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RESEARCH NOTE

Yeast extract elicited isoflavonoid accumulation and biosynthetic gene expression in *Pueraria candollei* var. *mirifica* cell cultures

Dolly Rani[1] · Thitirat Meelaph[2] · Wanchai De-Eknamkul[1,2] · Sornkanok Vimolmangkang[1,3]

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Abstract

Yeast extract (YE) has emerged as a potent biotic elicitor that can induce plant defense responses, leading to enhanced phytoalexin accumulation. Increased production of two isoflavones—daidzein and genistein—that are widely used in pharmaceutical industries was elicited using YE in suspension cell cultures of *Pueraria candollei* var. *mirifica*. Compared with the controls, cells treated with 2 mg/L YE for 21 days produced 11-fold increased amounts of daidzein and genistein in the suspension cultures (5.12 and 0.34 mg/g dry weight (DW), respectively). Furthermore, YE treatment significantly upregulated isoflavonoid biosynthesis, as revealed via gene expression studies. In particular, among genes involved in daidzein and genistein biosynthesis, the isoflavone synthase and isoflavone reductase genes were significantly upregulated and the chalcone isomerase and 2,7,4′-trihydroxyisoflavanone dehydratase genes were significantly downregulated; moreover, these changes were associated with the accumulation of these two isoflavones in suspension cell cultures. Overall, the results obtained in this study both emphasize the utility of YE for enhancing the in vitro production of the two bioactive isoflavones examined for pharmaceutical and nutraceutical utilization and advance our understanding of their biosynthesis in response to YE elicitation.

Key message

Yeast extract modulated the expression of genes involved in isoflavonoid biosynthesis in suspension cultures of *P. candollei* var. *mirifica* cells.

Keywords *Pueraria mirifica* · Phytoestrogen · Elicitation · Biosynthetic pathway

Abbreviations

YE	Yeast extract
MS	Murashige and Skoog
HPTLC	High-performance thin-layer chromatography
DW	Dry weight
CHS	Chalcone synthase
CHI	Chalcone isomerase
CHR	Chalcone reductase
IFS	Isoflavone synthase
HID	2,7,4′-Trihydroxyisoflavanone dehydratase
IFR	Isoflavone reductase

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✉ Sornkanok Vimolmangkang
sornkanok.v@pharm.chula.ac.th

[1] Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

[2] Research Unit for Natural Product Biotechnology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

[3] Research Unit for Plant-Produced Pharmaceuticals, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

Pueraria candollei var. *mirifica* (Airy Shaw & Suvat.) Niyomdham is a commercially important indigenous medicinal plant found in Thailand. It has been used in folklore practices since time immemorial as an individual agent or in amalgamations with other herbs. In Thailand, the roots of this plant have been used by local communities for over 100 years because of their rejuvenating properties in menopausal women and andropausal men (Wanadorn 1933). The major chemical constituents of *P. candollei* var. *mirifica* include chromenes, coumestans, and isoflavones (e.g.,

daidzein, genistein, and puerarin), all of which exhibit estrogenic activity (Chansakaow et al. 2000).

Isoflavones including daidzein (4′,7-dihydroxyisoflavone) and genistein (4′,5,7-trihydroxyisoflavone) are polyphenols that are commonly present in legumes, and they exhibit significant physiological and pharmacological functions (Dwiecki et al. 2009). Daidzein is a natural isoflavone phytoestrogen (Cassidy 2003). Genistein is a well-known phytoestrogen with multiple biological functions such as alleviating menopausal symptoms and minimizing cardiovascular risks in menopausal women (Mei et al. 2001).

Although isoflavones in *P. candollei* var. *mirifica* exert significant beneficial effects on human health, the key genes involved in isoflavone biosynthesis in suspension cell cultures of this plant are not well documented. Li et al. (2014) reported the gene expression profile of isoflavonone 7-*O*-glucosyltransferase from the roots of *Pueraria lobata* seedlings, and Jungsukcharoen et al. (2016) studied key enzymes related to isoflavonoid biosynthesis in *P. mirifica* tubers. The gene expression patterns of the six key enzymes involved in isoflavonoid biosynthesis, namely chalcone synthase (CHS), chalcone reductase (CHR), chalcone isomerase (CHI), isoflavone synthase (IFS), 2,7,4′-trihydroxyisoflavanone dehydratase (HID), and isoflavone reductase (IFR), were studied in the present study. Chalcone synthase and CHI participate in flavone, flavonol, anthocyanin, and isoflavonoid biosyntheses, whereas CHR and IFS are involved in isoflavonoid biosynthesis alone (Chu et al. 2014). 2,74′-Trihydroxyisoflavanone dehydratase is involved in the final step of daidzein, genistein, and puerarin biosyntheses (Li et al. 2014). Isoflavone reductase is involved in the final steps of biosynthesis of various isoflavonoid phytoalexins (e.g., vestitol, isoflavan) from daidzein (Chu et al. 2014; Jungsukcharoen et al. 2016).

