Characterization of MORE AXILLARY GROWTH Genes in Populus

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Abstract

Background: Strigolactones are a new class of plant hormones that play a key role in regulating shoot branching. Studies of branching mutants in Arabidopsis, pea, rice and petunia have identified several key genes involved in strigolactone biosynthesis or signaling pathway. In the model plant Arabidopsis, MORE AXILLARY GROWTH1 (MAX1), MAX2, MAX3 and MAX4 are four founding members of strigolactone pathway genes. However, little is known about the strigolactone pathway genes in the woody perennial plants.

Methodology/Principal Finding: Here we report the identification of MAX homologues in the woody model plant Populus trichocarpa. We identified the sequence homologues for each MAX protein in P. trichocarpa. Gene expression analysis revealed that Populus MAX paralogous genes are differentially expressed across various tissues and organs. Furthermore, we showed that Populus MAX genes could complement or partially complement the shoot branching phenotypes of the corresponding Arabidopsis max mutants.

Conclusion/Significance: This study provides genetic evidence that strigolactone pathway genes are likely conserved in the woody perennial plants and lays a foundation for further characterization of strigolactone pathway and its functions in the woody perennial plants.

Introduction

Plant architecture plays a major role in determining photosynthetic light use efficiency and biomass yield. Optimal plant architecture is critical for achieving maximum carbon capture per unit land area especially when plants are grown in high-density stands. One recent breakthrough in the field of plant biology was the discovery of strigolactones (SLs) as a new class of plant hormones controlling shoot branching [1,2]. More importantly, the synthesis of SLs in plants is regulated by the nutrient availability, particularly Pi deficiency, in soil and SLs exuded by roots serve as host recognition signals for symbiotic fungi [2–14]. Therefore, SLs are viewed as integrative signaling molecules that can couple nutrient availability and microbial symbiosis to the control of plant architecture and productivity. This breakthrough offers a new opportunity to dissect the interaction between plants and their environmental systems and to link this interaction to the control of plant architecture and productivity.

Substantial progress has been made in the last decade to understand the biosynthesis, signal transduction and physiological functions of SLs [reviewed by [12,15–28]]. MORE AXILLARY GROWTH1 (MAX1), MAX2, MAX3 and MAX4 are four founding members of strigolactone pathway genes in the model plant Arabidopsis [29–32]. While MAX1, MAX3 and MAX4 are key genes involved in SL biosynthesis, MAX2 is a key gene involved in SL signaling. MAX1 encodes a cytochrome P450 monoxygenase [30]. MAX3 and MAX4 encode two carotenoid cleavage dioxygenases (CCD), CCD7 and CCD8, respectively [29,31]. MAX2 encodes an F-box leucine-rich protein [32]. Loss-of-function mutations in each of these four MAX genes resulted in increased shoot branching [29–32]. Orthologues for MAX genes have been identified and corresponding mutants have been

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characterized in pea (\textit{ramosus}, \textit{rms}), rice (\textit{dwarf}, \textit{hdi}) and petunia (decreased apical dominance, \textit{dul}) (reviewed by [15–17,19–21,23]). In addition to \textit{MAX} genes, several other genes involving in SL biosynthesis or signaling have been identified. For example, \textit{D27} encoding a novel iron-containing protein is involved in SL biosynthesis [33,34]. 

\textbf{PLEIOTROPIC DRUG RESISTANCE1 (\textit{PDR1})} encoding an ATP-binding cassette transporter functions as a cellular SL exporter [35]. \textit{D14} encoding a protein of the α/β-fold hydrolase superfamily is involved in SL signaling and likely functions as a receptor for SLs [36–40]. D53, a member of class I Cip ATPase protein family, is a substrate of the SCF\textit{MAX} ubiquitination complex and the degradation of D53 protein is promoted by SL and is dependent on D14 and D3 (a rice ortholog of \textit{MAX2}) [31,42]. The degradation of \textit{BES1}, a positive regulator in brassinosteroid signaling pathway, is also dependent on \textit{MAX2} [43].

However, despite this important discovery, little is known about this new class of plant hormones in perennial woody plants. So far, among perennial woody plants, strigolactone pathway genes have only been recently studied in willow [44,45]. Because differences have been observed between monocots and dicots, between herbaceous and woody plants, and between annual and perennial plants, it has remained elusive whether the SL pathways are conserved in the perennial woody plants, such as \textit{Populus}. For example, studies in herbaceous plants (such as Arabidopsis and pea) suggested that SLs act as long-distance signaling molecules that can be transported from roots to shoots to exert functional control on shoot branching. However, the woody perennial plants, such as \textit{Populus}, are typically several orders of magnitude taller than annual herbaceous plants. This raises the question of whether SL can function similarly in \textit{Populus}.

