WHEAT LIGNANS AND CANCER PREVENTION

by

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B.S., Makerere University Kampala, 2001

M.S., Kansas State University, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Human Nutrition

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Abstract

Wheat lignans are phenylpropane dimers linked by β-β bonds with a 1, 4-diarylbutane structure. They are biosynthesized in the cell cytoplasm through action of enzymes of the phenylpropanoid pathway. Pinoresinol lariciresinol reductase (PLR) catalyzes the final steps of biosynthesis of wheat lignans. In epidemiological and clinical investigations, studies show that high plasma lignan amounts correlate with reduced risks of breast, colon, and prostate cancers. However, in some of the studies, the results are not consistent. More consistent results are observed when animal and cell culture models are used. Our previous studies in the Wang lab demonstrated that treatment of human colon cancer cells, SW480 with lignans results in a dose and time dependent inhibition of cancer cell growth. In the first paper, we investigated direct experimental cancer preventative characteristics of a wheat lignan, secoisolariciresinol diglucoside (SDG) vs. its metabolite enterolactone in human colon cancer SW480 cells. Treatment of cancer cells with 0-40 μM SDG or enterolactone resulted into inhibition of cancer cell growth as observed by reduction of cell numbers. The reduction appeared related to induction of S-phase cell cycle arrest rather than cytotoxic effect. Further analysis revealed that SDG was more stable in cell culture medium than enterolactone. HPLC-MS/ESI showed that enterolactone is the principle metabolite in cancer cells but undetectable SDG or its metabolites were in the cells treated with SDG. In the second paper, we investigated over expression of the PLR gene and enhancement of lignan levels in transgenic wheat. We transformed wheat cultivars (‘*Bobwhite’, ‘Madison’, and ‘Fielder’* respectively) with the *Forsythia intermedia* PLR gene under the regulatory control of the maize ubiquitin

promoter. Of the total 217 transgenic wheat lines, we successfully obtained 7 transformants with the inserted ubiquitin PLR gene as screened by PCR. Real-time PCR further indicated 109-117% PLR over expression over the transgenic control in 3 transformants of the 7 at T_0 generation. In addition, the levels of SDG, as determined by HPLC was found to be significantly elevated in one of the 3 positive transgenic plants. To the best of our knowledge, this is the first study reported that genetically engineered wheat with over expressed PLR enzyme enhancing phytochemical lignan has been successfully achieved.

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Approved by:

Major Professor
Weiqun Wang

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promoter. Of the total 217 transgenic wheat lines, we successfully obtained 7 transformants with the inserted ubiquitin PLR gene as screened by PCR. Real-time PCR further indicated 109-117% PLR over expression over the transgenic control in 3 transformants of the 7 at T_0 generation. In addition, the levels of SDG, as determined by HPLC was found to be significantly elevated in one of the 3 positive transgenic plants. To the best of our knowledge, this is the first study reported that genetically engineered wheat with over expressed PLR enzyme enhancing phytochemical lignan has been successfully achieved.

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Dedication

Dedicated to my father, John Patrick K'lara Nono (RIP) and my ever loving mother, Zeredy Tanga K'lara Nono. For your love that goes on and on and on and on……

CHAPTER 1 - WHEAT LIGNANS AND CANCER PREVENTION- LITERATURE REVIEW

Wheat Lignans and Cancer Prevention

Summary

Wheat lignans have phenylpropane dimers linked by β-β bonds with a 1,4-diarylbutane structure. They occur in significant amounts mainly as glycosides in aleurone layer of wheat kernels after biosynthesis in the cell cytoplasm through the phenylpropanoid pathway. In the phenylpropanoid pathway, wheat lignans are synthesized from simple phenyl derivatives such as corniferyl alcohols. The initial and final steps in this pathway involves deamination of phenylalanine catalyzed by phenylalanine ammonia lyase and reduction of pinoresinol to secoisolaciresinol by pinoresinol lariciresinol reductase respectively. On consumption, lignans are metabolized in the colon into enterolactone and enterodiol by the bacterial fermentation process. Enterolactone and enterodiol are important because they can be used as biomarkers in epidemiological studies. Epidemiological studies suggest that high circulating levels of lignan metabolites are correlated with reduced risks of chronic diseases such as cancer and cardiovascular disease. The same observation has also been observed in animal and cell culture models. For example, investigations in the mice induced to develop mammary carcinogenesis and treated with a wheat lignan at 0.01% (w/w) in diet showed reduced mammary carcinogenesis related biomarkers. Some of the mechanisms that are suggested for wheat lignan health protecting roles include anti-oxidant, anti-estrogenic, anti-proliferation, cell cycle induction, and apoptosis.

