DOI 10.1002/mnfr.200700233 Mol. Nutr. Food Res. 2007, *51*, 1518 – 1526

Research Article

Enhancing lignan biosynthesis by over-expressing pinoresinol lariciresinol reductase in transgenic wheat

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Lignans are phenylpropane dimers that are biosynthesized via the phenylpropanoid pathway, in which pinoresinol lariciresinol reductase (PLR) catalyzes the last steps of lignan production. Our previous studies demonstrated that the contents of lignans in various wheat cultivars were significantly associated with anti-tumor activities in APC^{Min} mice. To enhance lignan biosynthesis, this study was conducted to transform wheat cultivars ('*Bobwhite*', '*Madison*', and '*Fielder*', respectively) with the *Forsythia* intermedia PLR gene under the regulatory control of maize ubiquitin promoter. Of 24 putative transgenic wheat lines, we successfully obtained 3 transformants with the inserted ubiquitin-PLR gene as screened by PCR. Southern blot analysis further demonstrated that different copies of the PLR gene up to 5 were carried out in their genomes. Furthermore, a real-time PCR indicated ~17% increase of PLR gene expression over the control in 2 of the 3 positive transformants at T_0 generation. The levels of secoisolariciresinol diglucoside, a prominent lignan in wheat as determined by HPLC-MS, were found to be 2.2-times higher in one of the three positive transgenic sub-lines at T_2 than that in the wild-type (117.9 ± 4.5 *vs*. 52.9 ± 19.8 μg/g, $p < 0.005$). To the best of our knowledge, this is the first study that elevated lignan levels in a transgenic wheat line has been successfully achieved through genetic engineering of over-expressed PLR gene. Although future studies are needed for a stably expression and more efficient transformants, the new wheat line with significantly higher SDG contents obtained from this study may have potential application in providing additive health benefits for cancer prevention.

Keywords: Cancer prevention / Lignans / Pinoresinol lariciresinol reductase / Secoisolariciresinol diglucoside / Transgenic wheat

Received: June 25, 2007; revised: August 4, 2007; accepted: August 8, 2007

1 Introduction

Lignans are phenylpropane dimers linked by β-β bonds with a 1,4-diarylbutane structure [1, 2]. They occur naturally in a number of plant families, including the gramineae and oleaceae, which contain the monocots and eudicots, respectively [3, 4]. In monocots such as wheat, lignans are mostly located in the aleurone layer of seeds [5], and in eudicots such as *Forthysia*, lignans occur in the fruits and stems [6].

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Abbreviations: **PLR**, pinoresinol lariciresinol reductase; **SDG**, secoisolariciresinol diglucoside; **Ubi**, ubiquitin

The main lignan in wheat bran is secoisolariciresinol diglucoside (SDG). When consumed, SDG is oxidized by intestinal microflora to lignan metabolites, *e.g.* enterodiol and enterolactone. The biological importance of lignans and lignan metabolites has been previously reviewed [7–9]. Epidemiological studies show an inverse association between dietary intake of lignans and the risk of cardiovascular disease [10, 11]. Lignans also have potential protective roles against cancer in breast [12], prostate [13], and colon [14, 15]. A study done in rats showed that exposure of 10% flaxseed (SDG-rich plant seeds) or the equivalent SDG levels during suckling suppressed chemical carcinogen 7, 12-dimethylbenz(α)anthracene (DMBA)-induced mammary tumorigenesis [16]. In addition, *in vitro* cell culture studies demonstrated that enterolactone and/or enterodiol reduce growth and metastasis of breast cancer cells [17]. Furthermore, lignan metabolites have been shown to