As a first step towards exploring SL pathways in the model woody plant \textit{Populus}, we conducted a genome-wide search of sequence homologues of strigolactone pathway genes in the sequenced genome of \textit{Populus trichocarpa} [46]. By using Arabidopsis strigolactone pathway genes as templates, we identified \textit{Populus} sequence homologues for each of those four founding members of strigolactone pathway genes, namely \textit{MAX1}, \textit{MAX2}, \textit{MAX3} and \textit{MAX4}. We found that \textit{Populus} \textit{MAX} paralogous genes are differentially expressed across various tissues and organs. We showed that \textit{Populus} \textit{MAX} genes could complement or partially complement the shoot branching phenotypes of corresponding Arabidopsis \textit{max} mutants. These findings provide genetic evidence that SL pathways are likely conserved in the woody perennial plants.

\section*{Materials and Methods}

\subsection*{Plant Materials and Growth Conditions}

Arabidopsis wild type Columbia-0 (Col-0) and mutant \textit{max1-4} (SAIL_25_A05, ABRC stock #: CS862413) and \textit{max2-4} (SALK_028336) were obtained from the Arabidopsis Biological Resources Center (Columbus, Ohio). Arabidopsis \textit{max3-12} mutant has been previously described [47]. Seeds were surface sterilized by serial washing with 96% (v/v) ethanol, 20% (v/v) household bleach supplemented with 0.05% (v/v) Tween-20, and water, and placed at 4°C for 2 days. Seeds were subsequently plated on ½ Murashige and Skoog (MS) medium [48] supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar, and germinated in 12 h/12 h photoperiod at 23°C, approximately 90 μmol photons m$^{-2}$ s$^{-1}$. Seven-day-old Arabidopsis seedlings were transferred from ½ MS medium to soil and grown in growth chambers at 23°C, approximately 125 μmol photons m$^{-2}$ s$^{-1}$ with 14 h/10 h (long-day conditions) or 10 h/14 h (short-day conditions) photoperiods.

\section*{Cloning of \textit{Populus MAX} Homologous Genes}

The full-length open reading frame (ORF) of each \textit{PtrMAX} gene was determined according to the sequence information available at Phytozone [46,49]. Gene-specific primers were designed to amplify the full-length ORF of each \textit{PtrMAX} gene from cDNA library derived from RNA isolated from leaves and roots of \textit{Populus trichocarpa} plants. Subsequently, the full-length ORF of each \textit{PtrMAX} gene was introduced into the pENTR vector by using pENTR/D-TOPO Cloning Kit (Life Technologies). The cloned RT-PCR products were validated by sequencing and then transferred into plant Gateway destination vector pGWBS502\(\Omega\) (2×CaMV35S\(\Omega\)) [50] using LR clonase (Life Technologies). All primers used for cloning are listed in Table S1.

\subsection*{Genetic Complementation}

\textit{pGWBS502\(\Omega\) (2×CaMV35S\(\Omega\))} binary vectors containing \textit{35S::PtrMAX} plasmid were transformed into \textit{Arabidopsis} \textit{max} mutants via \textit{Agrobacterium tumefaciens} strain GV3101 mediated flower-dipping transformation [51,52]. T1 transformants were selected using 20 μg/L hygromycin B. A minimum of 20 independent transgenic lines were selected for each transgene. Two independent transgenic lines were used for further studies.

When plants reached maturity, the number of primary rosette-leaf branches was counted. A minimum of 10 individual plants per genotype were used.

\subsection*{RT-PCR}

To examine the absence or presence of \textit{Arabidopsis} \textit{MAX} transcript in the \textit{max} mutant, total RNA was extracted from 7-day-old seedlings using the Invisorb Spin Plant Mini Kit (Stratec Molecular). Two μg of total RNA were reversely transcribed to cDNA using Fermentas RevertAid Reverse Transcriptase (Thermo Scientific). For semi-quantitative RT-PCR, \textit{Arabidopsis} \textit{MAX}-specific primers spanning the full-length ORF of \textit{MAX} gene were used. PCR amplification of \textit{AtACTIN8 (AtACT8)} served as a control.

For the examination of expression of \textit{Populus} \textit{MAX} genes in \textit{Arabidopsis} transgenic lines, \textit{PtrMAX} gene-specific primers were used. All primers used for semi-quantitative RT-PCR are listed in Table S1.

