 h). As in single-node systems, primordia formation started after day 3, we analyzed gene expression during an extended time-frame, including day 0, day 3 (72 h) and day 5 (120 h) (**Fig. 7**).

Whereas the *MAX1.1* and *MAX1.2* were highly expressed in young and mature AXBs (**Fig. 3C**), both genes were downregulated in AXBs in water, like in decapitation, except for *MAX1.2* in young AXBs. In all cases, GR24-feeding counteracted the change in expression (**Fig. 7A**), suggesting feedback on *MAX1* gene expression. Both, the downregulation and the counter-effect of GR24, were statistically significant for *MAX1.1*. In both young and mature AXBs, *D14a* and *D14b* were significantly upregulated without GR24, unlike in decapitation, while GR24-feeding repressed this completely (**Fig. 7B**). In young AXBs, *MAX2a* and *MAX2b* showed a similar response

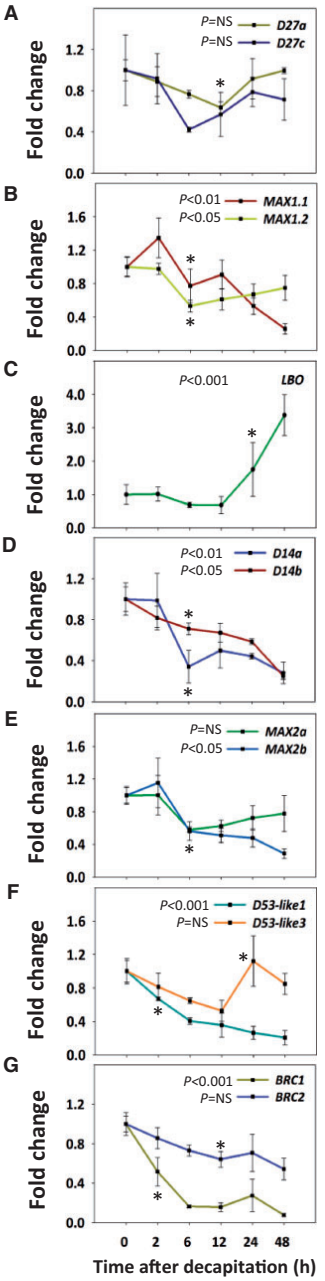

Fig. 5 Expression of SL pathway genes in AXBs proximal to the plant decapitation point. Gene expression (fold change) was analyzed at 0, 2, 6, 12, 24 and 48 h post decapitation. (A) *D27a* and *D27c*. (B) *MAX1.1* and *MAX1.2*. (C) *LBO*. (D) *D14a* and *D14b*. (E) *MAX2a* and *MAX2b*. (F) *D53-like1* and *D53-like3*. (G) *BRC1* and *BRC2*. Values represent the means of three biological replicates ±SE ($n = 6$ plants). Values were calculated relative to the AXBs at $t = 0$, set at 1. One-way ANOVA (*P*-value; NS, not significant). Asterisks indicate the first significant change in gene expression (Fisher's LSD test; *P*-value at least <0.05).

as the two *D14* genes in that both were significantly upregulated in water, while GR24 prevented this increase (**Fig. 7C**). Although in mature AXBs expression of *MAX2a* and *MAX2b* only slightly increased in the controls, GR24 had a statistically significant reducing effect on *MAX2a* (**Fig. 7C**).

D53-like1, encoding a putative repressor of SL signaling, was significantly downregulated in mature as well as in young AXBs, but GR24 prevented this decrease (**Fig. 7D**). This is in line with the decapitation experiments, where *D53-like1* was significantly downregulated already at day 1 and continued to decline up to 48 h (**Fig. 5**). Conversely, *D53-like3*, which is more closely related to *AtD53* than to *OsD53* (Supplementary Fig. S6), was upregulated in both young and mature AXBs in water, but GR24 prevented it in both cases (**Fig. 7D**). The increased expression of *D14a*, *D14b*, *MAX2a* and *MAX2b* in AXBs without GR24 could indicate that SL perception increased in response to diminished signal supply, reflecting homeostasis because GR24-feeding prevented upregulation of these genes. By contrast, the downstream target genes *BRC1* and *BRC2* were not significantly affected, except for *BRC1* in young AXBs.

In summary, the GR24-induced changes in expression of SL pathway genes in young and mature AXBs were quite similar, suggesting that the developmental stage is less important for the activation response. Although *MAX1.1*, *MAX1.2* and *D53-like1* were downregulated in the controls, *D14a*, *D14b*, *MAX2a*, *MAX2b* and *D53-like3* were upregulated. The only exception appeared to be *MAX1.2* in young AXBs, as it was not downregulated in controls. GR24-feeding counteracted these changes in all cases.

Discussion

The role of SL in shoot branching has been explored mainly in herbaceous plants (Gomez-Roldan et al. 2008, Umehara et al. 2008, Bennett and Leyser 2014, Marzec 2016, Waters et al. 2017, Barbier et al. 2019). This has yielded a wealth of data, showing that the studied species share the SL biosynthesis pathway that produces the universal precursor CLA, which is further converted to canonical and non-canonical SLs in a species-dependent fashion (Xie 2016, Iseki et al. 2018). The physiological relevance of this diversity has remained unclear (Zwanenburg and Blanco-Ania 2018). Although both canonical and non-canonical SLs are found in root exudates (Xie 2016, Iseki et al. 2018), tentative evidence shows that the SLs that regulate shoot branching are non-canonical (Yoneyama et al. 2018). How plants spatially and temporarily control the biosynthesis of the SLs that are involved in shoot branching has not been adequately addressed and remains an important research target (Kameoka and Kyozuka 2018).

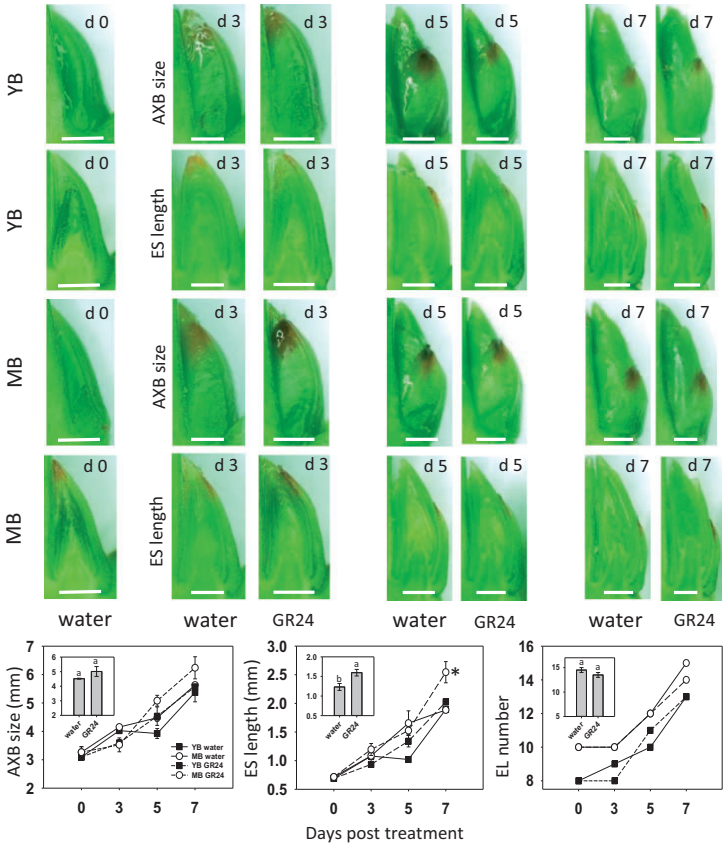

Fig. 6 AXB size, ES length and the number of embryonic leaves (EL) in young and mature AXBs (YB, MB) of single-node systems in water and GR24. Lengths of YB, MB and ES, as well as EL counts, were obtained as indicated in **Fig. 4A**. YBs and MBs: upper row photographs show example AXBs, and lower row photographs show longitudinally cut example AXBs at given days post isolation. The three line graphs show AXB size (left), ES length (middle) and EL number (right) at the indicated times after treatment with water and GR24. Insets: repeats of day 7. Different letters and asterisk indicate statistical differences (one-way ANOVA with post hoc Fischer's LSD test; *P*-value at least <0.05). Scale bars, 1.0 mm.

Especially, there is a need to better understand branching in trees, because their architecture, coupled to their superior CO_2 capture, is a critical element in mitigating climate change. To obtain more insight into the role of SLs in tree branching, we addressed the following questions. Are SL pathway and signaling genes conserved in the *Populus* genome? Is their expression spatially and functionally differentiated? Is the entire SL pathway operational in AXBs, independent of roots and shoot? Are SL biosynthesis and homeostasis affected by decapitation and GR24-feeding?

In addition to previously identified *Populus* homologs of SL biosynthesis and signaling genes (Czarnecki et al. 2014, Rinne et al. 2015, Muhr et al. 2016), we identified three homologs of *D27* (Supplementary Fig. S1), one of *LBO* (Supplementary Fig. S2) and three of *D53* (Supplementary Fig. S6). The existence of multiple copies in the *Populus* genome is a likely result of genome duplication (Tuskan et al. 2006). We found that the complete SL pathway of *Arabidopsis* and rice is conserved in *Populus* species and that in hybrid aspen the SL pathway genes show unique expression patterns (**Figs. 1**, **3**), which might relate to distinct features of tree branching. Firstly, the perennial lifestyle and the expansive shoot systems of trees (Tomlinson 1983, Millet et al. 1999, Barthélémy and Caraglio 2007, Ni et al. 2015, Rinne et al. 2015) require a modified branching strategy with a strong emphasis on mechanisms that act locally to control AXB outgrowth (**Fig. 8A**). Secondly, the AXBs, targets of SL signaling, are distinct in trees. In *Arabidopsis*, AXMs arise in axils of mature rosette leaves and produce simple scale-less buds (Grbić and Bleecker 2000, Long and Barton 2000). By contrast, in most trees, AXMs arise at a very early stage in the axils of emerging leaves and produce complex AXBs with an enclosed ES and sturdy bud scales (Garrison 1955, Paul et al. 2014, Rinne

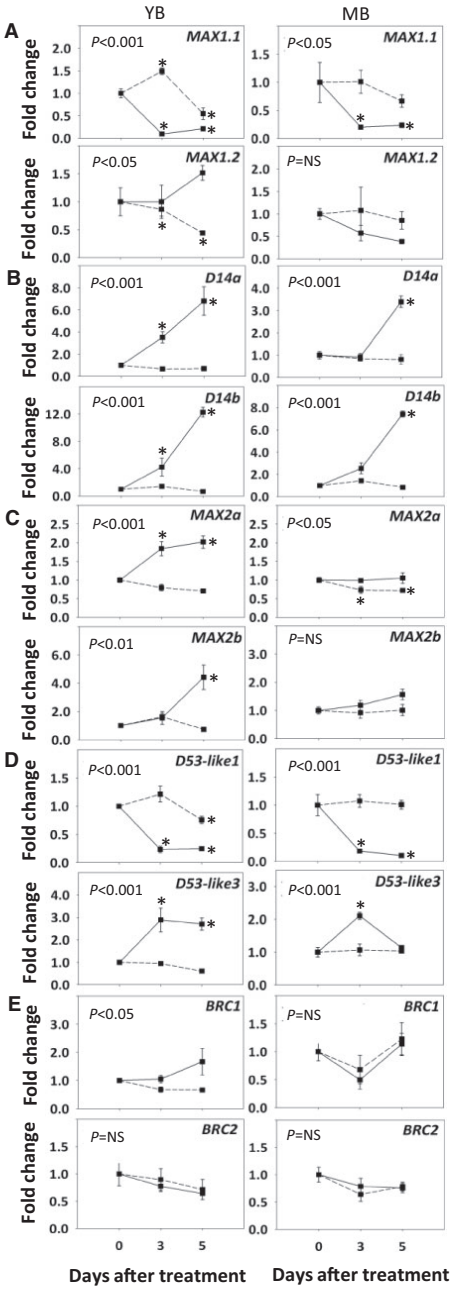

Fig. 7 Expression of SL pathway genes, downstream targets *BRC1* and *BRC2*, and the modulating effect of GR24. Young AXBs (YB, left column) and mature AXBs (MB, right column) on single-node systems in water control (–■–) and 10 μM GR24 (--■--). Gene expression was analyzed at 0, 3 and 5 d post isolation. (A) *MAX1.1* and *MAX1.2*. (B) *D14a* and *D14b*. (C) *MAX2a* and *MAX2b*. (D) *D53-like1* and *D53-like3*. (E) *BRC1* and *BRC2*. Values represent the means of three biological replicates ±SE ($n = 6$ plants). Values were calculated relative to the AXBs at $t = 0$, set at 1. Two-way ANOVA (*P*-value shows statistical significance between treatments; NS, not significant). Asterisks indicate significant differences with day 0 within each treatment (Fischer's LSD test; *P*-value at least <0.05).

et al. 2015). The outer scale presents a physical barrier that only gradually gives way (Supplementary Fig. S4).

Grafting experiments with herbaceous plants have shown that roots can act as the primary source for branch-inhibiting SLs (Beveridge et al. 2000, Morris et al. 2001, Turnbull et al. 2002, Simons et al. 2007). However, this does not necessarily reflect the situation in intact plants. Indeed, despite initial findings (Kohlen et al. 2011, Kohlen et al. 2012), xylem-transport of SL from roots to AXBs in intact plants has remained unconfirmed (Xie et al. 2015, Yoneyama et al. 2018). Nonetheless, our data indicate that AXBs in all likelihood receive CL and downstream products from elsewhere as, contrary to our initial assumption, AXBs themselves did not express *MAX3* and *MAX4*, but they did express the downstream biosynthetic gene *MAX1* (**Fig. 3B**). The few available studies on woody species did not detect *MAX3* and *MAX4* transcripts in AXBs and nodal bark, while *MAX4* was expressed only in wood tissue (Djennane et al. 2014, Muhr et al. 2016). Our data show that in hybrid aspen both genes are highly expressed in nodal bark tissues, and in addition in roots. Although root tips and bark tissues of source nodes expressed *MAX4* at similar levels, expression of *MAX3* was 65 times higher in the nodes. Moreover, in young nodes ('sink nodes'), *MAX3* and *MAX4* expressions were about 200 and 500 times higher, respectively, than in root tips (**Fig. 3B**). Based on our data, the bark of the AXB-associated nodes appears to be the main source of CL and downstream SL products. That in trees the nodes rather than the roots supply SLs to AXBs is a plausible conjecture, as it would allow for a more precise local control over branching of the expanding shoot system.

Young AXBs are active sinks that might import node-produced SLs along with sugars and other phloem-delivered compounds. As sugars can promote AXB outgrowth (Mason et al. 2014), a steady inflow of CL and SL-like compounds might be required to keep *BRC1* expression high in the maturing AXBs to prevent their outgrowth. Indeed, the expression of *MAX1* and *BRC1* steadily increases during AXB formation (Rinne et al. 2015). As *BRC1* and *BRC2* are class II TCPs, which repress cell cycling (Schommer et al. 2014), this suggests that during AXB formation SLs target *BRC1* to constrain the developing ES. Although the nodal bark expressed all SL biosynthesis genes, AXBs appeared to express *MAX1* and *LBO* (**Fig. 3C, D**), implying that they might convert imported CL and CLA, as well as MeCLA and other downstream products that require local conversion (**Figs. 1**, **8A**). That *LBO* is also expressed in the AXBs themselves is biologically meaningful, considering that its bioactive product, like MeCLA, is chemically unstable, providing

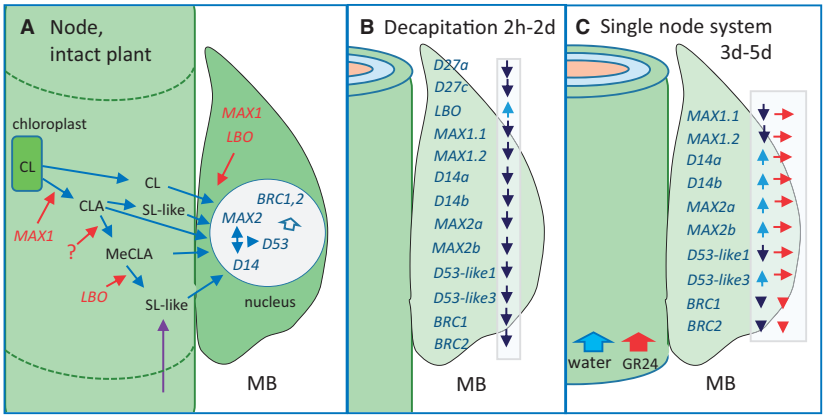

Fig. 8 Working models for mature AXBs (MBs) of proleptic hybrid aspen. (A) Node-to-bud signaling in intact plants. Nodes are dominant production centers of SLs, which are supplied to the AXBs. AXBs only express *MAX1* and *LBO*, which can convert imported CL, CLA, MeCLA (red arrow) into SL-like compounds that are hydrolyzed in nuclei by D14. Subsequent interaction with MAX2 results in ubiquitination and degradation of the transcription repressor D53. Downstream targets *BRC1* and *BRC2* inhibit AXB outgrowth. Roots hardly contribute SLs (purple arrow). Stippled lines delineate the node. (B) Proximal AXBs. All genes are initially downregulated (dark arrows) except for *LBO*, which is strongly upregulated (light blue arrow). (C) Single-node systems. *MAX1* genes are downregulated (dark arrows), but GR24-feeding (red arrow) prevents it through feedback (red arrows). *D14* and *MAX2* genes are upregulated (blue arrows), which is prevented by GR24-feeding (red arrows). *D53-like1* and *D53-like3* are, respectively, downregulated and upregulated (dark and blue arrows), but GR24-feeding prevents this (red arrows). *BRC1* and *BRC2* are modestly downregulated at day 3 (dark arrowheads), and GR24-feeding does not affect this (red arrowheads).

only weak inhibition of branching in a heterograft in *Arabidopsis* (Brewer et al. 2016).

Root tips also expressed *MAX3*, *MAX4*, *MAX1* as well as *LBO*, albeit at much lower levels than the nodes (**Fig. 3B–D**). Considering that e.g. *MAX2b* and *D53-like1* are hardly expressed in roots (**Fig. 3F, G**), root-produced SLs might serve specific root functions, including attraction of AM fungi in the rhizosphere. It is known that *Populus* roots can exude CLA, MeCLA as well as the canonical SL 4DO (Xie 2016). Although nodes of hybrid aspen expressed all SL pathway genes, including *D14* genes (**Fig. 3E**), *MAX2* genes (**Fig. 3F**) and *D53-like* genes (**Fig. 3G**), particularly *D14a* and *D14b* were expressed at much higher levels in the AXBs. The expression of *D14* in leaves (**Fig. 3E**) may take place in vascular tissues, as found for other species (Shen et al. 2007, Stirnberg et al. 2007, Zhou et al. 2013, Soundappan et al. 2015). As D14 is also present in the sieve tubes (Kameoka et al. 2016), by default it could move out of source leaves through mass flow toward sinks. Expression of SL pathway genes in vascular tissues may facilitate systemic bud–bud competition by modulation of auxin transport (Shinohara et al. 2013). Such systemic control by SL could also play a role in natural bud burst of hybrid aspen, for which AXBs require a pre-exposure to winter-chill that further upregulates the SL biosynthesis gene *MAX1* in AXBs (Rinne et al. 2018).

The role of LBO, which in *Arabidopsis* catalyzes the hydroxylation of MeCLA to the unidentified compound MeCLA+16D (Brewer et al. 2016), remains enigmatic in our study. Although expressed in all plant parts (**Fig. 3D**), its expression was highest in the nodes of the mature AXBs, which are poised for outgrowth, supporting its presumed role in inhibiting the outgrowth of para-dormant buds. Notably, expression of *LBO* in decapitation-activated AXBs was significantly increased after 1 d (**Fig. 5C**). As *BRC1* expression was already diminishing within 2 h, the increase in *LBO* expression might serve some as yet unidentified function. With the exception of *LBO*, the SL pathway genes were downregulated during the first 24 h in the bud activation process and followed by the start of ES elongation in the next 24 h (**Fig. 4B**).

The SL pathway has been shown to be subjected to homoeostatic control (Mashiguchi et al. 2009), like the GA pathway (Hedden and Thomas 2012). In single-node systems, the upregulation of receptor complex genes between days 3 and 5 could represent a response to diminished signal supply, considering the preceding downregulation of *MAX1* genes (**Fig. 7A**). Indeed, in support of this hypothesis, when signal supply was compensated by feeding GR24, the changes in the expression of the signaling genes were abolished (**Figs. 7B–D, 8C**). However, as most SL pathway genes may be subject to post-transcriptional and post-translational regulation (Zhou et al. 2013, Marzec and Muszynska 2015, Hu et al. 2017), this remains to be investigated.

It is unlikely that the initial triggers in decapitated plants and single-node systems are identical to those in natural branching because the constraints are different in all cases. For example, in decapitation experiments, the removal of the auxin-producing top part of the plant is crucial and could be the cause of AXB activation. However, in experiments with pea, auxin supply to the stump could not repress AXB outgrowth (Brewer et al. 2015). In hybrid aspen, the high expression of SL biosynthesis

genes in nodes of intact plants might prevent AXB activation in the intact plant, resulting in a proleptic branching style. Nonetheless, these high expression levels cannot prevent AXB outgrowth following decapitation. Sugar diversion (Mason et al. 2014, Kebrom 2017) might play a role also in decapitated hybrid aspen but is unlikely to be a factor in single-node systems that lack leaves. Although root-produced cytokinins (CK) are missing in this system, nodes might produce some CK as a result of the absence of a polar auxin transport stream (Nordström et al. 2004, Tanaka et al. 2006, Ferguson and Beveridge 2009).

Our data show that GR24-feeding cannot prevent outgrowth once AXBs are activated. As CK as well as GA can be locally produced in nodes and AXBs, it seems possible that they synergistically promote AXB activation, overriding SL effects by repressing *MAX2* and the downstream effects on *BRC1* (Ni et al. 2015). Indeed, we showed that decapitation upregulates GA biosynthesis genes in AXBs (Rinne et al. 2016), whereas SL pathway genes are downregulated (**Fig. 5**). GA not only represses SL perception, but it can also downregulate SL biosynthesis (Ni et al. 2015, Ito et al. 2017, Marzec 2017). Moreover, GA also reinvigorates symplasmic stem–bud connections by upregulating 1,3-β-glucanase genes (Rinne et al. 2011, Rinne et al. 2016), thereby potentially facilitating import of sugars and other nutrients to drive AXB outgrowth.

Conclusions

Nodes rather than distant roots may supply SL precursors and SLs to AXBs, whereas AXBs are sites of SL perception and *BRC1* action (**Fig. 8**). Mature AXBs can also synthesize SL-like compounds downstream of CL, but probably not CL itself as *MAX3* and *MAX4* are not expressed in AXBs, while *MAX1* and *LBO* are (**Figs. 3B–D, 8A**). As most SL pathway genes are downregulated by decapitation within hours, and ahead of ES elongation, SL might function in intact plants to inhibit AXB activation. Once activated, elongation of the ES might even be promoted by SL, as suggested by GR24-feeding of single-node systems. GR24-feeding data also support the notion that SL pathway genes are under homeostatic control. When apically produced auxin, root-produced cytokinins and leaf produced sugars are lacking, AXB still grow out despite high initial levels of SL gene expression in nodes, even after GR24-feeding. Although the initial triggers of AXB activation differ between intact plants, decapitated plants and single-node systems, the ensuing growth processes rapidly converge. SLs may restrain outgrowth only during AXB formation and para-dormancy in intact plants but cannot override the interacting factors that facilitate outgrowth of activated AXBs.

Materials and Methods

Plant material and sampling

Hybrid aspen (*P. tremula* × *P. tremuloides*) clone T89 was micro-propagated in vitro for 5 weeks in 20°C, planted in a mixture of soil/peat and perlite [4:1 (v/v)], fertilized with 4 g·l^{-1} Osmocote, grown in a greenhouse under long days (18 h light) at 20°C and 60% relative humidity, and watered twice a day. Natural daylight was supplemented by mercury-halide lamps with the lighting of 200–250 μmol·m^{-2}·s^{-1} (Osram) to maintain an 18 h photoperiod. The plants were replanted in 13 cm pots when they were ca. 60 cm high. Experiments were started when the plants had reached a height of 80–100 cm, and leaf production rates and elongation were stable. The plants were subdivided into three groups. Group one was kept in long-day (LD) conditions and decapitated at the BMP (**Fig. 2**), at around 40 cm below the apex, to eliminate apical dominance. The position of the BMP was as described by Rinne et al. (2015). Group two was kept in LD to collect various types of tissues and organs from intact plants. Group three plants were used for xylem-feeding experiments with single-node systems.

Measurements of AXB and embryonic shoot length, and embryonic leaf number

To record the developmental changes in AXBs proximal to the decapitation point AXB length was measured at 0, 2, 6, 12, 24, 48, 72 and 96 h post decapitation. At the same time points, the AXBs were cut longitudinally under a dissection microscope, and the length of the enclosed ES was measured from the top of the SAM to the middle of a line connecting the base of the outer scale (**Fig. 4A**). Lastly, comparable AXBs were fixed in 70% alcohol for assessing the neo-formation of leaves. Under a dissection microscope, the bud scales were peeled away, and the number of embryonic leaves was counted for each time point. Commonly the SAM contained one leaf buttress, which was included in the count.

AXB burst tests and GR24-feeding

To investigate the role of SL in AXB inhibition, we performed xylem-feeding experiments in combination with AXB burst tests under forcing conditions. As hybrid aspen is proleptic, the forced activation of AXBs represents a form of bud burst which, in contrast to sylleptic species and herbaceous plants, includes two processes, activation and outgrowth. For xylem-feeding, single-node systems without leaves were isolated from 6-week-old LD plants. The internode base was punched through pores in a Styrofoam sheet that was floated on water (control) or water supplemented with the synthetic SL analog (rac-GR24; Chiralix BV, The Netherlands) at a concentration of 10 μM. In preliminary experiment GR24 in concentrations of 1, 5 and 10 μM were tested, and 10 μM was chosen for the current experiments. In each treatment and time point, three replicates of young and three replicates of mature AXBs were used. AXB length, ES length and embryonic leaf number were recorded at days 0, 3, 5 and 7. The young buds and the mature AXBs below the BMP were harvested at days 0, 3 and 5 to analyze changes in the relative expression of SL pathway genes induced by decapitation, and by the combination of decapitation and GR24-feeding. The experiments were repeated at least twice.

Gene selection and identification

To examine the expression patterns of SL biosynthesis and signaling genes in 6-week-old intact plants, total RNA was extracted from different plant parts. These included the apex, young AXBs, the bark of the corresponding node of young AXBs ('sink node'), sink leaves, mature para-dormant AXBs, the corresponding node of mature AXBs ('source node'), source leaves and root tips (**Fig. 2**). In total, three AXBs above, and three below the BMP, as well as other tissues like indicated above, were collected from each of the six plants. Samples of two plants were pooled to obtain three biological replicates. Gene expression analyzes included *Populus* homologs of the *Arabidopsis* SL biosynthesis and signaling genes *D27a*, *D27b*, *D27c*, *MAX1.1*, *MAX1.2*, *LBO*, *MAX3*, *MAX4*, *D14a*, *D14b*, *MAX2a*, *MAX2b*, *D53-like1*, *D53-like2*, *D53-like3*, as well as the downstream target genes *BRC1* and *BRC2*.

To assess decapitation-induced changes in gene expression, mature AXBs proximal to the decapitation point at the BMP (**Fig. 2**) were collected at days 0, 2, 6, 12, 24 and 48 h post decapitation. For each time point, RNA was extracted from three biological replicates, pooled as described above. Sampling after day 1 (24 h) and day 2 (48 h) were carried out at the same time of the day to avoid potential diurnal effects on gene expression.

To assess the role of exogenous SL on gene expression in AXBs of single-node systems, they were incubated in water with or without GR24. AXBs were collected after 0, 3 and 5 d of treatment. Gene expression was assessed for the SL biosynthesis genes *D27a*, *D27c*, *MAX1.1* and *MAX1.2*, as well as the SL signaling genes *D14a*, *D14b*, *MAX2a*, *MAX2b*, *D53-like1*, *D53-like2*, *D53-like3* and downstream targets *BRC1* and *BRC2*.