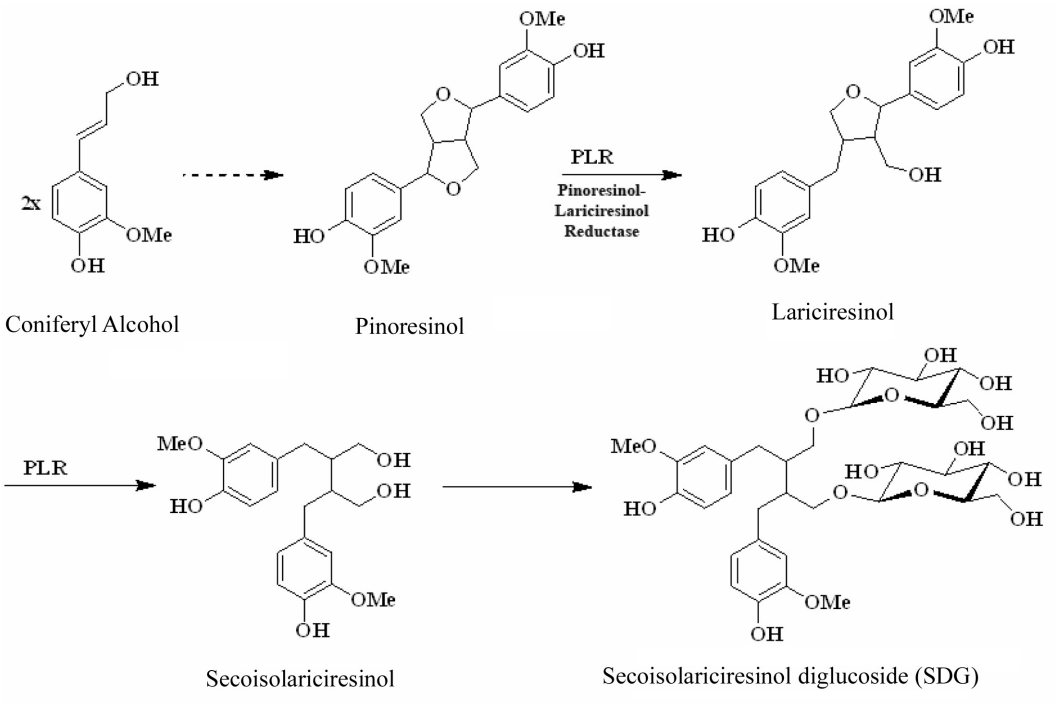

Figure 1. Schematic of lignan biosynthetic pathway with emphasis on the last enzymatic steps by pinoresinol lariciresinol reductase (PLR) leading to secoisolariciresinol diglucoside (SDG), a prominent lignan present in wheat (modified from Fujita *et al.* [3]).

reduce cell growth in human colon cancer SW480 cells [18]. It is interesting that the contents of lignans in wheat bran from various cultivars are correlated with anti-tumorigenesis in spontaneous Min mice with mutant adenomatous polyposis coli (APC^{Min}) [18, 19]. Lignans are abundant in flaxseed but not quite in wheat grains that usually contain about 4 – 50 μg/g [19]. Enhancement of the SDG biosynthesis in wheat plants, therefore, appears to be significant for cancer prevention.

Genetic engineering is one of the ways for genetic crop manipulation in order to enhance phytochemical synthesis, which has already been shown in many cases to improve agronomic and nutritional aspects of crop plants [20, 21]. The biosynthetic pathways to SDG occur via coupling of two coniferyl alcohol molecules to afford pinoresinol (Fig. 1). Then, pinoresinol undergoes sequential reduction by pinoresinol-lariciresinol reductase (PLR) to generate lariciresinol and secoisolariciresinol [3, 22]. Since PLR catalyzes the last steps of the lignan biosynthesis, it is postulated that over-expression of PLR gene by genetic engineering may enhance lignan contents. Although PLR gene had already been isolated from various woody plants such as *Forsythia intermedia* [3, 23, 24], the only known isolated PLR enzyme in monocots has been found from flaxseeds [25]. In wheat, however, the PLR gene and corresponding protein(s) have not been reported yet.

The purpose of this study is to enhance SDG biosynthesis in transgenic wheat by genetic transformation of *Forsythia intermedia* PLR gene. To the best of our knowledge, this is the first study trying to apply genetic engineering wheat for enhancement of lignan biosynthesis.

2 Materials and methods

2.1 DNA constructs

PLR cDNA (1.2 kb, GenBank accession number U81158) encoding (+)pinoresinol-(+)lariciresinol reductase in *Forsythia intermedia* was kindly provided by Dr. Norman Lewis from Washington State University (Pullman, WA). *Forsythi* PLR cDNA was initially cloned into pGEM® T Easy vector (Promega, Madison, WI). During amplification of PLR by PCR, *Bgl*I sites were appended to the 5′ and 3′ ends, respectively. The PCR product for PLR gene was then obtained following digestion by restricted enzyme *Bgl*I. The sequence of the *Forsythi* PLR gene was confirmed at the Gene Sequencing Facility, Department of Plant Pathology, Kansas State University (Manhattan, KS). The PLR gene was then inserted into *Bam*HI site (compatible ends with *Bgl*I) in pAHC17 plasmid under the control of the maize ubiquitin (Ubi) promoter (2.1 kb) as described by Christensen and Quail [26]. Restriction digestion with *Pst*I, *Eco*RI, and *Bam*HI, respectively, were used to confirm the correct directional insertions (data not shown). The PCR products by both primer sets (PLR F and R and Ubi-PLR F and R as denoted in Table 1 and illustrated in Fig. 2) were

Table 1. DNA sequences of the primers used in this study

Primers	Sequence	Product size (bp)
PLR F:	TCG TAG ACG TAG TAA TCA GCG CCA	539
R:	TCG AGC TCT TTC ACG GAG GCT AAA	
bar F:	CCT GCC TTC ATA CGC TAT TTA TTT	600
R:	CTT CAG CAG GTG GGT GTA GAG CGT G	
Ubi-PLR F:	GAT GCT CAC CCT GTT GTT TGG TGG TGT	583
R:	TGC CAA ATT GAC AGA GAC CTC CAA	
Real-time PCR F:	ATC CAA GAA CCC TCA ACA AGC TGG TGT	99
R:	TCC CAT GTC TGA ACA ATT CTC	