Biosynthesis of lignans

Very little information is available about the wheat phenylpropanoid pathway that occurs in the cell cytoplasm. However, earlier work on the contention of phenylpropanoid pathway has suggested the endoplasmic reticulum as the main organelle for this pathway (1-3). Enzymes of interest, phenylalanine ammonia lyase (E.C. 4.3.1.5) and pinoresinol lariciresinol reductase are among two of the enzymes involved in this pathway that catalyze initial and final steps respectively. Phenylalanine ammonia lyase catalyses the first step of the phenylpropanoid pathway involving deamination of phenylalanine to produce ammonia ion as a by-product while pinoresinol lariciresinol reductase catalyses the sequential reductive fission of pinoresinol to lariciresinol and then secoisolaciresinol. Lignans are biosynthesized to form monomeric or oligomeric lignans that are packaged as glycosides before release into the wheat aleurone layer during the seed development stage such as secoisolariciresinol diglucoside (SDG) (4). Monomeric lignans have been found in wheat, in tea (5) while an oligomeric lignan has been shown in flax (6). Our focus is primarily on the prominent monomeric lignan, SDG present in wheat.

Other monomeric wheat lignans in the phenylpropanoid pathway (Fig. 1) include pinoresinol, lariciresinol, and secoisolaciresinol. The sequential reductive fission of pinoresinol to lariciresinol and then to secoisolaciresinol is a key regulatory point in this pathway and is catalyzed by pinoresinol lariciresinol reductase as has shown in woody plants (7, 8) and in flax seeds (9). Very little information on the enzymatic actions of pinoresinol lariciresinol reductase. In the last decade, Norman and his colleagues at Washington State University have managed to isolate the pinoresinol lariciresinol reductase enzyme from *Forsthysia intermedia* (10, 11). Their work and that of others on identification of the DNA sequence of pinoresinol lariciresinol

reductase enabled us to isolate the very first partial sequence of the wheat pinoresinol lariciresinol reductase gene (Fig. 2) using primers designed based on the *Forsthysia intermedia* cDNA (see appendix B). Wheat pinoresinol lariciresinol reductase shares 50% sequence similar with the *Forsthysia* pinoresinol reductase gene. To the best of our knowledge, this is the first time the partial sequence of the wheat PLR sequence has been shown.

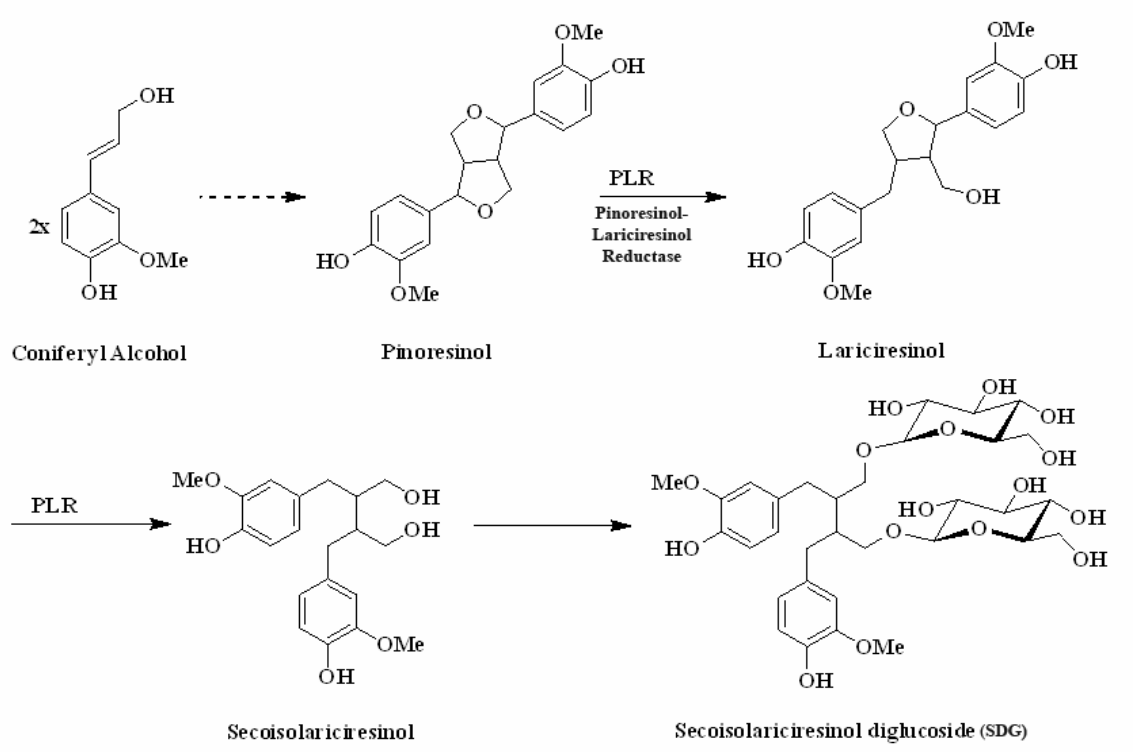

Figure 1:1 Phenylpropanoid pathway for lignan biosynthesis showing last stage lignan formation catalyzed by pinoresinol lariciresinol reductase