RNA extraction, cDNA preparation and quantitative RT-PCR analysis

Total RNA was extracted from 0.2 to 0.3 g of frozen tissue and grinded in a mortar with 500 µl extraction buffer (Qiagen RLT buffer containing 1% PVP-40), followed by an addition of a 0.4 volume KoAC (pH 6.5) and further homogenization. Subsequently, the solution was transferred to a 2-ml tube, incubated on ice for 15 min, and centrifuged at 12,000 rpm at 4°C for 15 min. The supernatant was transferred to a new 1.5-ml tube, and a 0.5 ml volume of 100% ethanol was added. The mix was transferred to RNeasy spin columns and further processed in accordance with instructions of the Qiagen Plant RNA isolation kit. Genomic DNA was eliminated using TURBO™ DNase kit (Invitrogen) treatment according to the manufacturer's instructions and cleaned using the total RNA purification system 'Purelink RNA mini kit' (Invitrogen). RNA was quantified with NanoDrop 1000, and the RNA quality was assessed with the Agilent 2100 Bioanalyzer system. One microgram of total RNA was reversely transcribed to cDNA with SuperScript® VILO™ reverse transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) was used to analyze transcript levels of all SL pathway genes. The reaction setup (20 µl total volume) was prepared using SYBR® select PCR master mix (Applied Biosystems). As a template, 2 µl of the cDNA (200 ng) were added. All the qPCR reactions were run with three biological replicates and analyzed in three technical repeats. Real-time qRT-PCR analyses were performed with the Applied Biosystems 7500 Fast Real-Time PCR system according to the manufacturer's instruction. Thermocycling conditions were set to 50°C for 2 min, 95°C for 2 min, 45 cycles of 15 s at 95°C and 60 s at 60°C. In addition, each PCR reaction included a negative control to check for potential genomic DNA contamination. PCR amplification of *Populus actin* served as a reference gene for normalizing the relative transcript level. For a complete list of primers and genes used for qRT-PCR see Supplementary Table S1.

Statistical analysis

Statistical analyses were carried out using analysis of variance (ANOVA) in combination with a post hoc test to determine significant differences between the subgroups. One-way ANOVA in combination with Fisher's LSD test was computed to monitor the decapitation-induced changes in transcript levels and to pinpoint the time within the 48 h trajectory when a significant change took place. To analyze the effect of GR24 on gene expression during the 7-d feeding experiment, two-way ANOVA (time and treatment as factors) was used in combination with Fisher's LSD multiple comparison test. The developmental changes during AXB activation (AXB length, ES elongation, leaf numbers) induced either by decapitation or isolation of the single-node systems and treated with or without GR24 were analyzed with one- or two-way ANOVA and combined with Fisher's LSD test. Computation was performed using Microsoft Excel data analysis (www.microsoft.com) and Minitab Statistical Software version 18.1 (www.minitab.com).

Bioinformatics

BLAST searches in GenBank, *P. trichocarpa* genome v3.0 and *P. tremula* × *P. tremuloides* (T89) v0.1 databases (http://www.ncbi.nlm.nih.gov/BLAST; http://www.phytozome.net; http://popgenie.org/) were used to identify SL biosynthesis and signaling genes. Gene-specific primer sequences for qPCR analysis were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Phylogenetic trees were created using the MEGA6 program (www.megasoftware.net) with the Neighbor–Joining method. Bootstrap support values are based on 1,000 replicates.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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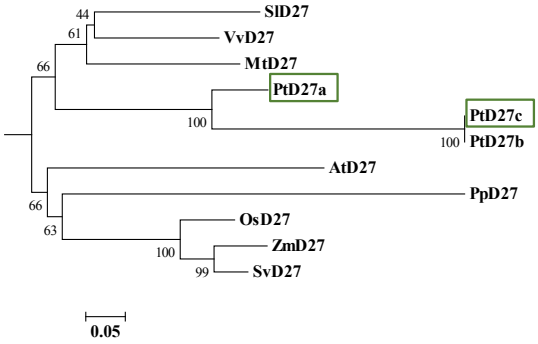
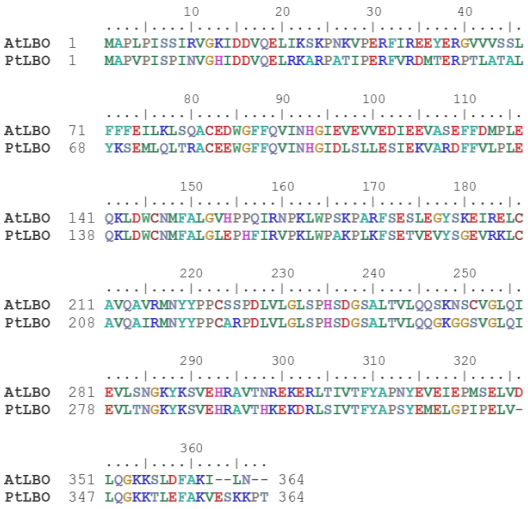
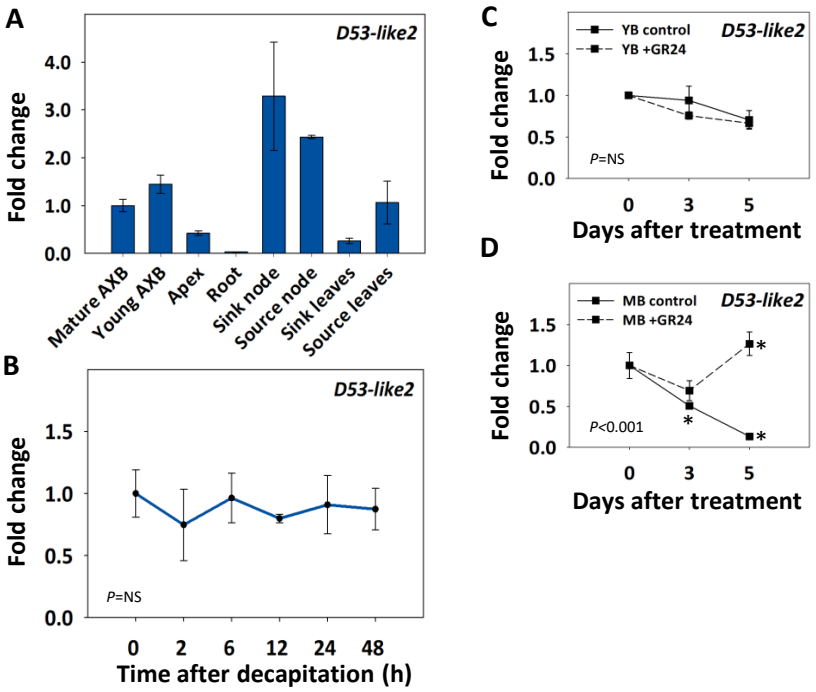


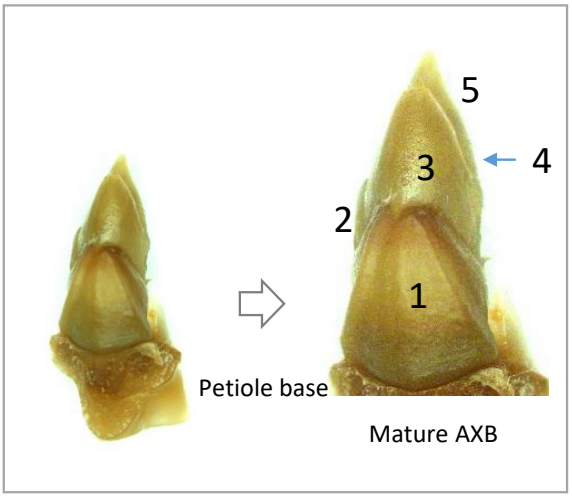
Supplementary Fig. S1. Phylogenetic analysis of DWARF27 family proteins. *DWARF27* (*D27*) in *Arabidopsis* and *Oryza* encodes an iron containing protein required for the SL biosynthesis. The sequence homologues were identified by Protein BLAST search in NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the sequences were retrieved from *Populus trichocarpa* genome (Tuskan et al. 2006; http://www.phytozome.net/) databases. The amino acid sequence alignment were performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). A phylogenetic tree was created using the MEGA6 program (www.megasoftware.net) with the Neighbor–Joining method. Bootstrap support values are based on 1000 replicates. The proteins used in the phylogenetic analysis were: AtD27 (At1g03055) is from *Arabidopsis thaliana*; OsD27 (Os11g0587000) is from *Oryza* sativa; ZmD27 (XP_008670838) is from *Zea mays*; VvD27 (XP_003634993) is *from Vitis vinifera*; MtD27 (AEW07379.1) is from *Medicago truncatula*); SbD27 (XP_0213168841) is from *Sorghum bicolor*; PpD27 (UniProt accession: A9SKY4) is from *Physcomitrella patens*; SlD27 (UniProt accession: C5Y5C4) is from *Solanum lycopersicum*; PtD27a (Potri.005G216400) and PtD27c (Potri.002G046500) are from *Populus trichocarpa.* The novel genes identified in this study are boxed.



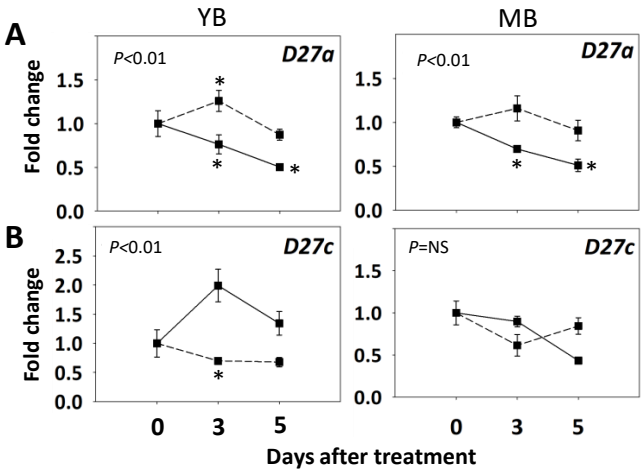
Supplementary Fig. S2. Amino acid sequence alignment of LBO proteins of *Arabidopsis* and *Populus*.



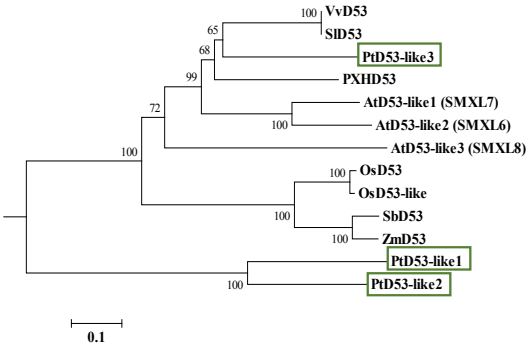
Supplementary Fig. S3. Expression patterns of *D53-like2* in hybrid aspen. (A) Expression in different plant parts. (B) Expression after plant decapitation in the AXB proximal to the decapitation point. (C, D) Expression over time in (C) young (YB) and (D) mature AXB (MB) of single node systems in water and in 10 µM GR24. Values represent the means of 3 biological replicates ±S.E. (n=6 plants), and are calculated relative to the corresponding AXB of intact plants. Effects of decapitation in (B) were not statistically significant (one-way ANOVA with Fisher's *post hoc* test; *P*=NS, not significant). Treatments with or without GR24 (C and D) were analyzed with two-way ANOVA and *post hoc* Fischer's LSD test. *P*-values show statistical significance between the treatments at indicated levels (NS, not significant). Asterisks indicate significant differences between day 0 and other time points within each treatment (*P*-value at least <0.05).



Supplementary Fig. S4. Mature AXB with decussate phyllotaxis. In hybrid aspen 5 bud scales enclose an embryonic or preformed shoot comprised of ~10 embryonic leaves. After bud activation the thin inner scales expand while the thick outer scale remains unchanged. Depicted is an AXB of a single node cutting that was fed water for 3 days. The enlargement shows the scale numbers. Scale 4 at the back side is not visible.



Supplementary Fig. S5. Expression patterns of *D27a* and *D27c* in hybrid aspen. (A) *D27a* and (B) *D27c* expression over time in young (YB) and mature AXBs (MB) of single node systems in water (—■—) and in 10 μM GR24 (··■··). Values are calculated relative to the AXBs at t=0, set at 1. Two-way ANOVA with *post hoc* Fischer's LSD test. *P*-values show statistical significance between the treatments at indicated levels (NS, not significant). Asterisks indicate significant differences between day 0 and other time points within each treatment (*P*-value at least <0.05).



Supplementary Fig. S6. Phylogenetic analysis of DWARF53 family proteins. DWARF53 (D53), a repressor of SL signaling in rice was used to identify sequence homologues in *Populus trichocarpa* by Protein BLAST search in NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the sequences were retrieved from *Populus trichocarpa* genome (Tuskan et al. 2006; http://www.phytozome.net/) databases. The amino acid sequence alignment were performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). A phylogenetic tree was created using the MEGA6 program (www.megasoftware.net) with the Neighbor–Joining method. Bootstrap support values are based on 1000 replicates. The proteins used in the phylogenetic analysis were: AtD53-like1/ SMXL7 (At2G29970), AtD53-like2/ SMXL6 (At1G07200), AtD53-like3/ SMXL8 (At2g40130) is from *Arabidopsis thaliana*; OsD53 (Os11g01330) and OsD53-like (Os12g01360) is from *Oryza* sativa; ZmD53 (PWZ44007) is from *Zea mays*; VvD53 (VIT_06S0004G06700) is *from Vitis vinifera*; SbD53 (XP_002441659) is from *Sorghum bicolor*; SlD53 (SOLYC09G055230) is from *Solanum lycopersicum*; PXH D53 (AQY56559) is from *Petunia X Hybrida*; PtD53-like1 (Potri.008G017600), PtD53-like2 (Potri.016G071800) and PtD53-like3 (Potri.009G046700) are from *Populus trichocarpa*. The novel genes identified in this study are boxed.

Supplementary Table S1. List of primers used in the gene expression studies.

Populus trichocarpa					
Protein	**Gene abb.**	**Locus name v2.0**	**Locus name v3.0**	**Forward 5′-3′**	**Reverse 5′-3′**
Strigolactone Biosynthesis genes					
DWARF27	*D27a*	POPTR_0002s04730	Potri.005G216400	GGCCCCTTTAGGACAGAAAA	TACAGGAGCCGCAAACTTCT
DWARF27	*D27c*	POPTR_0002s19530	Potri.002G046500	TCCTCCGCTACCTGAAGATG	GAAGCAGCTCCCCTTCTTTT
MORE AXILLARY BRANCHES3	*MAX3*	POPTR_0014s05590	Potri.014G056800	TCCATGACTGGGCATTTACA	ATCAAGCTTAATGCGGTTGG
MORE AXILLARY BRANCHES4	*MAX4*	POPTR_0006s25490	Potri.006G238500	TGCTTATGCTTGTGGAGCAC	TAGTGAGGGTGTTGGGGAAG
MORE AXILLARY BRANCHES1	*MAX1.1*	POPTR_0006s24320	Potri.006G226700	AAACGTTATGGCCCCATTTT	TGAGATGGGAGAGGGAACAC
MORE AXILLARY BRANCHES1	*MAX1.2*	POPTR_0018s07540	Potri.018G062100	CAGATGCCAACAGCTCAAGA	TCCAGGTGCTAACCAGATCC
LATERAL BRANCHING OXIDOREDUCTASE	*LBO*	POPTR_0010s02430	Potri.010G023600	AAGGTTGCCAGGGATTTCTT	CAAACATGTTGCACCAGTCC
Strigolactone signaling and perception genes					
DWARF14	*D14a*	POPTR_0002s11970	Potri.002G118900	TTATGAGGCTTGGGTCAAGG	GCGTTCGGCTAAATTCTCTG
DWARF14	*D14b*	POPTR_0014s01680	Potri.014G016500	GCATTCTCCCCTTTTTCACA	TTCAAGATTGGTGCATCGTC
MORE AXILLARY BRANCHES2	*MAX2a*	POPTR_0014s13910	Potri.014G142600	GTCGAGGGAGACTGCAGAAC	AGCAAGGCAGCTTAATCCAA
MORE AXILLARY BRANCHES2	*MAX2b*	POPTR_0011s07320	Potri.011G066700	GATTAAGCTTGTTCGCTGGC	TGGAAGGGTATGCTTCAAGG
BRANCHED1	*BRC1*	POPTR_0012s05660	Potri.012G059900	CATCATCGCGTAAAACTCCA	GTCGATTCTTCGACTGCACA
BRANCHED2	*BRC2*	POPTR_0010s14030	Potri.010G130200	ACTTGCTGCCACATCAAATG	AGTAGCCCCACTTGGAACCT
DWARF53	*D53-like1*	POPTR_0008s01850	Potri.008G017600	CTTCTGCCCTTGCAGAAATC	ATGGAGCATCCCATCTTGAG
DWARF53	*D53-like2*	POPTR_0016s07250	Potri.016G071800	GGGCAGTTTGATCCCAGTAA	TGCGTATTGCTTCATCTTGC
DWARF53	*D53-like3*	POPTR_0009s05140	Potri.009G046700	GAGGCGTTTTGCCTAGTGAG	CAATTCCTGACCCAACTCGT
Housekeeping gene					
ACTIN	*ACT*	POPTR_0001s31700	Potri.001G309500	CGATGCCGAGGATATTCAAC	ACCAGTGTGTCTTGGTCTACCC

Paper II

frontiers in Plant Science

ORIGINAL RESEARCH
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Dual Role of Gibberellin in Perennial Shoot Branching: Inhibition and Activation

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Shoot branching from axillary buds (AXBs) is regulated by a network of inhibitory and promotive forces, which includes hormones. In perennials, the dwarfed stature of the embryonic shoot inside AXBs is indicative of gibberellin (GA) deficiency, suggesting that AXB activation and outgrowth require GA. Nonetheless, the role of GA in branching has remained obscure. We here carried out comprehensive GA transcript and metabolite analyses in hybrid aspen, a perennial branching model. The results indicate that GA has an inhibitory as well as promotive role in branching. The latter is executed in two phases. While the expression level of *GA2ox* is high in quiescent AXBs, decapitation rapidly downregulated it, implying increased GA signaling. In the second phase, *GA3ox2*-mediated *de novo* GA-biosynthesis is initiated between 12 and 24 h, prior to AXB elongation. Metabolite analyzes showed that $GA_{1/4}$ levels were typically high in proliferating apices and low in the developmentally inactive, quiescent AXBs, whereas the reverse was true for $GA_{3/6}$. To investigate if AXBs are differently affected by GA_3, GA_4, and GR24, an analog of the branch-inhibitor hormone strigolactone, they were fed into AXBs of single-node cuttings. GA_3 and GA_4 had similar effects on GA and SL pathway genes, but crucially GA_3 induced AXB abscission whereas GA_4 promoted outgrowth. Both GA_3 and GA_4 strongly upregulated *GA2ox g*enes, which deactivate $GA_{1/4}$ but not $GA_{3/6}$. Thus, the observed production of $GA_{3/6}$ in quiescent AXBs targets $GA_{1/4}$ for GA2ox-mediated deactivation. AXB quiescence can therefore be maintained by $GA_{3/6}$, in combination with strigolactone. Our discovery of the distinct tasks of GA_3 and GA_4 in AXB activation might explain why the role of GA in branching has been difficult to decipher. Together, the results support a novel paradigm in which $GA_{3/6}$ maintains high levels of *GA2ox* expression and low levels of GA_4 in quiescent AXBs, whereas activation and outgrowth require increased $GA_{1/4}$ signaling through the rapid reduction of GA deactivation and subsequent GA biosynthesis.

Keywords: gibberellin, axillary branching, GA2-oxidases, GA3-oxidases, GA20-oxidases, GID1, strigolactone, hormones

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INTRODUCTION

Shoot branching is governed by a network of hormones that includes auxin, cytokinin (CK) and strigolactone (SL). How they interact to regulate axillary bud (AXB) activation and outgrowth still divides opinion (Ferguson and Beveridge, 2009; Hayward et al., 2009; Müller and Leyser, 2011; Puig et al., 2012; Rameau et al., 2015). Classic experiments established that a growing shoot apex can repress branching, a phenomenon known as apical dominance. The physiological explanation is that a proliferating apex produces a surplus of auxin that is send down the stem to inhibit AXB outgrowth, thereby promoting apical elongation. Removal of the apex releases AXBs from inhibition, triggering branching, but this can be prevented by supplying auxin to the cut stem (Thimann and Skoog, 1934; Phillips, 1975; Cline, 1991, 1997).

A current interpretation of these experiments is that the growing apex increases the relative amount of auxin in the polar auxin transport stream (PATS) of the main stem, thereby preventing AXBs from establishing their own auxin export path to the stem (Li and Bangerth, 1999; Bennett et al., 2006; Ongaro et al., 2008; Domagalska and Leyser, 2011). When auxin levels in the stem drop, export of auxin from the AXB to the stem is initiated, promoting AXB outgrowth. An alternative model proposes that auxin export is a consequence of AXB activation rather than a cause (Dun et al., 2006; Brewer et al., 2009; Ferguson and Beveridge, 2009). This is in line with the proposal of Cline (1997) that a fast initial enlargement of an AXB should be distinguished from the much slower outgrowth process. Experimental support comes from studies with garden pea (*Pisum sativum* L.), in which shoot decapitation triggers AXB enlargement ahead of the arrival of the auxin depletion front (Morris et al., 2005). Moreover, supplying auxin to the cut stem can prevent branching but not AXB enlargement. Finally, depleting stem auxin levels by auxin transport inhibitors does not affect initial AXB enlargement, but once AXBs have enlarged it promotes sustained outgrowth (Morris et al., 2005; Ferguson and Beveridge, 2009; Mason et al., 2014). In addition to the network of hormones, nutrients are important in AXB outgrowth in intact plants, as well as after decapitation when sugars are diverted to the larger AXBs, which are the strongest sinks (Mason et al., 2014; Kebrom, 2017).

The transcription factor BRANCHED1 (BRC1)/TEOSINTE BRANCHED1 (TB1) is an important branch-inhibitor (Aguilar-Martínez et al., 2007; Brewer et al., 2009; Dun et al., 2009; Leyser, 2009). Although *BRC1* was originally identified as the target of SL, it is now recognized to be a hub for branch-regulating signals, including various hormones and developmental as well as environmental cues (Wang et al., 2019). In Arabidopsis (*Arabidopsis thaliana*), BRC1 inhibits AXB outgrowth, probably by suppressing cell proliferation (Schommer et al., 2014), but in some circumstances it cannot prevent outgrowth (Seale et al., 2017). In rice (*Oryza sativa*), SL also induces degradation of the branch-promoting hormone CK through transcriptional activation of CK-oxidases (Duan et al., 2019). In accordance with this, AXB outgrowth in pea is accompanied by a reduction in SL biosynthesis and an increase in CK biosynthesis (Tanaka et al., 2006; Ferguson and Beveridge, 2009). Auxin also suppresses CK biosynthesis (Nordström et al., 2004). Thus, CK-induced outgrowth of activated AXBs may require low stem levels of auxin and SL.

While auxin, CKs and SLs are implicated in the regulation of AXBs, the role of gibberellins (GA) has remained obscure. This is unexpected as GAs promote many developmental processes, including germination, elongation, floral transition as well as AXB formation and dormancy release (Hazebroek et al., 1993; Richards et al., 2001; Yamaguchi, 2008; Rinne et al., 2011, 2016; Claeys et al., 2014; Zhuang et al., 2015). GA is often viewed as a branch-inhibitor because GA-biosynthesis and -perception mutants in Arabidopsis, as well as GA-deficient transgenic plants of various species have branched phenotypes. However, a complicating factor is that GA-deficiency or lack of GA perception not only increases branching but also reduces apical dominance (Scott et al., 1967; Talon et al., 1990; Murfet and Reid, 1993; Silverstone et al., 1997; Olszewski et al., 2002; Busov et al., 2003; Agharkar et al., 2007; Lo et al., 2008; Mauriat et al., 2011; Zawaski and Busov, 2014; Rameau et al., 2015). In contrast to the above, some studies suggest that GA promotes branching. In perennial strawberry, AXB outgrowth is diminished in a GA-biosynthesis mutant, while GA supply rescues the phenotype (Tenreira et al., 2017). Similarly, in the woody species *Jatropha* (*J. curcas* L.) (Ni et al., 2015) and hybrid aspen (*Populus tremula* × *P. tremuloides*) (Rinne et al., 2011), GA application promotes AXB outgrowth, whereas in *Rosa* sp. outgrowth requires GA biosynthesis (Choubane et al., 2012).

Only a small number of the more than 130 known GAs is biologically active, including GA_1, GA_3, GA_4, GA_5, GA_6, and GA_7 (King et al., 2001, 2003; Yamaguchi, 2008; Hedden and Sponsel, 2015). GA biosynthesis starts with plastid-localized geranylgeranyl diphosphate (GGDP), which is converted to *ent*-kaurene (**Figure 1**; Hedden and Phillips, 2000; Olszewski et al., 2002; Yamaguchi, 2008), and oxidized by cytochrome P450 mono-oxygenase in the endoplasmic reticulum to yield GA_{12} (Helliwell et al., 2001). From there, metabolites are shuttled through two parallel cytoplasmic pathways, the non-13-hydroxylation and 13-hydroxylation pathway, in which three groups of 2-oxoglutarate-dependent dioxygenases provide catalytic activity (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000; Olszewski et al., 2002; Hedden and Thomas, 2012). These include GA20-oxidases (GA20oxs) that produce GA precursors, GA3-oxidases (GA3oxs) that produce bioactive GAs, and GA2-oxidases (GA2oxs) that irreversibly deactivate precursors and bioactive GAs by 2β-hydroxylation (Thomas et al., 1999; Lo et al., 2008; Rieu et al., 2008a). Which of the two pathways is dominant depends on species, developmental stage, and organ type. For example, in rice, GA_1 dominates during vegetative growth but during anthesis it is GA_4 (Kobayashi et al., 1989; Hirano et al., 2008), whereas in hybrid aspen GA_4 regulates shoot elongation (Israelsson et al., 2004) and in Arabidopsis also flowering (Sponsel et al., 1997; Eriksson et al., 2006). GA signaling requires binding to the receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), which localizes to the cytoplasm and nucleus (Ueguchi-Tanaka et al., 2005; Willige et al., 2007; Hirano et al., 2008; Sun, 2010). Because GA_4 has the highest affinity to GID1

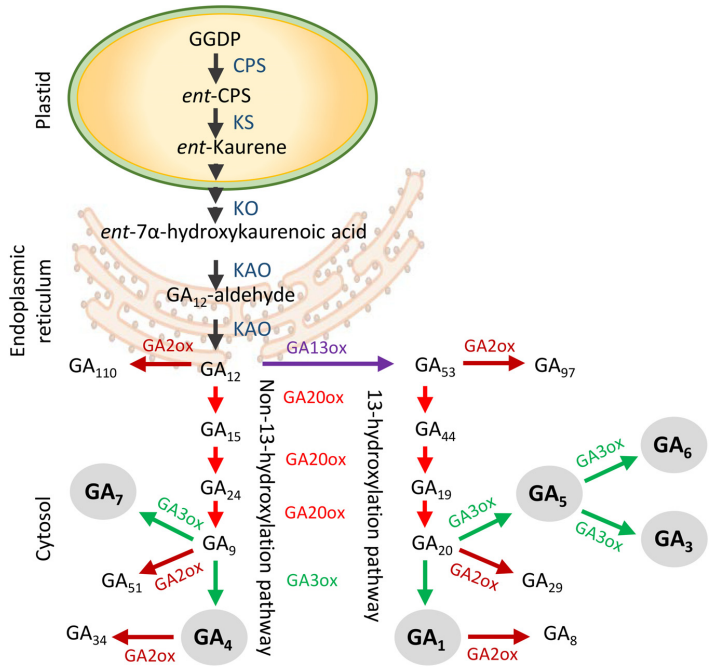

FIGURE 1 | Generalized scheme of gibberellin (GA) biosynthesis and deactivation in higher plants. GA biosynthesis starts in the plastids and is followed by the production of GA_{12} in the endoplasmic reticulum. In the cytoplasm GA_{12} is processed by GA20ox and GA3ox enzymes in two separate branches to produce bioactive GAs (gray circles). The non-13-hydroxylation yields GA_7 and GA_4, whereas the 13-hydroxylation yields GA_5, GA_6, GA_3, and GA_1. The GA2ox enzymes deactivate precursors and bioactive GAs. Abbreviations: GGDP, geranylgeranyl diphosphate; CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase.

(Nakajima et al., 2006), its effective concentration can be low. GA-GID1 binding enhances interaction with growth-repressor DELLA proteins, which are also present in the cytoplasm and nucleus (Sun, 2011; Davière and Achard, 2013). Subsequent interaction with ubiquitin E3 ligase complex $SCF^{SLY1/GID2}$ leads to ubiquitination and degradation of DELLA (Peng et al., 1997; Silverstone et al., 1998; Bolle, 2004; Ueguchi-Tanaka et al., 2007).

