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Expression of dirigent protein and Pinoresinol/Lariciresinol reductase genes of forsythia in transgenic potatoes.

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ABSTRACT

We tried to introduce two forsythia genes related in lignan biosynthesis, dirigent protein and pinoresinol/lariciresinol (P/L) reductase, into potatoes for accumulation of lignans in transgenic potatoes. We made binary vectors overexpressing dirigent protein gene and P/L reductase gene driven by a CaMV35S promoter and transformed into potatoes via Agrobacterium mediated transformation. And in order to control the metabolic flux of lignan biosynthesis pathway, we tried to inhibit chalcone synthase genes of potatoes by antisense inhibition technique also. We tried to use PCR screening method for selection of transgenic plants of different vectors. We tried to determine and compare lignan contents from different transgenic potato lines.

INTRODUCTION

Large respecting studies on plant metabolism provided that a vast and diverse assortment of organic compounds are produced by plants, but the great majority of which do not appear to participate directly in their growth and development. These substances, traditionally referred to as secondary metabolites or plant natural products. Interest in natural products was not purely academic but rather was prompted by their great utility as dyes, polymers, fibers, glues, oils, waxes, flavoring agents, perfumes and drugs. Recognition of the biological properties of myriad natural products has fueled the current focus of this field, namely, the search for new drugs, antibiotics, insecticides, and herbicides.

Plants as foods, in addition to their traditional nutritional values, contain certain non-nutritional phytochemicals such as Biochanin A, formononetin, daidzein, genistein, and the lignans matairesinol and secoisolariciresinol, that may exert long-term health-promoting affects in the human. Important pharmacological effects of plant in the human have been acknowledged and used in medicine for thousands of years.

The strong experimental evidence, suggest that both lignans and isoflavonoids are among the dietary factors affording protection against cancer and atherosclerosis (1,2).

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Lignans by definition, are generally as phenylpropane dimmer. The phenylpropane units are C-C linked, mostly through their C_3-side chains (tail to tail such as pinoresinol).

The lignans secoisolriciresinol (SECO) and matairesinol (MAT), precursors of the hormone-like mammalian lignans, are of particular interest due to their abundance in plants. They are widely distributed in plants accumulating as soluble components, such compound are known to exist as minor constituents of many plants where they form the building blocks for the formation of lignin in the plant cell wall.

As known that steroids are products of bacterial, fungal and plant metabolism while, according to present knowledge, flavonoids produced only by fungi and plants, whereas lignans are exclusively plant-derived chemicals.

Plant foods contain at least twelve thousand natural chemicals produced for structural, hormonal, attractants and chemoprotective purpose. A remarkable diversity has been described for naturally occurring phyto-oestrogens an ability to activate the oestrogen receptor and which have been shown to exert oestrogen effects on the genital tract of female animals. The lignans matairesinol and secoisolariciresinol have been found to possess oestrigenic, anti-oestrogenic, antioxidative, antiviral, antibacterial, insecticidal or fungistatic properties and have been shown to be antiproliferative in relation to many types of tumors in cell culture (3). At the last lignans- a widely distributed class of natural products are also found to have important roles in cancer chemotherapy and prevention, respectively.

Potato (*Solanum tuberosum* L.) is one of the widespread cultivation in top five leading word food crops. Due to its quality such as high productivity, high starch content and vitamin content especially B6, the potato has become one of the major food crop, and its utility is increasing every year, far more rapidly than that of any other crop.As edible plant, if in potato lignans gets accumulated - the beneficial compound, its value should be highly estimated as one of the important kind of functional food. In this study, we are trying to express dirigent protein gene and pinoresinol/lariciresinol reductase gene of forsythia in transgenic tobacco and potatoes. And in order to control the metabolic flux of lignan biosynthesis pathway, we tried to inhibit chalcone synthase genes of potatoes by antisense inhibition technique also.