1TCGAAGCCNCCAAATTCTTCTTCAACTCAAGCTTGTTGAAGCTATTAAAGG

52GCTGGAAATGTCAAGAGNNTTTTACCATCTGAGTTTGGAATGGATCCTGCAAA

123ATTTATGGATACGGCCATGGAACCCGGAAAGGTAGCACTTGATGAGAAGATG

156GTGGTAAGGAAAGCAATTGAAAAGGCTGGGATTCCTTTCACATATGTCTCTGC

209AAATTGCTTTGCTGGTTATTTCTTGGGAGGTCTCTGTCAATTTGGAAAATTCTT

263CCTTCTAGAGATTTTGTCATTATACATGGAGATGGTAACAAAAAAGCAATATAT

318AACAATGAAGATGATATAGCAACTTATGCCATCAAAACAATTAATGATCCAAGA

372ACCCTCAACAAGACAATCTACATTAGTCCTCCAAAAAACATCCTTTCACAAAAA

429AGTTGTTCAGACATGGGAGAAGCTTATTGGAAAGAACTGCAGAAAATTACACT

484CTCGAAGGAAGATTTTTTAGCCTCCGTGAAAGAGCTCGA

Figure 1:2 Partial sequence (~50% length of *forsthysia* 1.2 kb PLR sequence) of the wheat pinoresinol lariciresinol reductase gene

Bioavailability of lignans

The bioavailability of wheat lignans refers to the amount of wheat lignans that are absorbed into the blood stream after passage through the mammalian gut system (12). Consumption of wheat lignans and subsequent exposure to enzymatic and bacterial activity in the mouth, stomach, intestines, colon and cecal generates lignan metabolites, enterodiol and enterolactone. Bacterial activity in the colon results into fermentation of lignans into enterodiol, which is subsequently oxidized into enterolactone. Once formed, lignan metabolites are absorbed into the blood stream mainly through a passive mechanism. Excess unabsorbed lignan metabolites are excreted in urine and fecal matter (13).

Commercial foods rich in wheat lignans include shredded wheat cereal, toasted wheat bran flasks, and whole wheat bread (14). When these foods are consumed, the chewing action of the mouth physically breaks down wheat lignans into small swollenable particles. In addition to that, the first step of metabolism may involve removal of the attached sugars in the lignan glycosides; a reaction catalyzed by glycosidase. Glycosidase activities can occur in the food itself (endogenous or added during processing) or in the cells of the gastrointestinal mucosa or can be secreted by the colon micro flora (15). Colon micro floras are also important in wheat lignan fermentation process that occurs in the distal end of the digestive system.

Many factors are suggested to affect the overall efficiency of the bacterial fermentation for formation of lignan metabolites, enterolactone and enterodiol. Most important of all these factors is the food matrix in which the lignans are bound. Foods which are milled and finely crushed will provide a higher mammalian lignan source than others such as wheat bran or ground flax seeds (16). Besides milling, extrusion processes may be important in increasing accessibility of wheat lignans to the fermentation process. In addition, surgical procedures such as removal of

the distal section of the digestive gut such as in colon ileostomy may reduce overall enterolactone and enterodiol formation as seen with a rye diet (17).

Pharmacokinetics relates to the rate of availability and elimination of mammalian lignans from different organs within the body. A recent study on the availability of enterolactone and enterodiol after a dietary rich lignan meal (18) and after ingestion of purified SDG alone (19). In the later study, twenty healthy volunteers on a dose of purified SDG (1.31 μmol/kg body weight) show maximum blood plasma levels of enterolactone and enterodiol at 19.7 +/- 6.2 and 14.8 +/- 5.1 hrs respectively post ingestion. This indicates that enterodiol circulates faster than enterolactone. In their discussion, the authors suggest that both enterolactone and enterodiol are distributed through way of first order of kinetics. In addition, uptake of enterolactone and enterodiol has also been analyzed in vitro using different human epithelial cells (20). Jansen et al shows that conjugation and excretion of enterolactone and enterodiol in HT29, CCD841CoTr and CaCo-2 cells is complete after 8 hrs except for enterodiol in Caco-2 cells (20).