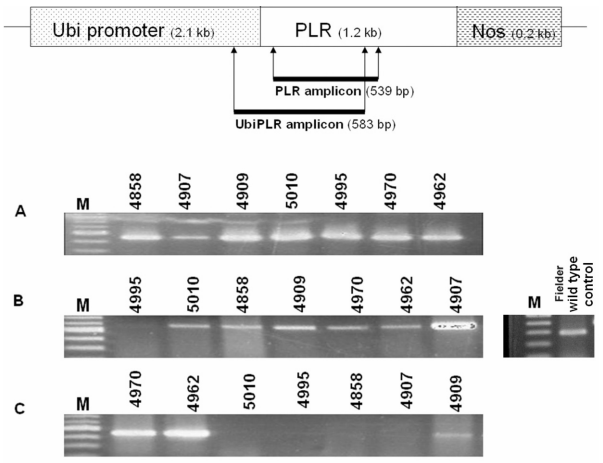

Figure 2. PCR screening analyses of genomic DNA extracted from various transgenic wheat plants. Top panel: schematic of gene construct in transformation pAHC17 plasmid. The gene of pinoresinol lariciresinol reductase (PLR) from *Forsythia intermedia* was constructed in pAHC17 under the control of the maize ubiquitin (Ubi) promoter. The gene cassette with the Ubi promoter, PLR transgene, and nopaline synthase (Nos) terminator is shown with the amplified fragments of PLR and Ubi-PLR for PCR screening analyses. Bottom panel: representative PCR screening of seven transgenic clones. The putative (T_0) transgenic wheat plants were analyzed by PCR-based analyses of genomic DNA using specific primers as denoted in Table 1 for *bar* (A), *Forsythia* PLR (B), and Ubi-PLR (C), respectively. A PCR profile generated using *Forsythia* PLR primer for genomic DNA extracted from the non-transgenic wild-type '*Fielder*' control is also indicated.

further confirmed by sequencing at the Gene Sequencing Facility, Department of Plant Pathology, Kansas State University (Manhattan, KS). The new constructed plasmid designated as pAHCUbi-PLR contains the Ubi promoter, opening reading frame from the *Forsythia* cDNA encoding PLR, and nopaline synthase (*nos*) terminator region (Fig. 2). In addition, plasmid pAHC20 contains the *bar* gene (2.0 kb) under the control of the maize Ubi promoter-intron [26]. The bar gene confers resistance to the herbicide glufosinate (Liberty®, Aventis, Research Triangle Park, NC). Both pAHCUbi-PLR and pAHC20 plasmids were used for wheat co-transformation.

2.2 Transformation procedure

Both pAHCUbi-PLR and pAHC20 plasmids were co-bombarded into embryogenic calli of wheat plants (*Triticum aestivum* L. cv. *'Bobwhite', 'Madison', and 'Fielder',* respectively). The method of co-transformation and selection of transgenic events have been described by Anand *et al.* [27]. Briefly, the premature seeds were surface sterilized with 20% sodium hypochlorite and 0.02% TWEEN-20. Immature embryos were then aseptically excised on CM4 medium to initiate somatic embryo formation. Somatic embryos that were proliferated in CM4 + osmoticum (0.2 M mannitol, 0.2 M sorbitol) were co-bombarded with pAHC20 and pAHCUbi-PLR plasmids at 1:1 ratio by using the particle inflow gun.

2.3 Selection and regeneration of transgenic wheat plants

The methods for selection and recovery of transgenic wheat plants were described by Alpeter *et al.* [28] with minor modifications. Briefly, wheat calli were placed on CM4 medium containing 5 mg/L glufosinate 16 h after co-bombardment. Cultures were kept in the medium of 10 mg/L glufosinate for 10 – 15 weeks. The growing clumps were transferred to shoot production medium (MSP) with 5 mg/L glufosinate selection until green shoots were observed [29]. The cultures were then re-transferred to elongation and rooting medium (MSE) containing 5 mg/L glufosinate but not 2,4-D for 2 – 3 weeks. Healthy looking plantlets obtained were transferred to soil and grown in environmentally controlled green house (16 h light at 600 $\mu E/m^2/s$).

2.4 Leaf painting assay

To examine the expression of the selectable *bar* resistance gene in the transgenic plants, leaf planting was done as previously described [27]. Briefly, freshly prepared solution of herbicide, Liberty® at 0.2% v/v was applied on the second/third youngest leaf using a cotton plug. The painted area was marked using a marker pen and visual observations were recorded 3 – 5 days after painting. Positive lines with resistant green leaves were selected for further PCR screening analysis.

