Journal of Sugarcane Research (2020) **10**, 152-157
https://doi.org/10.37580/JSR.2020.2.10.152-157

RESEARCH ARTICLE

Expression analysis of Cinnamyl Alcohol Dehydrogenase (CAD) involved in lignin biosynthesis of *Erianthus arundinaceus*

Lakshmi Kasirajan *, and Rabisha Valiyaparambath

Division of Crop Improvement, ICAR-Sugarcane Breeding Institute, Coimbatore, 641 007, India.

*Corresponding author: Email: K.Lakshmi@icar.gov.in

(Received 22 October 2020; accepted 25 December 2020)

Abstract
Erianthus arundinaceus has a great potential in bioenergy production and green-house gas mitigation due to its biological characteristic of huge biomass yield and stress tolerance. Our earlier transcriptome studies on a set of sugarcane genotypes differing for lignin content, showed that Cinnamyl Alcohol Dehydrogenase (CAD), was differentially expressed and of plays a major role in genetic regulation of lignin production, stem development and defense. This study aims to characterize the CAD gene and its expression in BL 21 *E. Coli* strain. In this context, understanding the regulatory functions of CAD gene would be more useful for achieving reduced lignin content and to have a cost-effective biomass conversion in sugarcane. Using bioinformatic tools like ExPasy and Prosite, the sugarcane CAD was found to belong to the Class I with affinity towards coniferyl aldehyde, sinapyl aldehyde with a predicted molecular weight of ~46kDa.

Keywords: Monolignols; Lignification; Protein; Secondary cell wall

Introduction

Second-generation ethanol (2GE) is produced by depolymerization of the carbohydrates (cellulose and hemicellulose) present in the plant cell wall (de Souza et al. 2014). Plant biomass mostly contains cellulose, hemicellulose, and lignin, which can be converted to biofuels as a source of renewable energy. Cell wall polymers have received significant attention in recent years because they are the major components in the plant biomass that is under consideration as a source of reduced carbon to partially replace fossil fuels (Henry 2010). Grasses with C_4 metabolism, such as Sugarcane, Erianthus, Sorghum, Miscanthus, and Switch grass, are the most efficient energy crops due to their perennial growth habit, maximize carbon sequestration and biomass accumulation (Hoang et al. 2015). Among these bioenergy crops, *Erianthus arundinaceus*, is considered the potential important genetic resource for bioenergy because of its biological characteristics of high fiber content, multiple pest resistance and abiotic stress tolerance (Hu 2017). Recently an increasing amount of research is now focusing on its biomass characteristics and conversion technologies towards biofuel. In-spite of the significance of Erianthus for bioethanol production, the current understanding about the genetic information on biomass traits is limited.

Lignin is a phenolic biopolymer of complex structure, synthesized by all plants. The biosynthesis of lignin starts with the synthesis of cinnamic acid from phenylalanine by phenylalanine ammonia lyase (PAL) in the cytosol (Lu et al. 2019). The building blocks of lignin are p-coumaryl, coniferyl, and sinapyl alcohols monomeric units, which later undergo dehydrogenative polymerizations by peroxidase (PER) and laccase (LAC) to form p-hydroxyphenyl (H), guaiacyl (G) and syringyl

(S) lignin, respectively (Chen and Dixon 2007). The relative proportion of each lignin unit varies with species, plant parts, and maturity. Cinnamyl alcohol dehydrogenase (CAD) catalyzes the conversion of the corresponding cinnamyl aldehydes to cinnamyl alcohols; this is the ultimate step inside the synthesis of monolignols before their polymerization and deposition in secondary cell wall. Hence CAD is a key player in phenylpropanoid biosynthesis (Baucher et al. 1996), which has been characterized in many plant species, including *Eucalyptus gunnii* (Hawkins and Boudet 1994); strawberry (Blanco-Portales et al. 2002); tobacco (Halpin et al. 1992), *Aralia cordata* (Hibino et al.1993) and poplar (Van Doorsselaere et al. 1995). Based on these reports the characteristic features of CAD vary between gymnosperms and angiosperms. Gymnosperm CAD is encoded by a single gene, and are highly specific on coniferyl aldehyde, than sinapyl aldehyde (O'malley et al. 1992; Galliano et al. 1993), while angiosperm CAD has multiple isoforms and have equal catalytic activity for both coniferyl and sinapyl aldehydes (Brill et al. 1999).

