Effect of PCB to Expression of Genes Involved in GA Biosynthesis During *Jatropha* Seedling

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Abstract

The expression of Gibberellin (GA) biosynthetic genes is of particular importance in promoting plant growth and development. Gibberellin inhibitors such as Paclobutrazol (PCB), interrupts ent-kaurene oxidase (KO) enzyme activity in the GA biosynthetic pathway which reduces plant height. Nonetheless, GA inhibitors may benefit harvesting and seed yield improvement. The affect of PCB on the expressions of *Jatropha* GA biosynthesis genes using semi-quantitative PCR was previously reported. However, the technique showed some limitations which was not able to determine the precise level of gene expression. In this study, the effects of PCB on plant height and GA content in *Jatropha* seedling were investigated. Gene expression of *ent-kaurene oxidase* (*JcKO*), *GA 20-oxidase1* (*JcGA20ox1*), *GA 20-oxidase2* (*JcGA20ox2*) and *GA 3-oxidase* (*JcGA3ox*) were determined by qPCR. Appearantly, PCB significantly reduced the plant heights shown by shortened stem length. The GA contents were significantly lower compared to the control. The studies on the GA biosynthesis genes revealed the incline and decline of relative mRNA abundance of *JcKO*, *JcGA20ox1* and *JcGA20ox2* during 0, 3 day after treatment (DAT) and one week after treatments, respectively. The *JcGA3ox* transcripts were not detected at 3 and 7 DAT. Principle component analysis (PCA) also comfirmed the importance of *JcGA20ox1* for plant height during seedling develoment. The comparisons of the phenotypes and transcriprion levels of the PCB treatments and those of control indicated that GA biosynthesis genes contributed to developmental processes with specific expression patterns during the plant growth.

Keywords: PCB; GA biosynthesis genes; *Jatropha* seedling

บทคัดย่อ

การแสดงออกของยีนที่เกี่ยวข้องในกระบวนการสร้างจิบเบอเรลลิน (GA) มีส่วนสำคัญในการพัฒนาและการเจริญเติบโตของพืช สารที่ยับยั้งกระบวนการสร้าง GA เช่น Paclobutrazol (PCB) จะยับยั้งการทำงานของเอนไซม์ ent-kaurene oxidase (KO) ในกระบวนการสร้าง GA ซึ่งส่งผลให้ความสูงของลำต้นพืชลดลง อย่างไรก็ตามการใช้ PCB อาจมีประโยชน์ในด้านการเก็บเกี่ยวและส่งเสริมให้มีปริมาณของผลผลิตมากขึ้น ในเบื้องต้นได้มีรายงานผลการศึกษาผลกระทบของ PCB ต่อการแสดงออกของยีนที่เกี่ยวข้องในกระบวนการสร้าง GA ของต้นสบู่ดำด้วยเทคนิค semi-quantitative PCR แต่เทคนิคนี้ไม่สามารถวัดระดับของการแสดงออกของยีนที่แม่นยำได้ ดังนั้นการศึกษานี้จึงได้ศึกษาผลกระทบของสาร PCB ต่อความสูงของต้นสบู่ดำ (*Jatropha curcas* L.) ปริมาณ GA และการแสดงออกของยีนที่เกี่ยวข้องในกระบวนการสร้าง GA คือ *ent-kaurene oxidase* (*JcKO*) *GA 20-oxidase1* (*JcGA20ox1*) *GA 20-oxidase2* (*JcGA20ox2*) และ *GA 3-oxidase* (*JcGA3ox*) โดยเทคนิค quantitative PCR (qPCR) ในต้นอ่อนสบู่ดำ ผลการทดลองพบว่า PCB ส่งผลให้ความสูงของลำต้นเตี้ยลงโดยมีลำต้นสั้นลง และมีปริมาณ GA น้อยกว่าชุดการทดลอง

