JOURNAL OF NATURAL PRODUCTS

Volume 68, Number 7 July 2005

Rapid Communications

The Transgenic *Arabidopsis* Plant System, *pER8-GFP*, as a Powerful Tool in Searching for Natural Product Estrogen-Agonists/Antagonists

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Received April 7, 2005

Abstract: The transgenic *Arabidopsis* plant system, *pER8-GFP*, may be used as a powerful tool in searching for natural estrogen-agonists/antagonists. Among selected plant extracts and natural products, the method was able to distinguish active extracts (e.g., *Glycine max* and *Pueraria lobata*) and pure compounds (e.g., 17β-estradiol (**1**), genistein (**10**), and daidzein (**11**)) and also to distinguish effects of structural changes on activity. Thus, this rapid sensitive system was proven to be suitable for the discovery of natural products with estrogenic activity.

In 2003, Hayashi and his colleagues established a large-scale assay using transgenic *Arabidopsis*, *Arabidopsis thaliana* (Brassicaceae), for screening plants for natural auxin signaling inhibitors. The yokonolides were identified as novel auxin inhibitors using this auxin-responsive reporter line.[1] This previous investigation supports the use of *Arabidopsis* constructs as useful assay systems. However, the related research all focuses on studies of plant cell signals, such as auxins.[2] The *Arabidopsis pER8-GFP* line, with an estrogen receptor-based transactivator XVE (pER8) system, was developed originally by Chua et al. to chemically regulate protein expression in the plant.[3] In the original paper on *pER8-GFP*, this transgenic plant harboring the human estrogen receptor was found to express GFP reporter protein in response to an estrogen, 17β-estradiol, and a possible antagonist, 4-hydroxytamoxifen, in a dose-dependent manner. It is of interest to determine whether the human estrogen receptor expressed in a plant system may recognize different chemical structures, including estrogen-agonists/antagonists and/or their precursors. This has stimulated the present investigation, which explores the *pER8-GFP* as a tool in screening for possible estrogen-agonists/antagonists from natural resources. This is the first study to use transgenic *Arabidopsis* for an estrogenic assay of reference to animals and humans.

On the basis of rational design based on the similarity of chemical structures, four plant methanol extracts as well as 13 pure compounds were assayed. The samples were classified into five groups, comprising estrogen, androgen, phytosteroids or steroid-like compounds, flavonoids, and isoflavonoids. The classifications and the compound types are listed as follows.

1. Estrogen. 17β-Estradiol (**1**) was active at 1 μM (Figure 1) as described in the past literature.[3]

2. Androgen. Testosterone (**2**), possessing a similar structure and in being only different in the ring A portion from estradiol, was active at a concentration of >10 μM. The activity of **2** is strong but weaker than that of **1**. It is suggested that *pER8-GFP* may recognize the structural differences between **2** and **1**.

3. Phytosteroids or steroid-like compounds. Phytosteroids are used as animal hormone precursors and have a steroid skeleton. However, β-sitosterol (**3**) was inactive at a concentration of 400 μM. Its long side chain might make it unable to bind to receptors. Vitamin D_3 (**4**), with a broken linkage in ring B of the steroidal skeleton and an additional side chain, was also inactive at a concentration of 400 μM.

4. Flavonoids. Two extracts were chosen for this assay. The methanol extracts of *Glossogyne tenuifolia* and the unripe flower buds of *Sophora japonica* were inactive at a concentration of 200 μg/mL. The former is an indigenous

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10.1021/np050121i CCC: $30.25
Published on Web 07/01/2005

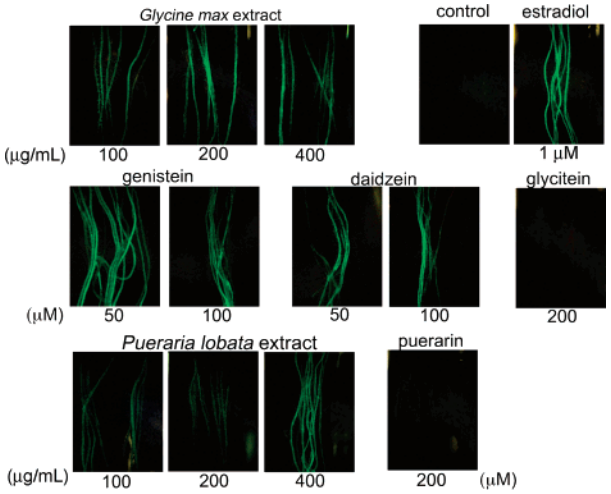


Figure 1. GFP expression of extracts and pure compounds from *Glycine max* and *Pueraria lobata*.

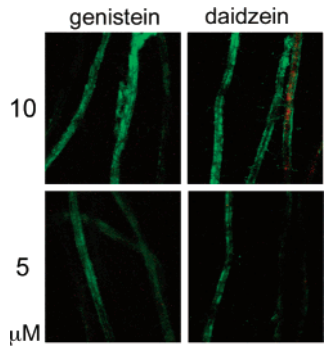


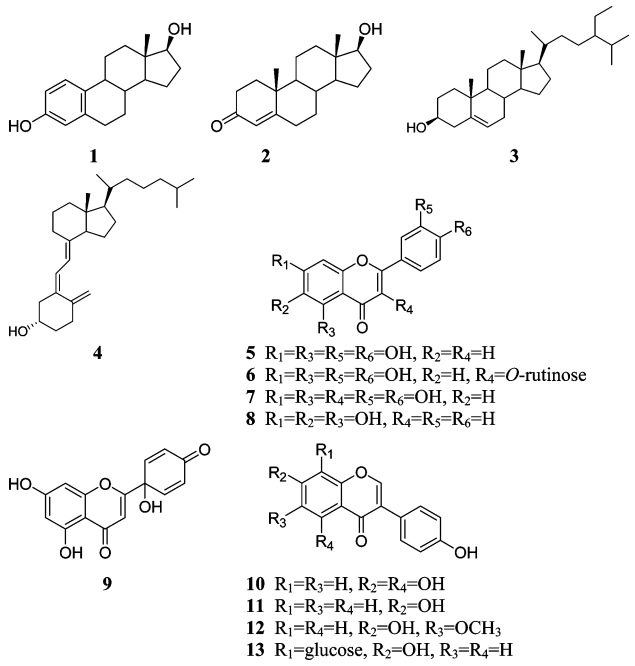
Figure 2. GFP expression of genistein and daidzein at concentrations of 5 and 10 μM.

