Evaluation of floral transcription factor genes involved in *Jatropha curcas* L. flower formation

Anupharb Seesangboon, Lucsame Gruneck, Prapassorn D. Eungwanichayapant, Jantrararuk Tovaranonte, Siam Popluechai*

School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand
**e-mail: siam@mfu.ac.th*

Abstract

Jatropha curcas L. (*Jatropha*) produces seed oil, a potential source for biodiesel production. Seed yield of *Jatropha* is an important factor for biodiesel production, despite few flower number productions leading to low seed yield production. This study attempted to identify floral transcription factor genes using bioinformatics approach, which have potentials in controlling *Jatropha* flowering process, and to investigated the effect of Benzyladenine (BA; 160 mg/L) on those genes (*UNUSUAL FLORAL ORGANS* (*UFO*), *SEPALATA 3* (*SEP3*) and *WUSCHEL* (*WUS*)). The expressions of these transcription factor genes were quantified using degenerated primers obtained from phylogenic tree analysis. It was found that BA induced high expression of *UFO, SEP3* and *WUS1* in flowers bud before blooming period (day 10 after treated). On the other hand, the genes were expressed lower than the control on days 14 and 20. The results suggested that degenerated primers can be used for further floral transcription factor genes expression analysis and BA can alter the expressions of the floral transcription factors.

Keywords: *Jatropha curcas* L., Benzyladenine, floral transcription factors, quantitative PCR.

Introduction

Jatropha curcas L. (hereafter referred to as *Jatropha*) is a member of Euphobiaceae family which originated in Central America. *Jatropha* seed contain is about 30-40% which can be used for biodiesel production (Kandpal and Madan, 1995; Fairless, 2007). *Jatropha* oil has been recognized as the suitable oil for biodiesel production (Raju and Ezradanam, 2002). However, seed yield of *Jatropha* is low, and as a result, it has low yield of biodiesel product (Sanderson, 2009).

Jatropha is a monoecious that produce male and female flower in the same of inflorescence (Haeller 1996; Liu et al., 2008). Generally, *Jatropha* produced female per male flower in the ratio of 1:22-27 (Alam et al., 2011). A plant growth regulator such as cytokinin, are well known in terms of floral sex determination regulator, depending on the plant species (Khryanin, 2002; Xiong et al., 2009). Benzyladenin (BA) is one of a cytokinin hormone which Bang-Zhen and Zeng-Fu (2011) reported that, treatment of *Jatropha* flowers with 160 mg/L of BA resulted in significantly increased the number of female flower (female: male ratio = 1:4) after compared with control (female: male ratio = 1:13.4).

Flower development is initiated when the plant meristem changes its identity from vegetative phase to reproductive phase (Diggle et al., 2011). There are many transcription factor related to floral organs development, UNUSUAL FLORAL ORGAN (UFO) and WUSCHEL

(WUS), which are required for floral organ identity genes expression. Moreover, *WUS* are up-regulated after treated with cytokinins. In addition, *SEPALATA 3* (*SEP3*) is also required for floral organ identity genes activation, thereby promoting the conversion from inflorescence to floral meristem (Diggle et al., 2011; Zik and Irish, 2003; Veit, 2009; Wellmer, 2006). This study attempted to identify the floral transcription factors involved in *Jatropha* flower formation using bioinformatics approach and observe the effect of BA on the expression of their genes.

Methodology

Chemicals treating and sample collection

Four years old *Jatropha* trees were used as models for this experiment. The *Jatropha* trial field of Mae Fah Luang University is locate in Mae Lao district, Chiang Rai, Thailand (19°46'43.5"N 99°41'24.9"E). The experiment was separated in to four blocks, two treatments (160 mg/L of BA and RO water) for each block, and 15 trees per treatment. Each plant was sprayed with 2 L of the chemicals. The inflorescence sizes, and the numbers of male and female flowers were recorded during the experiment (from April to August, 2014). The *Jatropha* floral meristems and flowers were collected daily for 40 days; the samples were kept at -20°C until use.