\subsection*{Quantitative RT-PCR (qRT)}

qRT was conducted to examine the transcript level of each \textit{PtrMAX} genes across various tissues and organs using a StepOnePlus (Applied Biosystems), Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and cDNA corresponding to 80 ng RNA in a total volume of 25 μl. Three biological replicates were used for qRT analysis. The following cycling conditions were used for PCR: 10 min at 95°C, 35 cycles of 15 s at 95°C and 60 s at 60°C. The transcript level was normalized against that of \textit{PtrACTIN5 (PtrACT5)}. Gene-specific qRT primers were designed using QuantPrime [53]. All primers used for qRT analysis are listed in Table S1.

\section*{Results}

\subsection*{Sequence Homologues of \textit{MAX} Proteins in \textit{Populus}}

\textit{MAX1}, \textit{MAX2}, \textit{MAX3} and \textit{MAX4} are four founding members of SL pathway genes in the model plant Arabidopsis. In order to identify sequence homologs of \textit{MAX} genes in \textit{Populus trichocarpa} (hereafter referred to as \textit{Populus}), we used amino acid sequences of

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Arabidopsis MAX proteins as templates to perform protein homology search in the fully-sequenced Populus genome. Based on the current annotation by TAIR (http://www.arabidopsis.org) [54] and Phytozome (www.phytozome.net) [49], Arabidopsis MAX1 (AtMAX1) has two transcript variants. The primary variant encoded by gene locus At2g26170.1 was used as a template for searching protein sequence homologues encoded by the Populus genome using the “Protein Homologs” search tool at Phytozome. The search identified two close sequence homologues encoded by loci Potri.006G226700 and Potri.018G062100, designated as PtrMAX1a and PtrMAX1b, respectively (Figure 1). Based on the current annotation by Phytozome, PtrMAX1a has

![Figure 1. Bioinformatic analysis of MAX1 proteins from Populus and Arabidopsis. (A) Amino acid sequence alignment. (B) Phylogenetic analysis of Populus sequence homologues of Arabidopsis MAX1 protein. (C) Amino acid sequence similarity and identity. doi:10.1371/journal.pone.0102757.g001](#)

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three transcript variants and PtrMAX1b has four transcript variants. The primary variants (e.g., Potri.006G226700.1 and Potri.018G062100.1) were used for subsequent analysis. PtrMAX1a showed approximately 84% similarity and 69% identity with AtMAX1 at the amino acid level. PtrMAX1b showed approximately 85% similarity and 69% identity with AtMAX1 at the amino acid level. PtrMAX1a and PtrMAX1b each other shared approximately 96% similarity and 90% identity at the amino acid level. The number of amino acids of PtrMAX1a (529 aa) and PtrMAX1b (529 aa) are comparable to that of AtMAX1 (522 aa). No other Populus proteins showed more than 43% similarity with AtMAX1 at the amino acid level.

The two closest Populus sequence homologues of Arabidopsis MAX2 (AtMAX2) were encoded by loci Potri.014G142600 and Potri.011G066700, designated as PtrMAX2a and PtrMAX2b, respectively (Figure 2). A third homolog, Potri.002G214300 consisting of 316 aa is only half the size of AtMAX2 (693 aa). Thus, it was not included in our further analysis. PtrMAX2a showed approximately 76% similarity and 61% identity with AtMAX2 at the amino acid level. PtrMAX2b showed approximately 71% similarity and 54% identity with AtMAX2 at the amino acid level.
PtrMAX2a and PtrMAX2b each other shared approximately 73% similarity and 57% identity at the amino acid level. The number of amino acids of PtrMAX2a (701 aa) and PtrMAX2b (671 aa) are comparable to that of AtMAX2 (693 aa). No other Populus proteins showed more than 32% similarity with AtMAX2 at the amino acid level.

Arabidopsis MAX3 (AtMAX3) has only one close sequence homologue in Populus encoded by loci Ptri.014G056800, designated as PtrMAX3 (Figure 3). PtrMAX3 showed approximately 77% similarity and 62% identity with AtMAX3 at the amino acid level. The number of amino acids of PtrMAX3 (613 aa) is comparable to that of AtMAX3 (629 aa). No other Populus proteins showed more than 35% similarity to AtMAX3 at the amino acid level.

