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Isolation of putative LAR (leucoanthocyanidin reductase) gene fragment differentially expressed in *Jatropha curcas* L. leaves

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Abstract

Jatropha curcas Linn. (Euphorbiaceae) is a shrub, which is widely distributed especially in tropical areas of Africa. Compounds that have been isolated from *J. curcas* leaves include flavonoids, sterols their glucosides and alkaloids. Many of both structural and regulatory genes included in secondary metabolite pathways have been cloned from several model plants. Leaves at four different growing stages were collected and cDNAs obtained were analyzed by PCR applying specific primers. All the reactions were performed three times and only repeatable fragments were taken into account for sequencing. Obtained sequences were compared in EMBL databases in order to identify their homology and their putative function. A total of 45 polymorphic fragments were collected. Twenty-six of them showed homology with ESTs (Expressed Sequence Tagged), while other 19 sequences showed to be homologous to genes involved in secondary metabolism and polyphenol pathways. The results obtained underline a different kinetic expression of the gene fragment isolated. They are especially expressed at different growing stage. In particular, applying LAR gene specific primers, an amplicon of 945bp was obtained. It is 98.4% homologous to the gene previously isolated in *V. vinifera* leaves. These interesting preliminary results are the first step for study and isolate other genes involved in polyphenol pathway in *Jatropha curcas*.

Key words: *Jatropha curcas*, LAR gene, Flavonol pathway

Introduction

Jatropha curcas L., one of the 175 species in genus *Jatropha* of the family Euphorbiaceae is a perennial small tree or large shrub native to tropical America and it is distributed throughout the tropics and subtropics of Asia and Africa (Natarajan et al., 2010).

Leaves and nuts of *Jatropha* (Figure 1) are toxic (they contain phorbol esters and curcin), nuts are sometimes roasted and dangerously eaten. However, a non-toxic variety is reported to exist in Mexico and Central America where is used for human consumption after roasting, and does not contain phorbol esters (Benge, 2006).

Jatropha is known as the physic or purging nut for its use as purgative/laxative and it is widely known as medicinal for treatment of a variety of diseases. Preparations of all parts of the plant, including seeds, leaves and bark, fresh or as a decoction, are used in traditional medicine and veterinary purposes. The methanol extract of *Jatropha* leaves showed potent cardiovascular action in animals and might be a source of beta-blocker agent for humans. The latex of *Jatropha* contains alkaloids including jatrophine, jatropham and curcain with anti-cancerous properties. The oil has a strong purgative action and is widely used to treat skin diseases and to soothe pain from rheumatism. The 36% linoleic acid (C18:2) content in *jatropha* kernel oil is of possible interest for skincare (Islam et al., 2011).

Compounds isolated from J. curcas leaves include the flavonoids apigenin and its glycosides vitexin and isovitexin, the catechin, the sterols stigmasterol, β -D-sitosterol and its D-glucoside (Mousumi and Bisen, 2008). Furthermore, *J. curcas* leaves contain steroid sapogenins, alkaloids, the triterpene alcohol, 1-triacontanol and a dimer of a triterpene alcohol (Staubmann et al., 1999). Many of both structural and regulatory genes have been cloned from several model plants, including maize, Antirrhinum, Tobacco, Petunia and Arabidopsis and have been expressed in genetically modified

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model plants and micro-organisms (Bogs et al., 2005; Dixon and Steele, 1999; Forkmann and Martens, 2001). Nowadays nothing has been reported about gene expression in this specie.

Figure 1. *Jatropha curcas* L.

Aerial parts containing organic acids (*o* and *p*-coumaric acid, *p*-OH-benzoic acid, protocatechuic acid, resorsilic acid), saponins and tannins. Amyrin, sitosterol and taraxerol were isolated from stembark. Latex contains curcacycline A, a cyclic octapeptide curcain (a protease) whereas curcin, a lectin Phorbolesters Esterases (JEA) and Lipase (JEB) were isolated from seeds. Roots are characterized from sitosterol and its d-glucoside, marmesin, propacin, the curculathyranes A and B and the curcusones A–D, the diterpenoids jatrophol and jatropholone A and B, the coumarin tomentin, the coumarino-lignan jatrophin as well as taraxerol (Garg et al., 2011).