plant of Taiwan and contains abundant flavonoids and contains 0.5% w/w luteolin (**5**) as a major constituent.[4] The flower buds of the latter, containing ca. 3% w/w rutin and quercetin, are a renowned Chinese medicine used in the treatment of cardiovascular diseases.

Five pure flavonoids were selected. Luteolin (**5**) (from *G. tenuifolia*), rutin (**6**) (from *S. japonica*), quercetin (**7**) (from *S. japonica*), and baicalein (**8**) (from the antioxidant and anticancer Chinese medicine *Scutellaria baicalensis*) were inactive at 400 μM. However, the unusual flavonoid protoapigenone (**9**), from the fern *Thelypteris torresiana*,[5] was active at a high concentration of >100 μM.

5. Isoflavonoids (Figure 1). The methanol extract of *Glycine max* (soybean) was active at a concentration of 50 μg/mL. Soybean is a daily food regarded as an abundant source of isoflavonoids, such as genistein (**10**), daidzein (**11**), and glycitein (**12**). Genistein and daidzein from *G. max* were active at the concentration of 5 μM. In comparison with the fluorescence expression of compounds **10** and **11** at 50 μM, each expression at 5 μM was relatively weak, but the activation of pER8 promoter was pronounced, as may be observed in the Figure 2. Thus, compound **10** was more active and had stronger fluorescence than that of **11**, on the basis of the experimental results. These data indicate that the major isoflavonoids of *G. max* are also the major active compounds. However, compound **12** was inactive. It is interesting to note that minor changes in structure led to modification in the resultant activity.

The methanol extract of *Pueraria lobata*, another folkloric Chinese medicine containing several isoflavonoid *C*-glycosides, was active at >100 μg/mL, but weaker than *G. max*. The inactivity of puerarin (**13**) from *P. lobata* might



be caused by the *C*-glycoside moiety. The results indicated that other active compounds might exist in this plant or that the compounds need to be altered to their active forms by in vivo metabolism.

Thus, on the basis of the aforementioned results, we have shown that *pER8-GFP* can be used as a tool in searching for natural estrogen-agonists/antagonists. This system is also suitable for the large-scale screening of crude plant extracts or to monitor bioactivity-guided isolation because it is a rapid and sensitive system. The use of this convenient and facile method to find pure compounds or extracts of natural products with estrogen-agonist/antagonist activity is proposed. By using rational designs in transgenic plants, they may be of promise to identify and isolate natural products interacting not only with an estrogen receptor but also with various nuclear receptors.

Experimental Section

Plant Material. *Glossogyne tenuifolia* Cass. (3.0 kg) was collected from Penghu Island, Taiwan, in July 2003. *Thelypteris torresiana* (Gaudich.) Alston (3.0 kg) was collected from Nantou County, Taiwan, in August 2003. *Sophora japonica* L. (600 g), *Glycine max* Merr. (600 g), and *Pueraria lobata* (Willd.) Ohwi (600 g) were bought from the I-Chen traditional Chinese medicine store, Kaohsiung, Taiwan, in December 2004. Botanist Dr. Hsin-Fu Yen (Researcher, National Museum of National Science, Taiwan) identified all species. The voucher specimens (Glossogyne001 for *G. tenuifolia*, YCWF-001 for *T. torresiana*, Sophora001 for *S. japonica*, Glycine001 for *G. max*, and Pueraria001 for *P. lobata*) were deposited at the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

17β-Estradiol (1), Testosterone (2), β-Sitosterol (3), Vitamin D_3 (4), Baicalein (8), Genistein (10), Daidzein (11), Glycitein (12), and Puerarin (13). These compounds were purchased from Sigma Chemicals Co. (St Louis, MO), TCI Chemicals Co. (Toyko, Japan), and Nagara Science Co (Gifu, Japan). All other chemicals were reagent or analytical grade.

Luteolin (5). This compound was isolated from *G. tenuifolia*.[4]

Rutin (6) and Quercetin (7). Rutin was extracted using boiling water and recrystallized from the buds of *S. japonica* in a conventional method.[6] Quercetin was obtained by the hydrolysis of rutin.

Protoapigenone (9). This was obtained from *T. torresiana*.[5]

***pER8-GFP* Reporter Assay.** *pER8-GFP* seedlings were grown on a vertically oriented GM agar plate[1] at 24 °C for 5 days in continuous light. The seedlings were transferred into 24-well microtiter plates containing GM liquid medium with/without chemicals in each well. The plate was incubated at 24 °C for 24 h to induce GFP protein. The GFP fluorescence in the root was observed with stereofluorescent microscopy (Olympus SZX12: *Ex* 460−490 nm, *Em* >510 nm), and photographs were taken by digital camera with the same exposure conditions.

Acknowledgment. We thank Dr. M. Inoguchi at Okayama University of Science for useful suggestions. We gratefully acknowledge the financial support from the National Science Council, Taiwan.

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NP050121I