Arabidopsis MAX4 (AtMAX4) has two close sequence homologues in Populus encoded by loci Ptri.018G044100 and Ptri.006G238500, designated as PtrMAX4a and PtrMAX4b, respectively (Figure 4). Both PtrMAX4a and PtrMAX4b have two transcript variants based on the current annotation by Phytozome. The primary variant (e.g., Ptri.018G044100.1 and Ptri.006G238500.1) were used for subsequent analysis. PtrMAX4a showed approximately 78% similarity and 65% identity with AtMAX4 at the amino acid level. PtrMAX4b showed approximately 78% similarity and 64% identity with AtMAX4 at the amino acid level. PtrMAX4a and PtrMAX4b each other shared approximately 97% similarity and 93% identity at the amino acid level. The number of amino acids of PtrMAX4a (557 aa) and PtrMAX4b (557 aa) are comparable to that of AtMAX4 (570 aa).

Figure 3. Bioinformatic analysis of MAX3 proteins from Populus and Arabidopsis. (A) Amino acid sequence alignment. (B) Phylogenetic analysis of Populus sequence homologues of Arabidopsis MAX3 protein. (C) Amino acid sequence similarity and identity.
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No other *Populus* proteins showed more than 42% similarity to AtMAX4 at the amino acid level.

In total, we identified two *Populus* sequence homologues each for Arabidopsis MAX1, MAX2 and MAX4, and one *Populus* sequence homologue for Arabidopsis MAX3.

Tissue and Organ Expression Patterns of *Populus* MAX Genes

Subsequently, we examined the expression patterns of each *PtrMAX* gene across various tissues and organs. We used qRT to examine the transcript level of each *PtrMAX* gene and normalized their transcript levels against the transcript level of *PtrACT5*. We made three interesting observations. Firstly, each *PtrMAX* gene...
was expressed at different levels with *PtrMAX1a* at the highest level and *PtrMAX4b* at the lowest level in most tissues and organs (Figure 5). Secondly, differential expression in terms of transcript level was observed between *PtrMAX* paralogous genes. For example, *PtrMAX1a* was expressed over 10-fold higher than *PtrMAX1b* across most tissues and organs. Similarly, *PtrMAX4a* was expressed over 10-fold higher than *PtrMAX4b* across most tissues and organs. In contrast, *PtrMAX2a* and *PtrMAX2b* were expressed at similar levels in most tissues and organs. Finally, differences in tissue and organ expression patterns were observed between *PtrMAX* paralogous genes. For example, *PtrMAX4a* was highly expressed in roots but the transcript of *PtrMAX4b* was undetectable in roots (Figure 5).

### Genetic Complementation of Arabidopsis max Mutants with Populus MAX Genes

In order to further test whether *PtrMAX* genes may function similarly as *AtMAX* genes, we conducted genetic complementation studies by heterologously expressing each *PtrMAX* gene in corresponding Arabidopsis *max* mutants. The expression of each *PtrMAX* gene was driven by the constitutive 35S promoter [55]. At least 20 independent transgenic lines were selected from each transformation and two independent transgenic lines were selected for further analysis. We used the number of primary rosette-leaf branches as a phenotype to determine whether heterologous expression of each *PtrMAX* gene could rescue the corresponding Arabidopsis *max* mutants.

For the test of *PtrMAX1* genes, we used a homozygous T-DNA insertional *max1* mutant allele designated as *max1-4* as the transformation background. In this mutant allele, a T-DNA is insertion in the 1st exon, 74 bp downstream of the start codon of MAX1 (Figure S1). RT-PCR analysis indicated that the full-length transcript of MAX1 was absent in *max1-4* mutant, suggesting that it is likely a loss-of-function allele (Figure S1). Consistent with the RT-PCR result, *max1-4* mutants displayed high number of primary rosette-leaf branches (Figure S1). For the complementation, the full-length ORF of *PtrMAX1a* and *PtrMAX1b* was each cloned into the binary plant expression vector pGWB502V (2×CaMV35SΩ) [50] and transformed into *max1-4* mutant background via *Agrobacterium*-mediated transformation. RT-PCR analysis indicated that in the transgenic lines (lines #7 and #8 for *PtrMAX1a*, and lines #2 and #6 for *PtrMAX1b*), *PtrMAX1a* or *PtrMAX1b* transgene was expressed in the Arabidopsis *max1-4* mutant background (Figure 6A, B). We counted the number of primary rosette-leaf branches and compared it with that of Columbia-0 (Col-0) wild type and...
max1-4 mutants which were grown side-by-side with transgenic lines under identical conditions. We found that the number of primary rosette-leaf branches in the transgenic lines was largely reverted to a similar number as in the wild type. Under our growth conditions, Col-0 produced about two primary rosette-leaf branches whereas max1-4 mutants produced about nine primary rosette-leaf branches. The PtrMAX1a transgenic lines #7 and #8 produced approximately two primary rosette-leaf branches and PtrMAX1b transgenic lines #2 and #6 produced about four primary rosette-leaf branches (Figure 6C). These results indicated that both PtrMAX1a and PtrMAX1b were able to complement or partially complement the shoot branching phenotypes of Arabidopsis max1-4 mutant.