After intake, enterolactone and enterodiol are excreted in urine as glucuronates (73-94%) or sulfate conjugates (2-10%), as free phenols in feacal matter (21). In animal models, the concentration of enterodiol and enterolactone excreted is dependent on the percentage amount consumed. For a diet supplemented with 0% -5% flax seeds, metabolite excretion is observed to increase exponentially and before it plateaus off at 5-10% (22).

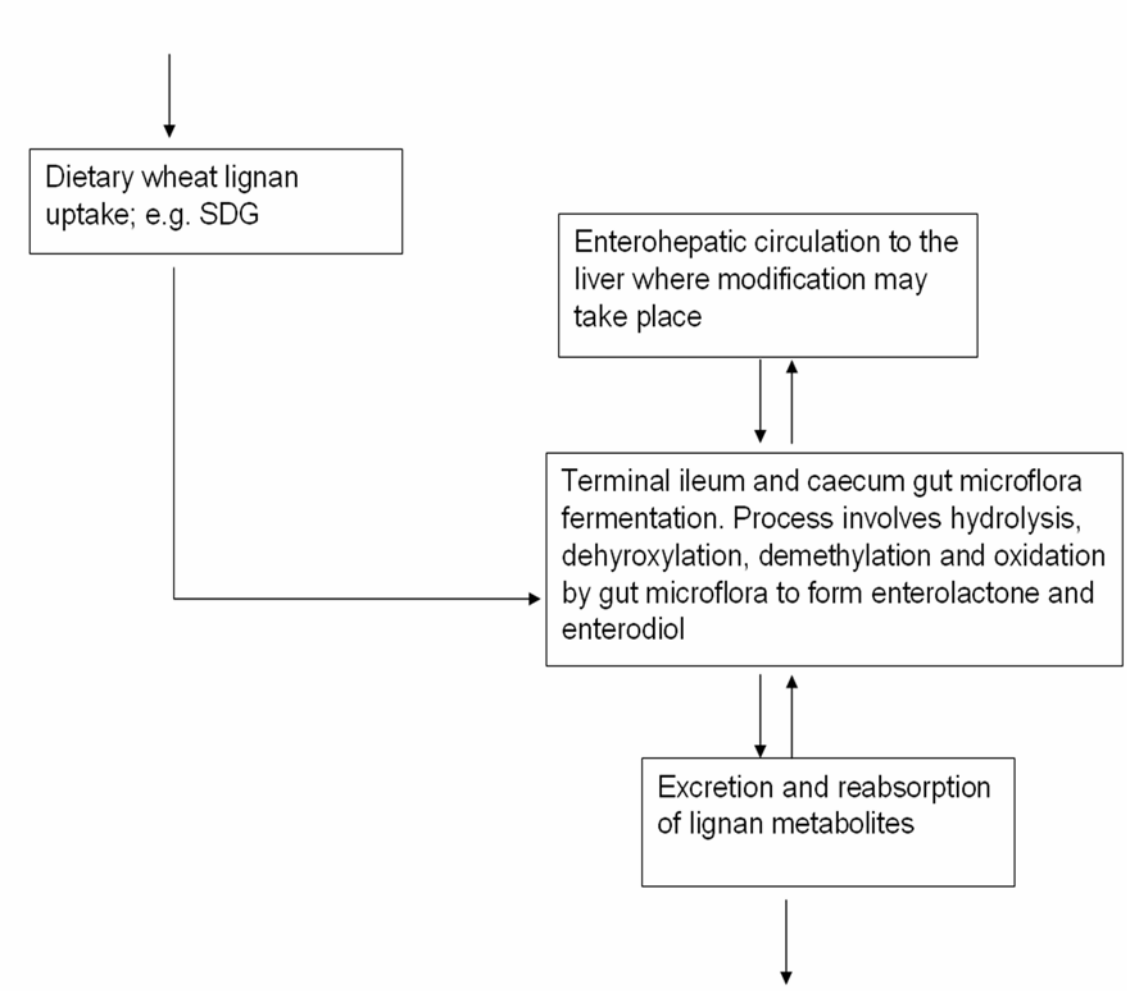

Figure 1:3 Lignan metabolism showing the fate of lignans through the digestive gut and its major metabolites, enterolactone and enterodiol

Lignans and cancer prevention studies

Many studies have established that there exists an inverse relationship between lignan consumption and reduced cancer risks. Lignan plasma amounts are directly correlated with reduced cancer risk as shown in some population studies and proved in animal and cell culture models

Epidemiological and clinical studies

Although conflicting data does exist, many epidemiological studies suggest an inverse relationship between wheat lignan consumption and various cancer risks including breast, colon, prostate and other cancers.