MATERIALS AND METHODS

Materials

Plant materials: Potato (cvs. Desiree), was maintained by subculture of nodal cuttings on sterile medium consisting Murashige & Skoog Salt Mixture (MS salt) supplied by Life Technologies, Inc, 30g/L sucrose, Vitamin mixture, 100mg/L inositol, 8g/L Agar for medium solidification, pH 5.8. The shoot cultures were grown in Petridish culture vassels in the culture room with a 16 h photoperiod of 2500-3500 lux by cool white lamp (Osram), at 23°C. For producing Microtubers, the shoot cultures were transplanted to medium as above described, but the content of sucrose was increased to 90g/L. For in vitro tuberization (temperature) 18°C was maintained in growth room.

Bacterial Strains and Culture Media:

Agrobacterium tumefaciens strain LB4404 containing appropriate helper and two Forsythia genes related in lignan biosynthesis, dirigent protein pinoresinol/lariciresinol (P/L) reductase were used for potato transformation (Fig 2).

Transformed *Agrobacterium* was cultured in YEP (Peptone: 10g/L, Yeast extract: 10g/L, NaCl: 5g/L, pH7.5, in need case adding 15g Agar for solidifying medium) medium containing 1ml/L, 50mg/ml antibiotic kanamycin and incubated at 28°C for overnight. Protocol- Minipreps was used in isolation and

purification of DNA and for PCR. The results were checked by running the PCR products in 1% Agarose gel.

Methods

Vector construction

We used PCR reactions for introducing NcoI site over the 5' ATG start and 3' stop codon. The pCambia 1301-CHS2AS gene was constructed by ligation of the potato chalcone synthase 2 gene with an antisense orientation between the CaMV 35S promoter and the 3' end of the nopaline synthase gene of plasmid pCambia 1301. The pCambia 1390-dirigent, P/L reductase genes were constructed by ligation of the *forsythia* dirigent protein gene(psd-Fi1) and P/L reductase(plr-Fi1) genes between the CaMV 35S promoter and the 3' end of the nopaline synthase gene of plasmid pCambia 1390-35S.

Transformation and selection

Transformed *Agrobacterium* were grown at the condition as described above and were used for potato transformation. Transfer 10 ml of YEP medium containing 10ul Kanamycin 50mg/ml in falcon tube 50 ml with stopper. Inoculate Cell (transformed *Agrobacterium*). Cap tube tightly and seal with parafilm paper. Incubate at 28°C for 5-6 h with agitation about 200rpm. Infect potato leaves by incubated transformed *Agrobacterium* for approx. 10 min. Plate infected potato leaves onto co-culture (CC) *(dry infected leaves with blotting sterilized filter)* solid medium and keep in darkness or in dispersed light. After 2 or 3 days transfer leaves onto PR (plant regeneration) medium contained antibiotics (Hygromycin or Basta) for selection and regeneration in cultural room.

PCR Screening of transgenic potatoes

PCR reaction was used as routine analytical tool for screening the transgenic potato among many Hygromycin and Basta selected shoots. The protocol of extraction of DNA was followed using molecular biotechnology (4). PCR was performed in a total 20ul using two each specific primers, the amplification profile consists of 94°C and 25 sec. for denaturation of DNA template; 55 °C for annealing and extension process took place at 72 °C for 2 min.

Determination of lignan content:

HPLC system Waters 501 with tunable Absorbance Detector 486, and μBondapak 3.9 x 300 was used for determining lignan content in potato transgenic lines. All solvent and chemicals used were reagent or HPLC grade

RESULT AND DISCUSSION

The monolignols are primarily converted into distinct classes of plant metabolites: the lignans and lignins. Lignins belong to a group of heterogeneous phynelpropane polymers, and the most metabolic flux through the phenylpropanoid biosynthetic pathway is directed to the production of the lignins. The lignins are structural component of cell wall where as lignans are generally defined as phenylpropane dimers and dimeric phenylpropanoid (C_6H_3) linked by 8-8' bonds. Lignan dimmers are found in ferns, gymnosperms and angiosperms, but in higher oligomeric forms also. Lignan formation utilizes coniferyl alcohol prominently, along with other monolignols, allylphenols, and phenylpropanoid monomers to a lesser extent.

