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The cinnamyl alcohol dehydrogenase family in flax: Differentiation during plant growth and under stress conditions

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ABSTRACT

Cinnamyl alcohol dehydrogenase (CAD), which catalyzes the reduction of cinnamaldehydes to their alcohol derivatives, is represented by a large family of proteins. The aim of the study was to identify the *CAD* isoforms in flax (*Linum usitatissimum* L.) – *LuCADs* – and to determine their specificity to enhance knowledge of the mechanisms controlling cell wall lignification in flax under environmental stresses. On the basis of genome-wide analysis, we identified 15 isoforms (one in two copies) belonging to three major classes of the CAD protein family. Their specificity was determined at the transcriptomic level in different tissues/organs, under *Fusarium* infection and abiotic stresses.

Considering the function of particular *LuCADs*, it was established that *LuCAD1* and *2* belong to Class I and they take part in the lignification of maturing stem and in the response to cold and drought stress. The Class II members *LuCAD3*, *LuCAD4*, *LuCAD5* and *LuCAD6* play various roles in flax being putatively responsible for lignin synthesis in different organs or under certain conditions.

The obtained results indicate that within Class II, *LuCAD6* was the most abundant in seedlings and maturing stems, *LuCAD3* in leaves, and *LuCAD4* in stems. Comparative analysis showed that expression of *LuCAD* genes in roots after *F. oxysporum* infection had the greatest contribution to differentiation of *LuCAD* expression patterns. Surprisingly, most of the analyzed *LuCAD* isoforms had reduced expression after pathogen infection. The decrease in mRNA level was primarily observed for *LuCAD6* and *LuCAD4*, but also *LuCAD1* and *8*. However, the induction of *LuCAD* expression was mostly characteristic for Class I *LuCAD1* and *2* in leaves. For cold stress, a clear correlation with phylogenic class membership was observed. Low temperatures caused induction of *CAD* isoforms belonging to Class I and repression of *LuCADs* from Class III.

1. Introduction

Cinnamyl alcohol dehydrogenase (CAD) is the final enzyme in the monolignols' biosynthetic pathway, and its characteristics and properties are widely described (Barakat et al., 2009; Goffner et al., 1992). In plants, CAD catalyzes the reduction of *p*-coumaricaldehyde, coniferylaldehyde and sinapylaldehyde to their alcohol derivatives which are then polymerized into lignin (Vanholme et al., 2010). *CAD* is one of the most popular genes to manipulate when it comes to obtaining plants with lowered lignin content (Vanholme et al., 2010, 2012). So far, *CAD* isoforms has been identified and described in several model plant species such as sorghum (Sattler et al., 2009), switchgrass (Saathoff et al., 2011), poplar (Barakat et al., 2009), *Arabidopsis* (Sibout et al., 2003), tobacco (Halpin et al., 1994), wheat (Qing-Hu, 2010) and melon (Jin et al., 2014).

In general, the CAD family can be divided into three classes depending on protein evolution, function and structure. The two main classifications presented by Barakat's and Guo's groups, have many similarities but also several noteworthy differences. CADs belonging to Class I, also named *'bona-fide'*, are considered as primary isoforms that are responsible for lignin synthesis (Barakat et al., 2009; Guo et al., 2010; Saathoff et al., 2012). Some proteins from class II are responsible for lignin synthesis in xylem, while others are hypothesized as being involved in plant defense mechanisms. CAD representatives from Class I and III seem to be conserved in terms of expression patterns, enzyme activity and substrate specificity (Guo et al., 2010). Anyway, this

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classification is still flexible and other models have been proposed (Jin et al., 2016; Rong et al., 2016; Saballos et al., 2009).

Many questions remain unanswered. For example, there are only suggestions that Class I refers only to CAD proteins responsible for xylem lignification, whereas representatives of the other two classes take part in the response to biotic and abiotic stresses (Barakat et al., 2011; Baxter and Stewart, 2013; Bukh et al., 2012). Up to now little has been done to at least preliminarily determine the tissue and functional specificity and the role of CAD isoforms which belong to all three classes.

Recently, some studies were announced, but they only touch the topic. For instance, the wheat CAD isoform TaCAD12 takes part in the response to the necrotrophic fungus *Rhizoctonia cerealis* (Rong et al., 2016). The treatment of melon fruits with plant hormones (ethylene (ET), auxin and abscisic acid (ABA)) showed that the expression of certain *CAD* isoforms was induced after the treatment (Jin et al., 2014). Still, it is only the beginning to determine the role and specificity of the different *CAD* isoforms in the lignification process, as well as the molecular background that induces/suppresses lignin biosynthesis in response to environmental factors.

The reason for lignin manipulation is that there is a need for new sources of biomass for the production of biofuels, animal feed, and components for biocomposites, as well as generation of new drugs. Carrying out many important functions in plants, lignin blocks processing of biomass or raw materials which generates a necessity for delignification which is an energy, time-consuming, expensive and polluting process (Baxter and Stewart, 2013; Vanholme et al., 2012). Flax derives raw materials rich in cellulose (shives and straw), oil and in bioactive compounds, making it a highly attractive material in the context of current needs (Preisner et al., 2014a,b). The drawback is that flax has a relatively high content of lignin, which makes it difficult to process. Flax is therefore in our opinion a good subject for research that will not only contribute to increased knowledge about the biosynthesis of lignin but also might diversify flax application.