The earliest work on association between wheat lignan intake and the risk of breast cancer was done by Adlercreutz and his colleagues (23). In that landmark publication, the authors found that lignan excretion high in vegetarian Asian women is accompanied by a low breast cancer risk while in omnivorous Boston women, lignan excretion was low accompanied with a high breast cancer risk (23). Today, at least 183 publications discuss the epidemiological results between lignan intake and breast cancer, a few with conflicting results (24). In a recent review article, the authors suggested that the inverse relationship between lignan consumption and breast cancer are more consistent in case-control studies vs. prospective studies, in pre-menopausal women vs. menopausal women, and in analysis of enterolactone vs. enterodiol (25). We recently published a book chapter (in press) where we emphasize essentially the same conclusions and add that the conflicting data are in part due to inadequate databases used in dietary lignan estimation as suggested by others (26). We further add here that estrogen receptor status and diet-gene interactions are essential in the analysis of epidemiological intervention data of dietary lignans in breast cancer (27). Perhaps, that is why Touillard and colleagues (2007) did

observe an inverse relationship between dietary lignan intake (< 1395 μg/day), enterolignan exposure and the risk of invasive breast cancer when they considered breast cancers that were estrogen and progesterone receptor positive for women who were post menopause (28).

In colon cancer epidemiological studies, the relationship between lignan intake and colon cancer is also summarized by a recent publication conducted in Netherlands that demonstrates that a substantial reduction of colorectal adenoma risk is associated with a high plasma level of lignan metabolites (29). In a Ontario based colon cancer population case control study involving 1095 cases and 1890 control subjects, the authors suggest that dietary lignan intake is associated with a significant reduction in colorectal cancer risk [OR (T3 vs. T1) = 0.73; 95% CI: 0.56, 0.94]. They did not observe any evaluated interactions between polymorphic genes that encode enzymes possibly involved in metabolism of phytoestrogens (CYPs, catechol O-methyl transferase, GSTs, and UGTs) and the risk of colon cancer (30).

In prostate cancer epidemiological studies, Arts and Hollman's review (31) suggest that the inverse relationship between lignan intake and prostate cancers is consistent when case-control studies are used and none when using prospective and nest case control studies. The same view is held by other authors (32, 33). In one of the few conflicting case control studies, a 2006 Swedish population-based case control study, the authors show conflicting results for a questionnaire-data for 1,499 prostate cases vs. 1,130 controls. They suggest no association between dietary intake of total or individual lignans and risk of prostate cancer. However, on further analysis, intermediate serum levels of enterolactone were associated with a decreased risk of prostate cancer (34).

In other cancer related epidemiological studies; women show a 50% (OR, 0.50; 95% Cl, 0.31-0.68; P=.04 for interaction) reduction in lung cancer risk when both hormone therapy use

and intake of enterolactone and enterodiol are analyzed together [ongoing US- base case control study (35)].

In the University of Texas M.D. Anderson study, no association is suggested between dietary lignan intake and reduction of testicular cancer in young men (36).

In conclusion, across all the cancers, the inverse relationship between wheat lignan consumption and cancer risk reduction are more observable in case control studies than in prospective cohort studies (25, 37) especially as seen in breast cancers. Perhaps, future studies should employ improved exposure assessment and multiple sampling techniques to ascertain positive associations. Also, the use of other biomarkers such as lignan antioxidant activity and sex hormone binding protein levels instead lignan metabolite levels for determination of associated cancer risks is suggested (38, 39).

Experimental animal and cell cultures studies

Many studies involving cancer inducible animal models and cell culture models test the potential benefits of lignans as anti-cancer agents by using either purified lignan preparations or foods rich in lignans. Unlike epidemiological studies, more consistent results are evident in experimental animal and cell culture studies. A summary of some of the important studies is shown below;

Table 1.1 Lignan cancer prevention studies in animal models

Source	**Causative agent and/ animal model**	**Effects on Cancer**	**Ref**
10% flax (or equivalent SDG)	NMU induced mammary tumor genesis in F344 rats	↓ number and size of mammary adenoma carcinomas	40
10 mg/kg enterolactone	DMBA induced mammary tumorigenesis	↓ tumor number	41
5% or 10% flaxmeal or SDG equivalent	AOM induced Sprague-Dawley rats	↓ number of crypts, foci and cell proliferation	42
10% flaxseed or SDG	Nude mice injected with MDA-MB-435	↓ lung and lymph metastases	43
0.02% secoisolariciresinol/ Matairesinol	Min Mice	no effect	44
73-293 μmol/kg SDG or 2.5-10% flax seeds	B16BL6 murine melanoma cells in C57BL/6 mice	↓ tumor number and size	45
0.01% SDG in diet	Azoxymethane induced F344 rats	abberant cryt foci	26