Most lignans are optically active and the first demonstrated example of stereoselective control of phenolic coupling was in the in vitro synthesis of (+) - pinoresinol. This overall reaction discovered in

Forsythia species. The formation of pinoresinol takes place under catalysing of laccase or laccase-like enzyme in the presence of dirigent protein. In this case we can assume that dirigent protein represents as a new class of proteins. The gene encoding the Forsythia dirigent protein has been cloned and functional recombinant protein expressed. Which is not homologous to any other protein.

Pinoresinol can be enatiospecifically converted into lariciresinol and secoisolariciresinol, followed by dehydrogenation to give matairesinol. Not only that pinoresinol/lariciresinol (P/L) reductase, also plays an important role in the conversion of pinoresinol into lariciresinol and secoisolariciresinol (5,6,7,8,9).

An attempt was undertaken to introduce *forsythia* genes related in lignan biosynthesis namely dirigent protein and pinoresinol/lariciresinol (P/L) reductase genes into potato. Figure 1 shows a proposed diagram of the lignan biosynthetic pathway of transgenic potatoes. To control the metabolic flux from anthocyanin biosynthesis to lignan, we tried to inhibit the chalcone synthase gene by antisense construct. The

Agrobacterium lines were individually or in combinations (mixed cultures) were transformed. Figure 3 shows the photograph of leaf disc transformation of potato. We tried to co-transform of three different vectors at same time by coculturing three different *Agrobacterium* lines. We have got the 2 plants of dirigent protein genes introduced, 18 plants of dirigent protein gene and chalcone synthase, 3 plants of dirigent protein gene and P/L reductase, 9 plants of dirigent protein gene, P/L reductase and chalcone synthase.

We screened transgenic plant by PCR method with specific primer set of each introducing genes, dirigent protein and P/L reductase genes of *forsythia* and chalcone synthase 2 gene of potato. For screening plants of chalcone synthase introduced, we used one primer of

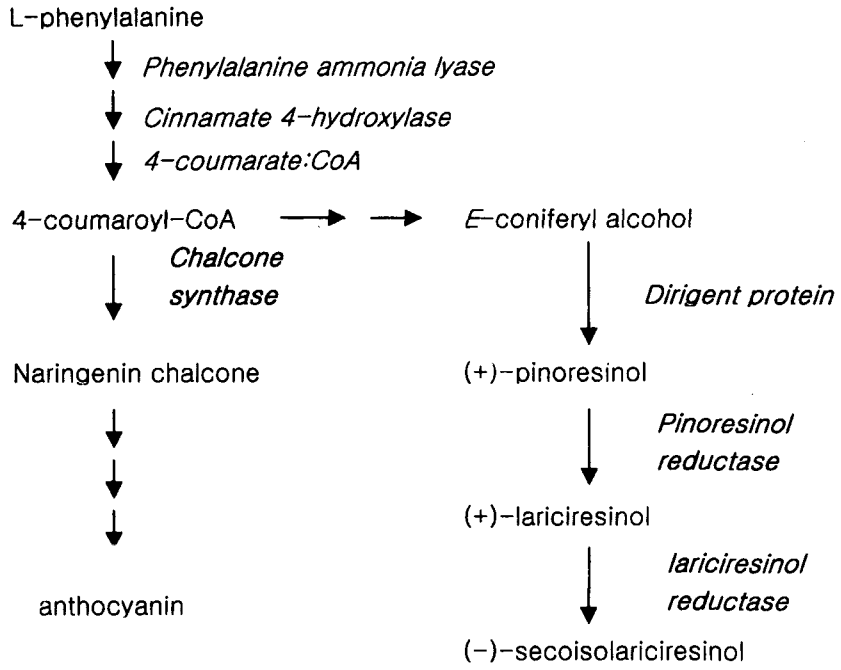

Fig. 1. A proposed diagram of the lignan biosynthetic pathway of transgenic potatoes. The enzymes in bold are encoded by genes expressed as transgenes in this study.