It is uncertain if the herbaceous branching models can be transferred directly to woody perennials, considering their different shoot size, lifespan, and AXB composition. In hybrid aspen nodal bark tissue might contribute to the regulation of AXB behavior, perhaps compensating for the inefficiency of long-distance transfer of root-produced strigolactone precursors (Katyayini et al., 2019). In hybrid aspen, AXBs are elaborate structures with sturdy scales that enclose a dwarfed embryonic shoot (ES) that arises over a developmental time span of 10 to 12 plastochrons (Rinne et al., 2015). However, deciduous perennials can show strikingly distinct branching styles, suggesting that even within them regulation of AXB outgrowth can differ. In sylleptic species, AXBs grow out in the same season, producing plastic branching patterns in response to environmental conditions (Wu and Stettler, 1998; Wu and Hinckley, 2001), whereas in proleptic species AXBs do not grow out in the same year (Hallé et al., 1978; Barthélémy and Caraglio, 2007). In hybrid aspen, AXBs cease development at the bud maturation point (BMP) and remain inactive until the next growing season (Paul et al., 2014). The AXBs can therefore be viewed as containing side shoots in which phytomer development is temporarily decoupled from stem elongation, which is postponed until the next growing season. In spring, the elongating stem of the ES telescopes out of the opening bud, allowing subsequent neoformation of leaves. Despite being locked in a developmentally quiescent state, the current year AXBs have a high potential for outgrowth, as shoot decapitation induces rapid outgrowth.

Previous analyses of several GA pathway genes in hybrid aspen suggested that GA-deficiency could explain the dwarfed nature of the ES, and that GA biosynthesis would be required for decapitation-induced elongation (Rinne et al., 2015, 2016). ES elongation might require GA_4 to regulate cell division and cell stretching, and to recruit GA_4-inducible 1,3-β-glucanases that optimize symplasmic conduits for nutrient and sugar import (Rinne et al., 2011, 2016). While different GA forms can have different developmental effects, the basis of this has not been investigated. To our knowledge, it has remained unknown which

GAs play a role during AXB quiescence and branching in hybrid aspen as well as other woody perennial species. The relative prominence of AXBs in hybrid aspen permitted us to carry out comprehensive analyzes of GA metabolite levels and GA-pathway transcripts.

The results support a novel paradigm of a dual role of GA in shoot branching, in which $GA_{3/6}$ and $GA_{1/4}$ have opposing tasks. AXBs produce $GA_{3/6}$ to maintain quiescence by upregulating *GA2ox* genes, which deactivate $GA_{1/4}$, keeping their levels low despite ongoing biosynthesis. AXB activation, in turn, is achieved by the instantaneous and strong downregulation of the *GA2ox* genes, boosting $GA_{1/4}$-induced signaling. Subsequent elongation is followed by $GA_{1/4}$ biosynthesis through *GA3ox2* and supported by GA precursor import from the node.

RESULTS

To understand the role of GA in shoot branching, we mapped the expression of all GA pathway genes in the major parts of intact plants, and in decapitation activated AXBs and associated nodes. The data were combined with analyses of GA intermediates and bioactive GAs. As GA and SL are thought to have opposite effects on AXB activation, we investigated how feeding of GA_3, GA_4 and the synthetic SL analog GR24 into AXBs of single-node cuttings influenced the expression of GA and SL pathway genes.

GA20oxs and *GA3oxs* Expression Is Organ- and Development-Related

The genome of *P. trichocarpa* contains eight *GA20ox* and three *GA3ox* genes (Tuskan et al., 2006; **Figure 1** and **Supplementary Figure S1**), but preliminary studies showed that *GA20ox2-2* was not expressed in hybrid aspen. Transcripts of the seven remaining *GA20ox* genes were present in young (developing) and mature (developmentally quiescent) AXBs (**Figure 2**). In decreasing order, the highest transcript levels were found for *GA20ox5*, *GA20ox8* and *GA20ox7,* whereas *GA20ox6*, *GA20ox3* and *GA20ox2-1* were little expressed, and *GA20ox4* only in leaves (**Figures 2A,B**). Of the highly expressed genes of this family, *GA20ox8* was the most generally expressed, but transcript levels were especially high in leaves. Whereas in bark tissue of nodes associated with sink leaves (denoted sink nodes) *GA20ox8* expression was high, it was almost completely absent in bark tissue of nodes at source leaves (denoted source nodes). Except for the AXBs, all other plant parts expressed *GA20ox* genes selectively, suggesting the various paralogs might have tissue-specific roles. That all *GA20ox* family genes were expressed in AXBs makes sense as AXBs harbor a complete, albeit dwarfed shoot system. Combining the transcript levels of all *GA20ox* paralogs showed that GA-precursor production was highest in sink leaves, followed by source nodes and associated AXBs. In contrast, roots and apices had low transcript levels (**Figures 2A,B**). Although transcript levels in young AXBs were approximately half of those in the mature quiescent AXBs, they were still almost three times higher than in apices and root tips.

Mature as well as young developing AXBs expressed *GA3ox1* and *GA3ox2*, but transcript levels of *GA3ox1* were significantly lower than those of *GA3ox2* (**Figure 2C** and **Supplementary Figure S1**). In apices, *GA3ox1* was virtually absent, whereas it increased in nodes and leaves during their maturation, reaching the highest levels in source leaves. In stark contrast, the expression of *GA3ox2* was very high in proliferating shoot apices, and high in growing root tips, sink nodes, sink leaves and AXBs. In the mature nodes and leaves *GA3ox2* expression was considerably reduced. The expression ratio of *GA3ox2*/*GA3ox1* showed that apices had the highest approximate ratio (1000), followed by tissues in sinks (20) and sources (0.25). Together the results reveal that, rather than being tissue specific, *GA3ox1* and *GA3ox2* are developmentally regulated, and that their physiological importance is reversed during tissue maturation. Thus, *GA3ox2* expression supports cell proliferation and growth, whereas *GA3ox1* is dominant in mature tissues.

In summary, the spatio-temporal expression patterns of the *GA20ox* and *GA3ox* family members show that source nodes and source leaves might stockpile GA precursors for delivery to AXBs, while AXBs themselves can produce precursors as well as bioactive GAs.

GA2ox Gene Expression Is Highest in AXBs and Source Leaves

In *P. trichocarpa*, the GA-deactivating *GA2ox* family is composed of seven genes (Gou et al., 2011; **Supplementary Figure S1**). *GA2ox2* was not expressed at measurable amounts in shoot tissues of hybrid aspen (not shown) and therefore was not included in the analyses. In decreasing order, the highest transcript levels were found for *GA2ox1*, *GA2ox4*, *GA2ox5, GA2ox6, GA2ox3,* and *GA2ox7* (**Figures 2D,E**). In all plant parts, *GA2ox1* had by far the highest transcript levels of the entire *GA2ox* family. The little expressed genes, *GA2ox5* and *GA2ox6*, were most highly expressed in source leaves. AXBs and source leaves stood apart by expressing most genes, and having the highest combined expression levels, around six times more than apices. Notably, the actively growing tissues, including apices, sink nodes and sink leaves, which expectedly are most active in GA signaling, all expressed *GA2ox* genes at a low level.

GID1 Receptor Gene Expression Is Highest in AXBs

We identified in hybrid aspen all four paralogs of the *P. trichocarpa GID1* genes, and named them *GID1A-1*, *GID1A-2*, *GID1B-1*, and *GID1B-2* (**Supplementary Figure S2**). In shoot tissues, transcript levels of *GID1A-2* were the highest, followed by *GID1B-1* and *GID1-A1*, whereas expression of *GID1B-2* was very low (**Figure 2F**). The combined transcript levels of *GID1* genes were clearly highest in AXBs and source leaves. In contrast, the expression was low in strong sinks, including proliferating apices, growing root tips, sink nodes and sink leaves. As growing tissues, but especially apices, expressed high levels of the proliferation-related GA-biosynthesis gene *GA3ox2* (**Figure 2C**), the lower *GID1* expression levels are expected to reflect high levels of

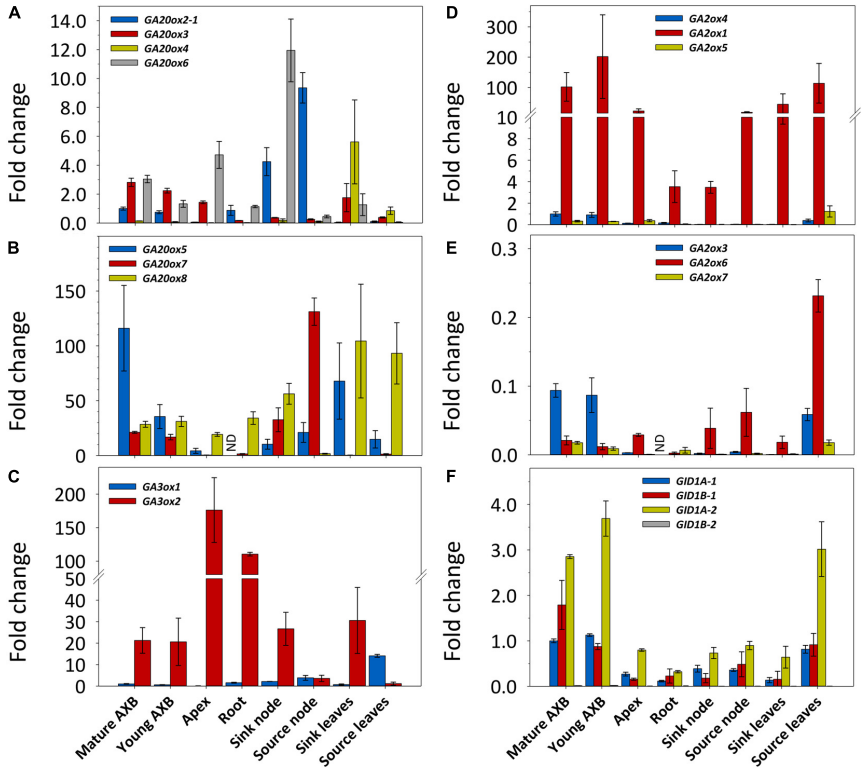

FIGURE 2 | Expression of gibberellin (GA) biosynthesis, deactivation and signaling genes in different plant parts in hybrid aspen. Relative expression (fold change) of *GA20ox* **(A,B)**, *GA3ox* **(C)**, *GA2ox* **(D,E)**, and *GID1* **(F)** family genes. The two larger gene families are depicted in two separate graphs with high **(B,D)** and little **(A,E)** expressed genes. Values represent the means of three biological replicates ± S.E. (*n* = six plants). ND, not detected. Fold changes are relative to reference gene expression in quiescent AXBs, set to 1. A moderately expressed gene within each family was selected for comparison: *GA20ox2-1*, *GA3ox1*, *GA2ox4*, and *GID1A-1*.

bioactive GAs because receptor abundance correlates negatively with GA levels (Middleton et al., 2012).

GA20ox and *GA3ox* Genes Are Not Early Activators of AXBs

Even though AXBs in hybrid aspen become quiescent when they reach the BMP, they maintained elevated transcript levels of *GID1* receptor genes (**Figure 2F**), indicating that they remain highly sensitive to GA even after completing development. Despite this, AXBs forestall outgrowth, likely through high expression of *GA2ox* genes to neutralize GA biosynthesis (**Figures 2D,E**).

To investigate if and how deactivation and GA biosynthesis changes during AXB activation and outgrowth we decapitated plants at the BMP, recorded the growth of the proximal AXBs over a 5-day period, and analyzed the changes in gene expression that occurred during the critical first 48 h. The BMP has been assessed before, based on the number of embryonic leaves (Rinne et al., 2015). Here, we determined the AXB growth by monitoring the dry weight increment of AXBs along the stem. A plateau in weight gain was reached at the end of zone 3, which is around AXB 12 (**Figure 3A**), in agreement with the earlier assessment based on embryonic leaf number (Rinne et al., 2015). Precise weight measurements revealed that decapitation not only significantly increased the weight of the proximal AXBs (zone 4), but also of the lower AXBs (zone 5 and 6), showing that all AXBs were activated (**Figure 3B**). Nonetheless, only the uppermost AXBs (zone 4) grew out, indicating that AXB activation is distinct from outgrowth, as suggested earlier (Cline, 1997).

Refining our previous suggestion (Rinne et al., 2016), we show here that net GA-biosynthesis is not the first step in decapitation-induced AXB activation. Although *GA20ox6* increased transiently at 2 h, and *GA20ox2-1* and *GA20ox4* at 48 h, these genes were little expressed in quiescent AXBs compared to *GA20ox5* and *GA20ox8*, which significantly decreased by 2 and 24 h, respectively (**Figure 4A**). Strikingly, the proliferation-related gene *GA3ox2*, serving *de novo* biosynthesis of GA, became significantly upregulated only between 12 and 24 h, in

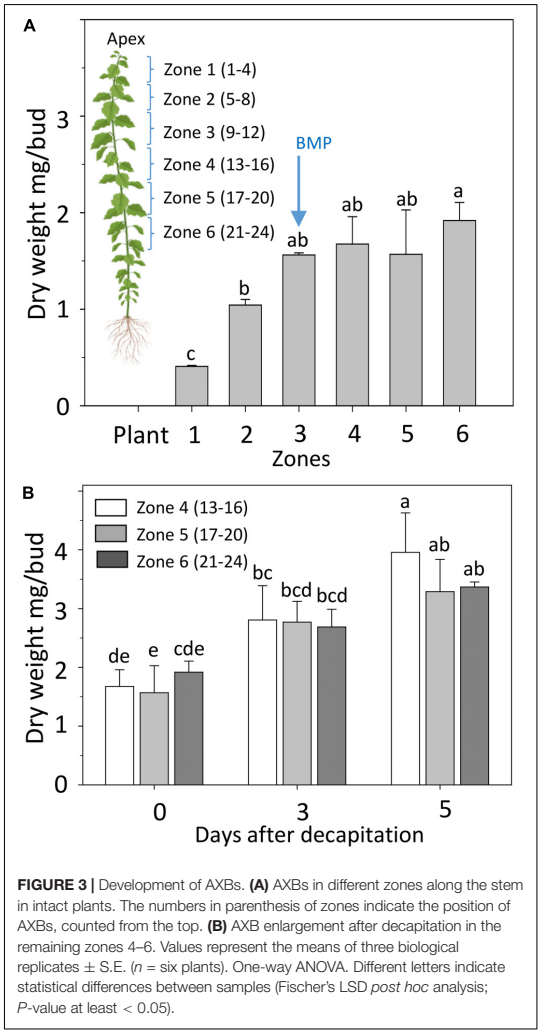

FIGURE 3 | Development of AXBs. **(A)** AXBs in different zones along the stem in intact plants. The numbers in parenthesis of zones indicate the position of AXBs, counted from the top. **(B)** AXB enlargement after decapitation in the remaining zones 4–6. Values represent the means of three biological replicates ± S.E. (n = six plants). One-way ANOVA. Different letters indicate statistical differences between samples (Fischer's LSD *post hoc* analysis; P-value at least < 0.05).

parallel with the downregulation of maturation-related *GA3ox1* (**Figure 4B**). In brief, *de novo* GA biosynthesis by *GA20ox* and *GA3ox* genes is not the initial factor that triggers AXB activation.

GA2ox Genes Are Early Responders During AXB Activation

All AXBs of intact plants expressed the GA-deactivating *GA2ox* genes, some at relatively high or very high levels (**Figures 2D,E**), but decapitation significantly downregulated them within a few hours (**Figure 4C**). This represented the first change induced by decapitation. The highly expressed gene *GA2ox1* was strongly downregulated between 2 and 6h post-decapitation, whereas the little expressed genes *GA2ox3*, *GA2ox4*, and *GA2ox7* were downregulated even earlier (**Figure 4C**). The remaining two little-expressed genes *GA2ox6* and *GA2ox5* responded later or not at all. This shows that the considerable levels of *GA3ox1* and *GA3ox2* expression in quiescent AXBs were counteracted by the high levels of *GA2ox1* expression. In other words, deactivation neutralizes biosynthesis in quiescent AXBs, whereas decapitation increases bioactive GAs by strongly reducing GA deactivation (**Figure 4C**). The significant parallel reduction in the expression of the *GID1* genes (**Figure 4D**) supports this conclusion, as it is well-known that transcription of *GID1* is reduced when levels of bioactive GAs rise (Middleton et al., 2012). That the expression of *GA20ox* genes did not increase in AXBs after decapitation, while the expression of *GA3ox2* was significantly elevated at 24 h, may indicate that additional GA precursors arrived from the nodes. In support of this, expression of *GA20ox2-1*, *GA20ox5* and *GA20ox7* in source nodes was high (**Figures 2A,B**), and decapitation transiently upregulated *GA20ox2-1*, *GA20ox3*, *GA20ox4*, and *GA20ox8* (**Figure 5A**). The putative pool of precursors in the nodes is unlikely to serve the production of bioactive GA in the source node itself, because the proliferation-related gene *GA3ox2* was little expressed, and further downregulated 2 h post-decapitation (**Figure 5B**). Although the maturation-related gene *GA3ox1* was transiently upregulated in source nodes between 2 and 6 h (**Figure 5B**), this was offset by the dramatic upregulation of *GA2ox1* and *GA2ox6*, the two major deactivating genes, as well as the little-expressed gene *GA2ox7* (**Figure 5C**). Moreover, the expression of the *GID1* receptor genes tended to increase in the nodes, suggesting a reduction in bioactive GA levels. Notably, the expression patterns of GA-biosynthesis, GA-deactivation and *GID1* receptor genes were almost opposite in nodes and activated AXBs (**Figures 4**, **5**).

Collectively, the results support the idea that nodal bark acts as a storage of GA precursors. The time frame of the events suggests that AXB activation is based on diminished deactivation of bioactive GAs in AXBs, making them available for GA signaling, whereas outgrowth relies on biosynthesis, assisted by delivery of node-produced GA precursors.

Xylem-Fed GA_3, GA_4, and GR24 Modulate GA- and SL-Pathways

Although often functioning redundantly, GA_3 and GA_4 are produced in separate biosynthetic branches. A biologically meaningful distinction is that GA_4 is deactivated by GA2oxs, whereas GA_3 is protected by a double bond at the C2, preventing 2β-hydroxylation (Nakayama et al., 1990). In hybrid aspen, GA_4 application to dormant AXBs triggers outgrowth, whereas GA_3 fails to do so, and a high concentration induces AXB abscission (Rinne et al., 2011). Another factor that affects AXB activation is SL, which acts as an inhibitor of outgrowth in hybrid aspen (Katyayini et al., 2019).

To investigate possible interference of these three hormone pathways, we fed them separately into single-node cuttings, monitored AXB behavior, and analyzed the expression of GA- and SL-pathway genes. As the simple act of isolating the single-node cuttings already activates the AXBs, these experiments test possible interference during AXB elongation.

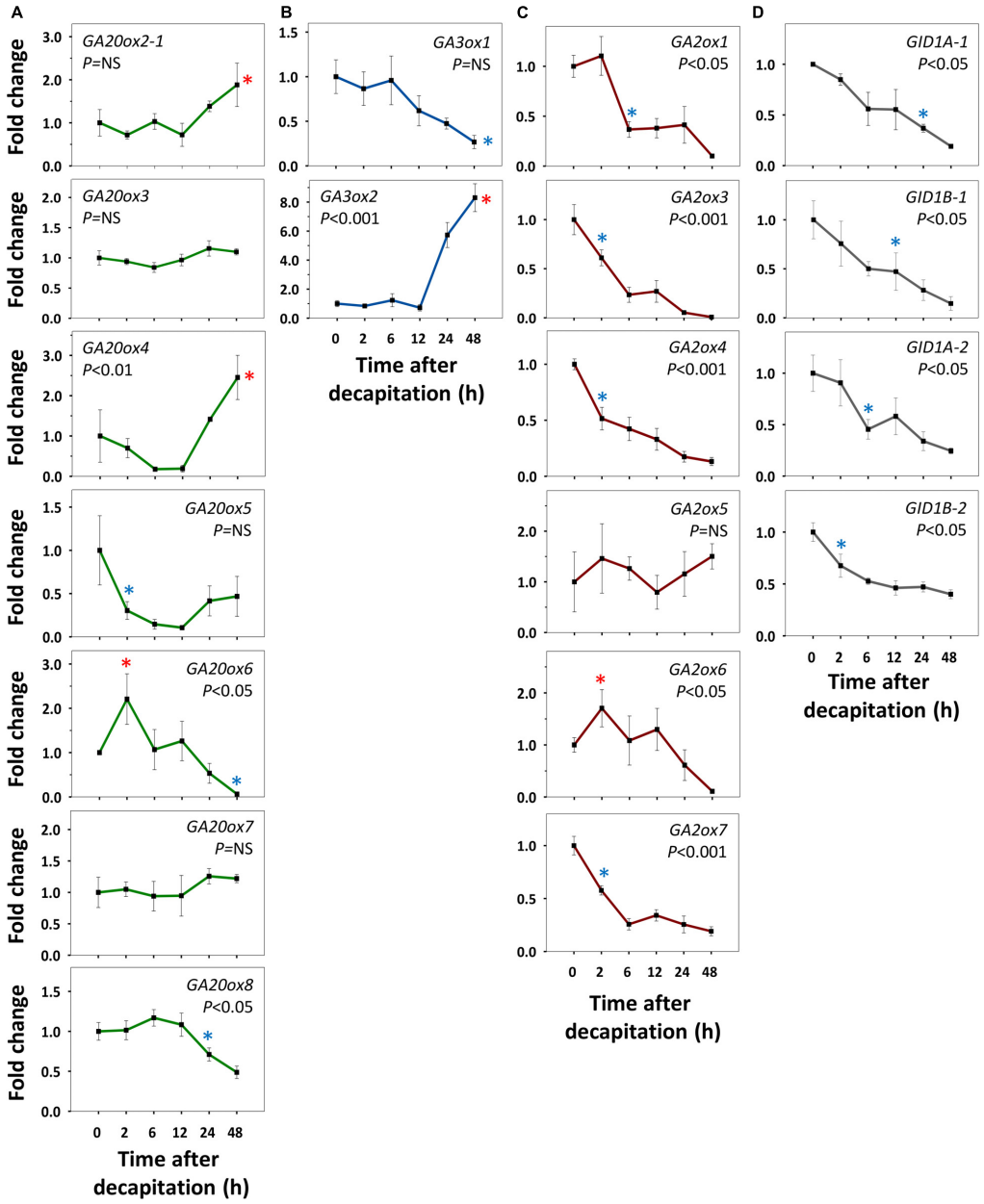

FIGURE 4 | Expression of gibberellin (GA) biosynthesis, deactivation and signaling genes in AXBs after activation by decapitation. **(A)** GA biosynthesis gene families encoding GA20-oxidases and **(B)** GA3-oxidases. **(C)** GA-deactivation gene family encoding GA2-oxidases. **(D)** GID1-receptor genes. Relative expression (fold change) was analyzed at indicated times after decapitation in three successive AXBs proximal to the decapitation point. Values represent the means of three biological replicates ± S.E. (n = six plants). One-way ANOVA (P-value; NS, not significant). Asterisk indicates the first significant decrease (blue) or increase (red), in gene expression in comparison to time 0 (Fischer's LSD *post hoc* analysis, P-value at least < 0.05).

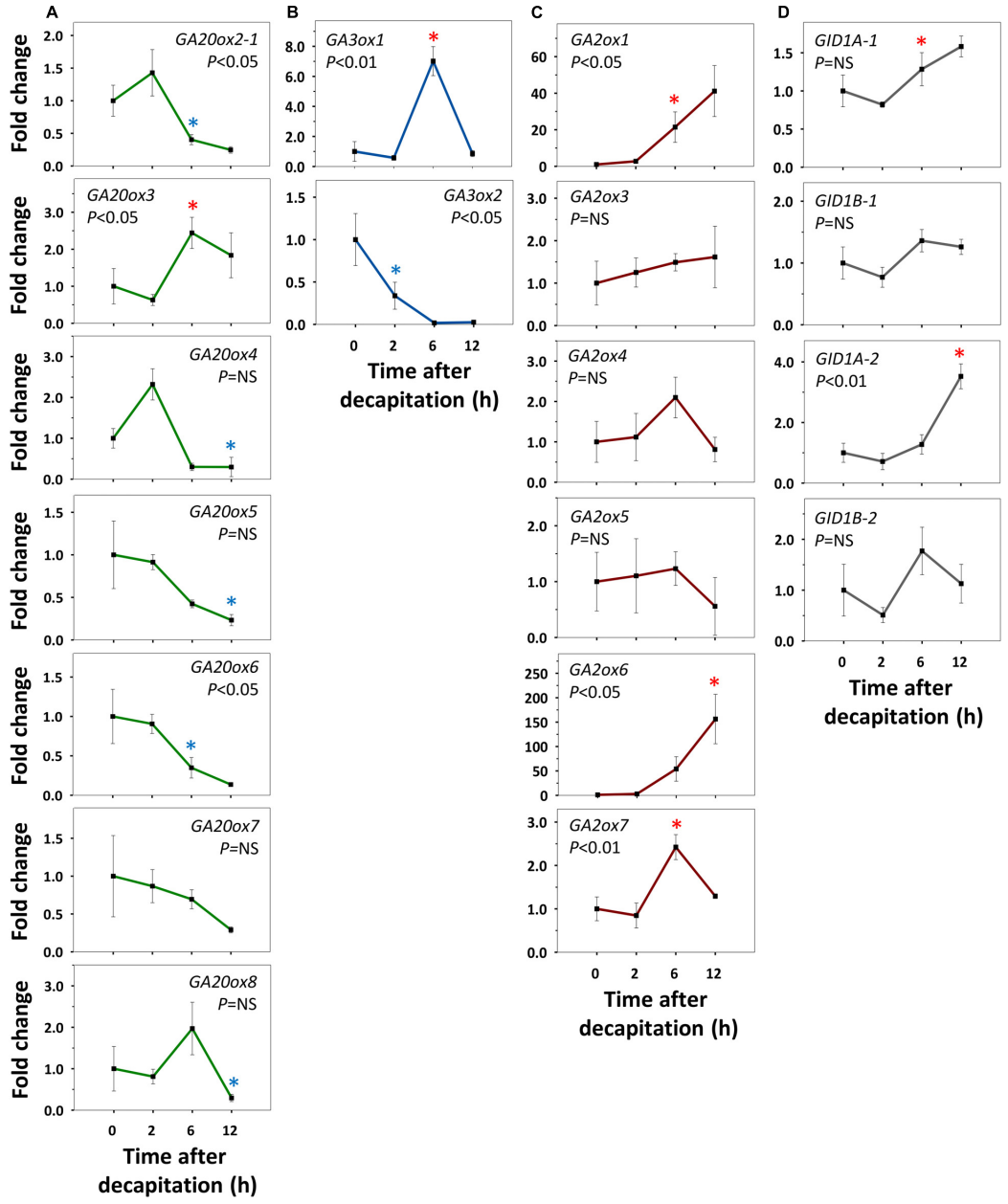

FIGURE 5 | Expression of gibberellin (GA) biosynthesis, deactivation and signaling genes in nodal bark after decapitation. **(A)** GA biosynthesis gene families encoding GA20-oxidases and **(B)** GA3-oxidases. **(C)** GA-deactivation gene family encoding GA2-oxidases. **(D)** *GID1*-receptor genes. Relative expression (fold change) was analyzed at indicated times after decapitation in three successive nodes proximal to the decapitation point. Values represent the means of three biological replicates ± S.E. (n = six plants). One-way ANOVA (P-value; NS, not significant). Asterisk indicates the first significant decrease (blue) or increase (red), in gene expression in comparison to time 0 (Fischer's LSD *post hoc* analysis, P-value at least < 0.05).