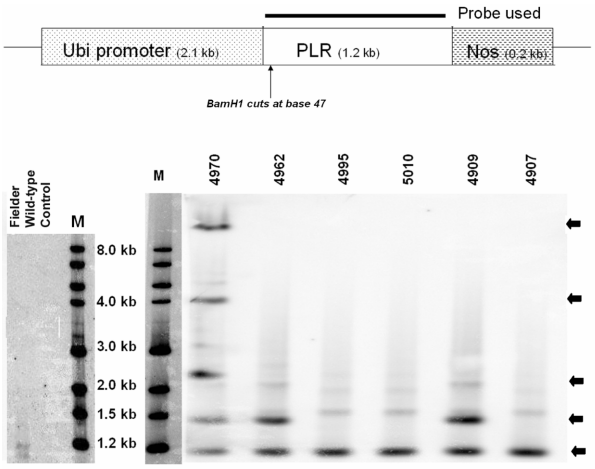

Figure 3. Southern blot hybridization of wheat genomic DNA resulting various integration patterns from different transgenic events. Top panel: *Forsythia* PLR inserted in the gene cassette with a unique *Bam*HI site at 47 bp. Bottom panel: representative gels of Southern hybridization. Wheat genomic DNA obtained from various independent transformants at T_0 were digested with *Bam*HI followed by hybridization with ^{32}P-labeled *Forsythia* PLR cDNA. The arrows indicate various copies of PLR gene that has different molecular weight at ~1.2, 1.5, 2.2, 4.0, and 10.0 kb, respectively. The endogenous wheat PLR gene was also detected when probed with the genomic DNA from the non-transformed wild-type '*Fielder*' control.

2.5 PCR screening analysis

As shown in Table 1, three primer sets were designed to screen *bar*, PLR, and Ubi-PLR combination genes, respectively, in transgenic wheat plants. Genomic DNA was extracted from leaves of transgenic wheat plants by using phenol chloroform extraction method [30, 31]. Briefly, 100 – 500 ng of genomic DNA from transgenic plants were screened by each of the three primer sets in a PTC-220 thermal Cycler (Hybaid Limited, Hastings, UK). Samples were denatured, annealed and extended at 94°C, 58 – 60°C, and 72°C for 1 min, 30 s, and 45 s, respectively, for 35 cycles. PCR products were visualized through 1.8% agarose gel electrophoresis by ethidium bromide staining. Only transformants that tested positive with Ubi-PLR primer sets were reported as a confirmation of transgenic success.

2.6 Southern blot analysis of transformed PLR gene

About 25 µg of extracted genomic DNA as mentioned above were fully digested with *Bam*HI and separated by electrophoresis in 0.8% agarose. *Forsythia* PLR contains a unique *Bam*HI site at 47 bp as indicated in Fig. 3. The genomic DNA fragments were then transferred to Hybond-N^+ nylon membrane using standard protocols (Amersham, Piscataway, NJ) and hybridized for 24 h with ^{32}P-dCTP-labeled *Forsythia* PLR gene. After hybridization, blotted membrane was exposed in a phosphor imager cassette and measured using the Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

2.7 PCR amplification and sequence of partial wheat PLR gene

Wheat genomic DNA was extracted as described above from a wild-type wheat cultivar '*Fielder*'. A primer set (PLR F and R as denoted in Table 1) was used for PCR amplification of a 539 bp PLR fragment at the same PCR conditions as mentioned above. The PCR product at 539 bp was purified using the montage DNA PCR purification kit (Millipore, Bedford, MA) and then inserted into a multiple cloning site of the pGEM® T Easy vector (Promega, Madison, WI) for ligation. The inserted wheat PLR gene fragment was isolated from the positive clones and sequenced at the Gene Sequencing Facility, Department of Plant Pathology, Kansas State University (Manhattan, KS). The sequence of partial wheat PLR gene was then compared to *Forsythia* PLR sequence in the GenBank at NCBI (National Centre for Biotechnology Information) by using NCBI Sequence Comparison Software at http://www.ncbi.nlm. nih.gov/

2.8 Real-time PCR quantification of PLR gene expression

To quantify the levels of PLR gene expression in the positive transgenic plants at T_0, total RNA was isolated from the leaf tissues by use of an isolation kit (Promega, Madison WI). The quantity of RNA was measured by spectrophotometric analysis at 260 nm. The quality and integrity of the extracted RNA was assessed by both spectrophotometric analysis at 230/260 ratio and gel electrophoresis in 1.0% agarose gels visualized by ethidium bromide staining under UV light. First strand cDNA synthesis was performed using 1 µg of RNA with reverse transcriptase under the recommended conditions of the ImProm-II™ Reverse Transcription System (Promega). The primer set (real-time PLR F and R as denoted in Table 1) was applied to amplify a 99-bp fragment of the PLR gene by using the Sybr green PCR master-mix1 (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed in the iCycler Thermal Cycler (Bio-Rad) with a classic amplification profile and the PCR product was then quantified by the iCycler Bio-Rad software. The reaction without cDNA product served as a negative control and the relative expression of PLR mRNA was normalized to a same amount of positive control GAPDH cDNA. The experiment was repeated in triplicate and the results were plotted as a relative log CT unit.