The polyphenolic compounds, known as condensed tannins or proanthocyanidins (PAs), are plant secondary metabolites synthesized via the flavonoid biosynthetic pathway. They occur in a wide range of plants and play an important role in defence against herbivores (Harborne and Grayer, 1993; Peters and Constabel, 2002). PAs can protect ruminants against pasture bloat (McMahon et al., 2000) and act as antioxidants with beneficial effects for human health including protection against free radical-mediated injury and cardiovascular disease (Bagchi et al., 2000; Lin et al., 2002; Milella et al., 2011a; Russo et al., 2012). Polyphenols were also used as marker in genomic and metabolomic comparision (Milella et al., 2011b) In red wine, PAs play an important role in wine quality, contributing to mouthfeel and colour stability (Glories, 1988). Thus, from both a nutraceutical and a food quality perspective, it is important to understand the mechanisms leading to the formation of PA polymers and how this is regulated by the plant (Bogs et al., 2005). PA biosynthesis is part of the flavonoid pathway that also produces anthocyanins and flavonols.

The objectives of this study were: isolate good quality RNA from leaves at different ripening stages; synthesize cDNA in *Jatropha curcas* L.; amplify expressed genomic sequences; identify some of them potentially involved in polyphenol biosynthetic pathways.

Materials and Methods

Plant Material

Leaves at four different growing stages were collected: young (Y), unripe (U), fully grown (F) and old (O). Leaves were immediately frozen in liquid nitrogen and stored at -80°C.

RNA Extraction and cDNA Synthesis

Total RNA was extracted starting from 0.5 g of leaf tissue with the following extraction buffer: 0.2 M Tris-HCl pH 8.5; 0.35 M NaCl; 7 M Urea; 0.2 M EDTA pH 8; 2% (w/v) SDS. To obtain highly purified RNA, during the cleaning steps, particular attention was placed. The synthesis of the cDNA was carried out following the protocol described in Ovesna et al. (2012).

Primer design and amplification

cDNAs obtained were analyzed by PCR applying specific primers related to polyphenol pathway (Table 1). They were obtained aligning different nucleic acid sequences of the same gene previously isolated in other species. Clustal V software was used to determinate the highest conserved gene regions and to design specific oligonucleotides (Higgins et al., 1992). PCR reactions were carried out in 50 µl final volume using 0.3 units of Taq polymerase, 10 mM Tris-HCl pH 8.1, 50 mM KCl, 250 mM dNTPs, 3 mM $MgCl_2$ and 2 mM primers, 0.5 ng of cDNA for 45 cycles with the following thermal profile: 94°C for 1.10 min, 44°C for 1.00 min, and 72°C for 2.00 min. Final elongation step was done at 72°C for 5.00 min. Amplified fragments were fractioned through 1.6% agarose gel containing 0.5 mg/ml of ethidium bromide. All the reactions were performed three times and only repeatable fragments were analyzed for sequencing.

Table 1. Some of the primers used for PCR amplification.

Primer	Sequence	N°bases	CG %
LAR for	5'-CATGGACAACACTCGATTAGCCTAC-3'	25	48
LAR rev	5'-TGTTGATGACAAAAGTAATGGGG-3'	23	40
CHI for	5'-ACACCTGCTGGAGTTGCCCA-3'	20	60
CHI rev	5'-AAAGAATGGGGCCCAGCCCA-3'	20	60
LDOX for	5'-CATTGGAGGAAGATGAGAGAATCATGAC-3'	28	42,8
LDOX rev	5'-GTCACAATCTGGCGCCAATCCT-3'	22	54,5

Sequencing and alignment of cDNA isolated fragments

Differentially expressed amplicons obtained were excised from the gel and eluted using "Quantum prep freeze 'N sequence DNA gel extraction spin columns" Bio-rad kit. The recovered fragments were cloned using pGEM-T Easy Vector Systems (Promega). Cloned cDNAs were sequenced using Sequenase kit applying Sp6 primer.