For the test of PtrMAX2 genes, we used a T-DNA insertional max2 mutant allele, max2-4, as the transformation background. The max2-4 allele had been previously described by Umehara et al. [2] and Ha et al. [56] but the genotyping and branching phenotype were not reported. These data are provided here as supplemental materials (Figure S2). In this mutant allele, a T-DNA insertion site was found within the 1st exon. RT-PCR analysis indicated that the full-length transcript of MAX2 was absent in max2-4 mutant, suggesting that it is likely a loss-of-function allele. Consistent with the RT-PCR result, max2-4 mutants displayed more shoot branches (Figure S2). For the complementation, the full-length ORF of PtrMAX2a and PtrMAX2b was each cloned into the binary plant expression vector pGWB502Ω and transformed into max2-4 mutant background (Figure 7). Compared with max2-4 mutants, the number of primary rosette-leaf branches in the transgenic lines was significantly reduced and in line #4 of PtrMAX2a, the number was reverted to a similar number in the wild type (Figure 7C). These results indicated that both PtrMAX2a and PtrMAX2b were able to complement or partially complement the shoot branching phenotypes of Arabidopsis max2-4 mutant.

For the test of PtrMAX3 gene, we used a T-DNA insertional max3 mutant allele, max3-12, as the transformation background. max3-12 was previously described by Umehara et al. [2] and the genotypic and phenotypic data had been reported by Li et al. [47]. The full-length ORF of PtrMAX3 was cloned into the binary plant expression vector pGWB502Ω and transformed into max3-12 mutant background. RT-PCR analysis indicated that in the transgenic lines (lines #2 and #4), PtrMAX3 transgene was expressed in the Arabidopsis max3-12 mutant background (Figure 8). Compared with max3-12 mutants, the number of primary rosette-leaf branches in the transgenic lines was significantly reduced but was not completely reverted to wild-type branch number (Figure 8C). These results indicated that PtrMAX3 was able to partially complement the shoot branching phenotypes of Arabidopsis max3-12 mutant.

For the test of PtrMAX4 genes, 35S:PtrMAX4a and 35S:PtrMAX4b transgenic lines were generated using Arabidopsis max4-1 mutant [31] as background. RT-PCR analysis indicated that in the 35S:PtrMAX4 transgenic lines, PtrMAX4a (lines #4 and #5) or PtrMAX4b (lines #3 and #9) transgene was expressed (Figure 9). Compared with max4-1 mutants, the numbers of primary rosette-leaf branches were significantly reduced in these transgenic lines but were not completely reverted to wild-type
branch number (Figure 9C). These results indicated that both PtrlMAX4a and PtrlMAX4b were able to partially complement the shoot branching phenotype of Arabidopsis max4 mutants.

Discussion

SLs are a new class of plant hormones controlling shoot branching. Due to the importance of shoot branching in the determination of photosynthetic light use efficiency and biomass yield in woody perennial trees, we wanted to examine whether the strigolactone pathway is conserved in the woody model plant Populus. We report the identification of sequence homologues of four founding members of SL pathway genes, namely MAX1, MAX2, MAX3 and MAX4. Furthermore, we provide genetic evidence that these Populus MAX genes likely function similarly in controlling shoot branching.

Due to recent genome duplication, in general, each Arabidopsis gene can have two paralogous genes in Populus. As such, we found two Populus paralogous genes for MAX1, MAX2, MAX3 and MAX4. Furthermore, we provide genetic evidence that these Populus MAX genes likely function similarly in controlling shoot branching.

For the complementation tests, we found that all PtrlMAX genes were able to rescue or partially rescue the shoot branching phenotypes of corresponding Arabidopsis max mutants (Figure 6, Figure 9), suggesting that both paralogous genes could function similarly. The expression analysis also indicated that PtrlMAX3 and PtrlMAX4a were expressed at the highest level in roots (Figure 5). These results are consistent with the view that roots represent one of the major biosynthesis sites for SLs (reviewed by [25]). This has been demonstrated in a grafting experiment where grafting wild-type Arabidopsis rootstocks to max1, max3, or max4 scions were able to restore a wild-type branching pattern to the mutant shoots [30].

