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Characterisation of the willow phenylalanine ammonia-lyase (*PAL*) gene family reveals expression differences compared with poplar

Femke de Jong [a,*], Steven J. Hanley [a], Michael H. Beale [b], Angela Karp [a]

[a] AgroEcology Department, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom
[b] Plant Biology and Crop Sciences Department, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

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ABSTRACT

Willow is an important biomass crop for the bioenergy industry, and therefore optimal growth with minimal effects of biotic and abiotic stress is essential. The phenylpropanoid pathway is responsible for the biosynthesis of not only lignin but also of flavonoids, condensed tannins, benzenoids and phenolic glycosides which all have a role in protecting the plant against biotic and abiotic stress. All products of the phenylpropanoid pathway are important for the healthy growth of short rotation cropping species such as willow. However, the phenylpropanoid pathway in willow remains largely uncharacterised. In the current study we identified and characterised five willow phenylalanine ammonia-lyase (PAL) genes, which encode enzymes that catalyse the deamination of L-phenylalanine to form *trans*-cinnamic acid, the entry point into the phenylpropanoid pathway. Willow *PAL1*, *PAL2*, *PAL3* and *PAL4* genes were orthologous to the poplar genes. However no orthologue of *PAL5* appears to be present in willow. Moreover, two tandemly repeated *PAL2* orthologues were identified in a single contig. Willow PALs show similar sub-cellular localisation to the poplar genes. However, the enzyme kinetics and gene expression of the willow PAL genes differed slightly, with willow *PAL2* being more widely expressed than its poplar orthologues implying a wider role for PALs in the production of flavonoids, condensed tannins, benzenoids, and phenolic glycosides, in willow.
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1. Introduction

The two main genera that comprise the Salicaceae, *Salix* (willows) and *Populus* (poplars and aspens), produce a wide range of secondary metabolites, of which those of the phenylpropanoid pathway are both abundant and diverse (Boeckler et al., 2011; Tsai et al., 2006). In addition to the promise shown by short rotation coppice willow as a fast-growing, dedicated biomass feedstock alternative to fossil fuels, this wide range of secondary products in this crop has the potential to be exploited by chemical and natural product industries (Karp, 2013; Karp et al., 2011). The phenylpropanoid pathway produces both the well-studied flavonoids, condensed tannins, and lignin, as well as the lesser studied benzenoids and phenolic glycosides (Babst et al., 2010; Boeckler et al., 2011; Shi et al., 2010, 2013; Tsai et al., 2006; Vogt, 2010). The entry point from primary metabolism into phenylpropanoid metabolism is the deamination of L-phenylalanine by phenylalanine ammonia-lyase (PAL, EC.4.3.1.24) to form *trans*-cinnamic acid (Vogt, 2010). PAL enzyme activity determines the flux through the phenylpropanoid pathway and the rate of phenylpropanoid production (Bate et al., 1994; Wang et al., 2014). Therefore, a better understanding of willow PAL expression and activity will aid the breeding and selection of willows for bioenergy and chemical product end-uses.

PAL is regulated developmentally and environmentally by transcriptional regulation through MYB, LIM and NTS transcription factors (Zhao and Dixon, 2011; Zhong and Ye, 2007). In addition, post-transcriptional regulation has been observed. In French bean phosphorylation of recombinant poplar PtrPAL by endogenous PAL-kinase was demonstrated to reduce PtrPAL stability (Allwood et al., 1999). Moreover, it was recently shown that in Arabidopsis, PAL activity is regulated post-transcriptionally through ubiquitination by Kelch repeat F-box (KFB) proteins to target PAL for degradation (Zhang et al., 2013). It has also been proposed that the flux through the phenylpropanoid pathway is regulated via metabolic channelling. For example, in *Nicotiana tabacum*, metabolic channelling towards lignin production occurs,

Abbreviations: 4CL, 4-coumarate-CoA ligase; C4H, *trans*-cinnamate 4 monooxygenase; KFB, Kelch repeat F-box; PAL, phenylalanine ammonia-lyase; SNP, single-nucleotide polymorphism; YFP, yellow fluorescent protein.