In the above studies, carcinogenesis or tumor markers in animal models is induced with either azoxymethane or N-nitroso N-methylurea (NMU) or 7, 12-dimethylbenz (α) anthracene (DBMA) (41-44, 47) treated along with lignan or lignan enriched diets. In one case, spontanous carcinogenesis animal model, Apcmin (mutated adenomatous polyposis coli gene) (45) were used. In two cases, human breast cancer cell lines or melanoma cancer cell lines xenografted into the animal model caused cancers (44, 46). In almost all animal models, lignan supplementation/treatment is started before induction of the carcinogenesis except in a few cases, where it was after carcinomas had been established. Wheat lignan or SDG equivalent diets (that is purified SDG of 73, 147 and 293 μ mol/ kg body equivalent to flaxseed at 2.5, 5 and 10% w/w) was the most common lignan diet used except two studies that used enterolactone injected intravenously (42) and secoisolaciresinol and matairesinol (44).

The results indicate that wheat lignans are chemo-preventative against initial stages of carcinogenesis as seen by reduction in number of early carcinogenesis related markers, such as number of ACF (aberrant crypt foci), size and number of adenoma, size and number of tumors and number of metastases.

Unlike the animal model experiments, the cell culture studies on human colon (47 – 49) prostate (50) and breast cancer cell lines (51) mostly use enterolactone and enterodiol. Our study suggested enterolactone inhibited a human colon cancer cell line, SW480 in a time and dose dependent manner (48). The effect may be additive when both enterolactone and enterodiol are combined (49). Enterolactone is also shown to induce apoptosis and inhibit growth in human colon cancer cell line, colo-201 (49). In this study, the expression of apoptosis suppressor protein and proliferation related PCNA protein is down regulated while apoptosis enhancing protein is up-regulated (49). In four other human colon tumor cell lines; LS174T, CaCo-2, HCT-15 and T-

84, enterolactone and enterodiol at 100 μM concentration reduced cell proliferation (39). In human breast cancer cell line (MCF-7), enterolactone at 10 nM significantly inhibited the growth of cells (51). At a lower dose (0.5-2 nM), the effect was stimulatory for cell proliferation; the dose amount used is the same as the levels of estrogen hormone estradiol circulating under normal conditions (1 nM). This and other studies suggested that enterolactone is agonist towards estradiol receptors in stimulated MCF-7 breast cancer cells at a low dose but antagonist at higher doses, hence indicating a possible mechanism by which it affects growth of estrogen sensitive cells (51). In prostate cancer cell lines (PC-3, DU-145, LNCAP), 10-100 μM enterolactone and enterodiol significantly inhibit growth of all cell lines (40). In this study, enterolactone (IC 50 of 57 μM) is more potent than enterodiol (IC 50 of 100 μM) (50).

Therefore, in conclusion, growth inhibitory effects in the cancer cell lines can be explained by several mechanisms; such as anti-oxidant, estrogenic and anti-estrogenic mechanisms among others.

Plausible mechanisms of lignans in cancer prevention

Many studies suggest that wheat lignans are chemo preventative agents (52) towards chronic diseases such as cancer through anti-oxidant, anti-estrogenic and other potential mechanisms.

Anti-oxidant activity

Wheat lignans are chemo-preventative agents that with high anti-oxidant activity (53). Reports ascertain that wheat lignans in physiologically important doses are 3-4 times more potent than vitamin E (54). SDG and its metabolites enterodiol and enterolactone have been reported to have scavenging ability towards free radicals (55, 56). However, comparison of anti-

oxidant activity between SDG and its metabolites, enterolactone and enterodiol indicated that SDG may have (58). It is further suggested that lignan's free radical scavenging ability may eliminate potential carcinogens such as reactive oxygen species through participation in phase 1 reactions. Free radical scavenging mechanism may explain for the plant lignans anti-oxidant role in 1) reduction of risks associated with coronary and cardiovascular diseases as in the Zupter elderly study (58) and as in Arts review (31), 2) reduction of lipoprotein intake into cells in atherogenic lesions.