Because preliminary tests with 1% methylene blue showed that it took more than 24 h before dye entered AXBs (not shown), the analyses were carried out at day 3 and day 5, well within the AXB elongation phase. AXB outgrowth tests showed that feeding of a relatively high concentration of GR24 neither inhibited nor promoted AXB burst, relative to the controls, while GA_4 significantly accelerated it, and GA_3 induced AXB abscission (**Supplementary Figure S3**). Gene expression analyses of AXBs showed that at the 3 d time point *GA20ox* genes were downregulated by both GA_3 and GA_4, except for the unresponsive *GA20ox3*, and there was no clear difference between the effects of GA_3 and GA_4 (**Figure 6A**). At the 5 d time point, the downregulated *GA20ox2-1*, *GA20ox4*, *GA20ox6*, and *GA20ox7* were upregulated by both GA_3 and GA_4. *GA20ox8* was unique in that it remained completely unaffected. Notably, it was downregulated by decapitation (**Figure 4A**). Overall, GA feeding showed that the expression of most *GA20ox* genes was under strong homeostatic control. Contrary to the downregulating

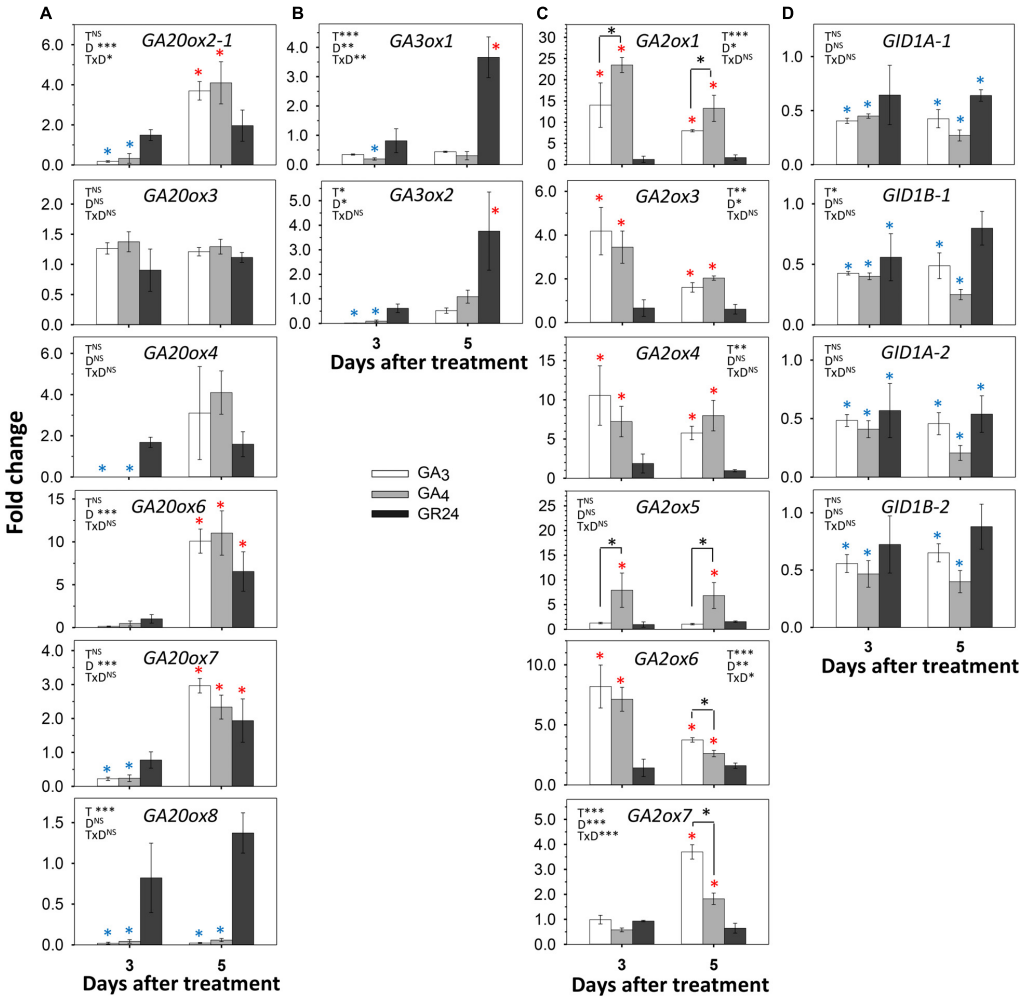

FIGURE 6 | Effect of GA_3, GA_4, and GR24 on expression of GA-pathway genes in AXBs. AXBs on single-node cuttings were fed with or without GA_3, GA_4 and GR24 at a concentration of 10 μM. **(A)** *GA20-oxidase* genes. **(B)** *GA3-oxidase* genes. **(C)** *GA2-oxidase* genes. **(D)** *GID1* genes. Values are calculated relative to control and represent the means of three biological replicates ± S.E. (*n* = six plants). The significance of factors in two-way ANOVA (T, treatments; D, duration in days; TxD, interaction) are indicated by asterisk(s) (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$). Asterisks above the bar indicate decrease (blue) or increase (red), relative to control, and above the hook differences between GA_3- and GA_4-treatments (Fischer's LSD *post hoc* analysis; *P*-value at least < 0.05).

effect of GA_3 and GA_4 at the 3 d time point, GR24 feeding tended to upregulate the expression of several *GA20ox* genes (**Figure 6A**) whereas at the 5 d time point *GA20ox6*, and *GA20ox7* were significantly upregulated, similarly to GA_3 and GA_4.

Of the *GA3ox* family genes, *GA3ox1* was significantly downregulated in AXBs by both GA_3 and GA_4 (**Figure 6B**). That *GA3ox1* expression remained low during the entire period was expected, as it was downregulated by decapitation and did not play a role in AXB activation (**Figures 4B**, **5B**). In contrast, *GA3ox2*, which is characteristically expressed in proliferating apices and upregulated in activated AXBs (**Figures 2C**, **4B**), was very strongly downregulated at day 3, although it recovered at day 5 (**Figure 6B**). Overall, GA feeding showed that *GA3ox* genes, especially *GA3ox2*, were homeostatically controlled. GR24 did not initially affect the expression of *GA3ox* genes, but at day 5 it significantly increased the expression of the maturation-related *GA3ox1* as well as the proliferation-related *GA3ox2*.

The GA-deactivating *GA2ox* genes were strongly upregulated by both GAs. The GA_4-induced upregulation of the major *GA2ox1* gene was almost 25-fold at day 3, while GA_3 was less effective (**Figure 6C**). In most cases, the expression levels decreased somewhat at day 5. However, *GA2ox5* expression continued to rise during GA_4 feeding, while this gene was unresponsive to GA_3. In contrast, the minor genes *GA2ox6* and *GA2ox7*, were more responsive to GA_3 than to GA_4 at day 5. The significant upregulation of *GA2ox* genes indicates that both GAs were effective, whereas GR24 had no effect, suggesting that GR24 does not promote GA deactivation in activated AXBs. *GID1* genes were significantly downregulated by GA_3 and GA_4 (**Figure 6D**). Interestingly, GR24 also reduced *GID1* expression almost to the same degree as the GAs, probably because it upregulated many GA biosynthesis genes (**Figures 6A,B**).

To assess if the reverse could also be the case, we tested how GA_3 and GA_4 affected expression of SL pathway genes (**Figure 7**). In the SL pathway, the gene *MAX1* encodes an enzyme that converts plastid-produced carlactone to the SL precursor carlactonoic acid (Abe et al., 2014). Of the two hybrid aspen paralogs *MAX1.1* and *MAX1.2*, the gene *MAX1.2* was downregulated by GA_3 and GA_4, especially by GA_3, both at day 3 and 5, whereas *MAX1.1* was downregulated only by day 5 (**Figure 7A**). The genes that encode the SL receptor, *D14a* and *D14b*, were strongly upregulated by GA_3 and GA_4, while *MAX2a* and *MAX2b* were moderately upregulated at day 3 (**Figures 7B,C**). Together this indicates that the GA-induced reduction of SL levels caused upregulation of *D14* and *MAX2* signaling genes through SL homeostasis. This might have transiently increased expression of *BRC1*, a downstream target of SL. In contrast, *BRC2* was slightly downregulated by both GAs (**Figure 7D**).

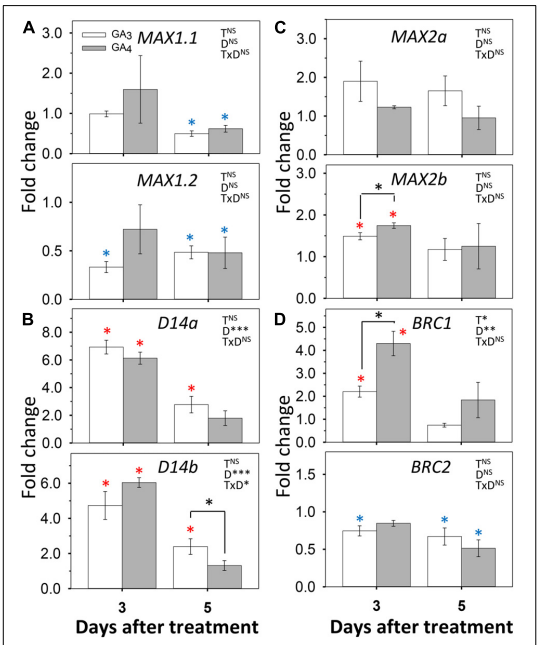

FIGURE 7 | Effect of GA_3 and GA_4 on selected SL-pathway genes in AXBs. AXBs on single-node cuttings were fed with or without GA_3 and GA_4 at concentration of 10 μM. Gene expression was analyzed after 3 and 5 days of treatment. **(A)** *MAX1.1* and *MAX1.2*. **(B)** *D14a* and *D14b*. **(C)** *MAX2a* and *MAX2b*. **(D)** *BRC1* and *BRC2*. Values are calculated relative to control and represent the means of three biological replicates ± S.E. (n = six plants). The significance of factors in two-way ANOVA (T, treatments; D, duration in days; TxD, interaction) are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$). Asterisks above the bar indicate decrease (blue) or increase (red), relative to control, and above the hook differences between GA_3- and GA_4-treatments (Fischer's LSD *post hoc* analysis; P-value at least < 0.05).

AXB Activation Increases the Ratio of GA_4/GA_1 to GA_3/GA_6

Gibberellin metabolites, precursors and bioactive molecules in intact and decapitated plants were analyzed using an establish method (Urbanová et al., 2013). This revealed the presence of spatio-temporal patterns in apices and AXBs of distinct zones along the stem (**Figure 8**). A notable finding was that apices contained bioactive GA of both branches of the GA pathway, although GA_1 was the dominant bioactive GA in apices, and levels of GA_4, GA_5, and GA_7 were significantly lower, at least by a factor 20. GA_6 was hardly detectable in apices, whereas GA_3 was below the detection limit of the LC-MS/MS method used. Although GA_1 and GA_4 levels were higher in apices than AXBs, these differences were not reflected at the level of precursors. In the case of GA_4, its immediate precursor, GA_9 was under the detection limit in apices, in contrast to GA_{24}, which was present at high levels. This could indicate that the pool of GA_9 is very small due to its rapid conversion to GA_4, GA_7 and the deactivation product GA_{51}. The GA20ox that produces GA_9 from GA_{24} could therefore be a rate-limiting enzyme in apices, but not in AXBs where these genes were well expressed. GA_1 levels in apices were about 40 times higher than the levels of its precursor GA_{20}, even though GA_1 was strongly deactivated to GA_8. This suggests that the GA_{20} pool is in a state of rapid flux in apices.

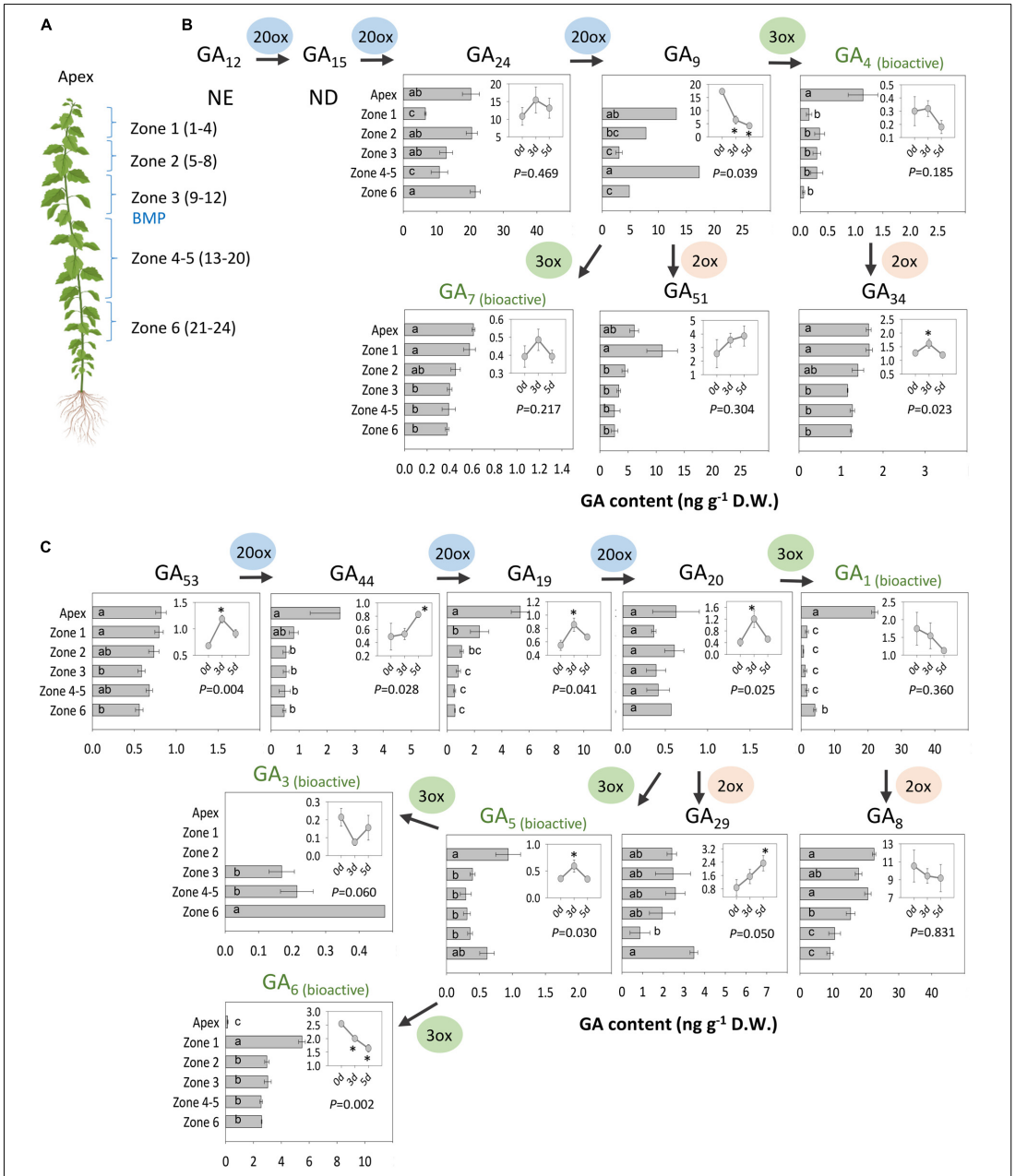

FIGURE 8 | Analysis of GAs. **(A)** Analyzed materials are indicated. The numbers in parenthesis of each zone refer to the position and the number of AXBs in each sample. GAs of the **(B)** non-13-hydroxylation and **(C)** the 13-hydroxylation pathway. Insets: Changes in GA levels 0, 3, and 5 days after decapitation in AXBs proximal to the decapitation point (*P*-value shown). Asterisks in insets indicate statistically significant change in GA levels. Values represent the means of three biological replicates ± S.E. (n = six plants). Different letters in bars indicate statistical differences in GA levels between the samples. NE, not estimated; ND, not detected. One-way ANOVA and pairwise *post hoc* analysis by Fischer's LSD test (*P*-value at least < 0.05).

In AXBs, GA_1 levels were ca.10-fold lower than in apices, while the level of the bioactive GA_4 was about 3- to 4-fold lower (**Figures 8B,C**). AXBs contained a considerable amount of GA_6, while GA_3 was produced at a much lower level, and only in mature AXBs (mature AXBs in zone 4-5) and aging (oldest AXBs in zone 6) (**Figures 8A,C**). GA-deactivation was especially prominent in the early 13-hydroxylation pathway, resulting in high levels of GA_{29} and, especially, GA_8. Whereas GA_1 content was low in AXBs, its deactivation product GA_8, was almost at the same level as in apices. When the *GA2ox* genes, responsible for this conversion, are abruptly downregulated, as observed after decapitation in AXBs (**Figure 4C**), GA_1 availability is expected to rise. In the non-13-hydroxylation pathway, most GA_9 was deactivated to GA_{51}, and comparatively little to the bioactive GA_4 and GA_7, both in apices and AXBs. Similarly, to GA_8, the GA_4-deactivation product GA_{34} was almost the same in AXBs and apices, suggesting that decapitation-induced downregulation of *GA2ox* expression in AXBs increases GA_4 availability.

Shoot decapitation only slightly affected GA content during the AXB elongation phase at 3 d and 5 d post-decapitation (**Figures 8B,C**, insets). The changes in the 13-hydroxylation pathway (**Figure 8C**) were more often statistically significant than those in the non-13-hydroxylation pathway (**Figure 8B**). In the latter, only the deactivation product GA_{34} increased significantly. In the 13-hydroxylation pathway, GA_1 also did not show any increase, even though all its precursors increased at day 3 and 5. The overall increase in precursors (GA_{53} to GA_{20}) resulted in a significant increase of the deactivation product GA_{29}. GA_3 and GA_6 were absent from apices, but were detected in AXBs, whereas decapitation lowered their contents, especially that of GA_6.

Interestingly, in a separate experiment under suboptimal greenhouse conditions, where plants tended to cease growth, GA_{20} levels and their deactivation products GA_{29} and GA_8 were higher in apices, while GA_1 levels were very low (**Supplementary Figure S4**). In these plants GA_3 was also detectable in apices, while GA_5 and GA_6 were under the detection limit. This highlights that GA_3 is not unique to AXBs *per se* but can be produced to restrict proliferation.

DISCUSSION

Shoot branching is regulated by a network of inhibitory and promotive forces. The present results obtained by combining gene expression profiling, metabolite quantitation and hormone treatments show that specific GAs promote branching, while others maintain AXBs in a quiescent state.

AXB Activation and Outgrowth Require Diminished GA-Deactivation

The differential expression of GA-pathway genes at the whole plant level appears to reflect the proleptic lifestyle of hybrid aspen, in which AXBs become quiescent once they reach maturity (Rinne et al., 2015). AXBs expressed most *GA20ox* genes at significantly higher levels than apices (**Figures 2A,B**). Nonetheless, the levels of bioactive $GA_{1/4}$ were significantly lower in AXBs than in proliferating apices (**Figure 8**). The obvious reason for this is that *GA2ox* genes were strongly expressed in AXBs, about 6-fold relative to apices (**Figures 2D,E**). As the encoded GA2ox enzymes irreversibly deactivate bioactive GAs by 2β-hydroxylation (Thomas et al., 1999; Olszewski et al., 2002; Middleton et al., 2012), the high level of *GA2ox* expression in AXBs can keep them quiescent. This is strongly supported by the fact that during AXB activation several *GA2ox* genes were rapidly and significantly downregulated, and subsequently also the four *GID1* receptor genes (**Figures 4C,D**). This indicates that GA availability had effectively increased because GID1 levels are known to diminish when GA levels increase due to homeostatic adjustment (Gallego-Giraldo et al., 2008; Hedden and Thomas, 2012; Middleton et al., 2012). Because bioactive GA levels reflect the balance between GA biosynthesis and deactivation (Phillips et al., 1995; Xu et al., 1999; Olszewski et al., 2002; Yamaguchi, 2008), the decapitation-induced reduction of GA deactivation increases its availability for signaling, even in the absence of increased biosynthesis.

The emerging picture is that quiescent AXBs are sensitized to GA, because relative to apices they have low levels of GA_1 and GA_4 despite the ongoing GA biosynthesis, but high levels of *GID1* expression. Thus, regardless of GA biosynthesis, the dwarfed ES of AXBs is GA deficient. The high *GA2ox* expression levels in AXBs appear to be developmentally controlled to keep AXB activation at bay and safeguard the proleptic nature of the shoot system. $GA_{3/6}$ can play a role in maintaining AXB quiescence (**Figure 8C**) by upregulating *GA2ox* genes, thereby deactivating $GA_{1/4}$, but not of itself (and $GA_{5/6}$) because it is not a substrate (Nakayama et al., 1990; Ito et al., 2017; Li et al., 2017). Thus, the specific presence of $GA_{3/6}$ in quiescent AXBs can effectively maintain them in a GA_4-deficient state. As GA_4 is involved in promoting cell division, elongation and energy metabolism (Hedden and Sponsel, 2015; Zhuang et al., 2015) and has the highest binding activity to GID1 (Ueguchi-Tanaka et al., 2005), keeping GA_4 low is necessary to prevent AXB activation and outgrowth. In addition, other factors may play a role in AXB quiescence, including SL (Katyayini et al., 2019) and BRC1-regulated ABA signaling (González-Grandío et al., 2017; Wang et al., 2019). After AXB activation, subsequent AXB elongation is supported by *de novo* biosynthesis of GA_1 and GA_4, initiated between 12 and 24 h through upregulation of *GA3ox2* (**Figure 4B**). In support of this, a previous study showed that this gene, originally named *GA3ox1*, is characteristically expressed in growing shoot apices (Israelsson et al., 2004). In short, our data support a model in which branching is initiated by a strong reduction of GA deactivation that raises the bioactive $GA_{1/4}$ pool to spearhead AXB activation, while additional $GA_{1/4}$ biosynthesis supports subsequent AXB elongation, as illustrated in **Figure 9**.

GA Biosynthesis Differs in Growing and Mature Tissues

The expression patterns of the GA biosynthesis genes were different for actively proliferating tissues (apices and roots), differentiated tissues (mature leaves), and developmentally

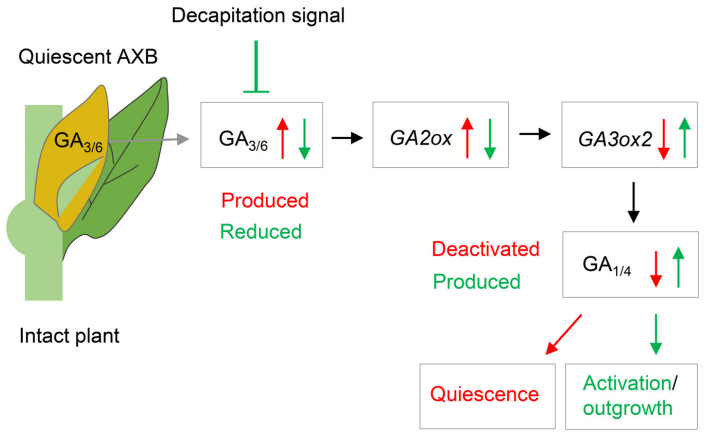

FIGURE 9 | Model of axillary bud (AXB) quiescence and activation. During their formation, AXBs accumulate $GA_{3/6}$, which upregulates *GA2ox* gene expression. GA2ox deactivates $GA_{1/4}$, thereby depriving the AXBs of $GA_{1/4}$-mediated signaling. Decapitation activates quiescent AXBs by rapidly reducing $GA_{3/6}$ levels and downregulating *GA2ox* transcription, thereby elevating $GA_{1/4}$ levels and signaling. Subsequently, the $GA_{1/4}$ biosynthesis gene *GA3ox2* is upregulated dramatically increasing GA levels and promoting AXB outgrowth. Red arrows and text indicate AXB inhibitory effects. Green arrows and text indicate AXB activating and growth promoting effects.

inactive tissues with high growth potential (AXBs) (**Figure 2**). For example, apices expressed *GA20ox* genes less than other tissues, but they highly expressed *GA3ox2*, whereas *GA3ox1* was hardly expressed. In contrast, quiescent AXBs expressed both *GA3ox* genes, whereas source leaves exclusively expressed *GA3ox1* genes. Thus, *GA3ox2* supports cell proliferation and growth at apices and root tips, whereas *GA3ox1* reflects tissue maintenance in source nodes and leaves. The fact that quiescent AXBs expressed both *GA3ox2* and *GA3ox1* appears to reflect their opposing developmental tendencies, as AXBs combine developmental stasis with high growth potential. As indicated above, the high levels of GA deactivation, maintained by the GA2ox-insensitive $GA_{3/6}$, are likely to be part of the developmental block that prevents AXB activation.

Although AXBs expressed all GA-pathway genes, their outgrowth is strongly dependent on a functional connection to the stem, especially nodal vascular tissue. The results suggest that nodal bark exported precursors to AXBs, because the *GA20ox* transcript levels in the AXBs were reduced soon after decapitation, whereas in the nodal bark they initially increased without increasing *GA3ox2* expression (**Figures 5A,B**). Transport of precursors and bioactive GAs (GA_3, GA_4, GA_9, GA_{12} and GA_{20}) is known to be crucial in directing development (Proebsting et al., 1992; Eriksson et al., 2006; Yamaguchi, 2008; Ragni et al., 2011; Dayan et al., 2012; Lange and Lange, 2016; Regnault et al., 2016; Binenbaum et al., 2018). The GA quantitation data support the idea that precursors are transported from nodes to the AXBs, as their levels increased in AXBs after decapitation, for example in case of GA_{20}, a key precursor of several bioactive forms of GA (**Figure 8C**). Such node-to-AXB delivery also plays a role in the SL-mediated control of AXB quiescence (Katyayini et al., 2019). Together, the analyses indicate that nodal bark tissue might affect AXBs by delivering SL and GA precursors.

GA and SL Pathways Are Buffered and Show Interference

During the AXB elongation phase, *GA2ox* genes responded strongly to GA feeding by upregulating their expression up to ≥20-fold at day 3. As the *GID1* expression levels were only reduced by about 2-fold, the upregulated *GA2ox* must have been effective in deactivating part of the supplied GA. Feeding GR24 did not affect the expression of *GA2ox* genes, but it did increase the expression of GA biosynthesis genes at day 5 (**Figures 6A,B**). A putative increase in GA levels by GR24 could explain why GR24 feeding reduced *GID1* expression levels to a similar degree as GA_3 and GA_4 (**Figure 6D**).

In hybrid aspen, SL pathway and perception genes are highly expressed in quiescent AXBs, but decapitation rapidly downregulated these genes as well as the downstream target gene *BRC1* (Katyayini et al., 2019). While $GA_{3/6}$, GA2ox as well as SL contribute to the quiescent state of AXBs in intact plants, their decrease in activated AXBs leads to elevated $GA_{1/4}$ levels through a reduction of GA2ox activity. Subsequent outgrowth might require CK in addition (Ni et al., 2017; Duan et al., 2019).

As feeding GA_3 and GA_4 reduced the expression of both *MAX1* genes (**Figure 7A**), GA represses SL biosynthesis, which supports earlier observations in other plant species (Ni et al., 2015; Ito et al., 2017; Marzec, 2017). Our data show that during the AXB elongation phase both GA_3 and GA_4 increased SL perception by upregulating *D14* genes and *MAX2b* (**Figures 7B,C**). This increase in SL perception and signaling genes presumably is a homeostatic response to a GA-induced

reduction in SL levels in the AXBs. In Arabidopsis, GA and GR24 converge on a large number of shared transcription targets (Lantzouni et al., 2017). However, in pea, SL can also independently of GA promote cell division in the stem (de Saint Germain et al., 2013). Here we found that GR24 increased the biosynthesis of GA during the AXB elongation phase. It is noteworthy that GR24 feeding can promote the elongation of the enclosed ES five to seven days post-decapitation (Katyayini et al., 2019), and the present data suggest this might involve GA. Whether these interferences between SL and GA pathways are direct or indirect remains to be established.

GA_3 and GA_6 Are Involved in AXB Development but Not in AXBs Outgrowth

In AXBs of intact plants, the gene *GA3ox1* could be linked to presence of GA_3 and GA_6. After decapitation, *GA3ox1* expression and GA_3 and GA_6 content decreased in AXBs (**Figures 4B**, **8C**) and were absent from apices (**Figure 2C**). This indicates that *GA3ox1* functions in the side branch of the 13-hydroxylation pathway that produces the deactivation-protected GA_3, GA_5 and GA_6. In contrast, *GA3ox2* converts precursors GA_9 and GA_{20} to GA_7, GA_4 and GA_1, in support of a previous study (Israelsson et al., 2004).

In apices GA_1 was more abundant than GA_4 (**Figure 8**), although GA_4 more efficiently promotes shoot elongation (Israelsson et al., 2004). However, plants can switch between pathways, depending on developmental phase or environmental conditions (Rieu et al., 2008b). For example, in a grass species GA_4 is produced during vegetative growth, while upon flowering it switched to GA_5 and GA_6 (King et al., 2001, 2003). That *GA2oxs* play a role in this, is supported by studies in *Jatropha*, where overexpression of *GA2ox6* induced a switch from the non-13-hydroxylation pathway (GA_4) to the 13-hydroxylation pathway (GA_3), and led to dwarfing (Hu et al., 2017). Our data suggest that the GA precursor GA_{20} can be converted to the growth-promoting GA_1 or the quiescence-related $GA_{3/6}$ (**Figure 8C**) dependent on developmental cues as well as environmental conditions. GA_3 accumulates in developing AXBs as well as in apices of stressed plants, while GA_1 levels remain low (**Supplementary Figure S4**). The effect of these cues on GA metabolism, and the distinct responses of plants to different bioactive GAs (Elfving et al., 2011; Rinne et al., 2011; Ni et al., 2015) warrant further investigation.