Total RNA extraction and first strand cDNA synthesis

The *Jatropha* flower samples were collected for total RNA extraction at three differences stages including days 10: flowers that had no sex organ differentiation, days 14: flowers that had sex organ development, and days 20: flowers developed sex organ. The total RNAs were isolated using the protocol modified from Chaudhary *et al*. (2011). The purity and concentration of total RNA was determined by the A260/280 ratio using spectrophotometer (NanoDrop® ND-1000). Then first strand cDNA synthesis was performed according to the manufacture procedure (RevertAid first stand cDNA synthesis kit, Thermo Scientific, #K1622). The cDNA products were kept at -20°C until use.

Phylogenic analysis and primer design

Neighboor-joining analysis was aligned by MEGA v6.0 bootstrap 500 times. The plant floral transcription factor sequences used for phylogenic analysis were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/) and Kazusa *Jatropha* genome database (http://www.kazusa.or.jp/jatropha/). The primer sequence was designed using Primer3 primer design web (http://bioinfo.ut.ee/primer3-0.4.0/) and the multiple nucleotide sequences were used for designing degenerated primer.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR reactions were carried out with CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, USA) using the SYBR green binding method. Each PCR reaction contained a total volume of 10 µL (2 µL of cDNA templates, 5 µL of 2x SensiFAST™ SYBR No-ROX mix (Bio Line, #Bio-98002, USA), 1 µL of 10 mM of each forward and reverse primers (table 1) and 2 µL of nuclease free water). The cycling conditions were consisted of an initial the cycle

95°C for 3 min, followed by 40 cycles of 95°C for 15s, and annealing temperature for 30s (Table 1). Finally, melting program was performed ranging from 65°C to 95°C with heating rate of 0.5°C per 3 seconds. The qPCR reactions were done triplicate and the negative controls (without template; NTC) were also performed for each primer pair. The expression analysis was calculated on Excel spread sheet based on relative quantification Livak's method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). Each target genes were normalized to *Actin* as an internal control. The mean relative expression data were subjected to the statistical analysis with Independent-sample t-test ($P<0.05$) using IBM SPSS statistics version 21.0 (Purchase Order: 10-58878).

Results

Phylogenic analysis of UFO, SEP3 and WUS

One UFO, one SEP3 and nine WUS protein coding sequences were homologued to *Jatropha* genome database (http://www.kazusa.or.jp/jatropha/). The relationship between those proteins coding sequences and model plants including; *Arabidopsis thaliana* and *Ricinus communis* were compared using Neighbour-joining phylogenic tree MEGA v6.0. The results showed that, one of *Jatropha* coding sequence, UFO (Jcr4S06464.20) and SEP3 (Jcr4S00712.120) protein sequences were similar to *R. communis*, 81.22% (EEF34248.1), and 92.82% (XP_002514893.1), respectively (Fig. 1a, 1b). Nine of proteins coding sequences of WUS were also similar to *R. communis*. In fact, *R. communis* is a plant in Euphobiaceae family, which is the same family of *Jatropha*. However, from the comparison of relationship of WUS protein to other plants was able to separate them into a two groups, defined as WUS1 and WUS2 (Fig. 1c, 1d).