Additionally, the CAD family can be generally divided into three classes. All Class I CAD members, and a bona fide CAD evolutionary branch, are associated with lignin synthesis (Zhang et al. 2006). Class II CADs, (sinapyl alcohol dehydrogenase (SAD), are associated with stress resistance. Class III CADs members may be redundant to Class I and Class III CADs, though their functions remain unclear (Yi et al. 2011). In this study we are particularly interested in CAD gene involved in lignin biosynthesis in *Erianthus arundinaceus,* as it can be grown on marginal lands with limited or no resources for the purpose of biofuel. In our previous study, gene encoding CAD and the coding sequence was isolated from *Erianthus arundinaceus* clone IK 76-81, designated as *EaCAD* (Kasirajan et al. 2020). The nucleotide sequence and the genetic information has been submitted in NCBI gene-bank (MN820707). In our present study, we attempted to express this gene in bacteria (*E. Coli* BL21) since the bacterial gadget is a convenient choice for the initial expression of genes.

Materials and Methods

Genetic material

In our previous study, (Kasirajan et al. 2020) gene encoding CAD and the coding sequence was isolated from *Erianthus arundinaceus* clone IK 76-81, designated as *EaCAD*, which is being used as the genetic material for this study.

Primer designing

The coding region of *EaCAD* gene fragment, which is bound by a start codon (ATG) in the 5' end and a stop codon (TAA) in the 3', end was amplified using gene specific primers

CADCDS Forward Primer: 5'TTGAATTCATGGGGAGCCTGGCGTCC 3' and

CADCDS Reverse primer - 5' TGAAGCTT TCAGTTGCTCGGCGCATC 3'

with *Eco*RI and *Hind*III restriction sites to facilitate directional cloning of *EaCAD* gene into pET-28a. The amplified product was double-digested with *Eco*RI/*Hind*III and the resultant fragment was cloned into the same sites of the pET-28a vector (Novagen). The engineered plasmid after confirmation of the presence of the insert, designated as (pET28a::EaCAD) and transformed into *E. coli* BL21 (DE3) cells (Novagen). Among the transformed colonies, two colonies were selected and grown in 5 ml of Luria bertani (LB) broth containing 100 µg/ml of kanamycin overnight at 37°C. An active culture was raised by inoculating 1% of the overnight grown colonies and grown at 200 rpm until an OD