ควบคุมอย่างมีนัยสำคัญ ($p \leq 0.05$) นอกจากนี้การศึกษายีนที่เกี่ยวข้องในกระบวนการสร้าง GA มีการแสดงออกที่เพิ่มขึ้นและลดลงของยีน *JcKO JcGA20ox1* และ *JcGA20ox2* ในวันที่ 0 3 และ 7 หลังจากได้รับสาร อย่างไรก็ตามไม่พบการแสดงออกของยีน *JcGA3ox* ในช่วงวันที่ 3 ถึง 7 จากการวิเคราะห์องค์ประกอบเพื่อบ่งชี้ถึงความสำคัญของยีน *JcGA20ox1* พบว่ามีความสำคัญต่อความสูงของต้นสบู่ดำในช่วงการเจริญของต้นอ่อน ทั้งนี้กระบวนการสร้าง GA มีความสำคัญต่อการพัฒนาและเจริญเติบโตของต้นสบู่ดำ และการแสดงออกของยีนที่เกี่ยวข้องในกระบวนการสร้าง GA มีแบบแผนการแสดงออกที่มีความจำเพาะในแต่ละระยะของการเจริญเติบโต

คำสำคัญ: พาโคลบิวทราโซล ยีนที่เกี่ยวข้องในการสังเคราะห์สาร GA ต้นอ่อนสบู่ดำ

Introduction

Jatropha curcas L. is an oil seed plant in the family Euphobiaceae which has been considered as a non-edible plant oil source for biodiesel production [1]. Although, the seed oil can be converted into a high-quality biodiesel fuel, the yield is considerably low since the ratio of female and male flowers are significantly low (1:22-27), and the plants are difficult to harvest due to their height [2].

Plant height is controlled by plant growth regulators. Auxin and Gibberellin (GA) are important phytohormones that regulate growth and influence plant development processes [3]. The biosynthetic pathway of GA is well characterized in Arabidopsis, for which it has been shown that GA 20-oxidase and GA 3-oxidase (GA3ox) are required in the final step of catalyzation to produce bioactive products. These enzymes convert GA_{53} to GA_{20} (by GA20ox) and GA_{20} to GA_1 (by GA3ox) in response to plant development [4], [5]. The effect of these phytohormones synthesis inhibitors on plant height were tested in *Jatropha*. Thongbai et al. (2007) found that the GA inhibitor was more effective in reducing plant height than the Auxin inhibitor [6]. Concurrently, Berova and Zlatev (2000) supported that treating plants with GA inhibitor inhibited GA synthesis, providing an effective plant height reduction [7]. Paclobutrazol (PCB) was also revealed to cause a remarkable reduction in plant height and increase seed yield [8].

The effect of PCB on *Jatropha* height during seedling link to the expression of GA biosynthesis genes was studied. Popluechai et al. (2012) investigated the effect of PCB on gene expression of *Jatropha* GA biosynthesis genes including *ent-kaurene synthase* (*JcKS*), *ent-kaurene synthase B* (*JcKSB*), *ent-kaurene oxidase* (*JcKO*), *GA 20-oxidase* (*JcGA20ox*), and *GA 2-oxidase* (*JcGA2ox*) using semi-quantitative PCR [9]. The study demonstrated that PCB down regulated the expression of *JcKS*, *JcKO*, and *JcGA20ox*. Nonetheless, due to the limitation of semi-quantitative PCR the level of gene expression in response to PCB treatment could not be precisely quantified. Reinvestigation of *Jatropha* genome database revealed two protein coding sequences of *JcGA20ox* were found, including *GA20ox1* (accession number Jcr4S03339) and *GA20ox2* (accession number Jcr4s06166). In addition, one protein coding sequence of *GA 3-oxidase* (*JcGA3ox*; accession number Jcr4S03817) was also found. In this research, the effect of PCB on gene expression of *JcKO*, *JcGA20ox1*, *JcGA20ox2* and *JcGA3ox* was studied using quantitative PCR (qPCR). The expression level will be discussed with GA concentration and height of *Jatropha* seedling.