* Corresponding author.
E-mail addresses: femke.dejong@rothamsted.ac.uk (F. de Jong), steve.hanley@rothamsted.ac.uk (S.J. Hanley), mike.beale@rothamsted.ac.uk (M.H. Beale), angela.karp@rothamsted.ac.uk (A. Karp).

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in which NtPAL1 shows coupling with *N. tabacum trans*-cinnamate 4-monooxygenase (NtC4H), which catalyses the next step in the pathway towards lignin (Achnine et al., 2004; Rasmussen and Dixon, 1999).

PAL encoding genes are generally well studied and are commonly found as small gene families comprising one to five members (Cochrane et al., 2004; Huang et al., 2010; Rawal et al., 2013; Reichert et al., 2009; Tsai et al., 2006), although in some plants such as in Eucalyptus (Carocha et al., 2015) and watermelon (Dong and Shang, 2013) the *PAL* gene family is larger than five members. The encoded proteins form a homo- or heterotetramer and the different *PAL* genes are thought to be involved in different branches of the phenylpropanoid pathway (Cochrane et al., 2004; Reichert et al., 2009; Tsai et al., 2006), an assumption now confirmed for the poplar *PAL* gene family (Kao et al., 2002; Shi et al., 2013; Tsai et al., 2006).

The *PAL* gene family in poplar (*Populus trichocarpa*) consists of five genes (*PtrPAL1–5*), which are separated into two groups by phylogenetic analysis (Tsai et al., 2006). Members of group A (*PtrPAL2*, *4* and *5*) are mainly expressed in xylem and root tips while group B genes (*PtrPAL1* and *3*) are more widely expressed (Tsai et al., 2006). In poplar this clear difference in expression of the *PAL* genes, combined with the co-localization of 4-coumarate-CoA ligase 2 (Ptr4CL2) and condensed tannins with PtrPAL1 suggests that, PtrPAL1 and 3 are predominantly responsible for the production of condensed tannins, flavonoids and other phenol metabolites, whereas PtrPAL2, 4 and 5 are predominantly responsible for the production of lignin (Kao et al., 2002; Tsai et al., 2006; Shi et al., 2013).

In contrast, in Arabidopsis, which has 4 *PAL* genes, *AtPAL1* and *AtPAL2* are predominantly expressed in most tissues with both *AtPAL3* and *4* expressed at lower levels (Cochrane et al., 2004). Using single, double, triple and quadruple *atpal* mutants, Huang et al. (2010) showed that there is redundancy in the role of individual AtPAL proteins. Given that all AtPAL proteins have a redundant role in the production of lignin and benzenoids, and that both AtPAL1 and 2 were shown to have a redundant role in flavonoid production (Huang et al., 2010), the different roles for individual PALs may not always conform to those suggested from the poplar studies.

Willow is an important biomass crop for the heat and power industries but could also be a potential feedstock for biofuels and other industrial products (Karp, 2013; Karp et al., 2011). Optimal growth is therefore essential. However, trees are perennial with long life cycles and are subject to continual environmental stresses for which they need protection. PAL activity determines the flux through the phenylpropanoid pathway contributing both to the production of lignin for growth, as well as the production of flavonoids, condensed tannins, and phenol glycosides for protection against high UV, visible radiation herbivores and pathogens (Karabourniotis et al., 2014; Tsai et al., 2006). In this respect, it is a key gene of interest with respect to the breeding of willows. To investigate PAL activity in willow we identified five *PAL* genes from the common osier, *Salix viminalis* L., a species frequently used in biomass breeding programmes, through homology searches with poplar *PAL* genes. Subsequently, we analysed, expression patterns, recombinant protein activity and subcellular localisation. Our results show that even though they are closely related to PtrPALs, willow PALs (SvPALs) display some differences in gene regulation and enzyme activity.