Yeast extract (YE) has been acknowledged as a potent biotic elicitor that can induce plant stress responses and the consequent enhancement of phytoalexin accumulation. Elicitation by YE significantly improved the amount of isoflavonoids in the suspension cell culture (Udomsin et al. 2019) of *P. candollei* var. *mirifica*. Exposure of a *P. candollei* var. *mirifica* cell suspension to YE (0.5 mg/L) enhanced deoxymiroestrol accumulation by twofold (191 µg/g DW), and elicitation using 1 mg/L YE increased isoflavonoid levels by 2.5–(514 µg/g DW) (Udomsin et al. 2019). In this study, we used a *P. candollei* var. *mirifica* cell suspension to assess the effects of YE on gene expression to understand the regulation of genes related to isoflavonoid production. Plant suspension cell cultures represent the best alternative to conventional methods for increasing the yields of bioactive compounds to cater to the escalating industrial demands of such natural low-molecular-weight molecules. Relative gene expressions of the key enzymes and accumulated levels of isoflavonoids in cell suspension cultures of *P. candollei* var. *mirifica* in response to YE elicitation were determined. To the best of our knowledge, this study represents the first analysis of gene expression in YE-elicited suspension cell cultures of *P. candollei* var. *mirifica*.

Calli were obtained from *P. candollei* var. *mirifica* stem explants using the protocol established in our previous report (Rani et al. 2018). Briefly, approximately 1-cm-long explants of 20-day-old *P. candollei* var. *mirifica* seedlings were excised and inoculated on Murashige and Skoog (MS; Murashige and Skoog 1962) medium supplemented with 200 mg/L KH_2PO_4, 1 mg/L thiamine HCl, 100 mg/L myo-inositol, and 0.2 mg/mL 2,4-dichlorophenoxyacetic acid for callus induction. The suspension cells obtained after 21 days were subcultured in fresh medium (20% seed volume).

Yeast extract (Merck, Darmstadt, Germany) was dissolved in ultrapure water (5 mg/mL) and autoclaved prior to use. The YE stock solution was added to the suspension cells after 6 days of subculture to achieve concentrations of 1, 2, and 3 mg/L. Untreated suspension cells were used as the controls. The suspension cells were collected 21 days after the addition of YE for further analysis based on our previous study (Rani et al. 2018), which reported that the maximal amount of biomass was obtained after approximately 28 days of subculture, after which the level started to decline.

Regarding gene expression analysis in elicited cells, total RNA was extracted from elicited and control suspension cells using approximately 100 mg of each sample after grinding in liquid nitrogen using an RNAprep Pure Plant Kit (Polysaccharides & Polyphenolics-rich) (TIANGEN, China), and samples were treated with DNase I to remove contaminating genomic DNA. The RNA concentration of each sample was determined using a NanoDrop spectrophotometer (Thermo Scientific, USA), and the quality was confirmed via agarose gel electrophoresis. Moloney murine leukemia virus reverse transcriptase and oligo dT primers were used to biosynthesize first-strand cDNA, which was prepared from 1 µg of total RNA using a RevertAid First-Strand cDNA synthesis kit (Thermo Scientific). Quantitative RT-PCR (qRT-PCR) was performed on a CFX Connect™ Real-Time System (Bio-Rad, USA) using the Luna® Universal qPCR Master Mix protocol (NEB, UK). Briefly, 100 ng of each cDNA template was mixed with 10 µL of the RT-PCR reaction mixture, which contained 5 µL of Luna Universal qPCR master mix and 0.25 µM of each primer. Gene-specific primer sequences for qRT-PCR are listed in Table 1. The thermal cycling conditions were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s; and one cycle of 95 °C for 10 s, 60 °C for 5 s, and 95 °C for 5 s. Gene expression was calculated relative to that of the elongation factor 1 (Elf1α) gene to minimize variations in cDNA template levels. The data were analyzed using CFX manager (Bio-Rad).