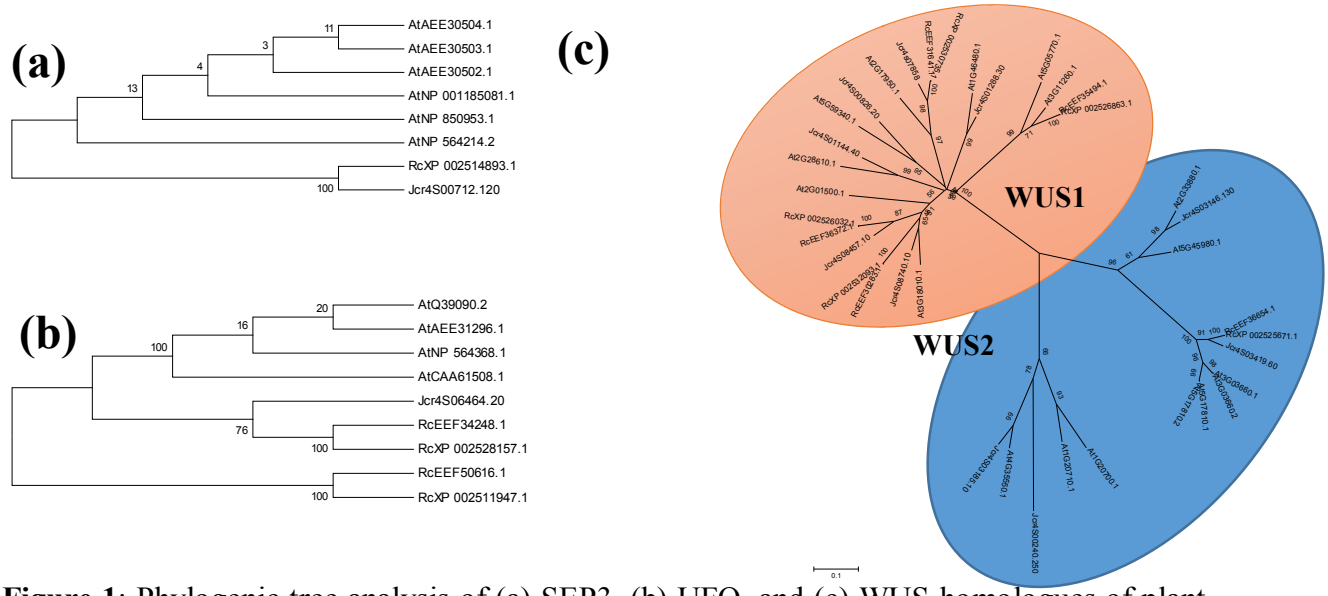

Figure 1: Phylogenic tree analysis of (a) SEP3, (b) UFO, and (c) WUS homologues of plant species in Genbank/Kazusa database. The accession started with At is the sequences from *Arabidopsis thaliana*, Rc from *Ricinus communis* and Jc from *Jatropha curcas*. The phylogenictree analysis tool using Neighbour-joining phylogenic tree, bootstrap value for 500 times (MEGA v6.0).