Although GA_3 is often used as a generic GA, it is different from GA_4 in important respects. The results show that in hybrid aspen GA_3 and GA_4 not only operate at distinct locations, their functions are also partly distinct. GA_4 feeding promotes AXB outgrowth, whereas GA_3 induces abscission in the non-dormant quiescent AXBs that form under long days (**Supplementary Figure S3**) as well as the AXBs that establish dormancy under short days (Rinne et al., 2011). GA_3 and GA_4 also induce different classes of 1,3-β-glucanases, destined for different subcellular locations (Rinne et al., 2011). Both GA_3 and GA_4 promote cell division, but GA_4 function requires histone deacetylases to transcriptionally block *GA2ox* (Li et al., 2017). Although required for apical growth, in the vegetative in the meristem dome itself GA_4 is absent, because its production is blocked, and a band of *GA2ox* expression below the meristem protects it from a damaging influx of GA_4 (Sakamoto et al., 2001; Jasinski et al., 2005; King et al., 2008; Bolduc and Hake, 2009). As GA_3 cannot be deactivated by GA2ox, GA_3 (as well as GA_5 and GA_6) can enter the meristem and induce floral transition in grasses, whereas GA_4 can only enter later, when the band of *GA2ox* expression is gone (King et al., 2003).

Because GA_3 can significantly upregulate *GA2ox* genes (**Figure 6C**), its accumulation in quiescent AXBs results in low levels of $GA_{1/4}$ due to deactivation, as both are substrates of GA2ox (Nakayama et al., 1990), thereby inhibiting GA_4-mediated AXB activation and elongation. Our finding that $GA_{3/6}$ were detected in quiescent AXBs and reduced by decapitation, matches our earlier finding that GA_3, unlike GA_4, cannot upregulate the growth-related α-clade 1,3-β-glucanases that optimize symplasmic conduits for transport to growing areas (Rinne et al., 2011).

CONCLUSION

A major finding was that hybrid aspen invests energy into producing and simultaneously deactivating $GA_{1/4}$ in quiescent AXBs, although they remain developmentally inactive until the next year. This seemingly wasteful strategy is an effective way to keep AXBs ready for rapid outgrowth in case the shoot apex is damaged or lost, allowing a new shoot to form before winter arrives. The results support a model in which SL and *GA3ox1*-mediated accumulation of $GA_{3/6}$ maintain AXBs in a quiescent state, with $GA_{3/6}$ upregulating *GA2ox* genes that deactivate $GA_{1/4}$. In turn, decapitation-induced AXB activation is triggered by a rapid downregulation of *GA2ox* genes, which shifts the balance between $GA_{1/4}$ biosynthesis and deactivation, increasing the $GA_{1/4}$ pool available for GA signaling. The initial $GA_{1/4}$ pulse is followed by increased *GA3ox2*-mediated *de novo* GA biosynthesis, and subsequent elongation of the AXB. The dual, opposing roles of $GA_{3/6}$ and $GA_{1/4}$ can explain why the role of GA in branching has been ambiguous.

MATERIALS AND METHODS

Plant Material and Sample Preparation

Hybrid aspen (*Populus tremula* × *Populus tremuloides*) clone T89 was micro-propagated *in vitro* and grown in a greenhouse under long days as previously described (Katyayini et al., 2019). When the plants were 80–100 cm tall, with stable leaf production and elongation rates, they were subdivided into three groups: (a) Intact plants for collection of tissues and organs for transcript analyses; (b) Decapitated plants (decapitated at the bud maturation point, ca. 40 cm below the apex), for transcript and GA analysis in AXBs, and transcript analysis of nodal bark; (c) Plants for xylem feeding of hormones into single-node cuttings. Samples for transcript and hormone analyzes were collected from six plants, with two plants pooled in three replicate

samples. Position of sampled buds and tissues is indicated in **Supplementary Figure S5**.

Quantification of GAs With Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

The samples (apices and AXBs) were harvested from different zones along the stem, as indicated in **Figure 3A**. For analysis, samples were immediately frozen in liquid nitrogen, and subsequently freeze dried. Sample preparation and quantitative analysis of GAs were performed by LC-MS/MS using $^2H_2$-labeled GA internal standards as described (Urbanová et al., 2013).

AXB Burst Tests and Feeding of GA_3, GA_4, and GR24

To investigate the effects of GA_3, GA_4 and the synthetic strigolactone GR24 on AXB outgrowth and gene expression, we performed xylem-feeding experiments under forcing conditions in growth chambers (18 h of light with a PPFD of 160−200 μmol m^{-2} s^{-1}, 20°C, and 60% relative humidity). Single-node cuttings were isolated from 6-week old plants. The internode base was punched through pores in a Styrofoam sheet, floated on water (control) or water supplemented with GA_3, GA_4 (Sigma-Aldrich) or racemic synthetic SL GR24 (Chiralix BV, Netherlands) at the effective 10 μM concentration (Katyayini et al., 2019). AXB burst was followed for 14 days and scored as Σ_{14}-values, as explained in **Supplementary Figure S3**.

Experiment Design and Gene Selection

For analysis of GA-pathway, total RNA was extracted from different plant parts as indicated (**Figure 2**). Gene expression analysis included hybrid aspen homologs of *P. trichocarpa* GA-biosynthesis genes *GA20ox2-1*, *GA20ox3*, *GA20ox4, GA20ox5, GA20ox6, GA20ox7*, *GA20ox8, GA3ox1*, and *GA3ox2*; GA-catabolism genes *GA2ox1*, *GA2ox3*, *GA2ox4*, *GA2ox5*, *GA2ox6*, and *GA2ox7*; GA-signaling genes *GID1A-1*, *GID1A-2*, *GID1B-1,* and *GID1B-2*. For phylogenetic analysis, see **Supplementary Figures S1, S2**.

To assess decapitation-induced expression changes, AXBs proximal to the decapitation point of the BMP were collected 0, 2, 6, 12, 24, and 48 h post-decapitation. Sampling after day 1 and day 2 was carried out at the same time of day to avoid potential diurnal effects on gene expression. Nodal bark tissues were collected 0, 2, 6, and 12 h after decapitation.

The effects of 10 μM GA_3, GA_4 and GR24 on gene expression in AXBs were investigated after xylem feeding of the hormones into AXBs of single-node cuttings. Samples were collected after 0, 3, and 5 days. Gene expression analysis included GA-biosynthesis *GA20ox2-1, GA20ox3*, *GA20ox4*, *GA20ox6, GA20ox7*, *GA20ox8, GA3ox1*, and *GA3ox2*; GA-catabolism genes *GA2ox1*, *GA2ox3*, *GA2ox4*, *GA2ox5*, *GA2ox6*, and *GA2ox7*; GA-signaling genes *GID1A-1*, *GID1A-2*, *GID1B-1* and *GID1B-2*. In addition, previously identified SL-biosynthesis and signaling genes *MAX1.1*, *MAX1.2*, *D14a*, *D14b*, *MAX2a*, and *MAX2b*, and the downstream target genes *BRC1* and *BRC2* (Katyayini et al., 2019) were analyzed after GA_3 and GA_4 feeding.

RNA Extraction and cDNA Preparation

Total RNA was extracted from 0.2 to 0.3 g of frozen tissue and grinded in a mortar with 500 μL extraction buffer (Qiagen RLT buffer containing 1% PVP-40), and further processed as described (Katyayini et al., 2019). The samples were transferred to RNeasy spin columns and further processed in accordance with instructions of the Qiagen Plant RNA isolation kit. Genomic DNA was eliminated using TURBO^{TM} DNase kit (Invitrogen) treatment according to manufacturer's instructions and cleaned using the total RNA purification system "Purelink RNA mini kit" (Invitrogen). RNA was quantified with NanoDrop 1000, and the RNA quality was assessed with the Agilent 2100 Bioanalyzer system. 1 μg of total RNA was reversely transcribed to cDNA with SuperScript® VILO^{TM} reverse transcriptase (Invitrogen).

Quantitative RT-PCR (qRT) Analysis

The reaction setup (20 μl total volume) for qRT was prepared using SYBR® select PCR master mix (Applied Biosystems). As a template, 2 μl of the cDNA (200 ng) were added. Real-time qRT-PCR analyses were performed with the Applied Biosystems 7500 Fast Real-Time PCR system according to the manufacturer's instruction. Thermocycling conditions were set to 50°C for 2 min, 95°C for 2 min, 45 cycles of 15 s at 95°C and 60 s at 60°C. Each PCR reaction included a negative control to check for potential genomic DNA contamination. For a complete list of primers and genes used for quantitative real time PCR (qRT-PCR) see **Supplementary Table S1**.

Statistical Analysis and Bioinformatics

Statistical analyses were carried out using analysis of variance (one- or two-way ANOVA) in combination with Fisher LSD *post hoc* test to determine significant differences between the subgroups. Computation was performed using Microsoft Excel data analysis[1] and Minitab Statistical Software version 18.1.[2]

BLAST searches in GenBank, *Populus trichocarpa* genome v3.0 and *Populus tremula* × *Populus tremuloides* (T89) v3.0 databases[3,4,5] were used to identify GA-biosynthesis, -catabolism and -signaling genes. Gene specific primer sequences for qPCR analysis were designed using Primer3.[6]

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

NK, PR, and CS designed the research. NK, PR, and DT performed the experiments. NK, PR, and CS analyzed and

[1]www.microsoft.com
[2]www.minitab.com
[3]http://www.ncbi.nlm.nih.gov
[4]http://www.phytozome.net
[5]http://popgenie.org
[6]http://bioinfo.ut.ee/primer3-0.4.0

interpreted the data. NK designed the illustration. All authors participated in writing and revising the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00736/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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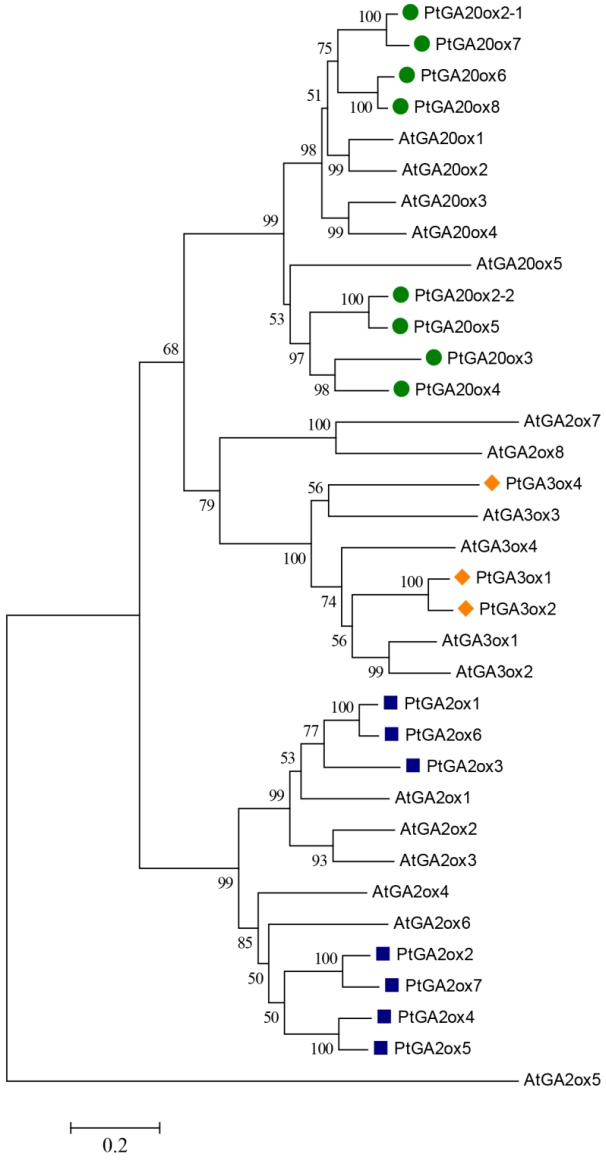
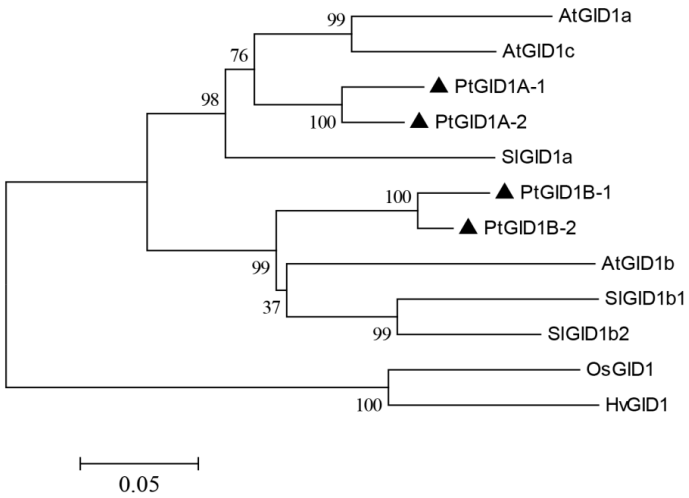
Supplementary Material



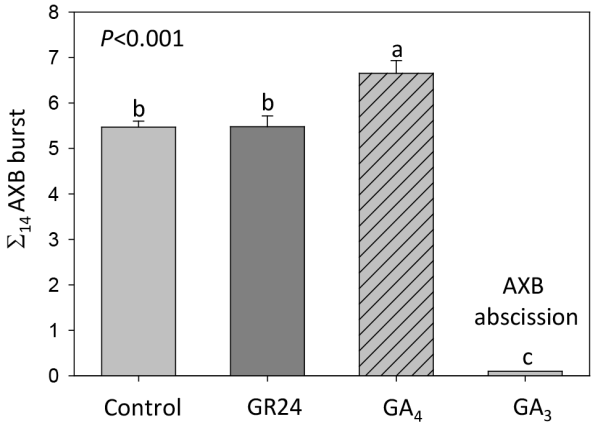
Supplementary Figure 1. Phylogenetic analysis of GA20-oxidases, GA3-oxidases and GA2-oxidases. The sequence homologues were identified by Protein BLAST search in NCBI

(http://www.ncbi.nlm.nih.gov/BLAST) and the sequences were retrieved from *Populus trichocarpa* genome (Tuskan *et al.*, 2006; http://www.phytozome.net/) databases. The amino acid sequence alignment were performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). A phylogenetic tree was created using the MEGA6 program (www.megasoftware.net) with the Neighbor–Joining method. Bootstrap support values are based on 1000 replicates. The proteins used in the phylogenetic analysis were: *Arabidopsis thaliana* AtGA2ox1 (At1g78440), AtGA2ox2 (At1g30040), AtGA2ox3 (At2g34555), AtGA2ox4 (At1g47990), AtGA2ox5 (At3g17203), AtGA2ox6 (At1g02400), AtGA2ox7 (At1g50960), AtGA2ox8 (At4g21200), AtGA20ox1 (At4g25420), AtGA20ox2 (At5g51810), AtGA20ox3 (At5g07200), AtGA20ox4 (At1g60980), AtGA20ox5 (At1g44090), AtGA3ox1 (At1g15550), AtGA3ox2 (At1g80340), AtGA3ox3 (At4g21690), AtGA3ox4 (At1g80330), AtGID1a (At3g05120), AtGID1b (At3g63010), AtGID1c (At5g27320); *Solanum lycopersicum* SlGID1a (Solyc01g098390), SlGID1b1 (Solyc09g074270), SlGID1b2 (Solyc06g008870); *Oryza sativa* OsGID1 (LOC_Os05g33730); *Hordeum vulgare* HvGID1 (A7MAQ4); *Populus trichocarpa* PtGA2ox1 (Potri.001G378400), PtGA2ox2 (Potri.002G191900), PtGA2ox3 (Potri.004G065000), PtGA2ox4 (Potri.008G101600), PtGA2ox5 (Potri.010G149700), PtGA2ox6 (Potri.011G095600), PtGA2ox7 (Potri.014G117300), PtGA20ox2-1 (Potri.002G151300), PtGA20ox2-2 (Potri.005G065400), PtGA20ox3 (Potri.005G184400), PtGA20ox4 (Potri.005G184200), PtGA20ox5 (Potri.007G103800), PtGA20ox6 (Potri.012G132400), PtGA20ox7 (Potri.014G073700), PtGA20ox8 (Potri.015G134600), PtGA3ox1 (Potri.001g176600), PtGA3ox2 (Potri.003g057400), PtGA3ox4 (Potri.018G033600). PtGA20oxs (●); PtGA2oxs (◆); PtGA3oxs (■).

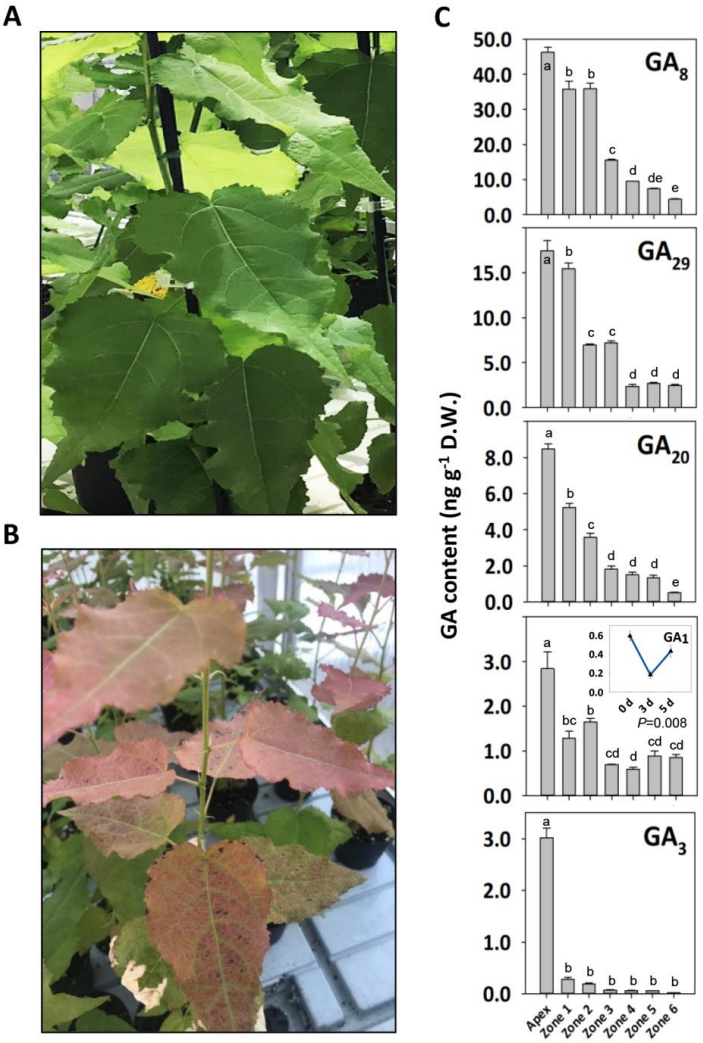


Supplementary Figure 2. Phylogenetic analysis of GID1, gibberellin receptor proteins. The sequence homologues were identified by Protein BLAST search in NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the sequences were retrieved from *Populus trichocarpa* genome (Tuskan *et al.*, 2006; http://www.phytozome.net/) databases. The amino acid sequence alignment were performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). A phylogenetic tree was created using the MEGA6 program (www.megasoftware.net) with the Neighbor–Joining method. Bootstrap support values are based on 1000 replicates. The proteins used

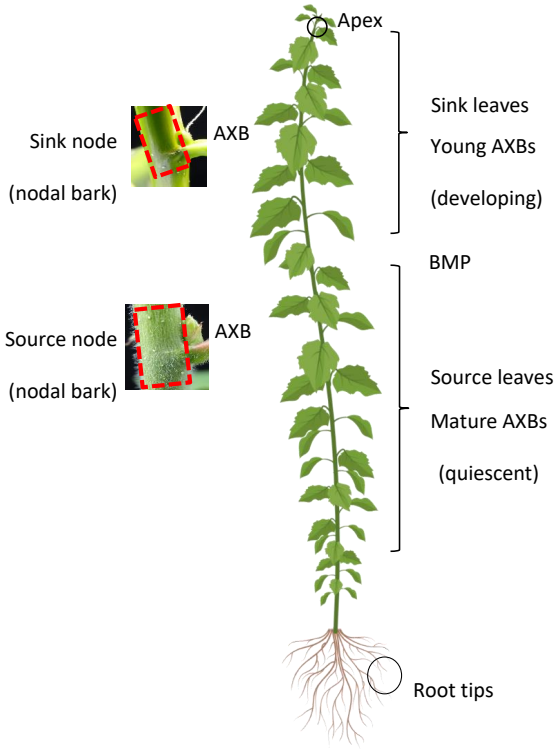
in the phylogenetic analysis were: *Arabidopsis thaliana* AtGID1a (At3g05120), AtGID1b (At3g63010), AtGID1c (At5g27320); *Solanum lycopersicum* SlGID1a (Solyc01g098390), SlGID1b1 (Solyc09g074270), SlGID1b2 (Solyc06g008870); *Oryza sativa* OsGID1 (LOC_Os05g33730); *Hordeum vulgare* HvGID1 (A7MAQ4); *Populus trichocarpa* PtGID1A-1 (Potri.005G040600), PtGID1B-1 (Potri.014G135900), PtGID1A-2 (Potri.013G028700), PtGID1B-2 (Potri.002G213100). PtGID1s (▲).



Supplementary Figure 3. Effect of hormones on AXB outgrowth. AXB outgrowth was studied in single node systems xylem-fed with or without 10µM GR24, GA_3 or GA_4. Σ_{14} values refer to timing of AXB burst. If AXB burst early, for example on day 1, AXB scores 14, if on day 14, score is 1. The values are means of two AXBs of 12-16 plants per treatment. One-way ANOVA (*P*-value). Different letters indicate statistical significance between the treatments (Fischer's LSD *post hoc* analysis; *P*-value at least <0.05).



Supplementary Figure 4. GA pathway switches to 13-hydroxylation under suboptimal environmental conditions. (A) Plants grown in optimal conditions (see analysis in Figure 8). (B) Red color as indicator of light stress. (C) GA analysis of plants shown in (b). Inset in GA_1: Changes in GA levels 0, 3 and 5 days after decapitation in AXBs proximal to the decapitation point (*P*-value shown). Asterisks in insets indicate significance change in GA levels. Different letters in bars indicate statistical differences in GA level between the samples. One-way ANOVA and pairwise *post hoc* analysis by Fischer's LSD test (*P*-value at least <0.05).



Supplementary Figure 5. Sampled materials. Young axillary buds in axils of sink leaves are associated with sink nodes, whereas mature buds in the axils of source leaves are associated with source nodes. Nodal bark of sink and source nodes was peeled off (stippled red box) under a dissection microscope, followed by analysis. BMP, bud maturation point; AXB, axillary bud.

Supplementary Table 1. *P. trichocarpa* genes, identifiers and primer pairs used for qPCR analysis.

Populus trichocarpa					
Protein	**Gene abb.**	**Locus name v2.0**	**Locus name v3.0**	**Forward 5′-3′**	**Reverse 5′-3′**
Gibberellin Biosynthesis genes					
GA3 oxidase1	*GA3ox1*	POPTR_0001s17680	Potri.001G176600	TGGCTCTCCTCTTGAGCATT	AACCATGTCAACCTCCTTG
GA3 oxidase2	*GA3ox2*	POPTR_0003s05610	Potri.003G057400	CCCTATCTCGCTCAATCTTTCC	AGTCAAGGTGCTTTTGGTGTAG
GA20 oxidase2-1	*GA20ox2-1*	POPTR_0002s15260	Potri. 002G151300	CGAAAAACCATGCCTTGAAT	GCCAAAGGATCTCCAGTGAG
GA20 oxidase3	*GA20ox3*	POPTR_0005s20660	Potri.005G184400	TCGGATCTCGTTGTGCTAGA	AGTTCCAATATGGCGAAGGA
GA20 oxidase4	*GA20ox4*	POPTR_0005s20650	Potri. 005G184200	GGCAATAAAGCAGGCTTCTG	TGTGATCATGGGCGAGACTA
GA20 oxidase5	*GA20ox5*	POPTR_0007s04360	Potri. 007G103800	AGCTTGCCCACAGAGTTCAT	GAGCAGTTGCAACCTCATCA
GA20 oxidase6	*GA20ox6*	POPTR_0012s14040	Potri.012G132400	ATTTCGACGCTTTTGTCGTT	GAGATTTTCTTGGCGTTTGG
GA20 oxidase7	*GA20ox7*	POPTR_0014s06960	Potri.014G073700	ATGGCACTCCGTTACTCCTG	CCACTGCTCTATGCAAGCAA
GA20 oxidase8	*GA20ox8*	POPTR_0015s14030	Potri.015G134600	ATCAAAACCATGCCATCCA	TGGTGTCGAAGAACTTGTGC
Gibberellin deactivation genes					
GA2 oxidase1	*GA2ox1*	POPTR_0001s38760	Potri.001G378400	TTCTTCTCATTACCGCTCTCTG	TCTACCCAGCCCACATCAC
GA2 oxidase2	*GA2ox2*	POPTR_0002s19260	Potri.002G191900	TGCCTTCCAGGTTTTAACGA	GGCAAGACCAGCTGTGGAG
GA2 oxidase3	*GA2ox3*	POPTR_0004s06380	Potri.004G065000	GGACCTCCTAACCCTTTTGG	TGGGTTTTCCTGAAAAATGG
GA2 oxidase4	*GA2ox4*	POPTR_0008s10100	Potri.008G101600	AGGTAGGGTTCGGAGAGCAT	GGTAGCGGGATCAGGTGTTA
GA2 oxidase5	*GA2ox5*	POPTR_0010s15950	Potri.010G149700	AATGGCCTATTTTGCTGCAC	TATCTCCAAGTCGCAGAGCA
GA2 oxidase6	*GA2ox6*	POPTR_0011s09770	Potri.011G095600	CAAGCCAGCACTTCAACAGT	ATTCCTCACATGCCTTGACC
GA2 oxidase7	*GA2ox7*	POPTR_0007s04360	Potri.014G117300	TTGCTTGCATGATGGTTTGT	GCCTCACGCTTTCAAATCTC
Gibberellin signaling genes					
GIBBERELLIN INSENSITIVE DWARF1	*GID1A-1*	POPTR_0005s04240	Potri.005G040600	ACCGTGGGACTAGCCTTCTT	ACAACCTCCGAGTTGACAGG
GIBBERELLIN INSENSITIVE DWARF1	*GID1B-1*	POPTR_0014s13170	Potri.014G135900	GATCATGTTGATCGCACCAC	GTGCTCAAGGGCTTTTCAAG
GIBBERELLIN INSENSITIVE DWARF1	*GID1A-2*	POPTR_0013s02980	Potri.013G028700	GGACCGAGATTGGTACTGGA	TAAACCAGCCACCACAACAA
GIBBERELLIN INSENSITIVE DWARF1	*GID1B-2*	POPTR_0002s22840	Potri.002G213100	GGGGAAAAAGCTTGAAGGAC	CAATTGCCAGTCTTGAACGA
Housekeeping gene					
ACTIN	*ACT*	POPTR_0001s31700	Potri.001G309500	CGATGCCGAGGATATTCAAC	ACCAGTGTGTCTTGGTCTACCC

Paper III

De novo transcriptomic analysis of axillary buds in response to decapitation reveals the key hormone pathways in Hybrid aspen

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Abstract

Shoot branching is a key feature of plant architecture regulated by axillary buds in a series of events. For decades, shoot branching has been studied using decapitation-induced axillary bud outgrowth, which is a crucial mechanism where the shoots continue its normal growth and development. Branching involves the interaction between several plant hormones. In this study, we used transcriptome analysis to investigate the genome-wide regulatory hormonal pathways and the genes involved in the regulation of shoot branching upon decapitation in *Populus* (*Hybrid aspen*). Here, we performed *de novo* RNA-Seq transcriptome expression analysis of axillary buds below bud maturation point at 0, 6, 12, 24, 48 and 72 h post-decapitation. In total, we identified 8977 differentially expressed genes (DEGs) between all the time points. Our results revealed several key hormone related genes that are differentially expressed upon decapitation, including the Jasmonic and Salicylate pathways. Gene ontology classification, enrichment analysis and KEGG analysis showed that axillary bud outgrowth is tightly regulated and show higher enrichment 24 h after decapitation. Several genes related to auxin cytokinin, abscisic acid, jasmonic acid, salicylic acid, brassinsteroids were differentially expressed at different time points post decapitation. These data provided insight into the molecular mechanisms of plant responses to decapitation and a starting point to understand the effect of hormones on shoot branching in *Hybrid aspen*.