Table 1 Primers for isoflavonoid biosynthesis genes involved in the daidzein and genistein biosynthetic pathways

Gene	Code from transcriptome data	Forward primer	Reverse primer
CHS	CL4228.2	GCAGAAGTTGGGTCTCAAA	CTGATTTCCTCCTCATCTCATC
CHI	U29926	ATTTCCCACCTGGCTCTA	ATTTCCCACCTGGCTCTA
CHR	U35087	ATAACTGCGTTCTCTCCTCT	GAGCCTCTGCAATCTCTTTC
IFS	CL4183.1	ATAACTGCGTTCTCTCCTCT	TTTGGCGTCCTTACCTTTC
HID	U42984	CTGTACCACGACACTGTTAAG	GGGTTGAAGAGCTGGAAAG
IFR	CL9120	CACAGCAGATAAAGGGAGATG	CATCGACGGTGCTGTATTT

CHS chalcone synthase, *CHI* chalcone isomerase, *CHR* chalcone reductase, *IFS* isoflavone synthase, *HID* 2,7,4′-trihydroxyisoflavanone, *IFR* isoflavone reductase

Twenty-one days after the addition of YE to the cell suspensions, cells from the triplicate treatment flasks and control cells were collected and dried at 50 °C for 48 h. Then, 100 mg of the cells were mixed with methanol (1 mL) and extracted for 30 min via sonication at 25 °C. The extract was concentrated to dryness and resuspended in methanol for further analysis. Stock solutions of daidzein and genistein (0.1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) were prepared in methanol, and concentrations equivalent to 200, 400, 600, 800, and 1000 ng were applied to high-performance thin-layer chromatography (HPTLC) plates. The standard and sample solutions were loaded onto precoated 20×10 cm^2 silica gel (60 F254, Merck, Germany) plates using an automatic Linomat 5 (CAMAG, Switzerland) applicator. The mobile phase was toluene:acetonitrile:ethyl acetate:water at a ratio of 55:31:13:0.5, and 250 µL of glacial acetic acid was added to the solution. The sample and standard were scanned using a CAMAG TLC scanner 3 controlled by VisionCATS in the absorbance mode at 254 nm.

Gene expression and HPTLC analyses of samples were performed using three independent sets of samples, and the analyses were repeated twice. The obtained values are presented as the mean ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA), Tukey's post-hoc test, Python (v. 2.7.15) stats models, and stats libraries. Statistically significant differences were indicated by $p < 0.05$.

In the present study, MS medium supplemented with 200 mg/L KH_2PO_4, 1 mg/L thiamine HCl, 100 mg/L myo-inositol, and 0.2 mg/mL 2,4-dichlorophenoxyacetic acid induced callus formation, and it was suitable for maintaining cell proliferation in cultures for long periods. The calli obtained in the modified MS medium continued to grow actively after several subcultures, and these were used for the subsequent experiments (Fig. S1a). Based on previous studies conducted by Korsangruang et al. (2010) and Udomsin et al. (2019), the YE concentrations used to assess gene expression and isoflavonoid accumulation in the present study were 1, 2, and 3 mg/L. No difference in cell morphology was observed after YE elicitation, and cells were collected after 21 days of treatment (Fig. S1b).