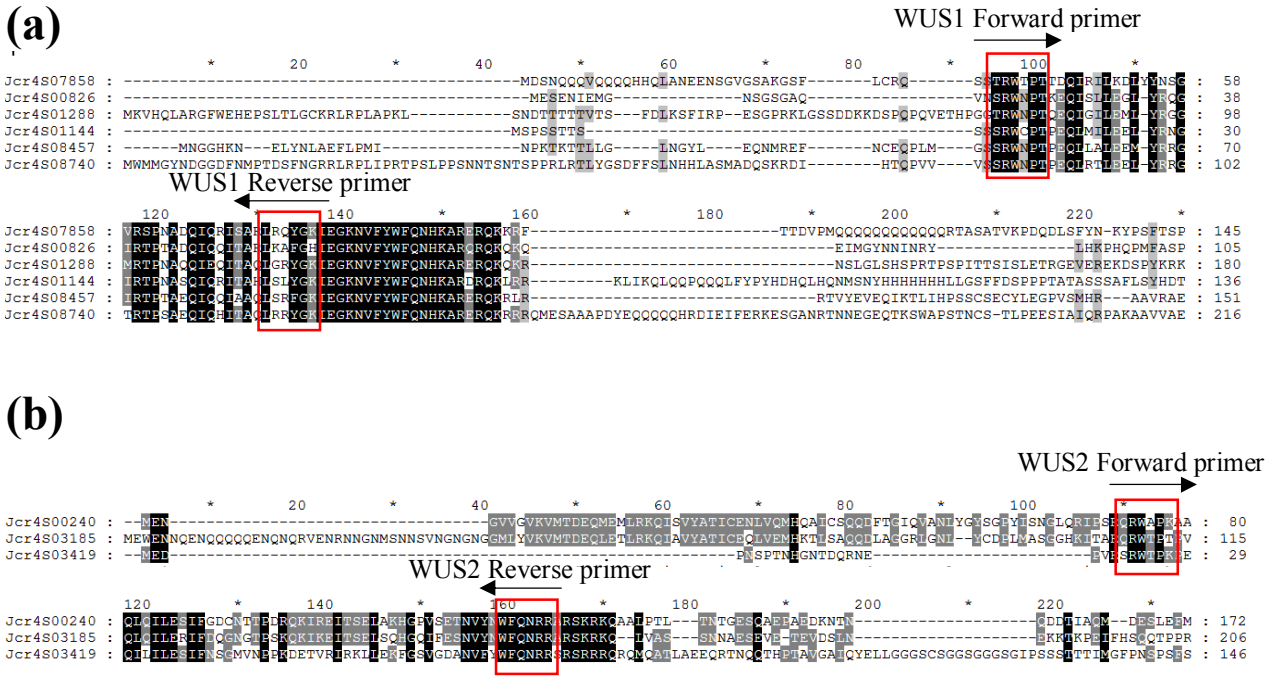

Figure 2: Multiple protein coding sequences alignment of WUS protein for design degenerated primer (a) WUS1 protein group and (b) WUS2 protein group. The primers were designed from the sequences in the red box.

Degenerated primer design

Degenerate primers might be used to amplify the specific DNA sequences for the known genes, or more than one of protein sequence of the genes that are known. So, degenerated primers of *UFO, SEP3, WUS1,* and *WUS2* were designed based on conserved region after relationship comparison using protein alignment analysis (http://www.ebi.ac.uk/Tools /msa/clustalw2/) (Fig. 2 and 3) (sequence of primers show on Table1). The amplification of those primers were checked with *Jatropha* DNA using PCR method. The results showed that, those genes were specific to the target sequences and could be used in this study (data not shown).