Materials and methods

Measurement of plant height

The seeds were prepared for the treatments as described previously [10]. Briefly, the soaked seeds were treated with water (control) and 600 mg/l PCB for 6 hours and then incubated at 38°C for 8 hours before germination. Plant height was investigated at 0, 3, 7, 11, 15, and 21 days after treatment. Plant samples were collected for GA quantification and total-RNA extraction in the same periods

GA extraction and quantification using HPLC

The whole plant samples were used for GA bioassay using High Performance Liquid Chromatography (HPLC) [11]. A five-gram sample of freeze dry fresh tissue from each treatment (at 0, 3, 7, 11, 15 and 21 DAT) was homogenized with 70% (v/v) methanol and stirred over night at 4ºC. The extract was filtered through a filter paper and the methanol evaporated under vacuum. The aqueous phase was adjusted to pH 8.5 with 0.1 M Phosphate buffer and then partition with ethyl acetate 3 times. After removal of the ethyl acetate phase, the pH of the aqueous phase was adjusted to 2.5 with 1 N HCl. The solution was then partitioned with diethyl ether 3 times, and passed through anhydrous sodium sulphate.

Table 1 Set of primers which related on GA biosynthesis

Gene name	Primer sequence (5'⟶3')	Accession number	Tm (°C)	Product size (bp)	Reference
JcActin-F	GAGCAGAGAGATTCCGATGC	Jcr4S06558	58.22	178	[12]
JcActin-R	GCAATGCCAGGGAACATAGT		58.23		
JcKO-F	TATATTGGGTGTGGGGGAGA	Jcr4s00030	63.79	257	[10]
JcKO-R	TTCCCTATCACTGGCAATCC		63.70		
JcGA20ox1-F	ATCGTGCCTCAACATCATCA	Jcr4S03339	64.07	133	[10]
JcGA20ox1-R	CATTAGGGGTTGGCTTTTCA		63.56		
JcGA20ox2-F	CGTATGCCACTTGCAGAGAA	Jcr4s06166	58.27	105	This study
JcGA20ox2-R	TTTCCACGGAAGTTTTGAGG		56.18		
JcGA3ox-F	CGATCCTGATAGAGCCAAGC	Jcr4S03817	57.92	138	This study
JcGA3ox-R	CTTGCCGGTAGCGAAAATAG		56.98		

After that, the ethyl ether phase was evaporated under vacuum and the dry residue containing hormone was dissolved in 2 ml of absolute methanol and stored in vials at 4ºC.

GA concentration in the sample solution was quantified using HPLC (Water®2996 Photodiode Array Detector, the column C_{18} 250x4.6 mm) at 208 nm. The mobile phase was acetonitrile-water (26:74 v/v), and 30 mM phosphoric acid (pH 4.0). The Symmetry C_{18} column was equilibrated 30 min for each mobile phase condition. The column temperature was maintained at constant 25±0.1ºC. The separation was carried out by isocratic elution with a flow rate 0.8 ml/min, and an injection volume of 10 µl was used for analysis.

Total-RNA extraction and cDNA synthesis

Total RNA from whole plant tissues at 0, 3, 7, 11, 15 and 21 DAT was extracted using RNA extraction protocol as described in Chaudhary et al., 2011 [13]. The Total RNA was quantified by NanoDrop Spectro photometer at absorbance ratio of A260/280 and A260/230. First strand cDNA was further synthesized from 2 µg of total RNA samples

using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas™) following the manufacturer's protocol.