Key words: *Hybrid aspen*, decapitation, *de novo* transcriptome, shoot branching, phytohormone

Introduction

Shoot branching is a highly plastic adaptive trait that regulates plant architecture from the complex regulation of axillary bud (AXB) outgrowth. During this process, axillary meristems (AXMs), that lie in the axils of the leaves to form the AXBs. The AXBs often undergoes immediate bud outgrowth and become lateral branch or can remain dormant or quiescent after AXB formation (Janssen, Drummond and Snowden, 2014; Considine and Considine, 2016; Rinne *et al.*, 2016). Though the molecular and biochemical mechanisms regulating the shoot branching are believed to be conserved between annuals and perennials, there is an evident distinction in the induction of AXMs, formation of AXBs and development of side shoots. Apical dominance is an inhibitory effect exerted by the shoot tip on the AXB outgrowth, whereas correlative inhibition induces the growth suppression by growing buds/shoots (Cline, 1991; Cline, 1997; Phillips, 1975; Thimann and Skoog, 1934; McSteen and Leyser, 2005). The development of AXB is completed at the bud maturation point (BMP) (Rinne *et al.*, 2016) in perennial *Hybrid aspen*- T89 where there are around 10-12 embryonic leaves (Katyayini, Rinne and van der Schoot, 2019). Decapitation releases the AXBs from dormancy and can grow out (Rinne *et al.*, 2015). The activity of AXB is correlated with various factors including, light intensity, soil nutrients, hormonal regulation, genetic factors, (Rameau *et al.*, 2015). Among which, plant hormones acts as a major determinant in controling the outgrowth of AXBs (Ongaro and Leyser, 2008; Evers *et al.*, 2011). Previous studies have shown the interaction between endogenous hormones, Auxin (AUX), cytokinin (CK), strigolactones (SL) and gibberellins (GA) in relation to branching (Ferguson and Beveridge, 2009; Leyser, 2009; Ni *et al.*, 2015; Rinne *et al.*, 2016; Katyayini, Rinne and van der Schoot, 2019).

Central to this hormonal network is AUX, synthesised in the shoot apex and transported basipetally via polar auxin transport stream (PATS) in the vascular parenchyma, acts to inhibit outgrowth of buds indirectly (Thimann and Skoog, 1933; Morris, 1977; Booker, Chatfield and Leyser, 2003; Blakeslee, Peer and Murphy, 2005). Indole-3-acetic acid (IAA) is the most abundantly existing auxin synthesized in the shoot apex and young leaves (Ljung, Bhalerao and Sandberg, 2001) where removal of shoot apex cuts down the major auxin source to inhibit the AXB outgrowth (Wolters and Jürgens, 2009). The key component controling shoot branching, PIN-FORMED auxin efflux carriers (PIN) acts to control the direction of PATS, especially PIN-FORMED1 (PIN1) (Paponov *et al.*, 2005; Wisniewska *et al.*, 2006). Loss of functional PIN1 protein results in the reduction in transport (Okada *et al.*, 1991).

Unlike auxin, Cytokinins (CKs) directly promotes bud outgrowth through long-distance acropetal transportation from tips of the roots to shoots in the transpiration stream of xylem (Nordström *et al.*, 2004; Chen *et al.*, 1985). Exogenous CK application stimulates the activation of cell-cycle related genes there by increasing the level of Endogenous CKs as they activate (Emery, Longnecker and Atkins, 1998; Schaller, Street and Kieber, 2014; Waldie and Leyser, 2018). However, it has been shown that CKs that promote AXB outgrowth after decapitation was biosynthesized in the nodal stems (Tanaka *et al.*, 2006). Understanding the role of action of CK in shoot branching has been demanding considering the large number of genes involved in each family (Hwang, Sheen and Müller, 2012). *Isopentenyl transferase* (*IPT*) gene encodes for a key enzyme in the synthesis of CKs in the early step of biosynthesis (Kakimoto, 2001; Takei, Sakakibara and Sugiyama, 2001). The *IPT* mutants (*ipt3,5,7*) impaired in CK biosynthesis have lower level of CKs and reduced branching phenotype (Miyawaki *et al.*, 2006; Müller *et al.*, 2015) and, *IPT1* and *IPT2* expression correlates with bud outgrowth suggesting the importance of CK in lateral bud outgrowth which is due to the increased CK accumulation locally (Tanaka *et al.*, 2006; Ferguson and Beveridge, 2009). In fact, CK signal is perceived by AHKs family proteins (Arabidopsis hystidine kinases) (Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Wulfetange *et al.*, 2011). These AHKs initiates a phosphorelay cascade, that targets the primary CK signaling response genes called *ARRs* (*Arabidopsis response regulators*) which are required for CK-mediated bud activation (Müller *et al.*, 2015; Waldie and Leyser, 2018). It was shown that cytokinin oxidase (CKX), the enzyme responsible for controlling the endogenous levels of CKs by irreversibly degrading the the active CK through inactivation (Werner *et al.*, 2001; Schmülling *et al.*, 2003; Werner *et al.*, 2003). Additionally, CK regulation is mediated by an activating enzyme, LONELY GUY (LOG), synthesizes active CKs suggesting the expression of CKs in a specific plant domain indicating its specific expression patterns (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009; Müller and Leyser, 2011).

Abscisic acid (ABA) has been shown to inhibit AXB outgrowth and reduced levels of ABA in AXBs promotes branching (Cline and Oh, 2006; Reddy *et al.*, 2013; Yao and Finlayson, 2015). ABA has been long known to be involved in dormancy (Tucker, 1977). Branching mediated by BRC1-mediated branching is inhibited by the ABA regulation, which acts downstream of ABA and the expression of *BRC1* was found to be not effective with ABA application(González-Grandío *et al.*, 2017; Wang *et al.*, 2019b; Yao and Finlayson, 2015). It was shown that fluridone

(ABA biosynthesis inhibitor) enhanced bud outgrowth in *Rosa hybrida* (Le Bris *et al.*, 1999). In general, the abundance of ABA in AXBs is dependent on a key ABA biosynthetic enzyme Nine-cis-epoxycarotenoid dioxygenase 3 (NCED3) (Urano *et al.*, 2009; Holalu *et al.*, 2020; González-Grandío *et al.*, 2017). The catabolic pathway is mainly established through hydroxylation pathway encoded by the gene *CYP707A* (Kushiro *et al.*, 2004; Pan *et al.*, 2021). ABA-responsive elements (*ABRE*s) play an important role in feedback regulation in ABA signaling (Wang *et al.*, 2019c) but its role in shoot branching still remins unclear.

Jasmonic Acid (JA) plays important roles in several biological processes, including regulation of plant growth and development and plant-resistance related pathways in response to biotic or abiotic stress as well as external damage (mechanical, herbivore, and insect damage) (Ruan *et al.*, 2019). JA biosynthesis has been widely studied in *Arabidopsis* and *L. esculentum* (Ruan *et al.*, 2019). There is not much known about the involvement of JA synthesis genes in shoot branching. In *Arabidopsis*, JA biosynthesis genes like, *lipoxygenase* (*LOX*), *allene oxide synthase* (*AOS*), *allene oxide cyclase* (*AOC*), *OPDA reductase3* (*OPR3*) help in regulating JA production (Wasternack, 2015). One of the important protein involved in JA signaling pathway is JASMONATE ZIM domain (*JAZ*), negatively regulates transduction of jasmonates in regulating plant development (Song *et al.*, 2022).

Brassinosteroids (BRs) was also identified as a positive regulator of shoot branching (Xia *et al.*, 2021). BR has been known to induce similar physiological responses as GA and IAA in plants (Zheng *et al.*, 2019). BR binding activates the cell surface receptor kinase called BRASSINOSTEROID-INSENSITIVE1 (BRI1) and leads to the activation of effector TFs BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESOR (BES1) playing a critical roles in BR signaling (Yin *et al.*, 2005; Wang *et al.*, 2012). The role of BR in shoot branching has been studied in tomato, where *BZR1* regulates BR which suppresses *BRC1* transcriptionally to promote outgrowth of AXBs (Xia *et al.*, 2021). However, the mechanism of BR synthesis and signaling in controlling shoot branching network needs better understanding.

Salicylic acid (SA) is traditionally known to be involved in plant immunity which functions in response to increase in SA upon pathogen attack (Tan *et al.*, 2020). The role of SA beyond plant immunity in regulating plant growth and development is very little studie. In several species, the level of SA in shoot is higher than in roots (Chen *et al.*, 1997; Rakhmankulova *et al.*, 2010). It was

shown that the level of SA in shoots increased upon pathogen attack in *Arabidopsis* (Bagautdinova *et al.*, 2022; Zhang *et al.*, 2010).

SL has been known to be involved in shoot branching apart from other biological processes. (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Recently we identified all the SL and GA pathway genes in *Populus* involved in shoot branching (Katyayini, Rinne and van der Schoot, 2019; Katyayini *et al.*, 2020). Our previous studies, for the first time, investigated the role of SL pathway genes in perennial shoot branching (Katyayini, Rinne and van der Schoot, 2019). We proved that the nodes rather than roots are the main hub for biosynthesis of SLs. AXBs are the center for SL perception which acts to inhibit the AXB outgrowth in intact plants. On the other hand, we also provided evidence that the dual role of GA in perennial shoot branching in our previous work (Katyayini *et al.*, 2020).

Until recently, most of the shoot branching studies have concentrated mainly on herbaceous species and little is known about the mechanism of branching involving other hormones like AUX, CK, ABA, JA, BA, SA in perennial woody plants apart from the recently reported SL and GA (Katyayini *et al.*, 2020; Katyayini, Rinne and van der Schoot, 2019). Advancement in transcriptome sequencing (RNA-Seq) paved the way for understanding complex plant responses (Martin *et al.*, 2013). High-throughput transcriptome sequencing is a powerful tool for identifying differentially expressed genes and pathways involved in the regulation of different biological process. Until now most of the studies on hormonal regulation pathway was based on array-based assays which were commonly used to study gene expression in model species, such as *Arabidopsis* and tomato (Cai and Lashbrook, 2008; Meir *et al.*, 2010). Only fewer transcriptome studies have detected novel genes and pathways involved in hormonal regulation in woody species like *Populus* (Wang *et al.*, 2019a). In order to advance in the current understanding of shoot branching in *Hybrid aspen*, we selected six different time points (0 h, 6 h, 12 h, 24 h, 48 h, and 72 h) after decapitation and sampled the buds below BMP at each time point. We performed transcriptomic analysis and subsequently compared the global expression profiles, that enabled the identification and characterization of the expression pattern of potential genes and molecular pathways involved in the hormonal regulation of shoot branching apart from earlier studied pathways like SL and GA (Katyayini, Rinne and van der Schoot, 2019; Katyayini *et al.*, 2020) such as, AUX, CK, ABA, SA, JA and BR. In addition, this study aimed that global transcriptome studies could be performed even in non-model species, lacking a genome sequence. Further detailed analyses of the genes

provided an insight on the regulatory network of hormones that control the outgrowth of AXBs in woody plants like *Populus*.

Materials and Methods

Plant material and experimental design

Hybrid aspen (*Populus tremula* x *Populus tremuloides*) clone T89 was micro-propagated *in vitro* for 5 weeks at 20°C, planted in a mixture of soil/peat and perlite (4:1 [v/v]) fertilized with 4 g L^{-1} Osmocote, and grown in a greenhouse under long days (18 h light) at 20 ºC, relative humidity 60%, and light intensity of 200-250 µmol$m^{-2}s^{-1}$ (Osram). The plants were watered twice a day and transplanted to 13 cm Ø pots when they were ~60 cm high. After another 6 weeks leaf production and elongation rates were stable, and plants were about 100-110 cm high. In these plants the BMP, determined as described (Rinne et al. 2015; Katyayini et al. 2019), was located at about 40 cm below the apex. Mature para-dormant AXBs were activated by removal of apical dominance through decapitation of the shoot at nodal position 12, immediately below the BMP.

RNA sampling

To investigate activation and outgrowth, six AXBs below the BMP for each of two plants (replicates) were harvested at control time point 0 h (CT0), Decapitation at 6 h (DecT6), 12 h (DecT12), 24 h (DecT24), 48 h (DecT48) and 72 h (DecT72). AXBs were immediately snap-frozen in liquid N_2 and stored at -80 ºC for later RNA isolation and transcriptome sequence analysis. Total RNA was extracted from 0.2-0.3 g of frozen tissue, ground using mortar and pestle with 500 µL extraction buffer (Qiagen RLT buffer, containing 1% PVP-40) followed by the addition of a 0.4 volume of KoAC (pH 6.5). Samples were homogenized for at least 30 s per step, transferred to 2-mL tubes, incubated on ice for 15 min, and centrifuged at 12,000 rpm at 4 ºC for 15 min. Supernatants were transferred to new 1.5-mL tubes and a 0.5 volume of 100% EtOH was added each time. Each mixture was pipetted up and down five times to obtain homogenized mixtures. The mixtures were transferred to RNeasy spin columns and processed in accordance with the instructions of the Qiagen Plant RNA isolation kit. TURBO™ DNase kit (Invitrogen) was used to remove genomic DNA contaminations from the mixtures, which were further cleaned using the total RNA purification system 'Purelink RNA mini kit' (Invitrogen). RNA was quantified with NanoDrop 1000, and RNA quality was assessed using the Agilent 2100 Bioanalyzer system.

cDNA library preparation and Illumina sequencing

RNA samples with RIN (RNA integrity Number) values above six were qualified to construct complementary DNA (cDNA) libraries. mRNA was isolated from magnetic beads containing oligo-dT and fragmented into short stretches. The cDNA strand was synthesized using random hexamer primers and short fragments were ligated with adapters and amplified by PCR. The libraries were assessed using the Agilent2100 Bioanalyzer and quantified with the ABI StepOnePlus Real-Time PCR system. The samples were sequenced at BGI Tech, Hong Kong, using the Illumina sequencing platform (Illumina HiSeq X Ten), generating paired end reads of 150bp.

***De novo* assembly of the transcriptome**

The *de novo* assembly was constructed using a bioinformatic pipeline (Supplementary Fig. S1) as described previously (Kovi *et al.*, 2016; Kovi *et al.*, 2017). Firstly, sequencing data quality was primarily assessed using program FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter sequences and low-quality reads were removed using the sickle program (https://github.com/najoshi/sickle/blob/master/README.md). The remaining high-quality clean reads were used for assembly. Briefly, filtered clean reads of control and decapitated samples, collected at different stages were used to construct a *de novo* assembly using the Trinity program (Trinity-v2.6.6). To analyze the completeness of the *de novo* assembly, we used the Core Eukaryotic Genes Mapping Approach (CEGMA) program version 2.4 (Parra, Bradnam and Korf, 2007). This CEGMA program "blasts" 248 widely conserved core eukaryotic genes (CEGs) against the *de novo* assembled transcriptome, aimed to assess presence and coverage of orthologs in the *h. aspen* transcriptome. After assessing the quality of *de novo* assembly, it was used as a reference to map individual reads from AXBs isolated from each replicate of the control and decapitated plants at time points 6, 12, 24, 48, and 72 h, using the Bowtie program. Transcript abundance was measured for each replicate and time point combination, expressed as the expected number of fragments per kilobase of transcript sequence per million mapped reads (FPKM) (Trapnell *et al.*, 2010) using RSEM version 1.1.11 (Li and Dewey, 2011).

Detection of differentially expressed genes (DEGs), sequence annotation and gene ontology (GO) enrichment analyses

Pairwise comparisons were carried out between all the time point combinations and DEGs were identified using the edgeR package (Robinson, McCarthy and Smyth, 2010) (https://www.r-project.org/). False discovery rate (FDR) was applied to determine the errors in the *p*-value threshold in multiple testing (Benjamini and Yekutieli, 2001). An FDR of ≤ 0.005 was used to determine the significant DEGs between the time points (Supplementary Fig. S2). As the statistical analyses have the criterium that genes have to be different between all time points, genes with diurnal rhythm (i.e. genes that are differentially expressed at time points 6 h and 12 h, but not at time points 24 h, 48 h and 72 h) are filtered out. In order to identify gene names and assign a gene ontology (GO) to the DEGs, the Blast2GO program (Conesa and Gotz, 2008) was used. Briefly, BLASTx was performed to search against the Viridiplantae database (extracted from NCBI) with an E-value threshold of 10e-06, followed by annotation with a cut-off value of 55 and GO weight Hsp-hit value of 20. To detect the over and under-represented gene ontologies, enrichment analyses was performed, where the significantly enriched GO terms were selected with a *p*-value of 0.01. The WEGO 2.0 tool (Ye *et al.*, 2018) was used to analyze the GO annotations of DEGs by comparing all the post-decapitation time points. In addition, the cluster of orthologous groups (COG) classification of differentially expressed genes (DEGs) was performed by eggNOG (v5.0) annotation (Huerta-Cepas *et al.*, 2019). Further KEGG pathway analyses were performed in the Blast2GO program (Conesa and Gotz, 2008). The tool REVIGO was used to primarily visualize and summarize the list of all the non-redundant GO terms in order to accurately divide the subset of GOs belonging to a subset and plotting according to semantic similarity (Supek et al., 2011).

Results

Transcriptome sequencing and *de novo* assembly

To obtain insights into decapitation-induced genome-wide gene regulation in mature AXBs, we performed Illumina RNA sequencing across 6 time points with two replicates. A total of 459.7 million high quality reads were generated from the cDNA libraries of below-BMP AXBs, isolated at time points 0, 6, 12, 24, 48, and 72 h post-decapitation (Table 1). The *de novo* transcriptome assembly generated by the trinity program contained 399,380 contigs, with an N50 of 1,871 bp (Table 1). The longest contig assembled size was 15,046 bp. The quality of *de novo* assembly was

tested using the CEGMA program to assess the coverage of Core Eukaryotic Genes (CEGs) in the transcriptome. The percentage of complete and partially complete CEGs was 97.18 % and 100 %, respectively (Table 2). The average number of orthologs per CEG in the assembly was 4.47, and the percentage of CEG that contained more than one ortholog was 98.34 (Table 2).

Quantification of differentially expressed genes

AXBs below the BMP of intact plants isolated at time 0 h acts as a control (CT0), whereas AXBs below the BMP isolated from decapitated plants at times 6, 12, 24, 48, and 72 h are represented as DecT6, DecT12, DecT24, DecT48, and DecT72, respectively (Fig. 1). For each sample, an average of 19 million reads were mapped to the *de novo* assembly, with an alignment rate around 85% (Table S1. A total of 8977 DEGs were detected by pairwise comparisons with a FDR < 0.005 (Fig. 2). Of all the DEGs expressed, 171 were up- and 128 down in CT0 vs DecT6, 451 up- and 341 down in CT0 vs DecT12, 225 up- and 256 down in CT0 vs DecT24, 519 up- and 713 down in CT0 vs DecT48, and 422 upregulated and 502 downregulated in CT0 vs DecT72 (Fig. 2A, B). The transcripts identified between CT0 vs DecT6 and CT0 vs DecT12 might have acircadian expression pattern. In order to mitigate this, we compared transcripts detected between CT0 vs DecT6 and CT0 vs DecT12 to CT0 vs DecT24, CT0 vs DecT48 and CT0 vs DecT72 transcripts and the common ones were included for further gene expression analysis. There were five DEGs and three DEGs common to all five comparisons in upregulated and downregulated genes, respectively. The maximum number of upregulated (713) and downregulated (351) DEGs were detected between CT0 and DecT48. To further understand the global gene expression profiles of the DEGs for each comparison, a heatmap was generated using the edgeR program (Fig. 3). The clustering showed that the genes from DecT48 and DecT72 clustered into a single branch and consists of more common upregulated genes, while the genes from CT0, DecT24, DecT6, and DecT12 were clustered together (Fig. 3). These results demonstrated that highest number of up- and down-regulated genes were detected during the ES and AXB elongation phase.

Functional annotation and gene ontology (GO) of DEGs

The DEGs, annotated with gene ontology (GO), were generated using WEGO software (Web Gene Ontology Annotation Plot). They were functionally classified into three categories: Cellular

Component, Molecular Function, and Biological Process, depicting the divergent functional classification of GO terms (Fig. 4). In the Cellular Component category, cell, cell part, organelle and membrane were the most highly represented groups, while binding protein binding and catalytic activity were predominated in the category Molecular Functions. In the Biological Process category, metabolic process, biosynthetic process, and cellular process were highly represented indicating the involvement of extensive metabolic activities during the bud activation. The DEGs involved in transcription regulator activity, catabolic process, cell cycle, cellular homeostasis, signaling and signal transduction were fairly represented.

To obtain a functional annotation of genes involved in bud activation and outgrowth, under- and over-represented GO terms were determined using Fischer's exact test in BLAST2GO program of all the DEGs. Gene ontologies were visualized and reduced using the REVIGO program (Supek *et al.*, 2011). During the first 6 h post-decapitation (CT0-DecT6) seven GO terms were enriched. Five of them were overrepresented: ER, organelle envelope, envelope, nuclear envelope, and endomembrane systems (Suppl. Fig. 3A). From six to 12 h post-decapitation (DecT6-DecT12) eight out of ten GO terms were overrepresented, namely cellular anatomical entity, membrane, DNA binding, biological regulation, regulation of biological process, ribosome, cytosol, and oxygen binding (Suppl. Fig. 3B). From 12 to 24 h post-decapitation (DecT12-DecT24), when elongation of the enclosed ES commenced (Fig. 1), enriched GO terms were clustered as membrane, localization, establishment of localization, transport, DNA binding, transporter activity and plastid (Suppl. Fig. 3C). From 24 to 48 h post-decapitation (DecT24-DecT48), when the AXBs started elongation (Fig. 1), enriched GO terms were grouped as catalytic activity, metabolic process, enzyme regulator activity, molecular function regulator, secondary metabolic process, peroxisome and microbody (Suppl. Fig. 3D). Finally, from 48 to 72 h post-decapitation (DecT48-DecT72), when AXBs were visibly elongated and the ES was considerably lengthened (Fig. 1), four out of six enriched GO terms were overrepresented i.e., reproductive process, multi organism process, pollination, and cell death (Suppl. Fig. 3E).

COG and KEGG classification of differentially expressed genes (DEGs)

To explore the potential function of the DEGs during AXB activation, we performed 'Clusters of Orthologous Groups of proteins (COG)' and 'Kyoto encyclopedia of genes and genomes (KEGG)' analysis. The DEGs were functionally assigned to COG categories using EggNOG5.0 software. A

total of 8826 DEGs were assigned to 25 functional categories (Fig. 5). The largest category of DEGs belonged to the 'function unknown' (2319 genes). 'Signal transduction mechanisms' was represented by 859 genes, transcription by 795 genes, post-translational modification, protein turnover, and chaperons by 671 genes, and carbohydrate metabolism (608 genes) (Fig. 5).

Further, we investigated the DEGs involved in AXB activation by using KAAS (KEGG Automatic Annotation Server) BLAST against the *Populus* database (Moriya *et al.*, 2007). A total of 3497 DEGs were classified into 363 pathways (Fig. 6). The KEGG pathways indicated major involvement of five categories: Category 1: Metabolic pathways (ko01100, 381 genes), Biosynthesis of secondary metabolites (ko00999, 200 genes). Category 2: cellular processes: Cell cycle (ko04110, 33 genes). Category 3: genetic information processing: RNA transport (ko03013, 46 genes), protein processing in endoplasmic reticulum (ko04141, 40 genes), ubiquitin mediated proteolysis (ko04120, 37 genes). Category 4: environmental information processing: plant hormone and signal transduction (ko04075, 26 genes). Category 5: organismal systems: circadian rhythm- plant (ko04712, 16 genes) and plant-pathogen interaction (ko04626, 16 genes).

DEGs related to hormone signaling pathways

Following decapitation, we investigated the time-dependent responses of genes that are central to the hormone signaling pathways of SL, GA, IAA, CK, ABA, JA, BR, and SA (Table 3).

In the SL-pathway, at 6 h post-decapitation, the expression of *MAX2a*, *D14a* and *MAX2b* was upregulated, but subsequently downregulated toward the 72-h time point (Fig. 7A). Further we noticed the strong upregulation of *MAX3* and *D53-like1* at 48 h and *MAX1.2* and *LBO* at 72 h and downregulation after 72 h of a set of genes that are associated with branch-suppression, including *D27a*, *D27c*, *D14a*, *D14b*, *MAX1.1*, *MAX2a*, *MAX2b*, and *BRC1* (Fig. 7A).

In the GA-pathway, the GA catabolic genes *GA2ox3*, *GA2ox2*, *GA2ox7*, *GA2ox4*, *GA2ox5*, *GA2ox6*, *GA2ox1*, and the signaling genes *GID1A-1*, *GID1B-1*, *GID1A-2*, and *GID1B-2 were* upregulation between at 6 h and then substantially downregulated toward the 72-h time point (Fig. 7B). In contrast, the GA biosynthesis genes showed an opposite pattern with a strong upregulation of *GA20ox5*, *GA20ox6 GA20ox8* at 48 h and *GA20ox2-1* and *GA20ox7* at 72 h., although they were also somewhat upregulated at 6 h. Significantly, the major GA-biosynthesis genes *GA3ox1* and *GA3ox2* were strongly upregulated at 72 h.

The DEGs associated with the CK biosynthetic and signaling pathway genes included *Populus histidine kinase* (*HK*), *isopentyl transferase* (*IPT*), *cytokinin oxidase* (*CKX*), *Lonely guy* (*LOG*), and the two-component response regulator *ARR-A* family (*ARR-A*) (Fig. 8). The transcript level of this precursor gene family was elevated in paradormant AXBs (CT0) and following decapitation at 6 h. The exception was *IPT3*, which was only upregulated at 72 h (Fig. 8A). Following the expression of precursor genes, the CK receptor genes *CKL1c*/*CKL01U* and *HK3a* were upregulated at 6-12 h, whereas *HK2* and *CRE1a*/ *CRE1b* followed from 24-72 h (Fig. 8B). The members of the CKX family, *PtCKX5a*, *PtCKX3a*, *PtCKX1b*, *PtCKX5b* tended to be differentially upregulated throughout the post-decapitation period, with high expression of *PtCKX3a* and *PtCKX1b* at 48 h (Fig 8C). *LOG* was identified as one of the important enzyme required for CK activation (Kurakawa et al., 2007). The LOG family genes, *LOG02U*, *LOG1* showed strong upregulation at 72 h whereas, *LOG7b* and *LOG01U* was highly upregulated at 48 h post-decapitation (Fig 8D). The expression level of *LOG8b*/*8c* was highly elevated at 6 h followed by *LOG06U* at 12 h after decapitation and was downregulated eventually. Most of the CK signaling genes from the ARR family showed a strong up regulation at 72 h except *PtRR3* at 6 h (Fig 8E).

The expression level of DEGs in the JA biosynthesis and signaling pathway, including *lipoxygenase* (*LOX*), *allene oxide cyclase* (*AOC*), *12-oxophytodienoate reductases* (*OPR*), *allene oxide synthases* (*AOS*), *CORONATINE INSENSITIVE1* (*COL1*) were analyzed (Fig 9A). The expression patterns of the JA biosynthesis gene *AOS* showed a strong upregulation 48 h. *OPR1* and *OPR2* were upregulated at 24 h, except for *OPR3* at 48 h (Fig 9A). At 48 h *LOX2* and *LOX3* were strongly upregulated like most of the genes in the biosynthesis pathway, whereas *LOX1* was downregulated at 12 h and onwards. Most of the biosynthesis genes were upregulated from 48 h. Expression of the signaling gene *COL1* was strongly downregulated at 72 h.

In order to identify the SA pathway genes that are specifically activated upon decapitation, we compared the gene expression profiles at different timepoints (Fig 9B). The *Isochorismate synthase* (*ICS*) gene, *ICS1/2*, is important in SA biosynthesis that showed a strong early upregulation at 12 h. While the *phenylalanine ammonia-lyase* (*PAL*) gene *PAL3/PAL4*, also involved in SA biosynthesis, was somewhat upregulated at 24 h.

To study the role of BR pathway genes in shoot branching, we analyzed the response of the genes involved in BR pathway after decapitation (Fig 9C). BR signals are perceived by the membrane localized receptor kinase BRASSINOSTEROID-INSENSITIVE1 (BRI1) which upon decapitation was hardly affected, and strongly downregulated at 72 h. Decapitation decreased expression of the BR biosynthesis gene *DET2*, but it led to a modest increase in the level of BR

biosynthesis genes *DET2*, *DWARF1* (*DWF1*) and *DWARF4* (*DWF4*) at 6 h and 12 h respectively. *DWARF3* (*DWF3*) was strongly upregulated at 12 h. Decapitation also resulted in the suppression of the expression of *BRASSINOSTEROID INSENSITIVE 2* (*BIN2*), a negative regulator of the signaling gene at 48 h. *BRASSINAZOLE-RESISTANT1* (*BZR1*) and *BRI1-EMS SUPPRESSOR1* (*BES1*), critical downstream components of BR signaling were upregulated from 12 h to 48 h and subsequently downregulated towards 72 h.