2.9 SDG identification and quantification by HPLC-MS

Sample extracts from transgenic wheat seeds at T_2 were quantified for SDG levels by HPLC and confirmed by MS.

Briefly, 10 – 30 transgenic or non-transgenic seeds (~0.2 – 0.8 g) were grounded and defatted by using hexane and then dried in the hood overnight. Defatted whole extracts were then homogenized under cold conditions with liquid nitrogen. The mixture was centrifuged and the supernatant was extracted for lignans by mixing with diethyl ether for three times. The upper organic phase containing the lignans was combined and evaporated to dryness. The residue was then re-dissolved in 100% methanol with 5 mM flavone as an internal standard and subjected to HPLC. HPLC procedure was performed according to our previous method [18] with a slight modification. Generally, samples were injected into a C18 column (5 μm, 250 × 4.6 mm, Alltech, Deerfield, IL) and eluted with a 5% ACN in pH 2.8, 0.01 mM phosphate buffer (solvent A) over 100% ACN (solvent B) at a flow rate of 1 mL/min. A gradient run of 0 – 10 min in 100% solvent A, 10 – 30 min in 0 – 100% solvent B and finally 30 – 40 min in 100% solvent B was determined as optimum. The SDG peak was detected by monitoring absorbance at 283 nm and identified by both retention time and mass spectrum comparison with a purified SDG (ChromaDex, Irvine, CA). A linear HPLC calibration curve was obtained for the concentrations between 0 – 100 μM. The SDG contents were calculated based upon the standard calibration curve following recovery adjustment by internal standard flavone and then expressed as μg/g in fresh seed samples.

HPLC-MS/ESI analysis was performed with Esquire 3000 plus mass spectrometer (Bruker Daltonics, Bremen Germany). Separations were achieved with a synergi RP C18 column (250 mm × 2 mm id, 5 μm) (Berlin, Germany) using ACN:water (containing 0.1% formic acid) for elution in a gradient from 0 min at 70% ACN:30% water to 3 min at 95% ACN:5% water, followed by isocratic elution with 95% ACN:5% water between 3 and 21 min, and finally 100% ACN from 24 to 25 min. The flow rate was 0.4 mL/min throughout. The MS/ESI traces recorded was positive ions from *m*/*z* 100 to1500. An MS software version 3.2 (Bruker Daltonics) was used to differentiate real peaks from background noise peaks.

2.10 Statistical analysis

All data were analyzed by the SAS statistical software, version 8.2. The real-time PCR determination and HPLC quantification were analyzed by one-way ANOVA protocol using the general linear model procedure followed by Fisher's protected least square difference. A probability of ≤ 0.05 is considered significant.

3 Results

3.1 Transgenic wheat plants

Two hundred seventeen putative transgenic lines were generated on selection medium containing 10 mg/L glufosinate. Out of the 217 putative transformants, 24 lines tested positive for the *bar* gene based on the leaf painting assay (data not shown). Three sets of the gene-specific primers (Table 1) were used for PCR screening analyses on these herbicide resistant lines. These primer pairs detected the *bar* (Fig. 2A), *Forsythia* PLR (Fig. 2B), and the combination of Ubi-PLR construction (Fig. 2C), respectively. All 24 herbicide resistant lines tested positive for both *bar* and *Forsythia* PLR genes and only 3 lines including #4909, #4962, and #4970 from transgenic wheat cultivar *‚Fielder'* tested positive for Ubi-PLR transgene (Fig. 2C). It should be noted that the wild-type *`Fielder'* also showed a positive band by PCR primers assigned for *Forsythia* PLR, suggesting a cross-reaction occurred from wild-type wheat PLR allele. Most of the transgenic lines such as #4858, #4907, and #5010 except for #4995 showed positive *bar* and PLR but negative Ubi-PLR combination, which appeared possibly due to the cross-reaction from the wild-type wheat PLR allele and thus used as the false positive controls. The line #4995 seemed to be a negative transgenic control because of its positive *bar* transgene but negative PLR and Ubi-PLR.