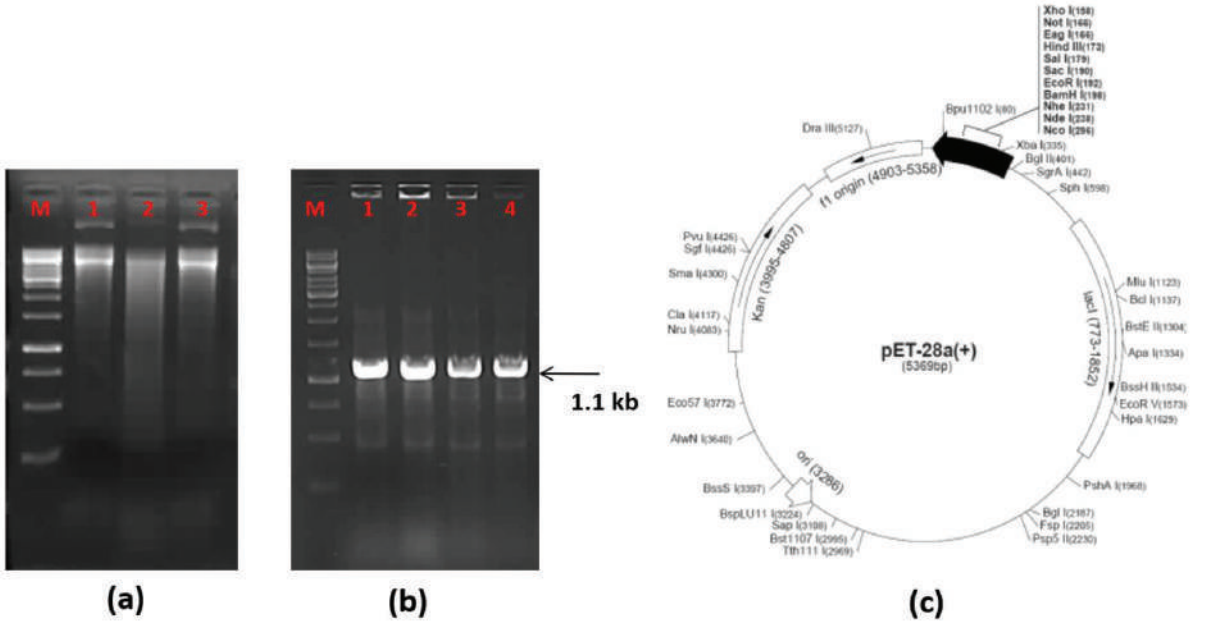

Figure 1 . (a) Plasmid DNA of vector pET 28a. (b) PCR amplification of CAD gene from plasmid DNA of ptz57R::EaCAD. Lane, M-1kb DNA ladder, lane 1-4 Biological replicates of PCR amplification. (c) vector map of pET28a showing the restriction site used for cloning.

of 0.5 at 37°C. Proteins were extracted and then subjected to denaturing SDS-PAGE TRIS-glycine gels (Novex, San Diego, CA). A Kaleidoscope pre-stained standard (Bio-Rad) was used as a molecular weight marker. The gels were stained with Coomassie blue R250.

SDS PAGE Analysis

Isopropyl thio-β-galactoside (IPTG) is a molecular mimic of allolactose which induces protein expression, while in pET28a the gene CAD is under the control of the lac operator. Prior to isopropylthio-β-galactoside (IPTG) induction, 1 ml of un-triggered culture IPTG induced cultures were incubated under continues shaking @ 200 rpm for 4 h at 28ºC for the expression of *COMT.* One ml of un-induced as well as induced cultures of different colonies were centrifuged at 6000 rpm for 15 min and the pellet was resuspended in 100 µl of SDS buffer and heated at 65°C for 5 min and loaded on 15% SDS-PAGE. Electrophoretic run was carried out for 2 h at 100 mA followed by Coomassie brilliant blue staining and destaining. The gel image was scanned for documentation.

Results and Discussion

Plasmid DNA was isolated from the vector

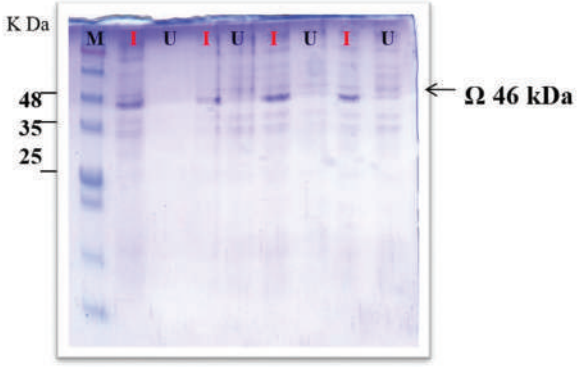

Figure 2. SDS PAGE analysis of CAD proteins isolated from E. coli BL21. Lane M- protein marker, lane 1,3,5,7 - crude proteins of transformed colony 1,2,3,4 of BL 21 cells containing the pET28a vector + CAD gene induced with 1mM IPTG, lane 2,4,6,8 - crude proteins of transformed colony 1,2,3,4 of BL 21 cells containing the pET28a vector + CAD gene un induced.