For the complementation tests, we found that all PtrlMAX genes were able to rescue or partially rescue the shoot branching phenotypes of corresponding Arabidopsis max mutants (Figures 6, 7, 8 and 9). For PtrlMAX1 and PtrlMAX2, we could identify transgenic lines in which the numbers of primary rosette-leaf branches were completely reverted to wild-type level (Figure 6, Figure 7), suggesting that PtrlMAX1 and PtrlMAX2 may function equivalently to Arabidopsis MAX1 and MAX2, respectively. On the other hand, although the numbers of primary rosette-leaf branches in PtrlMAX3 and PtrlMAX4 transgenic lines were significantly reduced when compared with Arabidopsis max3 and max4 mutants, respectively, the numbers were not completely reverted to wild-type level. There are several possible explanations. Firstly, the expression level of PtrlMAX3 and PtrlMAX4 in the transgenic lines may not be high enough to render complete rescue. Secondly, PtrlMAX3 and PtrlMAX4 may function similarly, but not equivalently, as Arabidopsis MAX3 and MAX4, respectively. PtrlMAX3 showed approximately 77% similarity and 62% identity with MAX3 (Figure 3) and PtrlMAX4 showed...
approximately 78% similarity and 65% identity with AtMAX4 at the amino acid level (Figure 4). It is unclear whether differences in amino acid sequence between \textit{Populus} and \textit{Arabidopsis} MAX3 and MAX4 may have contributed to such partial rescue phenotypes in the transgenic lines. Finally, it is also possible that there may be other MAX3 and MAX4 homologues in \textit{Populus} though no other \textit{Populus} proteins showed more than 35% similarity with AtMAX3 and no other \textit{Populus} proteins showed more than 42% similarity with AtMAX4 at the amino acid level. This deserves further investigation.

In addition to \textit{MAX} genes, several other genes are involved in SL biosynthesis or signaling such as D27 [33,34] and D14 [36,38–

![Figure 8. Genetic complementation of \textit{Arabidopsis} max3 mutants with \textit{Populus} MAX3 gene. (A) RT-PCR analysis of 35S:PtrMAX3 transgenic lines. (B) Number of primary rosette-leaf branches. Shown are average numbers of primary rosette-leaf branches from at least 10 individual plants ± S.E., * significant difference from max3-12, p<0.05. doi:10.1371/journal.pone.0102757.g008](image-url)
It would be helpful to identify sequence homologs of each of those genes in *Populus* and conduct genetic complementation studies similar to what have been described in this study in order to strengthen the view that strigolactone pathway exists and operates in the woody perennial plant *Populus*. Our preliminary analysis indicated sequence homologues of AtD14 (encoded by locus At3g03990) are present in the genome of *Populus*. For example, two proteins encoded by locus Potri.002G118900 and Potri.014G016500 showed about 89% and 88% similarity with AtD14 at the amino acid level. Our preliminary analysis indicated that sequence homologues of AtD27 (encoded by locus At1g03055) are also present in the genome of *Populus*. For example, a protein encoded by locus Potri.005G216400 showed about 59% similarity with AtD27 at the amino acid level. In order to fully assign SL pathway in *Populus*, future studies should focus on the functional characterization of these SL pathway genes (e.g. generating *Populus* transgenic lines), the determination of SL molecules, biosynthetic intermediates and derivatives, and the determination of the physiological roles of SLs in *Populus*.

In summary, this study provided a preliminary characterization of strigolactone pathway genes in the model woody plant *Populus*. Sequence homologues of those four founding members of Arabidopsis SL pathway genes have been identified in *Populus*. Gene expression analysis indicated that *Populus* MAX paralogous genes are differentially expressed across various tissues and organs. Genetic complementation studies indicated *Populus* MAX genes are able to complement or partially complement the shoot branching phenotypes of corresponding Arabidopsis *max* mutants. These findings lay a foundation for further characterization of SL pathway and its functions in woody perennial plants.

**Figure 9. Genetic complementation of Arabidopsis *max* mutants with *Populus* MAX genes.** (A) RT-PCR analysis of 35S:PtrMAX4a transgenic lines. (B) RT-PCR analysis of 35S:PtrMAX4b transgenic lines. (C) Number of primary rosette-leaf branches. Shown are average numbers of primary rosette-leaf branches from at least 10 individual plants ± S.E. *, significant difference from *max*-1, p<0.05.