The transcriptional profiles of the CHS, CHI, CHR, IFS, HID, and IFR genes, which are involved in isoflavonoid biosynthesis, were analyzed using qRT-PCR (Fig. 1) to determine whether YE elicitation altered signal transduction in suspension cells. The mRNA level of each gene studied was normalized to that of Elf1α. There was no significant difference in CHS expression between untreated and YE-treated suspension cultures (Fig. 2). Meanwhile, CHR expression was slightly upregulated following treatment with YE, although no significance was achieved. Chalcone synthase and CHR participate in the biosynthesis of several compounds, including lignans, stilbenes, and flavonoids (Fig. 1). Conversely, YE inhibited CHI gene expression in a concentration-dependent manner, whereas clearly induced IFS gene expression. The greatest increase in IFR expression was observed a YE concentration of 1 mg/L, with smaller but still significant upregulation noted at concentrations of 2 and 3 mg/L; these results suggest that the gene plays a major role in increased accumulation of isoflavonoids in the elicited cells. Presumably, YE induces the production of particular compounds by manipulating certain enzymes, as indicated by the downregulation of CHI and upregulation of IFS, the latter of which is involved in the final steps of daidzein and genistein biosyntheses. Meanwhile, YE treatment resulted in downregulation of the HID gene, which is involved in the final steps of isoflavonoid biosynthesis. This finding suggests that the production of substrates must be limited to achieve the final biosynthesis of daidzein and genistein (Fig. 2). Thus, the examined concentrations of YE were suitable for maintaining the balance of IFS upregulation and HID downregulation to support the biosynthesis of daidzein and genistein. The upregulation of IFR was also interesting because this gene, in addition to catalyzing the final steps of isoflavonoid biosynthesis, can induce the production of phytoalexins. The increased expression of IFS also resulted in increased production of 2,7,4′-trihydroxyisoflavanone, leading to increased daidzein accumulation. Because the production of these compounds must be