3.2 Detection of transgene by Southern blotting

To confirm the reliability of the PCR findings, Southern hybridizations were performed using *Forsythia* PLR probe as denoted in Fig. 3. All the individual T_0 lines with both *bar* and PLR transgenes including two false positive controls (#4907 and #5010) and a negative transgenic control (#4995) were screened after the digestion of their genomic DNA with restriction enzyme *Bam*HI that cut once in the respective PLR transgene cassette at 47. As showed in Fig. 3, one major *Forsythia* PLR gene hybridization band was found in both false positive controls and the negative transgenic control. Two major bands were observed in the Ubi-PLR positive lines #4909 and #4962 and even five major bands were noticed in the positive transgenic wheat line #4970. The molecular weight of the Ubi-PLR hybridization bands varied at ~1.2, 1.5, 2.2, 4.0, and 10.0 kb, respectively. In addition, the wild-type wheat cultivar *‚Fielder‘* had shown a weak band at ~1.2 kb, suggesting a possible cross-hybridization occurred between *Forsythia* PLR and wheat PLR gene.

3.3 Sequence of a partial wheat PLR gene and comparison with Forsythia PLR gene

Wheat genomic DNA from wild-type wheat cultivar *‚Fielder'* was used as a template for a PCR amplification by a pair of primers designed based upon *Forsythia* PLR gene. A 539-bp PCR product was obtained. After vector clean to remove all the unreadable N's from the sequence, an actual PCR product at 520 bp from the wheat genomic template was successfully sequenced and submitted to the GenBank with accession number at EU078326.

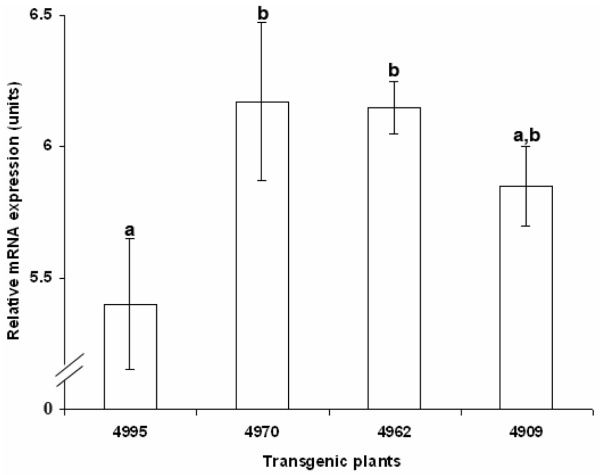

Figure 4. Quantitative real-time PCR for quantifying PLR transcript levels in independent transformants at T_0 obtained from various transgenic wheat clones. Results are means ± SD, n = 3. Means with different alphabetical letters differ significantly, $p \leq 0.05$.

By blast searching in the GenBank database of the NCBI webpage, the sequence of this new wheat PLR gene fragment is as much as 98% similarity to *Forsythia* PLR (U81158).

3.4 Real-time PCR quantification of transgene expression

Real-time PCR was used to quantify the expression of PLR gene in the three positive transgenic lines in comparison with the negative transgenic control #4995. As shown in Fig. 4, the relative expression of PLR gene in the transgenic lines #4962 and #4970 but not in #4909 was significantly higher than that in the negative transgenic control 4995. About 17% increase of PLR gene expression over the negative control was found in the two positive transformants at T_0 generation. The level of PLR gene expression in the wild-type wheat cultivar *'Fielder'* was also measured once, which had a comparable level to the negative transgenic control (data not shown).

3.5 Detection and quantification of SDG by HPLC and HPLC-MS

The SDG contents in the transgenic wheat seeds at T_2 generation were further determined by HPLC for a final measure of the functional transformation success. As shown in Fig. 5, a standard SDG peak (Fig. 5A) and the SDG peak in the seed extracts (Fig. 5B) were confirmed by MS with *m/z* at 704.04 $[SDG+H_2O]^+$ and 709.12 $[SDG+Na]^+$ (Fig. 5D) that was matched with the standard SDG (Fig. 5C). Figure 5E showed the SDG contents in the transgenic wheat lines #4970 I5, #4970 B1, #4970 A3, and #4909 E5, respectively, when compared with the wild-type *'Fielder'* control and a false positive control #5010 A2. The annotation of an alphabetic letter and an Arabic number following each wheat line represents various sub-lines in T_1 and T_2 generations, respectively. A significant increase in SDG contents was found in the transgenic sub-line #4970 I5 only, but not in other sub-lines. The contents of SDG were about 2.2-times higher in #4970 I5 than that in the wild type (117.9 ± 4.5 *vs*. 52.9 ± 19.8 μg/g, $p < 0.005$).

4 Discussion

Of the 217 putative transgenic wheat lines obtained after co-bombardment of pAHCUbi-PLR with pAHC20 plasmids, only 3 lines at T_0 were identified with a positive Ubi-PLR transgene by PCR screening. Southern blot further indicated one or multiple copies up to 5 of transferred PLR gene in those three transgenic plants. Real-time PCR quantification showed a significant increase in a relative expression of PLR gene in 2 of the 3 successful transgenic lines. Quantification of the SDG levels finally showed a significant increase in one of the transgenic sub-lines.