As the ABA pathway is often implicated in development and stress-resistance, we analyzed the expression pattern of the crucial ABA biosynthesis gene *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*). The *NCED* ortholog *NCED4* was downregulated upon decapitation, but the orthologs *NCED1/3/5* and NCED2 were strongly upregulated at 6 h post-decapitation and downregulated toward the 72 h time-point (Fig. 10A).

CYP707A counteracts ABA biosynthesis by encoding important ABA catabolism enzymes. *CYP707A2/CYP707A3* was highly upregulated at 6 h and downregulated after 12 h. The catabolism gene *CYP707A7* was strongly upregulated 24 h, whereas *CYP707A5* expression was modestly elevated (Fig. 10B).

ABA-responsive element (*ABRE*) function in the negative feedback regulation of the ABA signaling pathway. Most of the *ABRE*s were downregulated upon decapitation, but *ABRE2* and *ABRE5* were upregulated at 24 h (Fig 10C).

Enrichment analysis of DEGs in mature AXBs after decapitation

GO enrichment analysis was conducted using Fischer's exact test (p-value of 0.01) in the Blast2GO program and visualized in REVIGO. The GO terms under- or over-represented in the AXBs was extracted from the 8977 DEGs across the time points. Two GO terms were enriched in each comparison, CT0 vs DecT6 and DecT6 vs DecT12, including 'biogenesis', 'cellular component organization' and 'biological regulation', 'regulation of biological process', respectively (Fig 11A,11B). The semantic similarity analysis highlighted the increase in number of GO terms enriched between DecT12 vs DecT24 including, 'lipid cellular process', 'transport', 'localization', cellular metabolism', 'translation', 'cellular biosynthesis' (Fig 11C). The number of enriched GO terms decreased from 24 to 11 after 24 h of decapitation. Surprisingly, The GO terms 'metabolism' and 'transport' was also enriched between the time point DecT24 vs DecT48

together with the 'biosynthesis', 'response to stress' which was observed only after 24 h post decapitation (Fig 11D). After 48 h post decapitation, only 5 GO terms were enriched such as, 'cell death', 'multi-organism process', 'carbohydrate metabolism' (Fig 11E). It is quite evident from our results that GO terms show higher enrichment between DecT12 and DecT24 in between AXB activation (CT0-DecT6) and the start of ES elongation at DecT24 (Fig 11C), coinciding with the preparatory phase (Fig 1).

Discussion

The regulation of shoot branching is considered to be a key process in fitness and plant growth which involves a complex regulatory network (Wang *et al.*, 2019b). Shoot branching is generally known to be controlled by cross talk between various plant hormones. During the past decades, much of the emphasis have been given to AUX, CK and SL, as a key signal involved in AXB outgrowth (Domagalska and Leyser, 2011). The fate of the AXBs (outgrowth/dormancy) is decided based on the synergistic or antagonistic coupling between these hormones (Tan *et al.*, 2019). Here we study the role of AUX, CK, JA, BR, SA, ABA, essential for the control of shoot branching in *Hybrid aspen*, apart from the previously studied SL and GA (Katyayini, Rinne and van der Schoot, 2019; Katyayini *et al.*, 2020). In our current study, we demonstrate a comprehensive transcriptome-based analysis using RNA-seq method to identify the biological processes and hormonal pathways associated with shoot branching in perennial woody species, *Hybrid aspen*.

Here we identified 8977 DEGs in a pairwise comparisons between the time points, 0 h, 6 h, 12 h, 24 h, 48 h and 72 h. Among them a maximum number of 590 genes were up-regulated between CT0 and DecT48 and 713 genes were down-regulated at the same time point (Fig 2). In the recent studies, several up- and down-regulated genes were identified in active buds and xylem tissues during wood formation in *Populus* species (Chen, Chen and Zhang, 2015; Wang *et al.*, 2019a). However, these studies focused only on shoot branching in different species of *Populus* and wood formation but not on the AXB activation by decapitation.

In this study, KEGG functional classification identified many genes associated with ubiquitin mediated proteolysis which are known to be involved in hormone perception, degradation of hormone specific TFs to regulate hormone biosynthesis (Santner and Estelle, 2010). Additionally,

we also identified plant hormone signal transduction pathway genes which plays a key role in the process which is consistent with the studies from (Wang *et al.*, 2019a) in *Populus*.

Further GO enrichment analysis showed more enriched GO ontologies between 12 h to 24 h and 24 h to 48 h post decapitation which is in line with the elongation of ES stem in the AXB development (Katyayini, Rinne and van der Schoot, 2019).

To further understand the role of shoot branching in *Populus*, we analyzed the DEGs related to AUX, CK, SL, GA, JA, BR, SA and ABA. SL is one of the key regulator in shoot branching (Leyser, 2009). Our previous studies demonstrated that the expression levels of *MAX4*, *D27a*, *MAX1.1* and *BRC1* were highest in the below-BMP AXBs of intact plants (CT0) (Katyayini, Rinne and van der Schoot, 2019).The genes related to SL pathway were found to be differentially expressed in our data set, which signifies its involvement in shoot branching in *Populus*. On the other hand, we also mapped the expression levels of GA-pathway genes involved in biosynthesis, deactivation and signaling in our previous study (Katyayini *et al.*, 2020).

Cytokinin (CK) is the primary hormone that is a positive regulator of axillary bud outgrowth (Ni *et al.*, 2017). Many gene families were found to be involved in CK activity (Fig 8). The first step in CK biosynthesis is catalyzed by *IPT*, which is responsible for the biosynthesis of the precursors of active CKs (Zürcher and Müller, 2016). In this study, the expression of most of the *IPT*s (*IPT2,6,5,7,9*) downregulated after 48 h of decapitation (Fig 8A). While *IPT3* showed increased expression level upon decapitation which is consistent from the studies in *Arabidopsis* (Müller *et al.*, 2015)The reduced CK level was observed in loss-of-functional mutants of *IPT3* and *IPT5* response of *IPT3* is in line with the idea that auxin-mediated bud inhibition involves a reduction in the CK supply (Müller *et al.*, 2015). These results are consistent with the role of CK in positively regulating shoot branching. The expression level of *PtHK*s is similar to *IPT*s in terms that most of the genes in the family showed downregulation at 72 h (Fig 8B). The CK signaling receptors, *ARR*s acts as a negative regulator of cytokinin signaling also play an important role in shoot branching (Müller et al., 2015; Waldie and Leyser, 2018). The CK synthesis mediated by IPTs involved in activation of buds does not require ARRs in the activation process (Muller et al., 2015). In this study, most of the CK signaling genes *ARR*s were upregulated at 72 h which shows that the AXBs are CK resistant (Fig 8E). One of the important gene *ARR1* in *Arabidopsis*, a positive regulator in CK signaling (Sakai *et al.*, 2001) showed upregulation at 72 h which is a key regulator of CK responsive genes (Fig 8E). ABA is known to be an essential hormone in dormancy regulation, but its mechanism in bud

dormancy is not well characterized yet (Pan *et al.*, 2021). ABA acts as a negative regulator in bud activity (Yao and Finlayson, 2015). Accumulation of ABA in AXBs and related signaling pathways play significant role in branch inhibition (Mader, Emery and Turnbull, 2003; Holalu and Finlayson, 2017). It was shown that *NCED3*, one of the ABA biosynthesis genes is necessary for normal ABA accumulation in AXBs (González-Grandío *et al.*, 2017). This is in line with the evidence that *PtNCED3*, an important biosynthesis gene showed strong upregulation after 6 h post-decapitation (Fig 10A). *CYP707A* and *ABRE* are the genes that mediate ABA biosynthesis, catabolism and signaling, respectively. *ABRE* is also a key link in ABA signaling. In our results, we found that the expression of most of the *ABRE* genes showed downregulation at 72 h (Fig 10C), indicating that ABA signaling genes may play a role in suppressing AXB outgrowth.

BR plays a critical role in regulating plant growth and development (Singh and Savaldi-Goldstein, 2015). In this study, the BR signaling genes, *BRI1*, *BES1* and *BZR1* were upregulated between 12-48 h after decapitation and down regulated at 72 h (Fig 9B) which is similar to the studies in apical dominance in tomato. (Xia *et al.*, 2021).

Conclusion

To our knowledge, this is the first comprehensive transcriptome study by generating *de novo* assembly to identify the genes and pathways of the hormonal regulation that are differentially expressed in AXBs upon decapitation in *Populus*. The molecular response to decapitation in *Populus* is characterized by a significant increase in the expression of stress-related genes and a decrease in the expression of growth-related genes. This response is likely a result of the sudden removal of the shoot apical meristem, which leads to a disruption in the normal hormonal signaling pathways involved in plant growth and development. Thus, this study elucidates the regulatory mechanism of decapitation on axillary bud growth in *Populus* and further research is needed to explore potential applications for manipulating plant growth and stress tolerance.

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Figures

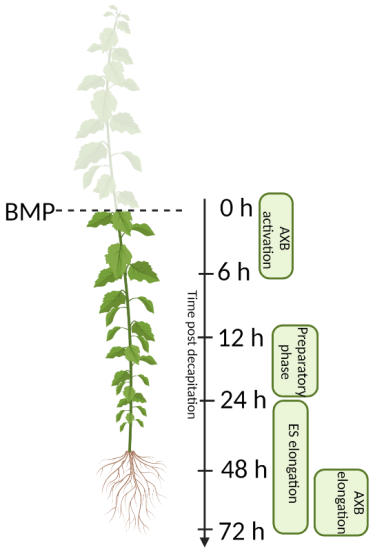


Figure 1. Harvesting timeline of mature axillary buds (AXBs) of proleptic h. aspen. Shoots were severed below the bud maturation point (BMP), and three consecutive AXBs immediately below it were analyzed at 0, 6, 12, 24, 48, and 72 h post-decapitation. AXB activation triggers the elongation of the enclosed embryonic shoot at 24 h, and AXB elongation at 48 h. Both continue beyond the 72-h time point, prior to burst. (Katyayini et al., 2019).

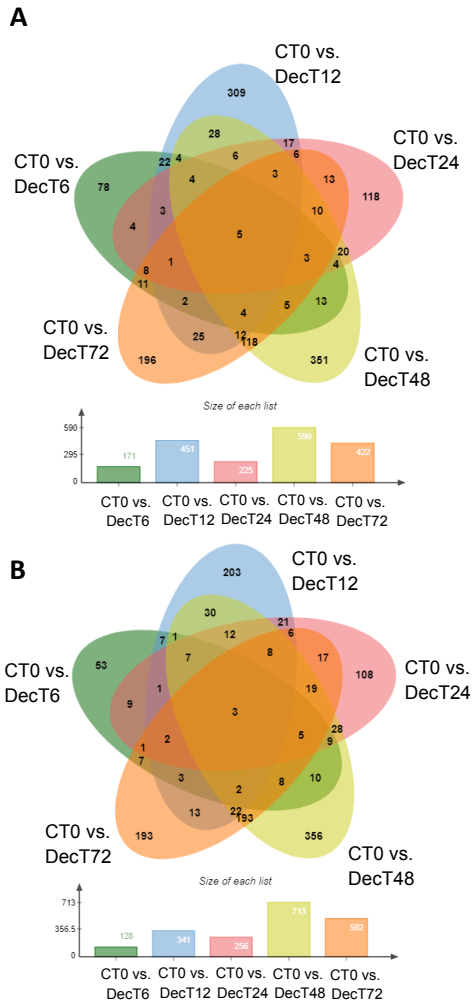


Figure 2. Transcriptional regulation of differentially expressed genes (DEGs) in AXBs at subsequent post-decapitation time points relative to the control time point, identified by de novo assembly. (A) Venn diagram representing the proportion of up- regulated genes per time interval (FDR ≤ 0.005). (B) Venn diagram representing the proportion of down-regulated genes per time interval (FDR ≤ 0.005).

CT0 (0 h, control, intact plant). DecT (post decapitation time point): DecT6 (6 h), DecT12 (12 h), DecT24 (24 h), DecT48 (48 h), DecT72 (72 h).

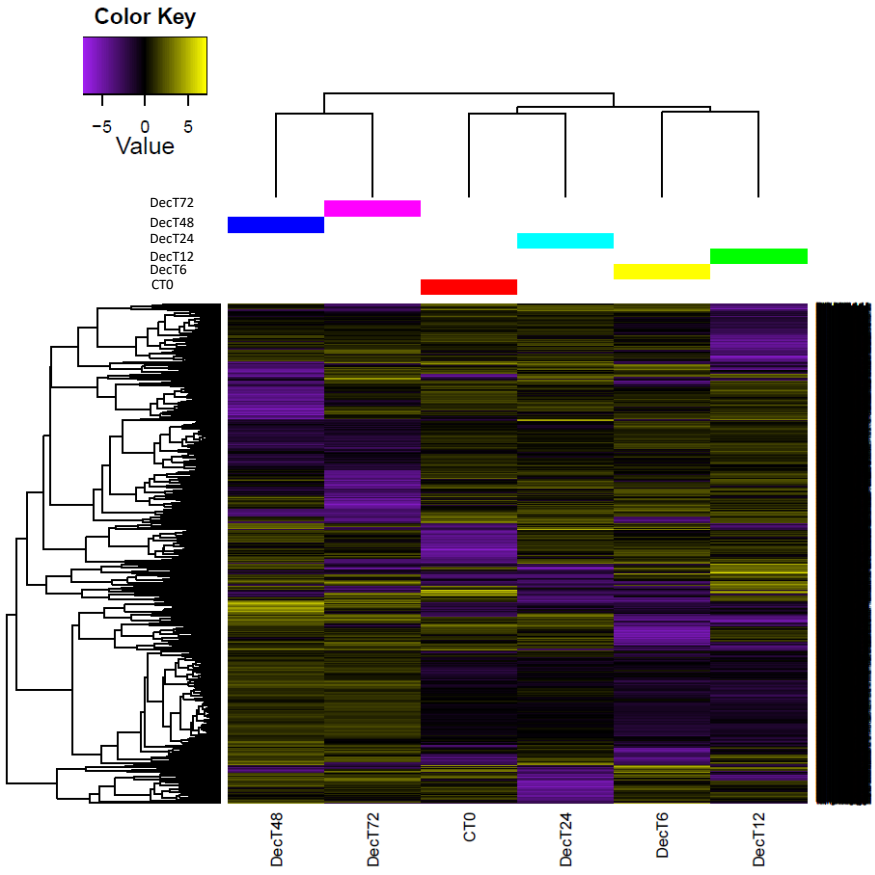


Figure 3. Heat map of differentially expressed genes (DEGs) in triple AXBs below the BMP in intact and decapitated plants at indicated time points identified by *de novo* assembly. Colored vertical bars indicate gene clusters, with yellow and purple indicating high and low expression levels, respectively. X-axis shows post-decapitation time points, with 'Dec' referring to decapitation and 'C' indicating control (non-decapitated).

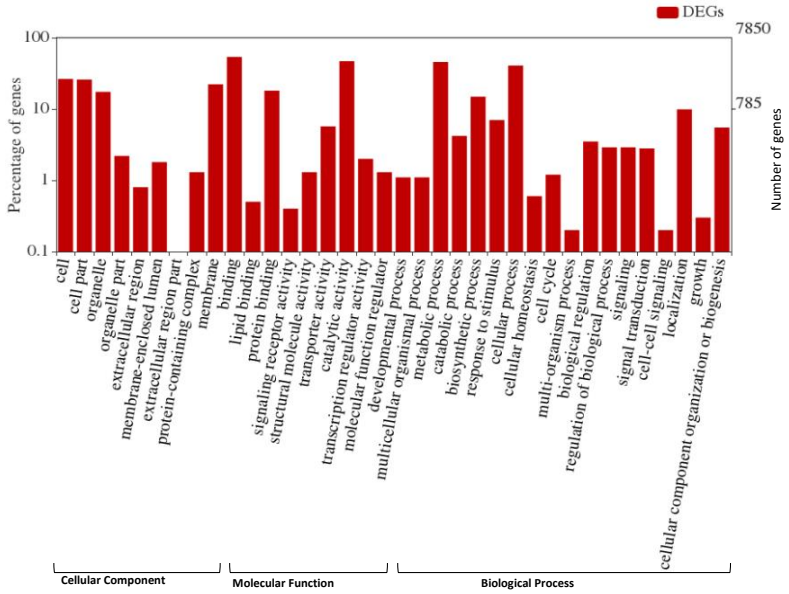
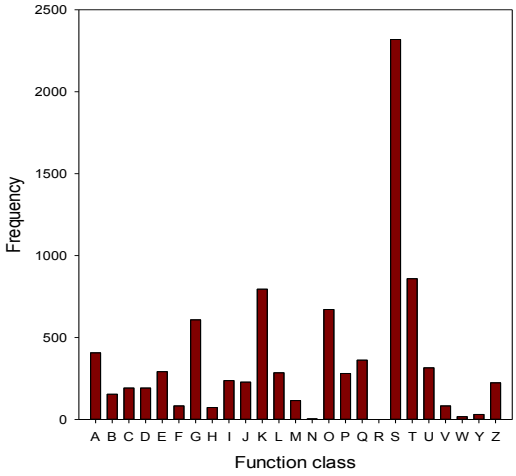


Figure 4. Gene Ontology classification of differentially expressed genes (DEGs) in AXBs post decapitation. The graph is generated by the WEGO tool (http://wego.genomics.org.cn) using the latest available GO. Three main categories are indicated: Cellular Component, Molecular Function and Biological Process. The left Y-axis represents the percentage of DEGs in each specific category for each of the main categories (log(10) scale). The right Y-axis indicates the number of DEGs per category (P<0.05)



A: RNA processing and modification
B: Chromatin structure and dynamics
C: Energy production and conversion
D: Cell cycle control, cell division, chromosome partitioning
E: Amino acid transport and metabolism
F: Nucleotide transport and metabolism
G: Carbohydrate transport and metabolism
H: Coenzyme transport and metabolism
I: Lipid transport and metabolism
J: Translation, ribosomal structure and biogenesis
K: Transcription
L: Replication, recombination and repair
M: Cell wall/membrane/envelope biogenesis
N: Cell motility
O: Post-translational modification, protein turnover, chaperones
P: Inorganic ion transport and metabolism
Q: Secondary metabolites biosynthesis, transport, and catabolism
R: General function prediction only
S: Function unknown
T: Signal transduction mechanisms
U: Intracellular trafficking, secretion, and vesicular transport
V: Defense mechanisms
W: Extracellular structures
Y: Nuclear structure
Z: Cytoskeleton

Figure 5. Cluster of Orthologous Groups (COG) classification of differentially expressed genes (DEGs) during decapitation-induced AXB activation. 8826 annotated DEGs were distributed over 25 COG functional categories. The functional COG classes are shown on the X-axis whereas gene frequencies are plotted on the Y-axis.

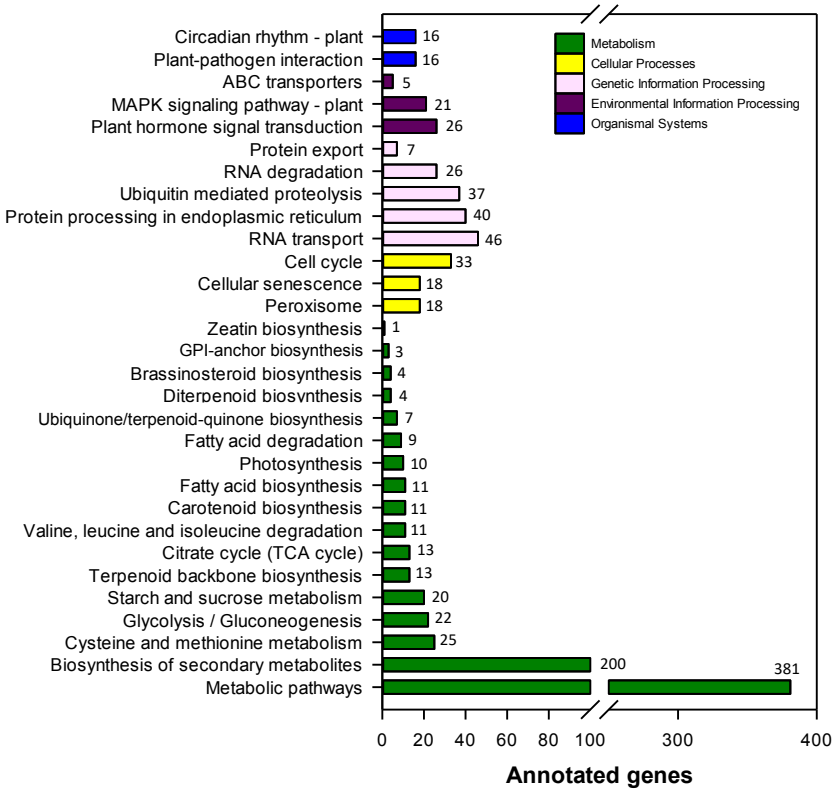


Figure 6. Kyoto encyclopedia of genes and genomes (KEGG)-based functional classification (KAAS) of differentially expressed genes (DEGs) during AXBs activation. The total number of DEGs was divided into five categories: Metabolism, Cellular Processes, Genetic Information Processing, Environmental Information Processing and Organismal Systems.

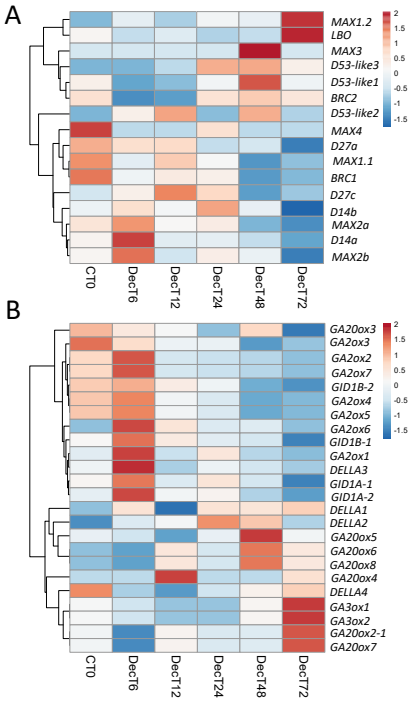


Figure 7. Heat maps of expression levels in fold changes per time point for hormone-related genes in response to decapitation detected by *de novo* assembly. (a) SL-related genes (b) GA-related genes (FDR < 0.005).

X-axis represents time points post decapitation compared to control at t=0 (CT0). The Y-axis represents the genes.

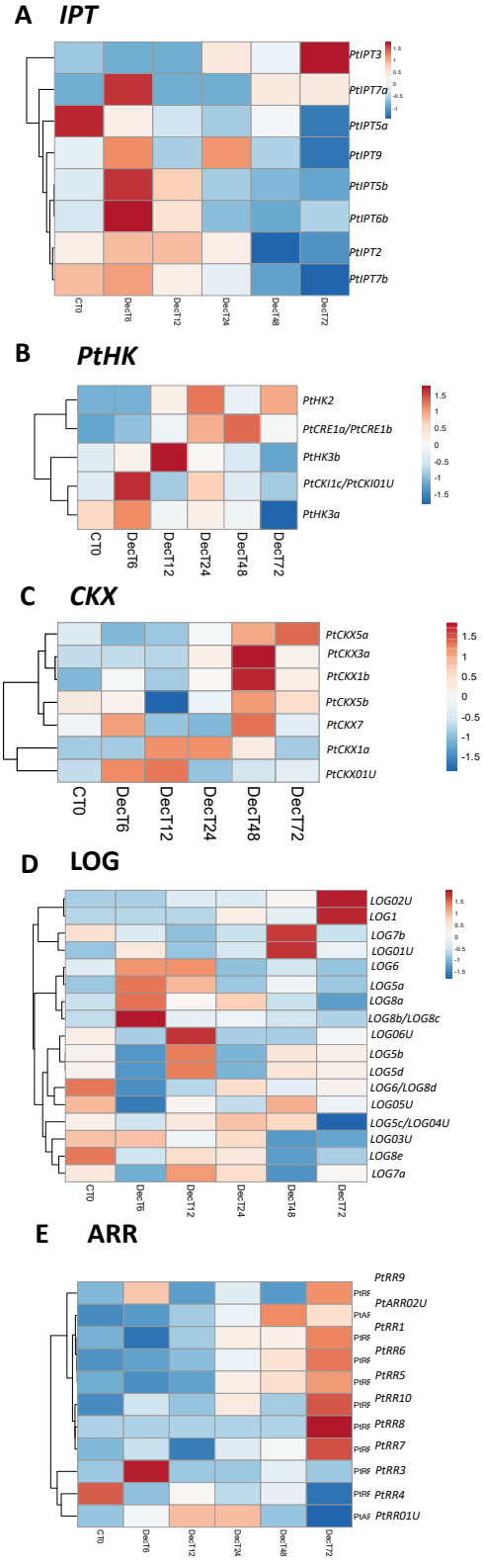


Figure 8. Heat maps of expression levels in fold changes per time point for CK-related genes in response to decapitation detected by *de novo* assembly. (a) CK biosynthesis genes, *IPT*s; (b) CK receptors, *HK*s; (c) CK catabolism genes, *CKX*; (d) CK regulation, *LOG*; (e) CK-signal transduction, *ARR*s (FDR < 0.005).

X-axis represents time points post-decapitation compared to control at t=0 and Y-axis represents the genes.

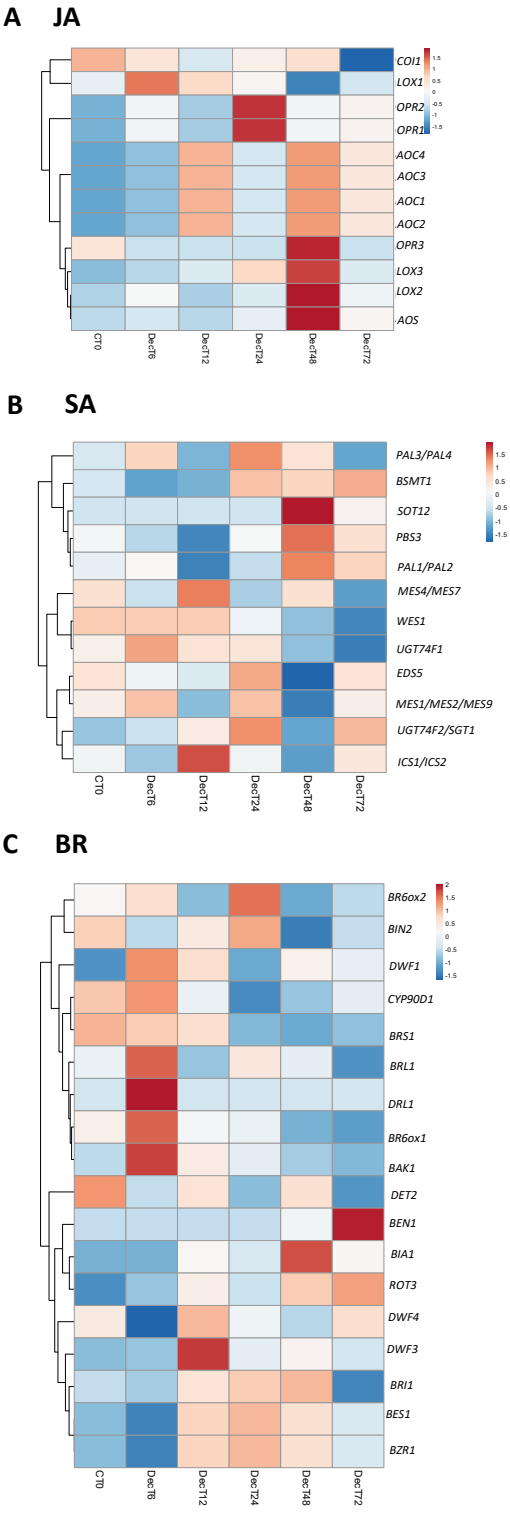


Figure 9. Heat maps of expression levels in fold changes per time point for hormone-related genes in response to decapitation detected by *de novo* assembly. (a) JA-related genes (b) SA-related genes and (c) BR-related genes (FDR < 0.005).

X-axis represents time points post decapitation compared to control at t=0 (CT0). The Y-axis represents the genes.