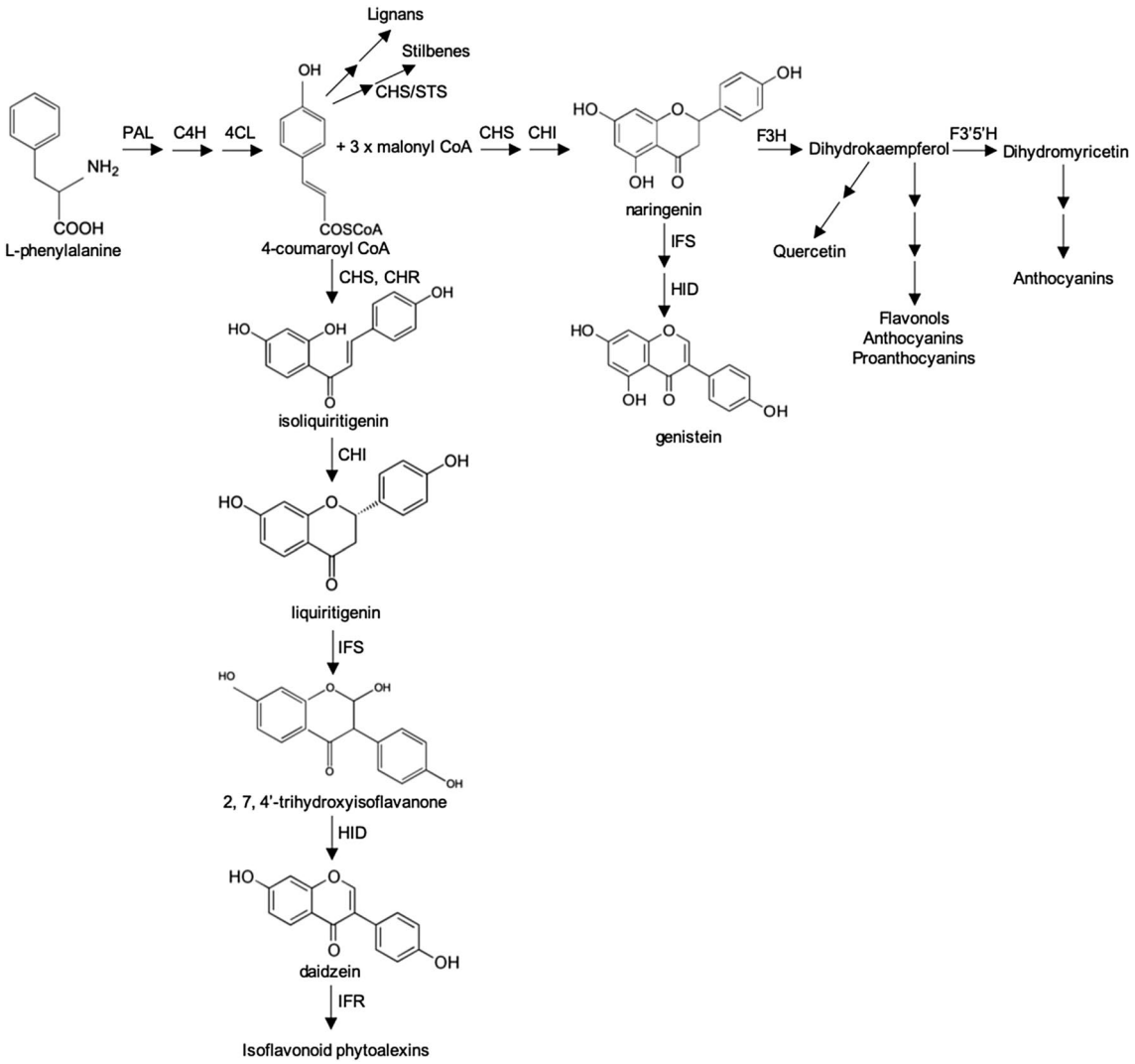

Fig. 1 Putative biosynthesis pathway in *Pueraria candollei* var. *mirifica*. *CHS* chalcone synthase, *CHI* chalcone isomerase, *CHR* chalcone reductase, *IFS* isoflavone synthase, *HID* 2,7,4′-trihydroxyisoflavanone, *IFR* isoflavone reductase, *STS* stilbene synthase, *F3H* flavanone 3-hydroxylase, *F3′5′H* flavonoid 3′5′-hydroxylase

balanced, IFR upregulation is needed to restore the biosynthesis of downstream products.

As presented in Fig. 3, different concentrations of YE (1, 2, and 3 mg/L) exerted distinct effect on the yields of the target isoflavones daidzein and genistein in the suspension cell cultures. The Rf values of daidzein and genistein were 0.48 and 0.58, respectively. The HPTLC fingerprint was changed by YE elicitation, as indicated by the decreased number of peaks (Fig. 3a, c) in treated cells compared with that in the control cells. Yeast extract at 1 mg/L did not enhance the production of daidzein, which was approximately equal to that in control cells. A steep increase in daidzein levels was observed when YE was used at a concentration of 2 mg/L. The amount of daidzein was quantified to be 5.12 mg/g DW, representing an 11-fold increase over the control levels (Fig. 3b). Increasing the YE concentration to 3 mg/L did not further increase the accumulation of daidzein. Meanwhile, genistein was not detected in control cells, whereas its levels were low (0.02 mg/g DW) in suspension cells treated with 1 mg/L YE. Similar to the findings for daidzein, genistein levels were increased to 0.34 and 0.3 mg/g DW in cells treated with 2 and 3 mg/L YE, respectively (Fig. 3d).

Despite growing interest in the application of elicitors, limited studies, mainly using cell cultures, have considered the possible effects of these compounds at the molecular level, particularly triggering the expression of genes involved in the biosynthesis of secondary metabolites. Therefore, the present study was conducted to substantiate the molecular and biochemical effects of YE on the accumulation of isoflavonoids in *P. candollei* var. *mirifica*.

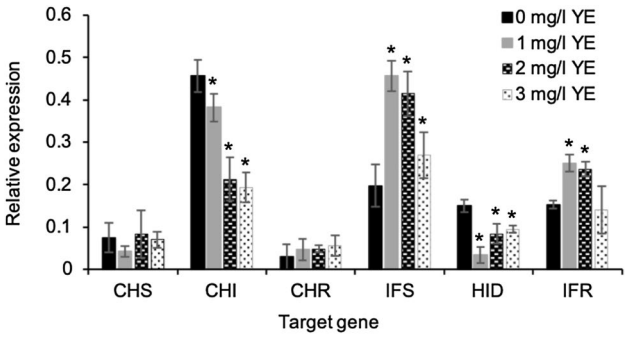

Fig. 2 Relative gene expression of different isoflavonone biosynthesis genes responsible for daidzein and genistein production in suspension cell cultures of *Pueraria candollei* var. *mirifica* treated with different concentrations of yeast extract for 21 days. The values are presented as the mean ± SE. Asterisks indicate significant differences in transcript levels compared with the controls at $p < 0.05$

It has been well documented that YE has a favorable effect on the production of isoflavones. Yeast extract (150 mg/L) increased isoflavonoid production by 20% in suspension cell cultures of *Pueraria tuberosa* (Goyal and Ramawat 2008). *Yeast extract* (100 mg/L) was also found to be the most successful in increasing the production of daidzein (2.21% DW) and genistein (0.293% DW) in suspension cell cultures of *Psoralea corylifolia* (Shinde et al. 2009).