Putative transformants were survived from conditioned-medium selection process and *bar* screening. PCR screening analysis further identified 24 transgenic plants out of 217 putative transformants for both *bar* and PLR positive genes. The wheat line #4995 that had positive *bar* but negative PLR and Ubi-PLR genes might be an escape, since the Southern blotting demonstrated the present of an endogenous wheat PLR. The line #4995 thus may not be a perfect negative control. Some transgenic plants such as #4858, #4907, and #5010 that carried positive *bar* and PLR genes but not Ubi-PLR could be used as the false positive controls. It should be noted that the positive PLR product in the false positive controls appeared to be synthesized from indigenous wheat PLR gene, since the wild-type wheat cultivar *'Fielder'* also showed a positive PLR band. It is unexpected that the indigenous wheat PLR gene could be homologous to *Forsythia* PLR. Because wheat is a monocot and *Forsythia* is an eudicot, it is usually predictable to perceive some divergence between their PLR genes. As the wheat PLR gene was recognized by the PCR primers assigned for *Forsythia* PLR gene in the PCR screening analysis and hybridized with *Forsythia* PLR cDNA in the Southern blotting, and as the sequence of the corresponded PCR product from wheat genomic DNA template shared 98% similarity to *Forsythia* PLR gene, this might suggest a homology, at least in part, between wheat and *Forsythia* PLR. Considering a vital role of lignans in plants as the precursors of cell wall lignin biosynthesis, it could be possible even for genetically diverse plants such as wheat and *Forsythia* to keep a homologous PLR as a conservative gene. However, the data accumulated in this study were suggestive, but not conclusive. A conclusion cannot be made until a whole-wheat

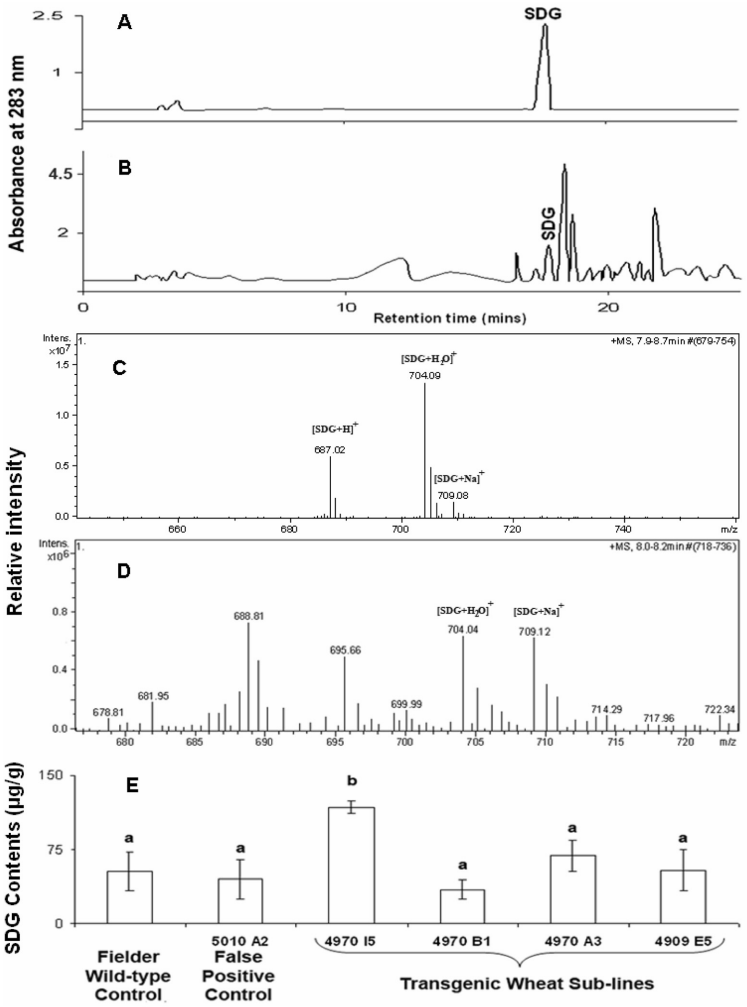

Figure 5. Quantification of SDG contents in transgenic wheat seeds from various sub-lines of the transgenic wheat plants at T_2. (A) HPLC chromatography of a standard SDG; (B) HPLC chromatography of a representative wheat seed extract; (C) MS spectrum of the standard SDG peaks, indicating 687.02 $[SDG+H]^+$, 704.09 $[SDG+H_2O]^+$, and 709.08 $[SDG+Na]^+$, respectively; (D) MS spectrum of the identified SDG peaks obtained from HPLC-separated wheat seed extract, indicating 704.04 $[SDG+H_2O]^+$ and 709.12 $[SDG+Na]^+$, respectively; (E) Quantifying SDG contents in various wheat seeds at T_2 from various transgenic wheat clones and non-transgenic '*Fielder*' controls. Results are means ± SD, $n = 3$. Means with different alphabetical letters differ significantly, $p \leq 0.005$.

