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Lignin and lignans in plant defence: Insight from expression profiling of cinnamyl alcohol dehydrogenase genes during development and following fungal infection in *Populus*

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ABSTRACT

Cinnamyl alcohol dehydrogenase (CAD) catalyses the final step in the biosynthesis of monolignol, the main component of lignin. Lignins, deposited in the secondary cell wall, play a role in plant defence against pathogens. We re-analysed the phylogeny of *CAD/CAD-like* genes using sequences from recently sequenced genomes, and analysed the temporal and spatial expression profiles of *CAD/CAD-like* genes in *Populus trichocarpa* healthy and infected plants. Three fungal pathogens (*Rhizoctonia solani*, *Fusarium oxysporum*, and *Cytospora* sp.), varying in lifestyle and pathogenicity, were used for plant infection. Phylogenetic analyses showed that *CAD/CAD-like* genes were distributed in classes represented by all members from angiosperm lineages including basal angiosperms and *Selaginella*. The analysed genes showed different expression profiles during development and demonstrated that three genes were involved in primary xylem maturation while five may function in secondary xylem formation. Expression analysis following inoculation with fungal pathogens, showed that five genes were induced in either stem or leaves. These results add further evidence that *CAD/CAD-like* genes have evolved specialised functions in plant development and defence against various pest and pathogens. Two genes (*PoptrCAD11* and *PoptrCAD15*), which were induced under various stresses, could be treated as universal markers of plant defence using lignification or lignan biosynthesis.

1. Introduction

Lignin is one of the most abundant biopolymers in nature after cellulose; approximately 30% of the organic carbon in the biosphere is in the form of lignin [1]. Lignin or lignin-like compounds are a major component of the secondary cell walls of vascular plants, including pteridophytes, some mosses, and algae [2,3]. Lignins are usually classified into three types (G, H, and S lignin), based on their content of monomer phenylpropanoid units [4]. The amount of lignin and its composition vary among cell types, taxa, and even within the same cell [5].

The pathway of lignin biosynthesis involves many enzymes, one of which is cinnamyl alcohol dehydrogenase (CAD). CAD is a multifunctional enzyme that catalyses the final reduction of cinnamylaldehydes to their corresponding alcohols. *CAD/CAD-like* genes have already been identified and studied in model species including *Arabidopsis thaliana* [6–9], *Oryza sativa* [10], and *Populus* spp. [11,12]. Various *CAD/CAD-like* homologs were also identified in non-model herbaceous [13–17], gymnosperms [18–20], and basal plants [21]. The expression profiles of *CAD/CAD-like* genes previously reported showed that the *CAD/CAD-like* genes might have evolved divergent functions. In *Arabidopsis*, CAD/CAD-like gene family is composed of nine genes (*AtCAD1–9*) [8,9]. Functional analyses of these genes in *Arabidopsis* have demonstrated that only two functions in plant development were involved in the biosynthesis of secondary cell walls in the xylem [6–8]. The other genes play a

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minor functional role in the lignification process in the stem and may function in plant defence against biotic and abiotic stresses through lignin and/or lignan biosynthesis.

A similar situation has been reported for the *CAD* gene family in poplar, in which different expression patterns suggest a divergence of function between *CAD/CAD-like* genes [11]. Four of these genes were expressed in mature leaves (*PoptrCAD7*, *PoptrCAD9*, *PoptrCAD12*, and *PoptrCAD13*), whereas only two (*PoptrCAD4* and *PoptrCAD10*) were expressed preferentially in the secondary xylem tissues from the woody stem of mature plants. Several *CAD/CAD-like* genes that were not associated with secondary xylem development in poplar were induced following herbivore damage of leaves; the others are induced or suppressed in different stem tissues [12]. Analysis of the promoter sequences of all poplar *CAD/CAD-like* genes has revealed several motifs that are involved in the modulation of gene expression under various developmental conditions and under biotic and abiotic environmental stresses [11].

While previous studies gave insight into the function of *bona fide CAD* and *CAD-like* genes, little is known on the function of these genes under biotic and abiotic stresses. It remains unclear whether the genes that are induced under herbivore stress [12] are also induced following other biotic stresses. It is also unclear whether the range of stress is crucial or the response is rather universal in the same tissue during specific stage of differentiation and maturation. To address these questions, we analysed the expression profiles of *CAD/CAD-like* genes in *Populus trichocarpa* (Torr. & Gray) during the first three months of development, and monitored the differentiation and maturation of xylem in stems. To check which of the poplar *CAD/CAD-like* genes are involved in defence mechanisms against biotic stresses, plants were inoculated with *Rhizoctonia solani*, *Fusarium oxysporum*, and *Cytospora* sp. These three fungal pathogens were chosen because they present different lifestyle and pathogenicity. The first two species are found worldwide and combine aggressive saprophytic activity with almost unlimited pathogenic capabilities. *Rhizoctonia* causes partial or complete girdling of emerged seedlings at or near the soil surface during the germination and emergence of seedlings [22,23]. In contrast, *Fusarium* isolates are host specific that induce various symptoms including yellowing and drying of leaves, wilting and death of branches, varying degrees of vascular discoloration, and overall plant stress [24,25]. *Cytospora* infects aboveground woody tissue [26–28] and causes plant canker and bark blight. This pathogen occurs commonly worldwide on fruit trees, hardwood, forest and shade trees, shrubs, and conifers.