A genomic response will result when leaking reactive oxygen species from the mitochondria stimulates redox sensor kinases that in turn activate transcription factors through the Nrf2 pathway (59). Through the Nrf2 pathway, active transcription of phase 2 metabolism enzymes will result and hence further consolidate the detoxification process. Hence, wheat lignans can essentially eliminate all these downstream pathways through elimination of reactive oxygen species. Infact, previous work of the Wang lab found that enterolactone was capable of inducing detoxification enzyme activity (60) most likely related to phase 2 metabolism.

Anti-estrogenic activity

Lignans as phytoestrogens act through estrogen receptor-mediated mechanisms. Structural similarity to estrogen receptors allows mammalian lignans to act as estrogenic antagonists competing with beta-oestradiol in the estrogen receptor complex. The levels of severity of a estrogen dependent cancer such as breast cancer is lower with high circulating levels of lignans and the reverse is also true. Inhibition of aromatase and 17 beta-hydrosyteroid dehydrogenase (important enzymes in estrogen synthesis) after a lignan ingestion, are suggested as strong evidence for this role (61). In relation to this, is the production of presenilin 2 in MCF-7 breast cancer cell lines elicited by mammalian lignan, enterolactone. Presenilin 2 production is

directly associated with inhibition of cellular proliferation as seen in breast cancer cells. A biphasic growth effect involving induction of cell proliferation at doses <10 μM enterolactone, and inhibition of the same at >10 μM has been observed in vitro (62).

In addition to this, lignans as phytoestrogens have been suggested to lower cholesterol through increased low density lipo-protein receptor activity (63). Wu et al (2006) also suggested that lignans ingestion from sesame may improve sex hormones through increased levels of sex hormone binding globulin in postmenopausal women (64).

However, estrogenic activity of lignans have been correlated with causing adverse effects in animals as reviewed by Rickard and Thompson (65) with some contradictions. Ward et al (2001) exposed flaxseed or SDG in diets of dams and observed no alteration of reproductive indices in male and female offsprings (66). In humans, Mitchell et al (2001) concluded that phytoestrogen dose had no effect in male semen quality (67).

Other potential Mechanisms

A recent study done in our lab showed that enterolactone, a mammalian phytoestrogen, causes a dose and time dependent inhibitory effect on the growth of SW480 cells due to S-phase cell cycle arrest (48). Other reports have also indicated a similar trend effect of lignans on cell cycle control points, and the apoptosis pathway. In the cell cycle, key control points involving tumor suppressor protein; the p53 protein, p21, p15 among others have been suggested to be involved in the growth inhibitory effect of lignans. Enterodiol is known to induce apoptotic cell death in MCF-7 tumors by increased expression of p21 protein levels and decrease of Bcl-2 levels (apoptosis suppressing protein), cyclin D1, and Rb protein expression (68). Rb protein is important in progression of cells into S-phase. In another study, secoisolariciresinol administration to D-galactosamine/lipopolysaccharide (LPS)-induced hepatic injury in mice

resulted in direct inhibition of apoptosis mediated by tumor necrosis factor alpha (TNF-alpha) (69).

Supplementation of min mice with a 10% rye (colon rye fermentation produces enterolactone) is significantly associated with normalized beta- catenin levels thus reducing the associated cell neoplasia in these min mice (70). In a study involving the TMCH mice (mice in which neoplasia is induced by Celiobacter rodentium) increased nuclear beta-catenin expression was recorded by both biochemical and histological approaches, accompanied by increases of cyclin-D1 and c-myc protein expression. When these mice were treated with lignans, levels of beta-catenin were normalized (71) resulting into regression of intestinal tumors.

Lignans also block TPA-induced phosphorylation of extracellular signal-regulated kinases (ERK) and UVB-induced phosphorylation of ERKs and JUN kinases via inhibition of AP-1 and NF-B transcriptional factors (72). Proto-oncoprotein c-fos, which constitutes part of the AP-1 transcription factor, is stated to be regulated by mammalian lignans in MDA-MB-468 breast cancer cells.

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CHAPTER 2 - CANCER PREVENTIVE CHARACTERISTICS OF LIGNAN PRECURSOR vs. LIGNAN METABOLITE IN HUMAN COLON CANCER SW480 CELLS

Cancer Preventive Characteristics of Lignan Precursor vs. Its Metabolite in Human Colon Cancer SW480 Cells

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[3]Abbreviations used: SDG, secoisolariciresinol diglucoside; HPLC- MS/ESI, high performance liquid chromatography- mass spectrometry /electron spray ionization.