PLR sequence in both gene and deduced protein is revealed.

It is interesting to look at the integration pattern of *Forsythia* PLR hybridization with wheat genomic DNA after *Bam*HI digestion, a single recognition site in *Forsythia* PLR gene at 47 bp. The various numbers of hybridization bands appeared to be related to the copy numbers of a PLR gene in the wheat. All the wheat lines tested including a negative transgenic control #4995 and two false positive controls #4907 and #5010 displayed an integrated hybridization band at ~1.2 kb, which seemed compatible with a smear band in the wild-type *'Fielder'*, indicating the endogenous copy of the wheat PLR. In comparison with the controls, however, the three positive transgenic wheat lines at T_0 demonstrated additional hybridization bands: at ~1.5 kb for both #4909 and #4962, and at ~1.5, 2.2, 4.0, and 10.0 kb, respectively, for #4970, suggesting the insertional copies of PLR gene. Plants #4909 and #4962 showed a similar hybridization pattern that might happen from a same transformation event. Plant #4970 had five major hybridization bands, which might come from different transformation events. Multiple copies from different transformation events usually occur due to unpredictable particle inflow gun as suggested by others [32, 33]. In addition, some weak bands presented in all the samples including the controls seemed to be coincided with the predicted weak binding of the partial 5′-side fragment of 47-bp PLR.

Real-time PCR showed a significant expression of PLR gene in both #4962 and #4970, but not #4909. The variation of a gene expression might not be merely associated with the copy numbers of a PLR gene from different transformation events. An identical expression of PLR was found between the transgenic plants #4962 and #4970, although they possessed a diverse copy number of PLR gene. That is to say, a copy number alone is probably not sufficient to account for the variation in the expression levels. In fact, many other factors such as insertion sites, biological variation, and/or gene silencing may affect insertional gene expression, especially in the ubiquitin-promoted gene expression in a transgenic plant as suggested by others [34, 35].

The contents of SDG in the wheat seeds at various T_2 sub-lines from the two transgenic wheat families (#4909 and #4970) that significantly over-expressed PLR gene were further examined. A considerable increase in the SDG

levels was found in one of the sub-lines (#4970 I5) in the #4970 family, averaging at 117.9 μg/g *vs*. 52.9 μg/g in the wild-type wheat cultivar *'Fielder'*. Such strong enhancement in lignan levels could result in a significant promotion for wheat products in cancer prevention. Our previous studies demonstrated that the contents of SDG in wheat bran from various wheat cultivars were correlated with anti-tumor activity in a spontaneous APC^{Min} mouse model [18, 19]. According to that correlation, the anti-tumor activity in the transgenic wheat sub-line #4970 I5, when its SDG contents were averagely raised from 52.9 to 117.9 μg/g, could be extrapolated to elevate from ~36 up to ~58%. Future studies to evaluate the anti-cancer activity of this novel SDG-rich transgenic wheat line are warranted.

It should be noted that neither #4909 nor other sub-lines in #4970 family showed a significant change in the SDG contents when compared with the wild-type or the false positive controls. The poor performance in lignan biosynthesis enhancement at T_2-seeds from those T_0-over-expressed transgenic plants might have been due to gene silencing, unstably expression, and/or inefficient transformation, etc. Indeed, a subsequent analysis of both PLR and *bar* genes by PCR was undetectable in the selected T_2 seeds including some sub-lines from both #4970 and #4909 families, suggesting the transgenic PLR might not be stably established in these sub-lines during random transmission. Furthermore, multiple enzymes are involved in the lignan biosynthesis and over-expression of a single enzyme may not be efficient if its precursor reactants are not just timely abundant. It is likely that a "pathway transformation" by transfer not only the last step enzyme PLR but also the early step enzyme(s) such as phenylalanine ammonia lyase, a well-known key-enzyme to control the initial step of the secondary metabolism in plants, may be much more effective for lignan biosynthesis enhancement.

Taken together, this is the first study to show a genetically transformed wheat line that has over-expressed PLR gene and thus enhanced SDG contents. Of the total 217 putative transgenic lines, 3 transformants with the inserted ubiquitin-PLR cassette were successfully obtained. Southern blotting further demonstrated insertional copies of PLR gene up to 5 in these three wheat genomes and a quantitative real-time PCR indicated over-expression of PLR gene significantly in 2 of the 3 transformants. The SDG contents were actually enhanced in one of the sub-lines. Although future studies are needed to establish a stably expression and more efficient transformants, the new wheat line with significantly higher SDG contents obtained from this study may have potential application in providing additive health benefits for cancer prevention.

The authors greatly appreciate the kind help of Dr. Norman Lewis, Washington State University, by providing PLR cDNA from Forsythia intermedia used in this study. This work was supported in part by a USDA Cooperative Project (KS 680-0199184) from the Agricultural Experiment Station, Kansas State University (Journal Contribution No. 06-199-J).

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