Here we reanalysed the phylogeny of *CAD/CAD-like genes* from land plants and analysed the expression of poplar *CAD/CAD-like* genes during plant development and following infection with three fungi pathogens. This analysis showed that *CAD/CAD-like* genes present various expression profiles during plant development and following infection with pathogens. Some of them were induced following infection and could be considered as markers of plant defence using lignification and lignans.

2. Materials and methods

2.1. Seed germination and seedling development

P. trichocarpa (Torr. & Gray) seeds originated from United States were bought from FLORPAK Młynki Seeds Store (Poland). The seeds were soaked in water, surface disinfected with 7% Clorox® (v/v) for 5 min, and washed three times (5 min each) in autoclaved distilled water. The seeds were germinated on 0.6% water agar (w/v; Difco, Detroit, MI, USA) in the dark for 48 h. Subsequently, they were transferred aseptically and placed between the walls of 300 × 30 mm glass test tubes and a roll of filter paper (17 × 13 cm in size; paper weight 69 g/m^2) placed inside them. The test tubes containing 100 ml of growth substratum (a sterile mixture of peat and perlite; 1:2, w/w) were moistened with 50 ml of liquid Hoagland's medium (Sigma-Aldrich, Doderich, Germany). Seedlings were incubated for three months in a growing room under light emitted by fluorescent tubes (Osram L36/W77 Flora; 100 μE/m^2/s) with a 16:8 light–dark cycle, 60% relative humidity, and a day to night temperature ratio of 24:20 °C. Material for RNA isolation was collected after 14, 30, 60, and 90 days of plant growth.

2.2. Phylogenetic analyses

CAD/CAD-like sequences used in this study include sequences from non-model and model species [12] including the basal angiosperm *Amborella trichopoda*. *Arabidopsis* CAD1 sequence was used to query the proteome of these species using TBlastN. Sequences that matched the query were retrieved, curated, and aligned using Muscle software [29]. Alignements were manually adjusted before analysis. Phylogenetic analysis was implemented in Phyml software [30] using the TG + G model and 100 bootstrap replicates.

2.3. Poplar plant inoculation with pathogens

Fungi used for plant inoculation were two-week-old mycelia that were grown on malt extract agar (MEA, Merck, Darmstadt, Germany) at 24 °C in the dark. The strains of *F. oxysporum* and *R. solani* were isolated from a forest nursery in Myslibórz Forest District (N 52°51′04″, E 14°51′44″); *Cytospora* originated from a shoot of *Populus* tree grown in Sołacki Park, Poznań (N 52°25′14″, E 16°54′11″). To inoculate poplar seedling, one piece (5 × 5 mm) of 2-week-old mycelial mat of each pathogen was placed into test tubes close to the zone between the shoot and root. Seventy-two hours after inoculation (AI), when symptoms of necrosis were visible, plant material (leaves and stems) was transferred to liquid nitrogen and stored at −80 °C until further use. Similarly, material from non-inoculated seedlings used as control was also collected and stored at −80 °C.

2.4. Stem anatomical analysis

Histological analysis of *P. trichocarpa* samples were performed as described by Bagniewska-Zadworna et al. [31]. The material was fixed immediately in 2% glutaraldehyde and 2% formaldehyde (overnight with one change of solution; pH 6.8; Polysciences, Warrington, PA, USA), rinsed three times with cacodylate buffer (0.05 M; pH 6.8; Polysciences), and dehydrated in a graded ethanol series (10–100%). Samples were embedded in Paraplast Plus (Sigma-Aldrich), sectioned with an HM 340E rotary microtome (Microm, Walldorf, Germany), and double-stained with Safranin O and Fast Green (Sigma-Aldrich). To analyse the xylem formation and the cell wall lignification, the slides were examined by light and fluorescence microscopy, at excitation wavelengths of 365 and 470 nm (Axioscope A1; Carl Zeiss, Jena, Germany).

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated using a modified TRIZOL extraction method as follows. Plant material (100 mg) was ground in liquid nitrogen using a mortar and pestle, transferred to a 1.5-ml Eppendorf tube, and resuspended in 1 ml of TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The samples were then vortexed and incubated at room temperature for 5 min with regular mixing. Cell debris was pelleted by centrifugation at 4 °C for 15 min at 12 000 rpm and the supernatant was extracted with 100 μl of chloroform. After centrifugation, the aqueous phase was recovered and RNA was precipitated with an equal volume of isopropanol for