Sequences were analyzed using BLASTN algorithm and compared with EMBL databases in order to identify their homology and putative function with those previously isolated.

Results and Discussion

The protocol applied for RNA extraction and cDNA synthesis demonstrated to be very effective in order to isolate good quality RNA and to generate cDNA starting from sampled tissues in *Jatropha curcas*.

Applying specific oligos, related to the polyphenol pathway, clear polymorphisms were observed. This result underlines a different kinetic of investigated gene expression. A total of 45 polymorphic fragments were collected, they included only the reproducible amplification products that showed polymorphisms among leaf growing stages. In order to confirm the size and the uniqueness of the isolated fragments (Figure 2), they were re-amplified. Twenty-six of them showed homology with ESTs (Expressed Sequence Tagged), while other 19 sequences showed to be homologous to expressed products involved in secondary metabolism and polyphenol pathway (Table 2).

The results reported in Table 2 shown the homologies found with genes involved in the flavonoid pathway. In particular, the fragments isolated from fully grown (F) stage shown homologies with different compounds involved in the synthesis of PAs and anthocyanins: leucoanthocyanidin reductase (LAR), Leuco Anthocyanidin Dioxygenase (LDOX) and Flavone synthase (FLS). Previous studies were focused on LAR gene, which plays a key role in catechin biosynthesis (Bogs et al., 2005). Using specific primers an amplicon of 945bp was obtained. It is 98.4% homologous to the gene previously isolated in *V. vinifera* leaves 1086bp (Bogs et al., 2005).

Table 2. Isolated fragment homologies.

Code	Protein	Growing stage	Access.
6C1-W2	Leuco Anthocyanidin Reductase (LAR)	F	L81312
5CY-Y2	Chalcone Isomerase (CHI)	U, F	Q949N3
20-Y2	Leuco Anthocyanidin Dioxygenase (LDOX)	F	Q9ST16
19-R2	Flavone synthase (FLS)	U, F, O	Q6R4C2