As observed in the present study, YE elicitation decreased the expression of CHS and CHI, and these expression changes did not adversely affect isoflavonoid production because these genes are involved in the early steps of isoflavonoid biosynthesis. Downregulation of these genes could have permitted the production of diverse compounds, and this would have adversely affected the production of daidzein and genistein. In contrast to the results obtained in the present study, YE elicitation was found to increase the expression of L-phenylalanine ammonia lyase and CHS transcripts in cell suspensions of *Medicago truncatula* (Suzuki et al. 2005). In another study, CHI expression was not significantly different between wild-type and transgenic *Arabidopsis*, suggesting that it is not a crucial enzyme for isoflavonoid biosynthesis (Li et al. 2014). Conversely, YE elicitation significantly downregulated CHI expression in the present study, leading to the biosynthesis of the desired products (daidzein and genistein) and possibly decreasing the diversity of compounds that could have otherwise been biosynthesized. Chalcone reductase participates in daidzein production by catalyzing the conversion of naringenin chalcone to isoliquiritigenin (Chu et al. 2014). The first committed step of isoflavone biosynthesis is catalyzed by IFS, and this gene is mainly found in soybeans and other leguminous plants. Isoflavone synthase is involved in the oxidation of 7,4′-dihydroxyflavanone (liquiritigenin) and 5,7,4′-trihydroxyflavanone (naringenin) to daidzein and

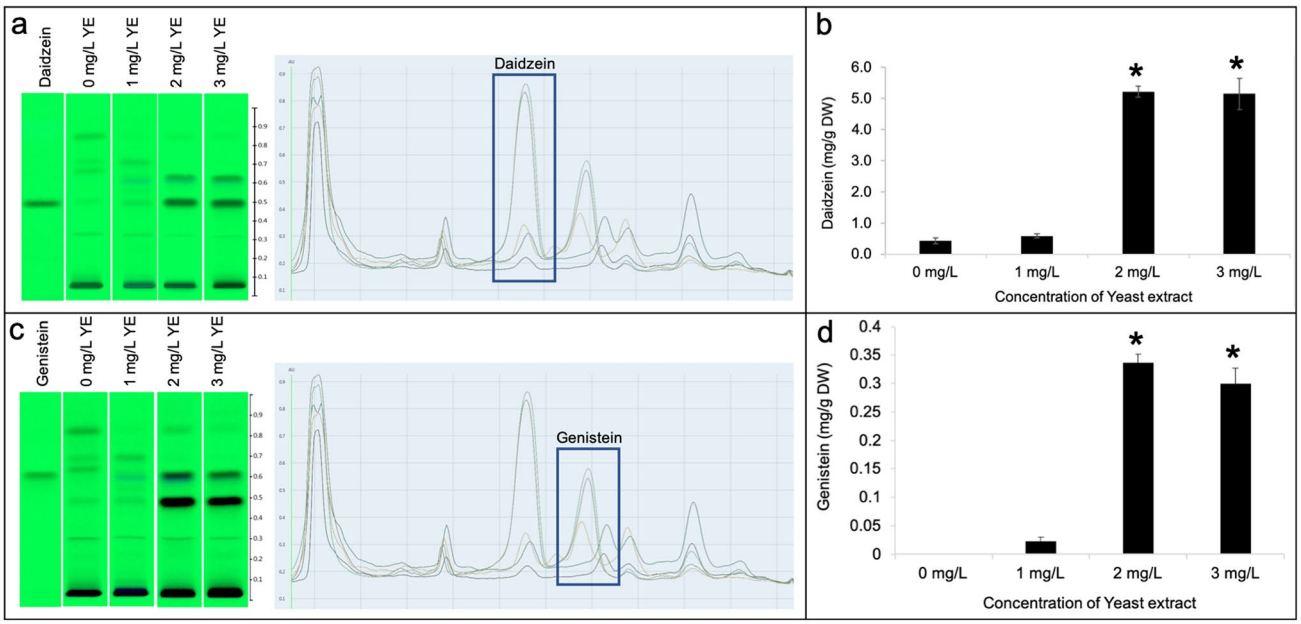

Fig. 3 High-performance thin-layer chromatography (HPTLC) fingerprint at 254 nm presenting the effects of yeast extract on cell suspension cultures of *Pueraria candollei* var. *mirifica*. **a** HPTLC profile of elicited suspension cells with standard daidzein. **b** Daidzein accumulation after elicitation in suspension cell cultures. **c** HPTLC profile of elicited suspension cells with standard genistein. **d** Genistein accumulation after elicitation in suspension cell cultures. The values are expressed as the mean ± SE. Asterisks indicate significant differences ($p < 0.05$)