Verification of gene expression by quantitative PCR

Genes involved in GA biosynthesis were tested by quantitative PCR (qPCR). *JcActin* was used as the internal control [12]. Primer sequences used in this study were designed from candidate genes in GA biosynthetic pathway. The primers for *JcKO* and *JcGA20ox1* were previously described [10]. The primers sequences were shown in Table 1. The qPCR reactions were performed on CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, USA) using the SYBR green binding method with three technical replicates for each biological replicate. The reactions were performed in a 96-well reaction plate consisted of: 1 µL of cDNA templates, 5µL of 2xSYBR green Master Mixed (2xSensi FAST™ SYBR No-ROX mix; Bio Line, USA), 1 µL of 10 µM of primer pairs and 3 µL of nuclease free water. The reactions were subsequently performed under the following conditions: 3 min at 95 °C for initial denaturation, followed by 40 cycles of 5 s at 95°C, 30 s at the optimal temperature for each primer. The gene expression levels were calculated on Excel spread sheet using ($2^{-\Delta Cq}$) formula for calculation [14].

Statistical analysis

Plant height and GA content were analyzed using paired samples t-test according IBM SPSS statistics version 21.0 (Purchased order: 10-58878) at confidence interval of 0.05. The mean data and the least significant difference (LSD) were also calculated. Pearson correlation was used for analysis of the relationship of GA content, height and gene expression. Gene expression in response to treatments was analyzed using Principle Component Analysis (PCA) of PAST-Paleontological statistics software program, version 3.06 (http://folk.uio.no/ohammer/past) [15].

Results and discussion

Differences in the growth of *Jatropha* appeared three days after PCB treatment (Figure 1). Paired sample t-test revealed significant differences ($p \leq 0.05$) in terms of plant height between control (untreated) and PCB treatment at 3 to 21 DAT. PCB not only affected plant height but also diameter growth (data not show), whereby leaves and internodes compressed into a shorter length compared to the control, as preliminarily observed by Sangsuriyaroj et al., 2012 [10].

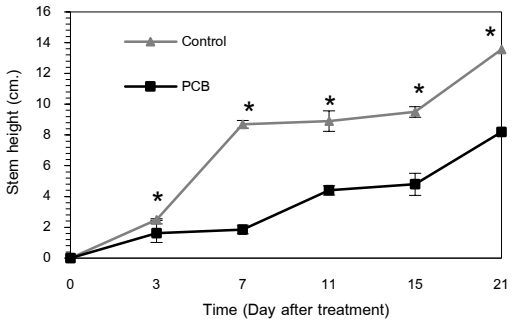

Figure 1. Plant height of *Jatropha* seedling treated with water and Paclobutrazol.*Statistically significant at the 5% level.

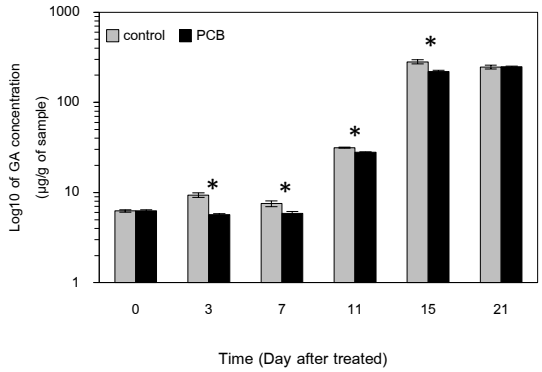

Figure 2 GA content of *Jatropha* seedling treated with water and Paclobutrazol. *Statistically significant at the 5% level.