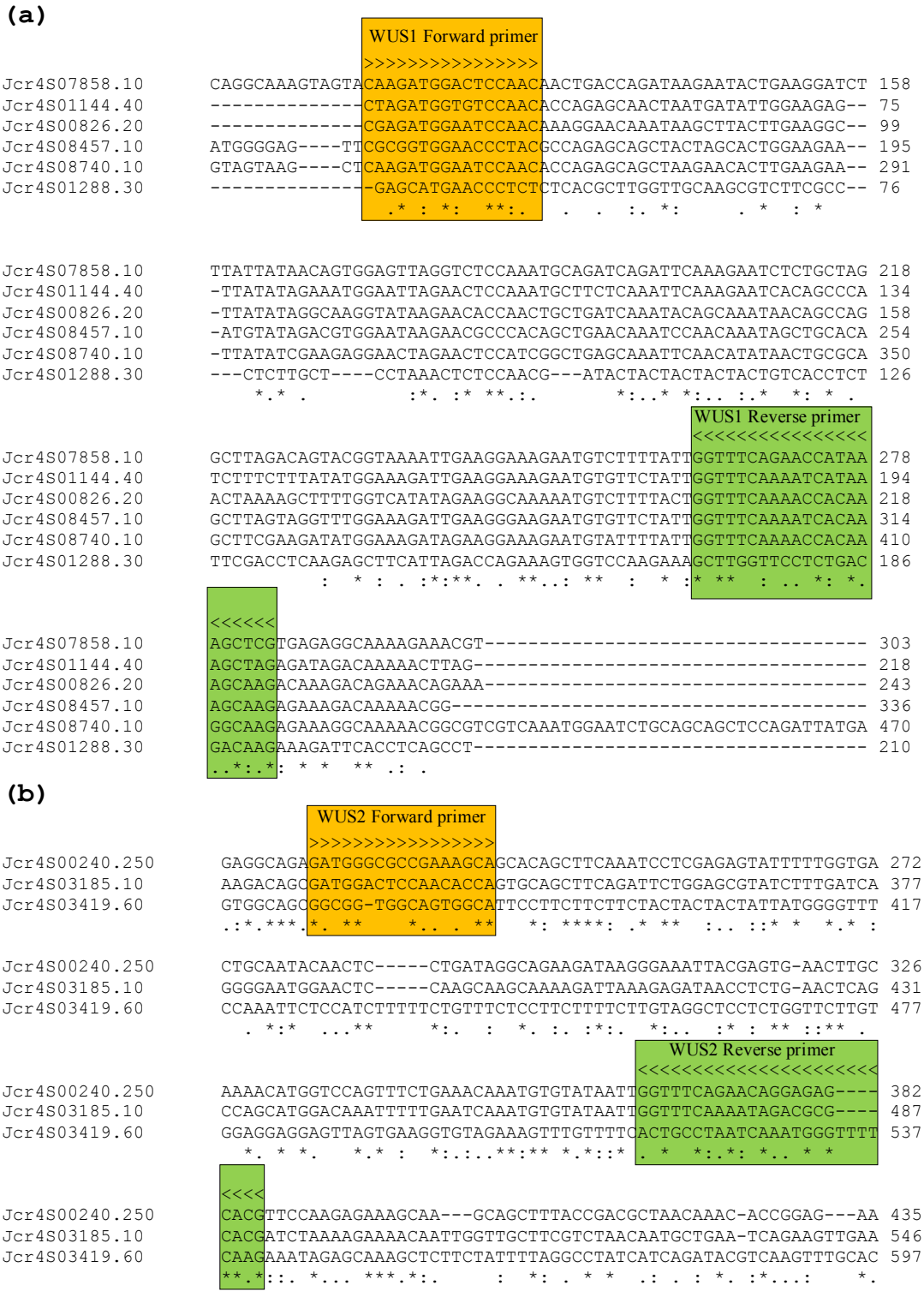

Figure 3: Nucleotide alignment; the conserved region in yellow box is forward primer and in green box is reverse primer (a) WUS1; (b) WUS2.

Table 1: Primers were used in this study

Name	Abbre.	Annealing temperature (°C)	GC%	length	Sequences 5' – 3'	Product size (bp)
UFO	*JcUFOF*	41	55.56	18	CCATGGATGGACGCTAGA	170
	JcUFOR		50.00	18	CGCGGTGATAAATGGATG	
SEP3	*JcSEP3F*	41	50.00	19	GCCCTGCAACGATCCCAAA	125
	JcSEP3R		55.56	19	TGGGTCCGCGTTGATCTGA	
WUS1	*JcWUS1F*	40	60.40	16	CDMGDTGGWVYCCDAC	169-172
	JcWUS1R		47.00	22	CKDGCYTTRTGRTTYTGRAACC	
WUS2	*JcWUS2F*	50	59.30	18	GATGGRMBCCRAMASCWG	166
	JcWUS2R		50.80	22	CKTGMBYKYCKRTTYTGRAACC	

The expression analysis

The *Jatropha* flowers of each treatment was collected after treated, on days 10, 14 and 20, for total RNA extraction and quantification of the gene expression of *SEP3, UFO, WUS1* and *WUS2*. The genes expression showed that, at days 10, after treated with BA, *UFO,* and *WUS1* were highly expressed and significantly different from those controls. Furthermore, the expression of *UFO* in control flower was higher than those in treated flowers on days 14. On days 20, *SEP3* and *UFO* were highly expressed in control than those in treated flowers. However, *WUS2* was not expressed (Fig. 4).