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**Supporting Information**

**Figure S1** Arabidopsis *max1-4* mutant. (A) T-DNA insertion site. (B) PCR genotyping. (C) RT-PCR analysis. (D) Shoot branching phenotypes. (E) Number of primary rosette-leaf branches. Shown are average numbers of primary rosette-leaf branches from at least 10 individual plants ± S.E. *, significant difference from Col-0, p<0.05.

(TIF)

**Figure S2** Arabidopsis *max2-4* mutant. (A) T-DNA insertion site. (B) PCR genotyping. (C) RT-PCR analysis. (D) Shoot branching phenotypes. (E) Number of primary rosette-leaf branches. Shown are average numbers of primary rosette-leaf branches from at least 10 individual plants ± S.E. *, significant difference from Col-0, p<0.05.

(TIF)

**Table S1** A list of primers used in this study.

(DOC)

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Author Contributions

Conceived and designed the experiments: OC JY XW. Analyzed the data: OC JY XW J-GC.

References

1. Gomez-Roldan V, Ferma S, Brewer PB, Puech-Pages V, Dan EA, et al. (2008) Strigolactone inhibition of shoot branching. Nature 455: 189–194.
2. Umehara M, Hanada A, Yoshida S, Akiyama K, Araki T, et al. (2008) Inhibition of shoot branching by new terpenoid plant hormones. Nature 455: 195–200.
3. Akiyama K, Matsuzaki K, Hayashi H (2005) Plant strigolactones induce rhizobial branching in arbuscular mycorrhizal fungi. Nature 435: 824–827.
4. Besserer A, Puech-Pages V, Kiefer P, Gomez-Roldan V, Jannou A, et al. (2006) Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. Plant Biol 8: 1239–1247.
5. Yoneyama K, Xie XN, Kasimoto D, Sekimoto H, Sugimoto Y, et al. (2007) Nitrogen deficiency as well as phosphorus deficiency in sorghum promotes the production and exudation of 5-deoxystrigol, the host recognition signal for arbuscular mycorrhizal fungi and root parasita. Plant Cell Physiol 48: 125–132.
6. Yoneyama K, Yoneyama K, Takeuchi Y, Sekimoto H (2007) Phosphorus deficiency in red clover promotes exudation of orobanchol, the signal for mycorrhizal symbionts and germination stimulant for root parasita. Plant Physiol 143: 1031–1038.
7. Lopez-Raez JA, Charnikovitcha T, Gomez-Roldan V, Matsuova R, Kohlen W, et al. (2008) Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytol 180: 874–883.
8. Umehara M, Hanada A, Magome H, Takeda-Kamiya N, Yamaguchi S (2010) Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice. Plant Cell Physiol 51: 1118–1126.
9. Kohlen W, Charnikovitcha T, Liu Q, Bours R, Domagalska MA, et al. (2011) Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host Arabidopsis. Plant Physiol 155: 974–987.
10. Mauclerc-Gail E, van den Ouwehand C, Goormachtig S, Berekema T, Vuytrole M, et al. (2012) Strigolactones are involved in root response to low phosphate conditions in Arabidopsis. Plant Physiol 160: 1329–1341.
11. Yoneyama K, Xie XN, Kim HI, Kusumoto D, Sekimoto H, et al. (2012) How do nitrogren and phosphorus deficiencies affect strigolactone production and exudation? Plant Cell Physiol 53: 1175–1187.
12. Brewer PB, Kohli H, Beveridge CA (2013) Diverse roles of strigolactones in plant development. Mol Plant 6: 16–28.
13. Czarnecki O, Yang J, Weston DJ, Tuskan GA, Chourey K, et al. (2013) A dual role of strigolactones in phosphate absorption and utilization in plants. Int J Mol Sci 14: 7681–7701.
14. Foo E, Yoneyama K, Hugill CJ, Quittenden LJ, Reid JB (2013) Strigolactone and the regulation of pea symbioses in response to nitrate and phosphate deficiency. Mol Plant 6: 141–152.
15. Beveridge CA, Kyozuka J (2010) New genes in the strigolactone-related shoot branching pathway. Curr Opin Plant Biol 13: 54–59.
16. Xie XN, Yoneyama K, Yoneyama K (2010) The strigolactone story. Annu Rev Phytopathol 48: 93–117.
17. Domagalska MA, Leyser O (2011) Signal integration in the control of shoot branching. New Phytol 190: 545–549.
18. Wang YH, Li JY (2011) New genes in the strigolactone-related shoot branching pathway. Curr Opin Plant Biol 14: 94–99.