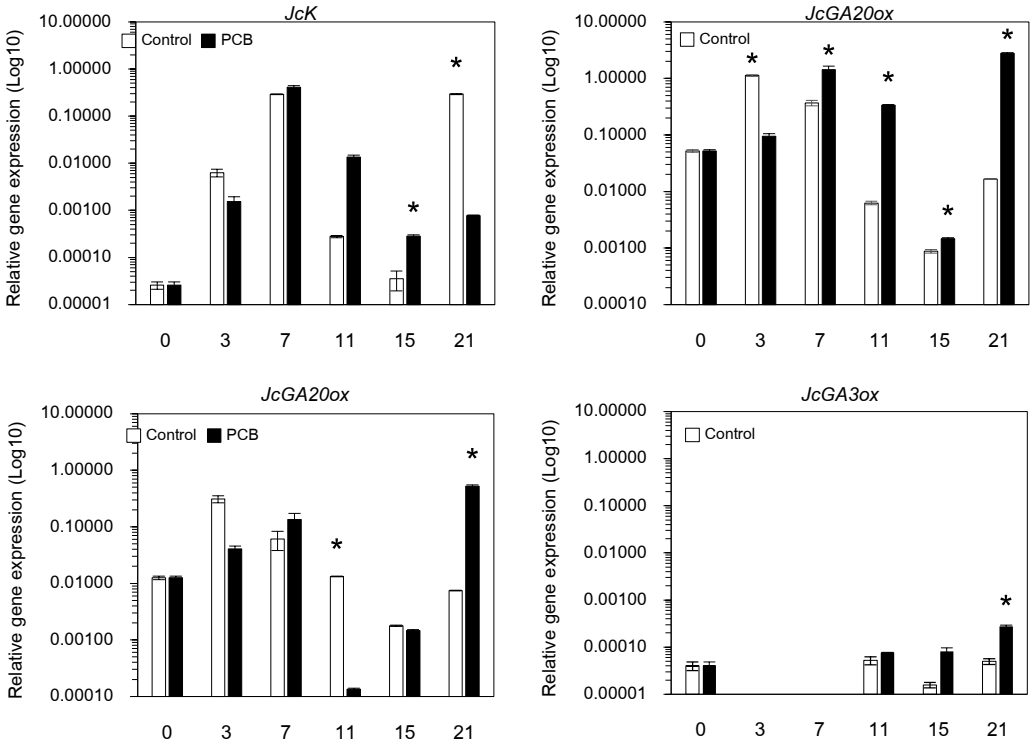

Figure 3 Quantification of the expression of genes involved in Gibberellins biosynthesis pathway by qPCR**.** Results are shown as relative expression of genes at difference time point between control and PCB treatment. Values are Log10 of mean relative expression ($2^{-\Delta ct}$) ± standard error of mean (*n*=3). *Statistically significant at the 5% level.

These results depicted that reduction in plant height of *Jatropha* was effectively retarded by PCB application in which GA synthesis was inhibited. Similar results in Jatropha height reduction were reported [8], [16], [17].

The GA quantification using HPLC showed that GA content in PCB treatment was significantly reduced from 3 to 15 DAT ($p \leq 0.05$). However, no significant difference of the GA content was observed at 21 DAT (Figure 2). The results were in accordance with the studies in *Jatropha* and rice [6], [16], [18].

The relative mRNA abundance of *JcKO*, *JcGA20ox1* and *JcGA20ox2* were studied during the development of seeds. The expression of those genes were seen throughout the experiment (Figure 3). Treating the seeds with 600 mg/L of PCB effected the genes encoding the enzymes required in the GA biosynthetic pathway. The relative mRNA abundance of *JcKO*, *JcGA20ox1* and *JcGA20ox2* genes increased during seed germination at 0 and 3 DAT, whereas transcripts of *JcKO*, *JcGA20ox1*, *JcGA20ox2* genes were gradually decreased after one week in both treatments. The expressions of the genes were higher in the PCB treatment. Positive correlation was observed for *JcKO* and *JcGA20ox1* in control during two weeks after treatment, whereas *JcKO* was negatively correlated to *JcGA20ox2*. Though there was no significant difference in any DAT (Table 2). Increase in transcript accumulation during a few days after treatment indicated a requirement for GA precursors at the early stages of seed germination [19]. However, high expressions of *JcKO* and *JcGA20ox1* in PCB treatment are probably because PCB competed with ent-kaurene for interacting with KO enzyme [20], as it plays a crucial role in interacting with the KO enzyme, thereby reducing its activity required for substrate conversion [21], [22]. It was obviuosly the positive and negative correlations of *JcKO* with *JcGA20ox1* and *JcKO* with *JcGA20ox2* might be resulted from the changes in flux through the GA biosynthetic pathway (Table 3). Moreover, the interaction between the inhibitor and the enzyme also affected the concentration of GA in *Jatropha* which was reduced. This interaction resulted in a shorter vegetative stem. In order to modulate metabolic flux toward GA biosynthesis, the expressions of *JcGA20ox* and *JcGA3ox* in the PCB treatment were stimulated, indicating negative feedback regulation of the GA biosynthesis pathway in *Jatropha* [23].