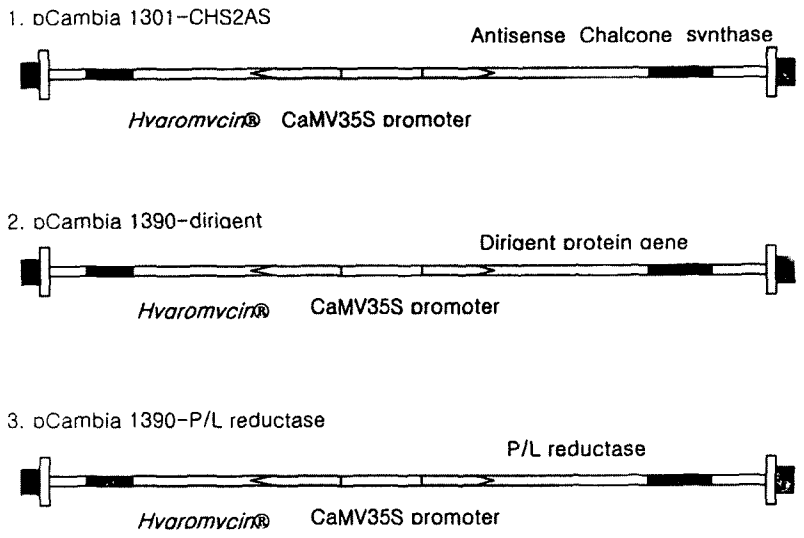

Fig. 2. Construction of vectors used in this study. pCambia 1301-CHS2AS represents antisense expression of potato chalcone synthase2 gene. pCambia 1301-derigent represents expression of forsythia dirigent gene psd-Fi1. pCambia 1301-P/L reductase represents expression of forsythia pinoresinol/lariciresinol reductase gene plr-Fi1.

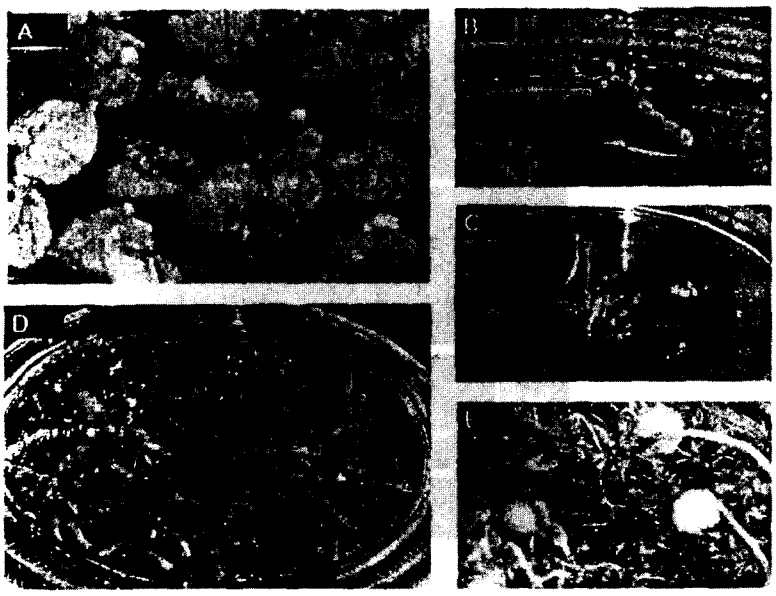

Fig. 3. Leaf disc transformation of potato. A) Calli induction from leaf cut area, for 2 weeks after coculture. B) and C) Regenerated potato shoots in PR (plant regeneration) media for 1~2 months after coculture. D) Transgenic potato lines cultured in vitro. E) In vitro tuberization of transgenic potatoes.