19. Seto Y, Kamesaka H, Yamaguchi S, Kyozuka J (2012) Recent advances in strigolactone research: chemical and biological aspects. Plant Cell Physiol 53: 1843–1853.
20. Tsuchiya Y, McCourt P (2012) Strigolactones as small molecule communicators. Mol Biotechnol 48: 464–469.
21. Mason MG (2015) Emerging trends in strigolactone research. New Phytol 190: 975–977.
22. de Saint Germain A, Boutron S, Boyer FD, Rameau J, et al. (2014) Novel insights into strigolactone distribution and signalling. Curr Opin Plant Biol 15: 383–389.
23. Rasmussen A, Depuydt S, Goormachtig S, Geelen D (2013) Strigolactones fine-tune the root system. Planta 238: 615–626.
24. Waters MT, Nelson DC, Scafidi A, Flematti GR, Sun YKM, et al. (2012) Specialisation within the DWF4 protein family confers distinct responses to strigolactones and strigolactones in Arabidopsis. Development 139: 1293–1295.
25. Hamiaux C, Drummond RS, Janssen BJ, Ledger SE, Cooney JM, et al. (2012) DADD2 is an alpha/beta hydrolase likely to be involved in the perception of the plant branching hormone, strigolactone. Curr Biol 22: 1032–1036.
26. Nakamura H, Nao YL, Murashige T, Yoneoka T, Fuku H, et al. (2013) Molecular mechanism of strigolactone perception by DWF4. Nat Commun 4: 2613.
27. Jiang L, Liu X, Xiong G, Liu H, Chen F, et al. (2013) DWF4 acts as a repressor of strigolactin signalling in rice. Nature 504: 401–405.
28. Zhou F, Liu X, Zhu L, Ren Y, Zhou K, et al. (2013) Strigolactone/MAK2-induced degradation of D35 regulates strigolactone signalling. Nature 504: 406–410.
29. Wang Y, Sun Z, Zhu W, Jia K, Yang H, et al. (2013) Strigolactone/MAK2-induced degradation of brassinosteroid transcriptional effector BES1 regulates shoot branching. Dev Cell 27: 601–618.
30. Ward SP, Salmon J, Hanley SJ, Karp A, Leyser O (2013) Using Arabidopsis to study shoot branching in biomass willow. Plant Physiol 162: 800–811.
31. Salmon J, Ward SP, Hanley SJ, Leyser O, Karp A (2014) Functional screening of willow alleles in Arabidopsis combined with qTL mapping in willow (Salix) identifies SxMAX4 as a coppering response gene. Plant Biotechnol J 12: 480–491.
32. Tuukkan S, Difazio S, Jansson S, Bohlmann J, Grigoriev I, et al. (2006) The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). Science 315: 1596–1604.
33. Li Z, Czarnecki O, Chourey K, Yang J, Tuskan GA, et al. (2014) Strigolactone-repressed proteins revealed by iTRAQ-based quantitative proteomics in Arabidopsis. J Proteome Res 13: 1359–1372.
34. Murashige T, Skoog F (1962) A revised medium for growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–477.
35. Goodstein DM, Shiu S, Howson R, Neupane R, Hayes RD, et al. (2012) Phytozeome: a comparative platform for green plant genomics. Nucleic Acids Res 40: D1178–1186.
36. Nakagawa T, Suzuki T, Murata S, Nakamura S, Hino T, et al. (2007) Improved gateway binary vectors: High-performance vectors for creation of fusion constructs in Transgenic analysis of plants. Biosci, Biotechnol, Biochem 71: 2095–2100.
37. Holsters M, Silva B, Van Vliet F, Genetello C, De Block M, et al. (1989) The functional organization of the nopaline A. tumefaciens plasmid pTiC58. Plasmid 21: 212–230.
38. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743.
39. Arvidson S, Kwasniewski M, Riano-Pachon DM, Mueller-Roeber B (2008) QuantiPrime - a flexible tool for reliable high-throughput primer design for quantitative PCR. BMC Biotechnol 8: 46.
40. Lamesch P, Bernardini TZ, Li D, Swarbrick D, Wilks C, et al. (2012) The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. Nucleic Acids Res 40: D1202–D1207.
41. Bennett PN, Chua NH (1999) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 115: 939–950.
42. Han CV, Leyva-Gonzalez MA, Osakabe Y, Tran UT, Nishiyama R, et al. (2014) Positive regulatory role of strigolactone in plant responses to drought and salt stress. Proc Natl Acad Sci U S A 111: 851–856.