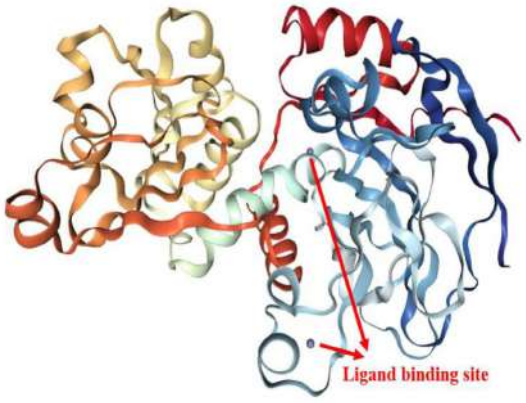

Figure 3. The 3D structure of EaCAD protein showing the ligand binding sites

pt57R::EaCAD by alkaline lysis method and used as the template for amplification of the coding sequence of CAD with gene specific primers and with high fidelity LA-TAQ Polymerase. The expected size of the amplicon (~ 1100 bp) was gel eluted according to the instructions of Qiagen gel elution protocol. The eluted fragment was confirmed through sequencing. Which proved that the *EaCAD* cDNA is a 1.1kb fragment which codes for 365-amino acids. The expression vector pET28a and the eluted fragment (*EaCAD*) was subjected to double digestion with *Eco*RI and *Hind*III for 4 hrs at 37˚C, and ligated with T4DNA ligase in such that the open reading frame of the CAD is unaltered (Fig. 1). The ligation mixture was transformed into BL21 host for protein expression. The SDS PAGE displayed the protein expression pattern of transformed colonies (Fig. 2). A comparative domain analysis with protein sequence of *EaCAD* and sorghum CAD family members showed that the Erianthus CAD is 95% like SbCAD2. While *EaCAD* differed from the other isoforms of CAD by position among different CAD and PROSITE domains distribution throughout the full-length protein sequences sites. The three-dimensional structure of *EaCAD* was predicted with RCSB PDB (http://www.rcsb.org/pdb). It showed a medium chain alcohol dehydrogenases catalytic domain with 195 residues in two fragments and a NAD(P) binding rossmann domain with 157 residues. The results showed two possible binding sites for Zn, which is being highlighted in the 3D model as ligand binding sites (Fig. 3).

Compared to the uninduced samples, induced samples showed a thicker band on the molecular weight of ~46kDa range which was exactly like the predicted molecular weight obtained from the bioinformatics studies. *EaCAD* is similar, to the results reported by (Ma 2010), in which a cDNA encoding a CAD was isolated from wheat, designated as TaCAD1. TaCAD1 was expressed in *E. coli* as a His-tagged protein with a relative molecular mass of about 42.5 kDa. TaCAD1 protein was composed of the catalytic and nucleotide-binding domains, and highly expressed in stem, during later maturation stage. Genome-wide analyses of Arabidopsis, rice, and sorghum showed that CAD-like genes exist in multiple isoforms, and the genetic information on bona fide CAD enzymes involved in lignin biosynthesis are limited. In gymnosperms there is one CAD enzyme (O'malley et al. 1992), while there are two or three CAD isoforms in each dicot species. For example, in Arabidopsis CAD codes for three isoforms (AtCAD5, AtCAD4, and AtCAD1) among which AtCAD1 play a pivotal role in lignin biosynthesis (Sibout et al. 2005). Recent study of Oryza gh2 gene showed that gh2 protein had strong CAD and SAD activities (Zhang et al. 2006), which demonstrated that the same CAD gene products in angiosperm plants can synthesize both coniferyl and sinapyl alcohol. While gymnosperm CAD is highly specific for the degradation of coniferyl aldehyde due to the existence of SAD, which have been proved specifically to participate in the degradation of sinapaldehyde and can hardly be detected in

other angiosperm plants such as Arabidopsis and Oryza (Li et al. 2001). The fact that gymnosperm plants contain mainly G monomers whereas angiosperm plants consist of G and S monomers may be a consequence of this distinction (Baucher et al. 1996), but more specific reasons are underexplored. Our results of *EaCAD* is similar, to that of other CAD genes from angiosperms and hence the protein sequence of CAD from Erianthus might be useful for subsequent research on lignin modification for improved biomass conversion.

Conclusion

Cinnamyl alcohol dehydrogenase (CAD) catalysis the reduction of hydroxyl cinnamaldehydes to the cinnamyl alcohols, prior to their transport and polymerization in the cell wall. Suppression of a single lignin-related CAD gene could result in substantial changes in lignin composition, such as changes in the S:G-ratio and also it has been identified that it activates compensatory mechanism in the fibre cell wall (Sibout et al. 2003). The Loss of function or down-regulation of CAD generally leads to reduction of lignin content with no much loss of mechanical strength, hence, the CAD gene cloned, and the expression characterized in this study can be utilized for gene silencing which affects lignin biosynthesis and cell wall chemical composition as well as towards product diversification

References

Baucher M, Chabbert B, Pilate G, Van Doorsselaere J, Tollier MT, Petit-Conil M, Cornu D, Monties B Van Montagu M, Inze D, Jouanin L, Boerjan W. 1996. Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. Plant Physiology. 112(4): 1479-1490.

Blanco-Portales R, Medina-Escobar N, López-Ráez JA, González-Reyes JA, Villalba JM, Moyano E, Caballero JL, Muñoz-Blanco J. 2002. Cloning, expression and immunolocalization pattern of a cinnamyl alcohol dehydrogenase gene from strawberry (Fragaria x Ananassa cv. Chandler). Journal of Experimental Botany. 53(375): 1723-1734.

Brill EM, Abrahams S, Hayes CM, Jenkins CL, Watson JM. 1999. Molecular characterisation and expression of a wound-inducible cDNA encoding a novel cinnamyl-alcohol dehydrogenase enzyme in lucerne (*Medicago sativa* L.). Plant Molecular Biology. 41(2): 279-291.