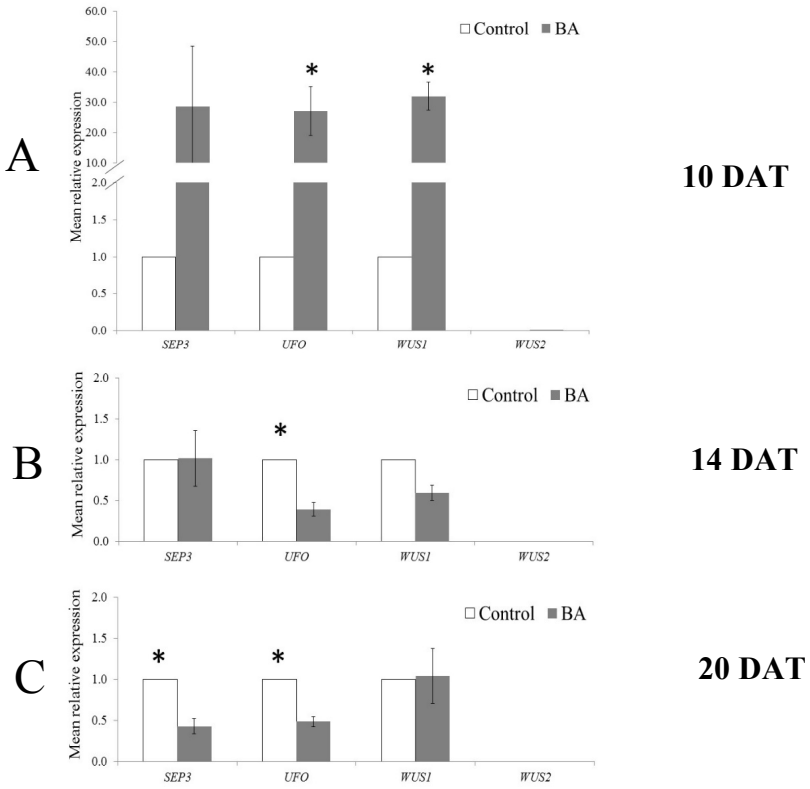

Figure 4: The mean relative expression normalized by internal control of genes might be involved in floral organ development. The mean relative expression values were analyzed by statistical analysis using Independent-sample t-test (P<0.05) **(A)** 10 days after treated (10 DAT), **(B)** 14 days after treated (14 DAT), and **(C)** 20 days after treated (20 DAT).

Discussion

To elucidate the effect of BA on floral transcription factor genes in *Jatropha*, three floral transcription factors were selected including; SEP3, UFO, and WUS based on proteins, which reported in literature of *Arabidopsis*. Gene expression of those transcription factor genes were performed by using quantitative PCR method and the results showed that, the expression of *SEP3, UFO* and *WUS1* were significantly higher than those in control ($P<0.05$). These results are consistent to previous reported by Diggle et al. (2011); whose found that UFO and WUS are also required to activate floral organ identity genes group and cytokinins will up-regulate homeobox meristem genes (WUS). In addition, *SEP3* also appears to be involved in floral organ identity genes activation in *Arabidopsis*. Adversely, *WUS2* was not expressed in both control and treatments, which might be from the protein coding sequences of *WUS2* that was not affected by BA. During flowers blooming (days 14 after treated), *UFO* in control flowers was significantly expressed higher than those in treated flowers, and after 20 days of treatment with BA, *SEP3* and *UFO* in control flowers were significantly expressed higher than those in treated flowers. The results indicated that after treated with 160 mg/L of BA was able to stimulated *SEP3*, *UFO*, and *WUS1* before flower blooming; whereas, during flower blooming and after blooming, their expression was decreased (lower than the control) (Fig. 4). Interestingly, the gene expression occurs during flower blooming were decrease in this study. Thus, the expression level of transcription factors during each time period and the number of flower which is affected by BA will be studied in the future.