2. Results

2.1. Cloning phenylalanine ammonia lyase

Homology searches using *PAL* gene sequences identified putative *S. viminalis* homologues for four of the five poplar *PAL* genes, which were named according to their poplar orthologues, *SvPAL1* for *PtrPAL1* (Potri.006G126800; 95.7% nucleotide identity), *SvPAL2* for *PtrPAL2* (Potri.008G038200; 95.2% identity), *SvPAL3* for *PtrPAL3* (Potri.016G091100; 95.9% identity), and *SvPAL4* for *PtrPAL4* (Potri.010G224100; 93.5% identity). No orthologue was detected for *PtrPAL5* (Potri.010G224200). In addition, two tandemly repeated copies of *SvPAL2* were detected on a single willow contig (100% nucleotide identity) and were named *SvPAL2–1* and *SvPAL2–2*. For all *S. viminalis PAL* genes, orthologues were found in the recently released *Salix purpurea* genome (97–99% homology). Consistent with *S. viminalis*, no putative *S. purpurea* orthologue of *PtrPAL5* was identified. In contrast, to *S. viminalis SvPAL2–1* and *SvPAL2–2*, in *S. purpurea SpPAL2–1* and *SpPAL2–2* do not share 100% nucleotide identity, with one SNP resulting in an early stop codon in SpPAL2–1 amino acid sequence (S-Fig. S2, Online Resource 2). The genes encoding *SvPAL* were between 2160 and 2136 bases long encoding for proteins between 711 and 719 amino acids with calculated molecular weights between 77.6 and 77.9 kDa and calculated pI values ranging between 6.03 and 6.56 (Table 1). To confirm the calculated molecular weight an SDS–PAGE gel analysis was done with purified recombinant 6His-SvPAL protein, showing two bands of around 90 and 76 kDa, respectively (Fig. 1), likely responding to 6His-SvPAL (band 1) and SvPAL (band 2). SvPALs shared between 70–84% amino acid sequence identity with those from Arabidopsis and 81–85% amino acid sequence identity with tobacco (*N. tabacum*). Phylogenetic analysis of the corresponding amino acid sequences showed that the SvPALs cluster together with SpPALs and PtrPALs in two distinct groups (Fig. 2), the first (group B) comprising Sv/Sp/PtrPAL 1 and 3, along with AtPAL1 and AtPAL3 and the second (group A) comprising Sv/Sp/PtrPAL2 and 4 and PtrPAL5.

To further compare the poplar and willow *PAL* genes, the expression of the *SvPAL* genes was analysed in young leaves, stem, phloem, xylem, mature fully expanded leaves and roots. Due to the 100% nucleotide identity of *SvPAL2–1* and *SvPAL2–2*, gene expression of these two genes could not be analysed separately. As shown in Fig. 3, qRT-PCR analysis showed that *SvPAL1*, *2*, and 3 are highly expressed in roots and to a lesser extent in young and mature leaves. *SvPAL1*, *2* and *3* were expressed at a similar, low level in phloem tissue. However in xylem and stem tissue *SvPAL2* gene expression was roughly twice that of for *SvPAL1* and *3*. Expression of *SvPAL4* was much lower than the other willow PAL genes.

To confirm PAL kinetic activity and subcellular localisation, full-length *SvPALs* were cloned using primers designed to homologous willow sequences. *SvPAL1*, *2* and *3* were successfully cloned from cDNA of willow shoot-tip (comprising leaves and stem). As *SvPAL4* expression was only detected in young leaves, cDNA from willow young leaves was generated for cloning *SvPAL4*. However,

Table 1
SvPAL protein properties. The kinetic parameters (±standard deviation), with L-phenylalanine as substrate, were calculated from three separate measurements with three technical repeats each.

Isozyme	SvPAL1	SvPAL2	SvPAL3	SvPAL4
CDS length	2160	2136	2148	2136
Exon	2	2	2	2
Intron length	741	1088	794	709
aa length	716	711	715	711
Predicted molecular weight (Da)	77.885	77.473	77.842	77.659
pI	6.56	6.43	6.03	6.19
Enzyme kinetics done at pH	8.8	8.8	9.0	
Kinetics				
K_m (μM)	81.6 ± 0.30	32.35 ± 0.50	88.41 ± 0.55	
V_{max} (pkat/μg protein)	15.5 ± 0.43	7.13 ± 0.43	27.16 ± 1.32	
k_{cat} (s^{-1})	22.79	8.35	28.98	
k_{cat}/K_m (s^{-1} M^{-1})	279,495	258,242	327,748	

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