So far, one complete sequence of flax *CAD* has been published together with its promoter and one partial *CAD* sequence. The first two, were identified by our team; however, no further analyses of their expression profiles or specificity has been conducted (Preisner et al., 2014a,b). This gave us a good start to continue previous studies on *CAD* and lignification in flax and to perform a genome-wide analysis of *CAD* genes in flax (*LuCAD*).

Thus, the main purpose of the study is to identify of *CAD* isoforms in flax and to determine expression profiles in different tissues during flax growth, and under stress conditions. The novelty of the manuscript concerns a combination of bioinformatics and gene expression analysis tools to gather the knowledge about crop plants with potential rapid applications. The molecular background of growth of such plants and development is still relatively poor in comparison to the model plants or grasses.

Recently, Eom and collaborators identified four *CAD* genes in flax and conducted *in silico* analyses of them (Eom et al., 2016). Additionally, they measured their expression only in flax suspension culture cells, seeds, cotyledons and young leaves. However, these results are not meaningful in terms of *LuCAD* specificity during plant growth and development, as stem lignification starts later, around the time when the first true leaves are unfolded, while fiber is lignified after flowering (Preisner et al., 2014a,b).

Here we present altogether 15 isoforms of *CAD* in flax (one in two copies). The specificity of *LuCADs* was verified by determining their relative expression level in different plant tissues during flax growth and development, as well as under abiotic stresses and *Fusarium* infection. In contrast to Eom's group we analyzed expression patterns in *in vitro* cultures and at four stages of flax development *in vivo*: 6-week old plants, 8-week stems, 12-week stems and fiber. The collected data will contribute to increased knowledge about the mechanisms that control lignification under the influence of various biotic and abiotic stresses and will also help to annotate the flax genome (Wang et al., 2012).

2. Material and methods

2.1. Plant material

Linum usitatissimum L. cultivar Nike was used in all experiments. It is a fibrous flax variety relatively resistant to *Fusarium* infection. *In vitro* plants and seedlings were cultured in a Fitotron growth chamber on a basal Murashige and Skoog medium (MS) with 1% sucrose and 0.8% agar, pH 5.8, under stable conditions: temperature 22 °C/16 °C, day/night 16/8 h. Seeds were vernalized for a week in darkness at 4 °C, then sterilized with 96% ethanol and rinsed in sterile distilled water. After transferring onto MS medium, germination was conducted in the darkness for 4 days, and then seedlings were cultured for 8 days in a Fitotron. The whole seedling was used for analysis. For stem and fiber analysis, plants were cultivated in the experimental field of the Genetic Biochemistry Department at the Faculty of Biotechnology, University of Wroclaw in 2015. The tissue was collected from the mid-stem section from 5 plants in the 8th and 12th week after sowing and separated into lignified stem parts (mostly xylem and fiber) and remaining delignified stem residues called throughout the text stem residues. The tissues were stored at −80 °C for further analysis.

2.2. Identification of CAD isoforms

The genome-wide analysis of the flax genome was done using known *CAD* sequences from *Arabidopsis thaliana* (*AtCAD4, AtCAD5, AtCAD9*), *Nicotiana tabacum* (*NtaCAD1*) and *Oryza sativa* (*OsCAD7*) (Wang et al., 2012). Flax genome was screened for *LuCADs* with the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov) and Phtyozyme (https://phytozome.jgi.doe.gov). Mutual comparison of identified scaffolds carrying *CAD* genes to exclude possible repetitions of genes and verification whether the obtained sequences indeed code for *CAD* was performed with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) and NCBI BLAST. The promoter sequences were predicted using the TSSP On-line tool (Using RegSite Plant DB, Softberry Inc. http://www.softberry.com) and PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002; Solovyev et al., 2010). The latter was also used for *in silico* analysis of promoter sequences to identify *cis*-acting regulatory elements. The coding and protein sequences of identified genes were detected using the Fgenesh gene-finder (http://www.softberry.com) (Solovyev et al., 2006).

2.3. Analysis of gene and protein sequences

The theoretical molecular weight (MW) and isoelectric point (pI) of particular proteins were predicted by the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Conservative motifs and residues characteristic for alcohol dehydrogenases were identified using the Motif scan tool (MyHits http://myhits.isb-sib.ch/ and http://web.expasy.org/compute_pi/) and NCBI's Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al., 2015). The visualization of gene structure was predicted using Gene Structure Display Server (GSDS) v2.0 (http://gsds.cbi.pku.edu.cn/) (Hu et al., 2015). Subcellular localization was computed using: subCELlular LOcalization predictor CELLO v.2.5 (http://cello.life.nctu.edu.tw/), the Distill Prediction of Protein Structural Features tool (http://distill.ucd.ie/distill/) and MultiLoc2-HighRes (http://abi.inf.uni-tuebingen.de/Services/MultiLoc2) (Baú et al., 2006; Blum et al., 2009; Boden and Hawkins, 2005). The phylogeny analysis was done on a set of 60 CAD proteins (full list of sequences is presented in Supplementary Data Sheet 1) aligned using the MUSCLE software method, analyzed using PhyML (software based on the maximum-likelihood

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