genistein, respectively (Chu et al. 2014). Interestingly, in contrast to the findings for CHS and CHI, the CHR and IFS genes, which are important enzymes for flavonoid biosynthesis and isoflavonoid production, were upregulated by YE elicitation, resulting in the enhanced production of daidzein and genistein. 2,7,4′-Trihydroxyisoflavanone dehydratase expression was reduced by YE elicitation in the present study, although the expression of the gene was higher in cells treated with 2 or 3 mg/L YE than in those treated with 1 mg/L YE; this result suggests that the substrate amount must be regulated (balanced) to ensure the final biosynthesis of daidzein and genistein. Isoflavone reductase plays a crucial role in responding to biotic and abiotic environmental stresses. Elevated IFR levels were observed in YE-treated cells in the current study. Isoflavone reductase is a downstream enzyme responsible for the accumulation of specific isoflavonoid phytoalexins (Chu et al. 2014). Upstream enzymes influence a larger number of pathway end-products than downstream enzymes; thus, the expression levels of downstream genes are more crucial for the production of the desired isoflavones. In the present study, it was also observed that the generated amounts of daidzein and genistein were reduced when the YE concentration was increased from 2 to 3 mg/L, which may be attributable to the fact that plants cannot tolerate high levels of elicitation. Consequently, the amounts of the desired isoflavones were reduced despite the increased expression of certain biosynthetic genes.

Because daidzein and genistein are plant defense molecules, elicitation could be an efficient approach for increasing the production of isoflavones in vitro. Yeast extract elicitation induced the enzymatic and genetic activation of isoflavonoid production in *P. lobata* cell cultures (Park et al. 1995). In the present study, cells treated with 1 mg/L YE did not show enhanced daidzein production compared with control cells and showed only slight genistein production. A noteworthy increase in daidzein and genistein production was observed when YE was used at a concentration of 2 mg/L. The results suggest that elicitation using 2 mg/L YE was optimal for daidzein and genistein production under the study conditions. In addition, elicitation with YE was associated with changes in the HPTLC profiles of the cells, as indicated by the decreased number of peaks (Fig. 3a, b). These results indicated that YE may have direct effects on the genes involved in isoflavonoid biosynthesis, in line with the findings for gene expression. These findings confirmed that YE increased daidzein and genistein production through effects on genes involved in isoflavonoid biosynthesis. By contrast, Korsangruang et al. (2010) reported that YE at all examined concentrations weakly induced isoflavonoid production in suspension cell cultures of *P. candolle*i var. *mirifica*. Park et al. (1995) reported that the treatment of suspension cell cultures of *P. lobata* with YE resulted in rapid declines in the amounts of isoflavonoid conjugates. Notably, we directly determined the amounts of daidzein and genistein as opposed to the total isoflavonoid content, and only a few bands were observed on the HPTLC profile, indicating that fewer compounds were produced in the YE-elicited cells. Thus, it is possible that the total isoflavonoid content was reduced by YE elicitation despite the increased production of daidzein and genistein. The data indicate that elicitation using YE would be useful if the required end-products are daidzein and genistein as opposed to all isoflavonoids collectively.

A simple and feasible elicitation process using YE was proposed in the present work for enhancing the yield of the health-promoting phytoalexins daidzein and genistein in suspension cells of *P. candolle*i var. *mirifica*. Yeast extract, which has advantages such as low cost, lack of toxicity, and biocompatibility, makes the recommended elicitation protocol more commercially appealing for the scale-up production of the two bioactive isoflavones. Notably, this study also highlighted the preliminary molecular mechanisms responsible for the enhanced biosynthesis of daidzein and genistein in suspension cells following YE elicitation. These transcriptomic elements can help overcome impediments in isoflavonoid production in *P. candolle*i var. *mirifica*. Furthermore, functional genomics should be used to characterize the role of such transcriptomic elements in isoflavonoid production.

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