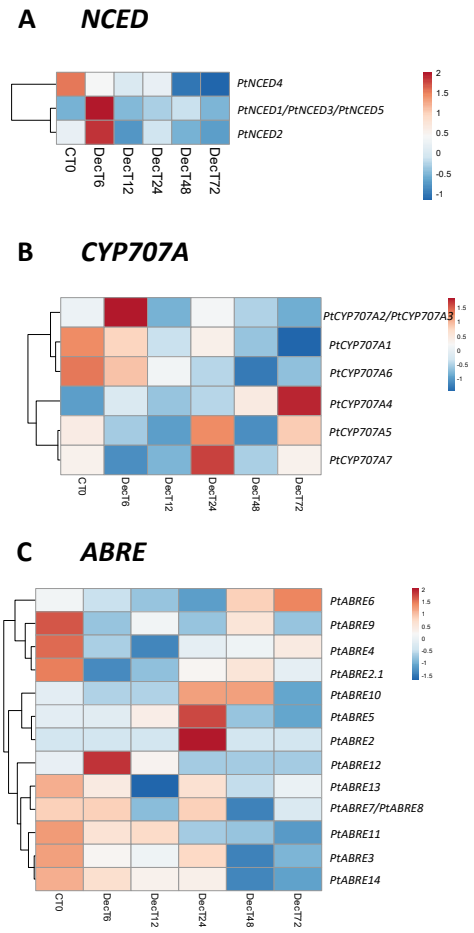
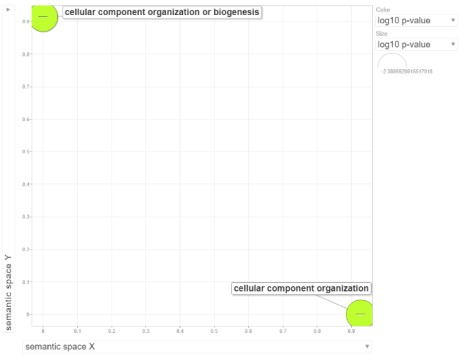


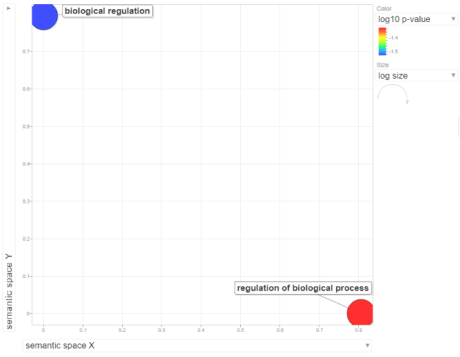
Figure 10. Heat maps of expression levels in fold changes per time point for ABA-related genes in response to decapitation detected by *de novo* assembly. (a) ABA-biosynthesis genes, *NCED* (b) ABA-catabolism genes, *CYP707A* and (c) ABA-signaling gene, *ABRE* (FDR < 0.005).

X-axis represents time points post decapitation compared to control at t=0 (CT0). The Y-axis represents the genes.

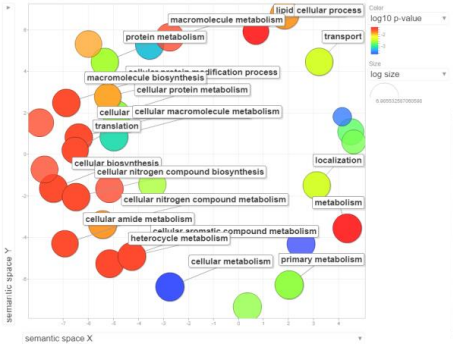
A


cellular component organization or biogenesis
cellular component organization
semantic space X
semantic space Y
log10 p-value
log10 p-value

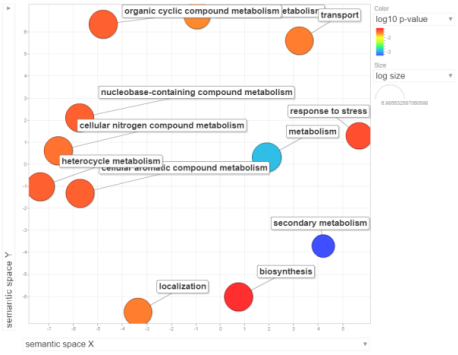
B


biological regulation
regulation of biological process
semantic space X
semantic space Y
log10 p-value
log size

C


lipid cellular process
macromolecule metabolism
protein metabolism
transport
macromolecule biosynthesis
cellular protein metabolism
cellular
cellular macromolecule metabolism
translation
cellular biosynthesis
cellular nitrogen compound biosynthesis
localization
cellular nitrogen compound metabolism
metabolism
cellular amide metabolism
heterocycle metabolism
cellular metabolism
primary metabolism
semantic space X
semantic space Y
log10 p-value
log size

D


organic cyclic compound metabolism
transport
nucleobase-containing compound metabolism
response to stress
cellular nitrogen compound metabolism
metabolism
heterocycle metabolism
secondary metabolism
biosynthesis
localization
semantic space X
semantic space Y
log10 p-value
log size

E

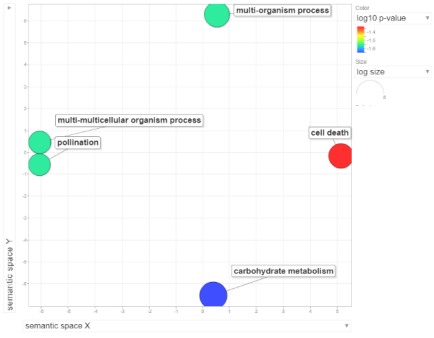
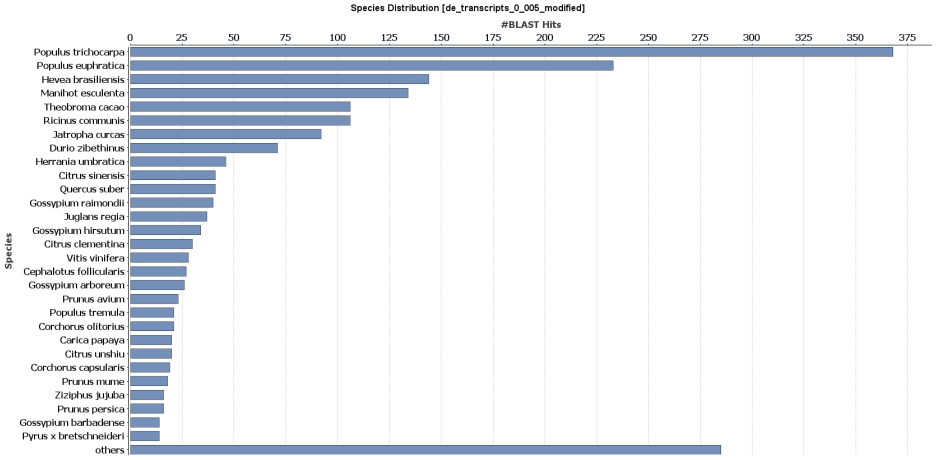

multi-organism process
multi-multicellular organism process
pollination
cell death
carbohydrate metabolism
semantic space X
semantic space Y
log10 p-value
log size

Figure 11. Gene ontology (GO) enrichment analysis constructed with REVIGO for all the DEGs associated in response to decapitation between time points. The scatter plot of showing the significance of GO term for DEGs in (A) CT0 vs DecT6 (B) DecT6 vs DecT12 (C) DecT12 vs DecT24 (D) DecT24 vs DecT48, and (E) DecT48 vs DecT72 shows the cluster representatives (i.e., terms remaining after the redundancy reduction) in a two-dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities. Bubble color indicates the *p*-value for the false discovery rates (FDRs) and the bubble size specify the frequency of the GO term (more general terms are indicated as larger size bubbles). Bubble color indicates the log10 p-value (red is lower significance; blue is higher significance). Larger bubbles indicate that it contains more general terms or more enriched genes, and small bubbles indicate fewer specific GO terms.



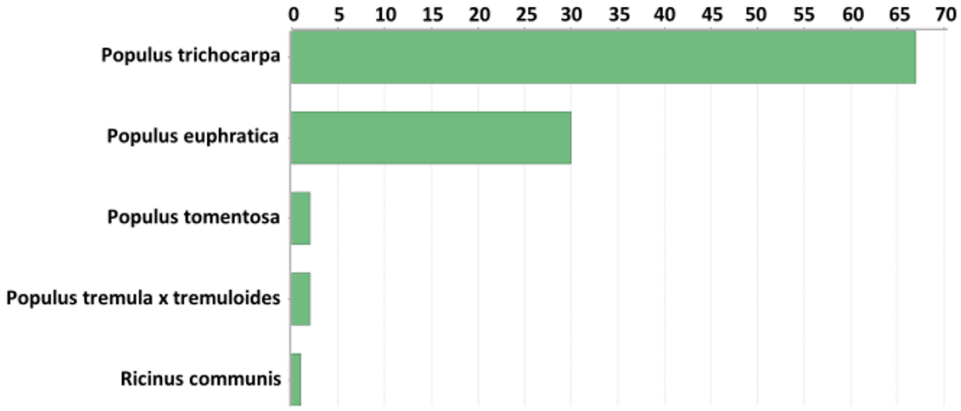


Fig. S1 The top blast hit-species distribution *de novo*-based assembly

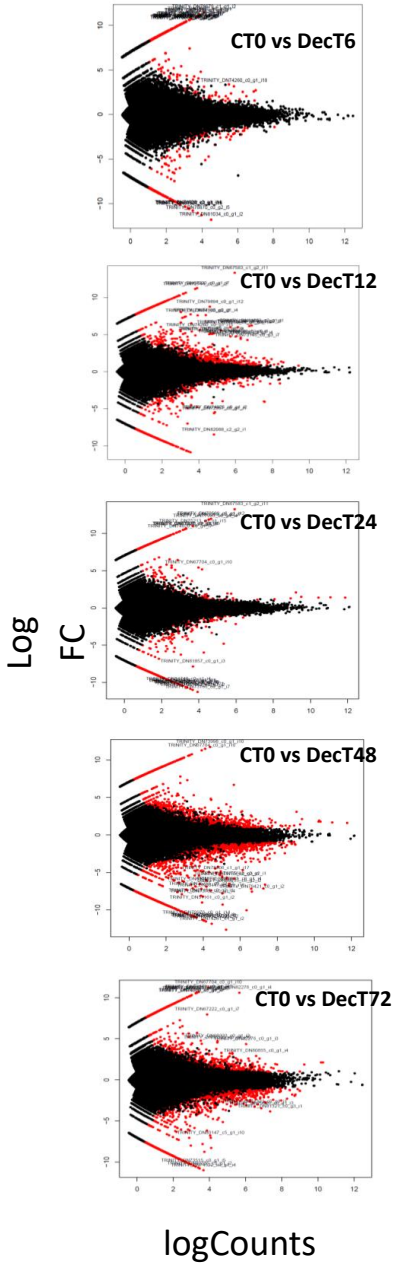


Fig. S2 Pairwise comparisons of gene expression between control and at different decapitated time points. Red points indicate differentially expressed genes (DEGs) with a false discovery rate (FDR) of 0.005. FC, fold-change. logCounts indicate counts per million mapped reads.

Tables

Table 1. Statistics from the de novo transcriptome assembly data analysis.

	Assembly statistics
Min. contig length (bp)	201
N50 (bp)	1,871
Max. contig length (bp)	15,046
Total no. of contigs	399,380
Sum of the reads	459,745,595

Table 2. Statistics of the completeness of de novo assembly based on 248 CEGs by CEGMA.

Out of 248 CEGs1	Statistics
% of fully represented	97.18
% of at least partially represented	100
Average number of orthologs per CEG	4.47
% of detected CEGs with more than 1 ortholog	98.34

1 CEGs: Core Eukaryotic Genes

Table 3. List of TRINITY IDs and expression values (FPKM) of all the genes studied

Gene	Transcript ID	CT0	DecT6	DecT12	DecT24	DecT48	DecT72
SL							
D27c	TRINITY_DN71513_c0_g2_i4	11,99	16,055	21,955	18,66	7,86	10,15
MAX4	TRINITY_DN52966_c0_g1_i3	1	0	0	0,5	0	0
MAX3	TRINITY_DN93306_c0_g1_i1	0	0	0	0	1	0
D27a	TRINITY_DN67807_c0_g1_i2	46,61	39,31	40,65	21,01	23,275	6,435
D14a	TRINITY_DN55251_c0_g1_i3	148,64	251,845	118,17	123,38	95,315	58,13
MAX2a	TRINITY_DN62165_c0_g1_i1	353,5	383,5	338,5	347,5	294	279
D14b	TRINITY_DN55251_c0_g1_i1	158,07	185,655	163,33	206,62	155,685	91,37
D53a	TRINITY_DN71306_c1_g1_i5	8,355	1,865	2,975	7,14	14,42	6,975
MAX1.1	TRINITY_DN72588_c0_g1_i14	551,245	259,54	466,71	316,8	45,1	129,46
D53c	TRINITY_DN80696_c0_g1_i7	546,965	547,49	584,545	754,04	757,205	674,88
MAX1.2	TRINITY_DN72588_c0_g1_i6	0	2,79	0,805	3,495	2,95	10,035
LBO	TRINITY_DN63292_c0_g1_i2	7	3,5	5	3	3,5	15
MAX2b	TRINITY_DN66053_c0_g1_i6	41,17	53,575	34,66	42,73	38,88	25,385
BRC2	TRINITY_DN78768_c0_g2_i2	36,505	13,915	15,715	35,715	39,22	35,09
BRC1	TRINITY_DN65257_c0_g1_i3	197,98	120,98	142,28	136,575	56,815	71,33
D53b	TRINITY_DN78836_c0_g1_i3	24,67	31,535	36,02	25,035	35,61	26,295
BR							
BR6ox2	TRINITY_DN78494_c1_g1_i4	13,46	17,355	4,695	24,745	2,985	7,015
BRI1	TRINITY_DN81398_c2_g2_i5	406,645	391,93	488,46	513,145	524,21	341,84
BIA1	TRINITY_DN75537_c3_g1_i4	0	0	23,77	12,585	55,21	24,19
DWF1	TRINITY_DN80158_c0_g1_i4	1342,155	1864,34	1730,32	1386,07	1661,19	1586,225
ROT3	TRINITY_DN67470_c0_g1_i2	0	7,285	24,525	11,645	32,065	37,39
BIN2	TRINITY_DN78801_c3_g1_i2	145,91	80,94	127,41	157,97	41,505	82,505
BES1	TRINITY_DN73967_c1_g1_i4	52,23	48,425	63,645	65,07	62,33	56,01
DRL1	TRINITY_DN28437_c0_g1_i2	0	1,73	0	0	0	0

DWF4	TRINITY_DN75078_c0_g1_i10	11,24	6,24	12,69	10,29	8,82	11,9
BRL1	TRINITY_DN81161_c0_g1_i5	55,26	75,165	46,235	61,36	54,07	40,34
BR6ox1	TRINITY_DN77119_c0_g1_i10	16,485	24,715	15,095	14,02	8	6,67
BAS1	TRINITY_DN63302_c1_g4_i2	0	0	0	0	0	0
CYP90D1	TRINITY_DN76441_c0_g1_i10	27,745	30,205	20,74	11,33	15,395	19,84
BRS1	TRINITY_DN74415_c1_g1_i3	58,09	55,97	53,67	36,56	34,81	37,245
BAK1	TRINITY_DN70007_c0_g1_i3	154,7	213,15	178,56	165,48	150,895	146,345
DWF3	TRINITY_DN75450_c0_g1_i14	0	3,215	133,175	33,94	55,915	22,57
BEN1	TRINITY_DN97729_c0_g1_i1	0	0	0	0	0,5	2
DET2	TRINITY_DN75753_c2_g1_i2	29,26	20,5	25,97	18,945	26,11	17,16
BZR1	TRINITY_DN73967_c1_g1_i4	52,23	48,425	63,645	65,07	62,33	56,01
JA							
LOX3	TRINITY_DN79651_c0_g1_i13	380,505	424,695	483,74	666,145	834,45	485,595
LOX2	TRINITY_DN81997_c0_g1_i6	2810,095	3897,175	2755,46	3299,08	6372,025	3725,53
OPR2	TRINITY_DN79828_c0_g2_i1	0	2,64	0,705	7,205	2,62	3,17
AOS	TRINITY_DN67649_c0_g1_i7	52,74	75,2	48,66	114,245	374,23	149,105
OPR3	TRINITY_DN75420_c0_g2_i2	13,48	0	0	0	30,945	0
COI1	TRINITY_DN77367_c1_g3_i1	705,38	677,105	626,285	664,985	682,62	549,9
AOC1	TRINITY_DN49203_c0_g1_i1	7	8,5	19,5	11	20,5	16
AOC2	TRINITY_DN49203_c0_g1_i1	7	8,5	19,5	11	20,5	16
AOC3	TRINITY_DN49203_c0_g1_i1	7	8,5	19,5	11	20,5	16
AOC4	TRINITY_DN49203_c0_g1_i1	7	8,5	19,5	11	20,5	16
LOX1	TRINITY_DN78097_c3_g1_i5	11,21	15,695	13,56	12,075	7,375	10,2
OPR1	TRINITY_DN79828_c0_g2_i1	0	2,64	0,705	7,205	2,62	3,17
SA							
EDS5	TRINITY_DN64104_c0_g1_i4	13,18	10,5	10,015	15,25	5,26	12,77
WES1	TRINITY_DN73638_c0_g1_i1	137,615	137,82	138,53	106,115	76,67	56,945
SOT12	TRINITY_DN68006_c0_g1_i2	0	0	0	0	6,645	1,86
PAL3/PAL4	TRINITY_DN81486_c8_g2_i2	1632,545	1943,145	1473,17	2076,08	1882,825	1441,49
UGT74F2/SGT1	TRINITY_DN81468_c0_g1_i12	3,5	5	7,5	11,325	3,28	10,4

ICS1/ICS2	TRINITY_DN73586_c0_g1_i41	11,265	8,61	15,18	11,06	7,56	11,56
MES4/MES7	TRINITY_DN49824_c0_g1_i1	41,885	35,445	47,12	34,22	41,615	30,935
PBS3	TRINITY_DN68277_c0_g1_i5	37,46	24,775	11,19	38,275	62,005	45,61
BSMT1	TRINITY_DN69946_c3_g1_i1	7,235	2,18	3,385	16,385	15,175	16,87
PAL1/PAL2	TRINITY_DN81085_c3_g1_i4	1964,29	2095,46	1262,425	1725,03	2728,875	2421,885
MES1/MES2/MES9	TRINITY_DN74521_c0_g1_i11	5,98	7,32	4,075	7,295	2,925	6,42
UGT74F1	TRINITY_DN77634_c0_g1_i1	193	238,5	208,44	205,77	131,46	96,56
CKX							
PtCKX3a	TRINITY_DN63614_c0_g1_i1	69,17	66,97	55	258,365	625,02	248,85
ptCKX1b	TRINITY_DN64525_c0_g1_i3	0	9,935	1,685	10,57	27,15	12,265
PtCKX5a	TRINITY_DN77476_c0_g2_i1	9,5	3	4,49	13,45	23,94	28
PtCKX5b	TRINITY_DN77476_c0_g3_i1	86,29	83,51	46,375	78,4	102,04	91,265
ptCKX1a	TRINITY_DN77960_c1_g1_i13	0	0	3,85	3,865	2,12	0
PtCKX7	TRINITY_DN60295_c0_g2_i1	71,945	79,57	66,055	65,32	81,755	70,2
PtCKX01U	TRINITY_DN79962_c0_g1_i21	12,93	31,185	32,435	10,235	13,755	16,36
PtHK							
PtCKI1c / ptCKI01U	TRINITY_DN59586_c0_g1_i3	0,7	4,5	0	2,5	1,17	0
PtHK2	TRINITY_DN71841_c0_g1_i18	0	0	10,93	21,155	7,87	18,445
PtHK3a	TRINITY_DN79011_c1_g1_i1	292,545	335,845	244,43	261,67	244,205	120,09
PtCKI1b	TRINITY_DN100462_c0_g1_i1	0	0	0	0	0	0
PtCRE1a/PtCRE1b	TRINITY_DN80629_c1_g1_i7	91,045	101,535	138,5	183,975	201,71	142,715
PtHK3b	TRINITY_DN82043_c2_g2_i10	210,63	234,93	320,71	229,495	204,625	165,735
IPT							
PtIPT2	TRINITY_DN71421_c0_g1_i10	18,79	21,575	22,27	19,215	9,255	11,245
PtIPT5a	TRINITY_DN65394_c2_g1_i7	15,15	8,52	5,89	5,39	7,615	2,425
PtIPT9	TRINITY_DN80885_c1_g1_i12	293,095	337,325	276,46	335,42	277,325	250,835
PtIPT7a	TRINITY_DN60967_c1_g1_i2	0	1,62	0	0,16	1,19	1
PtIPT5b	TRINITY_DN65394_c2_g1_i9	24,595	59,9	42,6	16,865	13,98	10,64
PtIPT6b	TRINITY_DN60967_c1_g1_i1	4,5	15,88	9	2,34	1,31	2,5
PtIPT3	TRINITY_DN66301_c1_g1_i3	0,5	0	0	2,5	1,84	6,005

PtIPT7b	TRINITY_DN58427_c0_g1_i3	285,365	297,96	251,08	225,765	169,05	137,315
PtRR							
PtRR8	TRINITY_DN97273_c0_g1_i1	0	0	0	0	0	2
PtRR1	TRINITY_DN61829_c0_g2_i4	39,335	21,42	48	79,545	78,91	111,16
PtRR7	TRINITY_DN64157_c3_g1_i14	98,01	114,02	77,985	125,005	136,215	193,11
PtRR3	TRINITY_DN80538_c1_g2_i4	0	9,895	0	0	2,385	0
PtRR6	TRINITY_DN74186_c0_g1_i4	11,61	17,24	26,27	56,33	77,145	108,5
PtRR4	TRINITY_DN79250_c0_g1_i10	40,945	25,5	31,73	27,265	30,13	21,01
PtRR10	TRINITY_DN72979_c0_g1_i15	84,485	108,945	100,565	133,57	102,2	168,87
ptARR02U	TRINITY_DN61829_c0_g2_i6	7,165	9,08	15,195	24,54	42,795	33,835
PtRR9	TRINITY_DN65625_c1_g1_i5	2,37	15,775	0,295	7,485	0	18,845
ptARR01U	TRINITY_DN35884_c0_g1_i2	0,5	1	1,5	1,5	0,5	0
PtRR5	TRINITY_DN79250_c0_g1_i12	24,5	18,175	21,96	52,075	58,895	71
LOG							
LOG02U	TRINITY_DN56685_c0_g1_i2	0	0	0,5	0,5	1	3
LOG6	TRINITY_DN65526_c1_g1_i17	1,86	4,225	4,275	0,87	1,5	0,965
LOG7b	TRINITY_DN80152_c0_g2_i2	3,045	1,2	0	0,655	5,655	0,655
LOG6/LOG8d	TRINITY_DN65526_c1_g1_i11	43,275	10,315	18,705	34,045	24,055	28,575
LOG01U	TRINITY_DN62511_c0_g1_i5	0	1,5	0	0,5	3,095	0,86
LOG05U	TRINITY_DN55001_c0_g1_i3	23,33	12,56	19,915	16,68	23,7	18,975
LOG03U	TRINITY_DN75965_c0_g1_i16	41,265	41,15	25,37	37,255	4,24	6,695
LOG1	TRINITY_DN67350_c0_g1_i1	0	0	0	2,5	1,3	6,54
LOG8a	TRINITY_DN65591_c0_g2_i1	1	2,5	1,5	2	1	0,5
LOG8e	TRINITY_DN77887_c0_g1_i1	33,85	18,49	27,41	25,965	13,15	16,7
LOG7a	TRINITY_DN64282_c1_g1_i1	7,91	2,04	10,855	8,79	1	6,72
LOG06U	TRINITY_DN75965_c0_g1_i2	2,265	0	5,47	0	0	1,67
LOG5c/LOG04U	TRINITY_DN65904_c0_g2_i3	505,57	472,61	510,705	530,675	524,41	421,705
LOG5b	TRINITY_DN71303_c2_g3_i1	5	2,5	7	3	5,5	5,105
LOG5d	TRINITY_DN71303_c2_g3_i1	5	2,5	7	3	5,5	5,105
LOG8b/LOG8c	TRINITY_DN59015_c0_g1_i4	16,215	30,89	18,78	19,225	16,705	15,5

LOG5a	TRINITY_DN64282_c1_g1_i9	0	1,965	1,57	0	0,71	0
GA							
DELLA1	TRINITY_DN76975_c0_g1_i4	554	650	503	649	648	668
DELLA2	TRINITY_DN76975_c0_g1_i8	389	488	525	642	602	454
DELLA3	TRINITY_DN75123_c1_g1_i4	686	849	624	683	647	619
DELLA4	TRINITY_DN75123_c1_g1_i7	320	248	229	256	283	303
GA20ox2-1	TRINITY_DN61451_c0_g2_i1	28	17	30	25	26	41
GA20ox3	TRINITY_DN64308_c1_g1_i2	64	59	57	49	62	44
GA20ox4	TRINITY_DN63236_c0_g1_i1	0	0	2	0	0	1
GA20ox5	TRINITY_DN37367_c0_g1_i1	3	2	0	3	9	4
GA20ox6	TRINITY_DN68844_c0_g1_i7	235	227	277	250	305	275
GA20ox7	TRINITY_DN61451_c0_g2_i1	28	17	30	25	26	41
GA20ox8	TRINITY_DN68844_c0_g1_i7	235	227	277	250	305	275
GA2ox1	TRINITY_DN73739_c0_g1_i3	64	173	46	104	40	26
GA2ox2	TRINITY_DN56403_c0_g1_i3	21	29	11	10	9	8
GA2ox3	TRINITY_DN62277_c1_g1_i7	98	69	44	43	3	16
GA2ox4	TRINITY_DN73001_c1_g1_i7	22	27	15	12	6	7
GA2ox5	TRINITY_DN73001_c1_g1_i7	22	27	15	12	6	7
GA2ox6	TRINITY_DN73739_c0_g1_i6	0	7	4	1	1	0
GA2ox7	TRINITY_DN56403_c0_g1_i3	21	29	11	10	9	8
GA3ox1	TRINITY_DN60990_c0_g1_i9	3	1	0	0	3	8
GA3ox2	TRINITY_DN60990_c0_g1_i9	3	1	0	0	3	8
GID1A-1	TRINITY_DN74628_c0_g1_i7	299	398	261	325	251	169
PtGID1A-2	TRINITY_DN74628_c0_g1_i8	796	1290	840	896	650	516
GID1B-1	TRINITY_DN73406_c1_g1_i3	630	951	689	540	496	248
GID1B-2	TRINITY_DN73406_c1_g2_i4	248	258	223	204	151	139
NCED							
PtNCED1/PtNCED3/PtNCED5	TRINITY_DN70553_c0_g1_i2	19,42	162,36	22,4	32,33	41,94	19,93
PtNCED2	TRINITY_DN68124_c0_g1_i3	329,55	577,52	181,27	282,245	215,445	192,02
PtNCED4	TRINITY_DN64824_c0_g1_i1	944	620	512	560	210	166

CYP707A							
PtCYP707A1	TRINITY_DN65573_c0_g1_i11	13,395	10,97	5,2	8,785	4	0
PtCYP707A2/PtCYP707A3	TRINITY_DN69437_c0_g1_i5	14,5	22,015	11,005	14,88	12,235	10,875
PtCYP707A6	TRINITY_DN73769_c0_g1_i14	17,4	14,7	10,115	6,655	2,125	5,5
PtCYP707A5	TRINITY_DN73769_c0_g1_i24	53,22	35,115	27,735	69,305	25,245	61,545
PtCYP707A7	TRINITY_DN72858_c0_g1_i7	32,395	7,905	13,67	54,84	17,645	32,355
PtCYP707A4	TRINITY_DN72858_c0_g1_i16	38,435	91,44	62,955	73,745	138,08	231,79
ABRE							
PtAREB13	TRINITY_DN70752_c1_g1_i5	34,945	31,235	18,175	31,635	25,495	28,405
PtAREB5	TRINITY_DN74550_c0_g1_i16	21,885	21,39	32,51	62,88	8,22	0,96
PtAREB2	TRINITY_DN83013_c0_g1_i1	0	0	0	2	0	0
PtAREB10	TRINITY_DN79800_c2_g1_i11	1,595	0,9	1,4	4,87	5,445	0
PtAREB11	TRINITY_DN70752_c1_g1_i4	51,315	40,83	42,68	22,125	21,39	14,77
PtAREB3	TRINITY_DN63956_c0_g2_i2	72,83	62,005	60,345	67,67	43,635	49,035
PtAREB4	TRINITY_DN74550_c0_g1_i11	24,495	7,845	3,26	11,53	12,6	15,53
PtAREB12	TRINITY_DN74197_c0_g1_i11	4,375	15,605	6,225	0	0	0,305
PtAREB9	TRINITY_DN79800_c2_g1_i10	19,805	0	6,935	0	10,685	0
PtAREB14	TRINITY_DN63956_c0_g2_i1	190,455	173,195	157,86	158,78	93,085	102,545
PtAREB6	TRINITY_DN74197_c0_g1_i12	30,51	24,77	22,025	17,5	38,955	44,85
PtAREB7/PtAREB8	TRINITY_DN68166_c0_g2_i6	5,925	6	3,48	5,835	2	4,28
PtAREB2	TRINITY_DN64367_c0_g2_i4	31,35	14,795	18,135	23,575	26,155	21,725