In contrast, the mechanism for the maintenance of GA homoeostasis was different in the control treatment. Transcripts of *JcKO*, *JcGA20ox* and *JcGA3ox* genes were higher than that of the PCB treatment at 3 DAT and reduced during the second week after treatment. The results suggested that there might be sufficient JcKO, JcGA20ox and JcGA3ox enzymes to produce bioactive GAs in the GA pathway as previously reported in *Arabidopsis* [24]. The reduction and elevation of gene expression was suggested to be correlated with the changes in bioactive GA level. Additionally, negative correlation between *JcKO* and *JcGA3ox* was found at 11 and 15 DAT in PCB treatment. The accumulation of *JcGA3ox* transcript levels was also reported to be under feedback control by the pathway of GA response [25].

Furthermore, the expressions of *JcKO*, *JcGA20ox1*, *JcGA20ox2* and *JcGA3ox* at 21 DAT were in accordance with Pearson's correlation (Table 3). *JcKO* was positively correlated to *JcGA20ox1, JcGA20ox2,* and *JcGA3ox* (consistently shown on the height) except in the control, where *JcKO* was highly expressed and appeared to be negatively correlated to *JcGA20ox1, JcGA20ox2,* and Jc*GA3ox*. This indicated that there was a specific expression patterns of GA biosynthetic genes during plant growth and development. The stem height is

consistent with the transcription profiles, indicating that *JcGA20ox1*, *JcGA20ox2*, and *JcGA3ox* were predominantly expressed in PCB treatment, presumably due to PCB reduction response. In PCB treatment, *JcGA3ox* was significantly and positively correlated to height (Table 4) indicated that *JcGA3ox* might involve in stem elongation of *Jatropha* seedling. Similar finding was also reported in pea that *PsGA3ox* preferentially expressed in stems and affected GA concentration and thereby growth [26],

Table 2 Pearson's correlation analysis of the expression of gene *JcGA20ox1, JcGA20ox2*, and *JcGA3ox* compared with *JcKO* in control treatment.

Genes	Day after treatment (DAT)					
	0	3	7	11	15	21
JcGA20ox1	0.002	0.964	0.000	0.505	0.872	-0.008
JcGA20ox2	0.135	-0.109	0.647	0.843	-0.235	-0.886
JcGA3ox	-0.312	N/A	N/A	-1.000**	-0.992	-0.999*

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 3 Pearson's correlation analysis of the expression of gene *JcGA20ox1, JcGA20ox2*, and *JcGA3ox* compared with *JcKO* in PCB treatment.

Genes	Day after treatment (DAT)					
	0	3	7	11	15	21
JcGA20ox1	0.002	-0.908	0.639	-0.283	0.779	0.814
JcGA20ox2	0.135	0.415	1.000*	-0.980	0.779	0.367
JcGA3ox	-0.312	N/A	N/A	-0.441	-0.952**	0.998*

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 4 Pearson's correlation analysis of the expression of gene *JcKO, JcGA20ox1, JcGA20ox2*, *JcGA3ox* and GA content compared with height**.**

	Control	PCB
JcKO	0.608	-0.268
JcGA20ox1	-0.448	0.656
JcGA20ox2	-0.458	0.712
JcGA3ox	0.277	0.890*
GA content	0.676	0.852*

*. Correlation is significant at the 0.05 level (2-tailed).