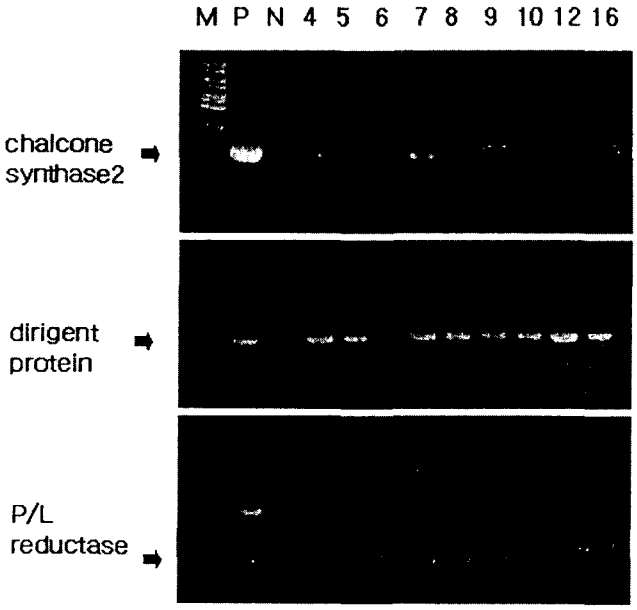

Fig. 4. PCR screening of transgenic tobacco lines co-transformed with pCambia 3301-CHS2AS ,pCambia 1390-derigent, pCambia 1390-P/L reductase gene. M, DNA size marker; P, positive control; N, negative non-transformed; 4~16, different transgenic lines.

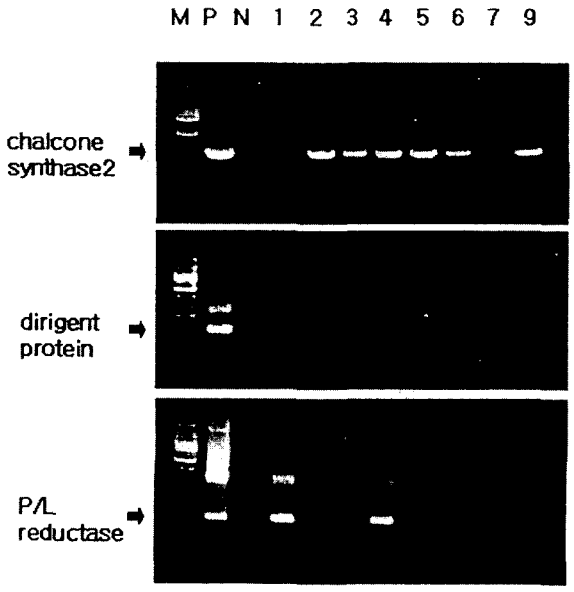

Fig. 5. PCR screening of transgenic potato lines co-transformed with pCambia 1301-CHS2AS ,pCambia 1390-derigent pCambia 1390-P/L reductase gene. M, DNA size marker; P, positive control; N, negative non-transformed; 1~9 different transgenic lines.

Table 1. Co-transformation profile of three different Ti-vectors , pCambia 1301-CHS2AS ,pCambia 1390-derigent, pCambia 1390-P/L reductase gene, of transgenic tobacco.

Agrobacterium lines	Response							
	4	5	6	7	8	9	10	12
pCambia3301-CHS2AS	+	-	-	+	+	+	+	+
pCambia-dirigent	+	+	-	+	+	+	+	+
pCambia-P/L reductase	+	+	-	+	+	+	+	+

1-9: Regenerants

Note: (+) transformed, (-) Nontransformed

Table 2. Co-transformation profile of three different Ti-vectors , pCambia 1301-CHS2AS ,pCambia 1390-derigent, pCambia 1390-P/L reductase gene, of transgenic potatoes.

Agrobacterium lines	Response							
	1	2	3	4	5	6	7	9
pCambia1301-CHS2AS	-	+	+	+	+	+	-	+
pCambia-dirigent	-	-	-	-	-	-	-	-
pCambia-P/L reductase	+	-	-	+	-	-	-	

-1-9: Regenerants

Note: (+) transformed, (-) Nontransformed

CaMV35S promoter region, to distinguish from chalcone synthase gene of itself. The obtained data is shown in figure 4 and 5. In tobacco, we have all of 6 lines transformed. But in potato transformation we failed to get all transformed lines of three different vectors, we have only 2 lines of P/L reductase incorporated and 6 lines of chalcone synthase gene among 9 lines of hygromycin selected. Table 1 and 2 shows that the co-transformation profile of tobacco and potato transformation with mixed co-culture of different *Agrobacterium* lines. We did not have all three genes introduced lines of potatoes yet and we are still selecting transgenic lines of potatoes. Sooner orlater, we are going to do the chemical analysis of lignan, pinoresinol and secoisolariciresinol from transgenic potatoes.

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