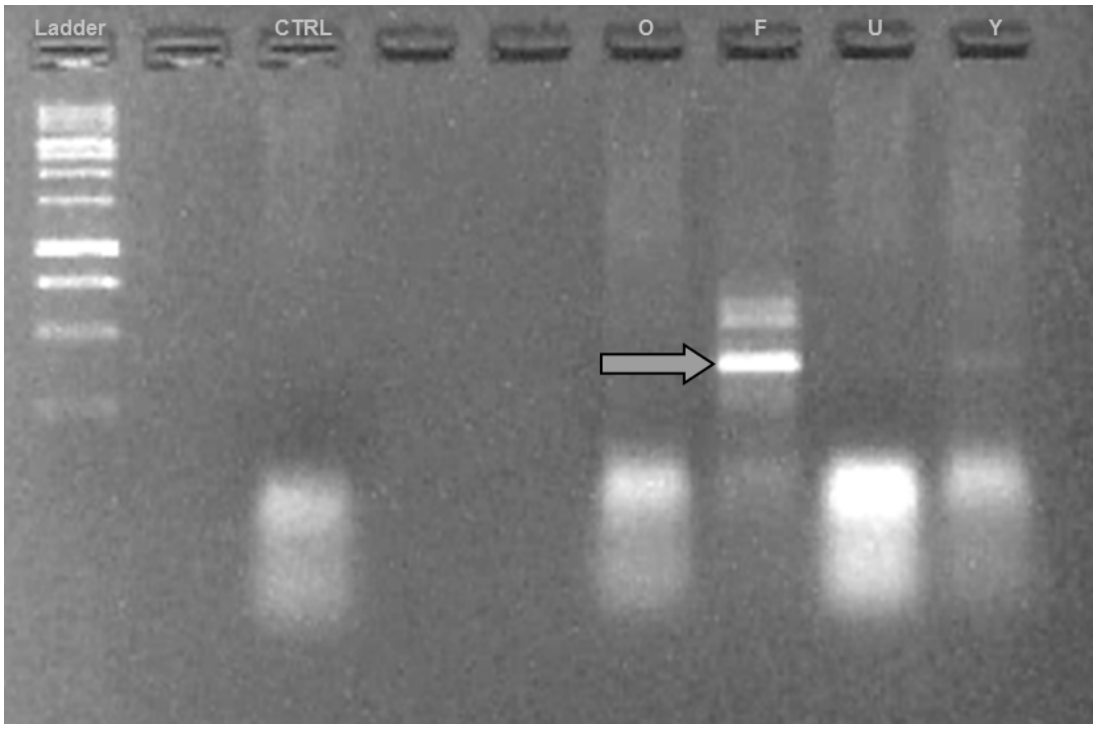

Figure 2. LAR amplification product (indicated by arrow) obtained by PRC reaction.

In this study, we show that the cDNA coding for LAR gene is expressed during PA synthesis in *J. curcas* and are regulated in a temporal manner and it is genotype dependent. Synthesis of PA polymers is believed to occur by addition of an intermediate derived from a flavan-3,4-diol (such as leucocyanidin) to a flavan-3-ol terminal unit (such as catechin or epicatechin) with sequential addition of further extension subunits as the polymer lengthens. Taking into account the stage of the leaves analyzed it is possible to see that the LAR expression is present only in the ″F″ stage. Unlike, the FLS gene it is expressed in all stages evaluated except than Y stage (young leaf).

These interesting preliminary results are the first step for study and isolate other genes involved in polyphenol pathway in *J. curcas*.

cDNAs will be insert in a plasmid and expressed *in vitro* to confirm protein structure and biological function.

Is particularly important to isolate and characterize genes regulating the biosynthesis of secondary metabolites of interest, such as the isolated fragment showing homology with the sequence of the LAR enzyme.

Whereas the flavan-3-ol biosynthesis had traditionally been considered to be the product of leucoanthocyanidin reductase (LAR), converting leucocyanidin to catechin with an epimerase then converting catechin to epicatechin (Stafford, 1990) and since LAR activity has been reported in several plants and its activity correlated with PA accumulation (Stafford, 1990; Joseph et al., 1998; Marles et al., 2003), could be interesting to aimed further studies to investigate the level of expression of several genes involved in flavonoids patway in different stages of growth and in particular assess expression of the LAR gene in fully grown (F) stage.

Conclusion

The flavonoid biosynthetic pathway is one of the most important metabolic systems in plants. The development of genomics datasets for organisms that include Arabidopsis, the moss *Physcomitrella patens* (http://www.moss.leeds.ac.uk/), and the model legume *Medicago truncatula* (http://www.noble.org/medicago/), are also offering opportunities to examine this metabolic model system from entirely new perspectives. As with any good model, each new piece of information appears to raise a number of unanticipated and intriguing questions. At the same time, new tools are providing the opportunity to consider flavonoid biosynthesis, not as an assemblage of independent components, but as part of a large, complex, and tightly orchestrated metabolic network. The ability to now consider flavonoid enzymes in *Jatropha curcas*, for the very first time, and to examine the

interdependence of the pathways of secondary metabolism using genomic, proteomic, and metabolic profiling methods are likely to move us much more rapidly toward this end. Moreover, others studies of these aspects and detailed knowledge of the mechanisms regulating these physiological processes may be a key to understand and then manage those processes for obtaining products that can be used in areas such as nutraceutical and/or producing compounds of vegetable active against some diseases. It is quite clear that, even for much-studied "old" pathways like flavonoid biosynthesis, these are exciting times.

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