Besides, no expression of *JcGA3ox* was observed at 3 and 7 DAT, reflecting a tissue-specific localization and stages of development. Nonetheless, no significant correlation of height to those genes was found in the control. Moreover, height of *Jatropha* showed a significantly positive correlation with GA content in PCB treatment (Table 4). Presumably, PCB caused reduction in height as GA concentration

decreased, by retarding the expression of GA biosynthesis genes (*JcGA20ox1*, *JcGA20ox2* and *JcGA3ox*) through GA biosynthetic pathway.

Principle Component Analysis (PCA) was done for determining of the effects of PCB to gene expression profiles. The PCA plot of the genes involved in GA biosynthesis showed two components for the expression profiles of the genes, 96.76% and 3.09% respectively (Figure 4). The first principle component (component 1) was strongly correlated with *JcGA20ox1* (PCA score of 2.30) which was placed far from the remaining genes due to its differential expression pattern in response to both treatments This could be stated that based on the principle component score of 2.30 that this component 1 is mainly an estimation of *JcGA20ox1*, while *JcKO* is the only one of the genes that increased in the second principle component (PCA score of 0.38). According to the gene expression levels, these could be confirmed the effects of PCB on the expressions of the genes in the GA biosynthesis pathway. It would follow that having high *JcGA20ox1* and *JcKO* transcripts tend to have a lot of substrates and enzymes available for bioactive GA synthesis. Nevertheless, the transcription levels were maintained by the feedback regulation as previously mentioned. In addition, PCA has demonstrated the importance of *JcGA20ox1* for plant height which are contributed to seedling development. As their loss of function of GA20-oxidase has been tested in *Arabidopsis* [27].

Conclusions

In this study, we demonstrate that PCB reduced plant height by altering the gene expression of *JcKO*, *JcGA20ox1*, *JcGA20ox2* and *JcGA3ox*. The information can be used for developing semi-dwarf of *Jatropha* in the near future.

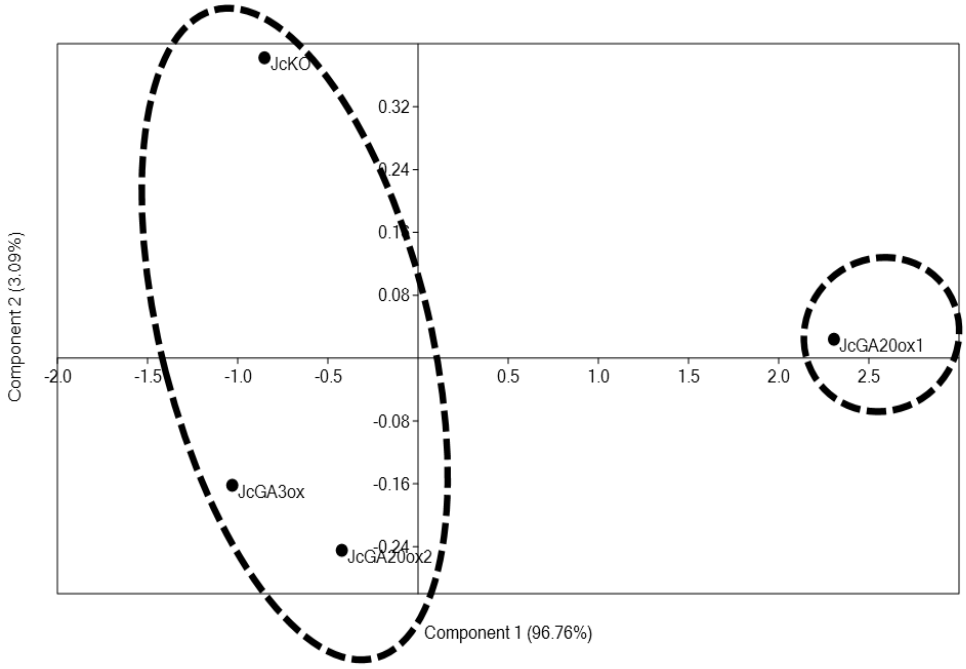

Figure 4 Principle component analysis (PCA) showing genes involved in GA biosynthesis distribution among component 1 and 2.

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