Conclusion

In this study, the effect of BA to floral transcription factor genes was investigated. Degenerated primers from protein alignment analysis were used for gene expression analysis. The results showed that transcription factor genes in flowers treated with BA for 10 days were significantly expressed higher than those in control. On the other hand, those genes were expressed lower than the control flowers after day 14 and day 20 during flower blooming. Accordingly, the effect of BA on flower blooming, the expression level of transcription factors along with time period and the number of flower which affected by BA will be further investigated.

Acknowledgements

The study was supported by National Research Council of Thailand (Grand number: 2557A3070 2007).

References

Alam, N.C.N., Abdullah T.L. and Abdullah N.A.P. (2011). Flowering and fruit set under Malaysian climate of *Jatropha Curcas* L. *Am J Agric Biol Sci, 6*, 142-147.

Bang-Zhen, P., & Zeng-Fu, X. (2010). Benzyladenine Treatment Significantly Increases the Seed Yield of the Biofuel Plant Jatropha curcas. *Journal of Plant Growth Regulation, 30*(2), 166-174. doi: 10.1007/s00344-010-9179-3

Chaudhary S., Shah P., Vyas M.K., Kumar V., Srivastava N., Katudia K.H., Vaidya K., Chikara S.K. (2011) Establishment of the protocol for high quality RNA isolation from *Jatropha curcas*. *Asian J Exp Biol Sci, 2*, 715-720

Diggle, P. K., Di Stilio, V. S., Gschwend, A. R., Golenberg, E. M., Moore, R. C., Russell, J. R., & Sinclair, J. P. (2011). Multiple developmental processes underlie sex differentiation in angiosperms. *Trends Genet, 27*(9), 368-376. doi: 10.1016/j.tig.2011.05.003

Fairless, D. (2007). Biofuel: the little shrub that could-maybe *Nature* (Vol. 449, pp. 652-655). England.

Heller J. (1996) Physic nut *Jatropha curcas* L. promoting the conversation and use of underutilized and neglected crops. 1. Gatersleben: Institute of plant genetics and crop plant research. International Plant Genetic Resources Institute, Rome

Kandpal, J. B., & Madan, M. (1995). Jatropha curcus: a renewable source of energy for meeting future energy needs. *Renewable Energy, 6*(2), 159-160. doi: http://dx.doi.org/10.1016/ 0960-1481(94)00081-G

Khryanin, V. N. (2002). Role of Phytohormones in Sex Differentiation in Plants. *Russian Journal of Plant Physiology, 49*(4), 545-551. doi: 10.1023/A:1016328513153

Liu H., Deng Y., Liao J. (2008). Floral organogenesis of three species of *Jatropha* (Euphorbiaceae). *J Syst Evol, 46*, 53-61

Raju A.J.S., Ezradanam V. (2002). Pollination ecology and fruiting behavior in a monoecious species, *Jatropha curcas* L. (Euphorbiaceae). *Current Sci, 83*,1395-1398

Sanderson, K. (2009). Wonder weed plans fail to flourish *Nature* (Vol. 461, pp. 328-329). England.

Veit, B. (2009). Hormone mediated regulation of the shoot apical meristem. *Plant Mol Biol, 69*(4), 397-408. doi: 10.1007/s11103-008-9396-3

Wellmer, F., Alves-Ferreira, M., Dubois, A., Riechmann, J. L., & Meyerowitz, E. M. (2006). Genome-wide analysis of gene expression during early Arabidopsis flower development. *PLoS Genet, 2*(7), e117. doi: 10.1371/journal.pgen.0020117.eor

Xiong, G., Li, J., & Wang, Y. (2009). Advances in the regulation and crosstalks of phytohormones. *Chinese Science Bulletin, 54*(22), 4069-4082. doi: 10.1007/s11434-009-0629-x

Zik, M., & Irish, V. F. (2003). Global identification of target genes regulated by APETALA3 and PISTILLATA floral homeotic gene action. *Plant Cell, 15*(1), 207-222.