Chen F, Dixon RA. 2007. Lignin modification improves fermentable sugar yields for biofuel production. Nature Biotechnology. 25(7): 759-761.

de Souza AP, Grandis A, Leite DCC, Buckeridge MS. 2014. Sugarcane as a bioenergy source: history, performance, and perspectives for second-generation bioethanol. Bioenergy Research. **7:** 24–35.

Galliano H, Cabané M, Eckerskorn C, Lottspeich F, Sandermann H, Ernst D. 1993. Molecular cloning, sequence analysis and elicitor-/ozone-induced accumulation of cinnamyl alcohol dehydrogenase from Norway spruce (*Picea abies* L.). Plant Molecular Biology. 23(1): 145-156.

Halpin C, Knight ME, Grima-Pettenati J, Goffner D, Boudet A, Schuch W. 1992. Purification and characterization of cinnamyl alcohol dehydrogenase from tobacco stems. Plant Physiology. 98(1): 12-16.

Hawkins SW, Boudet AM. 1994. Purification and characterization of cinnamyl alcohol dehydrogenase isoforms from the periderm of eucalyptus gunnii hook. Plant

Physiology. 104 (1): 75-84.

Henry RJ. 2010. Evaluation of plant biomass resources available for replacement of fossil oil. Plant Biotechnology Journal. 8(3): 288-293.

Hibino T, Shibata D, Umezawa T, Higuchi T. 1993. Purification and partial sequences of Aralia cordata cinnamyl alcohol dehydrogenase. Phytochemistry. 32(3): 565-567.

Hoang NV, Furtado A, Botha, FC, Simmons BA, Henry RJ. 2015. Potential for genetic improvement of sugarcane as a source of biomass for biofuels. Frontiers in Bioengineering and Biotechnology. 3:182.

Hu Y, Zhang L, Hu J, Zhang J, Shen F, Yang G, Zhang Y, Deng S, Qi H, Yan J, Bai S. 2017. Assessments of E*rianthus arundinaceus* as a potential energy crop for bioethanol and biomethane production. BioResources. 12(4): 8786-8802.

Kasirajan L, Valiyaparambth R, Kubandiran A, and Velu J. 2020. Isolation, cloning and expression analysis of cinnamyl alcohol dehydrogenase (CAD) involved in phenylpropanoid pathway of *Erianthus arundinaceus*. 3 Biotech. 10(1):11.

Li L, Cheng XF, Leshkevich J, Umezawa T, Harding SA, Chiang VL. 2001. The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell. 13(7): 1567-1586.

Lu J, Shi Y, Li W, Chen S, Wang Y, He X, Yin X. 2019. RcPAL, a key gene in lignin biosynthesis in *Ricinus communis* L. BMC Plant Biology. 19(1):181.

Ma QH. 2010 Functional analysis of a cinnamyl alcohol dehydrogenase involved in lignin biosynthesis in wheat. Journal of Experimental Botany. 61(10): 2735–2744.

O'malley DM, Porter S, Sederoff RR. 1992. Purification, characterization, and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (*Pinus taeda* L.). Plant Physiology. 98 (4): 1364-1371.

Sibout RA, Eudes, Mouille G, Pollet B, Lapierre C, Jouanin L, Séguin A. 2005. CINNAMYL ALCOHOL DEHYDROGENASE-C and -D are the primary genes involved in lignin biosynthesis in the floral stem of Arabidopsis. Plant Cell. 17(7): 2059-2076.

Van Doorsselaere J, Baucher M, Chognot E, Chabbert B, Tollier MT, Petit-Conil M, Leplé JC, Pilate G, Cornu D, Monties B and Montagu MV, Inze D, Boerjan W, Jouanin L. 1995. A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid O -methyltransferase activity. Plant Journal. 8:855–864.

Yi Xu, Schlarbaum SE, Liang H. 2011. Investigation of genome structure of a cinnamyl alcohol dehydrogenase locus in a basal angiosperm hardwood species, L*iriodendron tulipifera* L., reveals low synteny. Journal of Systematics and Evolution. 49: 396-405.

Zhang K, Qian Q, Huang Z, Wang Y, Li M, Hong L, Zeng D, Gu M, Chu C, Cheng Z. 2006. GOLD HULL AND INTERNODE2 encodes a primarily multifunctional cinnamyl-alcohol dehydrogenase in rice. Plant Physiology. 140(3): 972-983.