Abstract

Our previous study demonstrated that lignan metabolites, enterolactone and enterodiol, inhibited colonic cancer cell growth by inducing S-phase cell cycle arrest and apoptosis. However, the lignan present in our diet is glucoside precursors such as secoisolariciresinol diglucoside (SDG). This study is thus focused on the cancer preventative impact of SDG in human colonic SW480 cancer cells. Treatment with SDG at 0-40 μM resulted into a dose and time dependent decrease in cell numbers which was comparable to enterolactone. The cell growth inhibition by SDG seemed not mediated by cytotoxicity, but induced cell cycle arrest at S-phase. Furthermore, HPLC analysis indicated SDG in medium for 48 hrs was much more stable than enterolactone (95% stability for SDG vs. 57% for enterolactone). When the cells were treated by enterolactone at 40 μM for 48 hrs, the intracellular levels of enterolactone detectable was 8.3×10^{-8} nmol/cell. However, the intracellular levels of SDG or its known metabolites as measured by HPLC-MS/ESI were undetectable. Taken together, these findings provide novel characteristics of dietary lignan on colonic cancer cell growth. The stability and intracellular level analysis may enhance our understanding of bioavailability of dietary lignans for cancer preventation.

Introduction

Potential cancer preventive effects of precursor lignan, secoisolariciresinol diglucoside (SDG) have been suggested to be mediated through its metabolites, enterolactone and enterodiol (1-3) or even its aglycone, secoisolariciresinol (4, 5). Enterolactone and enterodiol are formed anaerobically after the bacterial colon fermentation process (6) of SDG. Specialized bacterial strains, *Peptostreptococcus sp*. SDG-1 and *Eubacterium* sp. SDG-2 convert SDG into enterodiol (7) which may be oxidized to enterolactone. Besides the bacterial fermentation process,

enterolactone and enterodiol can also be formed by other cell types and organelles. A recent study showed that liver microsomes can generate lignan metabolites from other lignans, secoisolariciresinol and matairesinol (8).

Many studies associate diet supplementation of SDG with cancer preventive properties in animal models. Our recent study showed that SDG levels in wheat bran was correlated to anti-tumor activities in four wheat cultivars (9). Others also found that SDG at 0.01% significantly reduced azoxymethane-induced formation of abberant crypt foci (ACF) in mice (10). In addition, experimental animal studies show that dietary supplementation with 73-293 μmol/kg SDG inhibits experimental metastasis of B16BL6 murine melanoma cells in C57BL/6 mice (11).

Anti-estrogenic activity of lignans has been suggested as a potential mechanism of anti-cancer potential of lignans in especially estrogen dependent cancers (12). Structure similarity to estrogens allows lignans such as SDG to bind with the β-estrogen receptor and therefore inhibit 17 β-estradiol activity as seen with decreased aromatase activity in studies involving lignan supplemented diets (13-15). Other potential mechanisms by which lignans are cancer preventive are discussed in current review (16). Important among suggested mechanisms, is the inhibitory effect of lignans on DNA synthesis (17). Inhibition of DNA synthesis may cause programmed cell death or apoptosis (18).

In this study, we investigated direct cancer preventive characteristics of SDG in human colon cancer cells, SW480. The effect of SDG on inhibition of cancer cell growth was studied through cell cultures. Through chromatographic techniques, SDG was also investigated for medium stability compared to enterolactone. Finally, HPLC- MS/ESI was used to find out whether SDG or enterolactone were taken up intracellularly into human cancer cells treated with lignans hence their bioavailability for cancer preventive effects.

Materials and methods

Cell culture and treatment

The human colon cancer cells, SW480, were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle Media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis MO). The cells were cultured in either 6-well plates or 200 ml volumetric flasks at 37 0C in 5% CO_2 until 60-80% confluence. At this confluence, cells were treated with SDG and enterolactone, at 0-40 μM for 24 and 48 hrs, at which time the cells had reached < 100% confluence. SDG (99% purity) was purchased from ChromaDex (Irvine, CA) while enterolactone was purchased from either Sigma-Aldrich (St Louis, Mo) or ChromaDex. The lignans were dissolved in DMSO and mixed with fresh medium to achieve the final concentration of DMSO at 0.2%, a concentration that did not alter cell growth or cell cycle measurements compared with the DMSO-free media. After treatment cells were detached and counted by hemacytometer as our previously reported (9).

Cytotoxicity Assay

The cell viability was measured in adherent cells by trypan blue staining. The viable cell numbers in treated cells were compared with that in vehicle controls.

Cell cycle analysis

Cell cycle analysis was done as previously described (9). Briefly, cells were fixed in ethanol, centrifuged and the pellet resuspended in phosphate buffer saline solution, pH 7.4, containing 20 g/L propidium iodide and 5000 U/L of RNase (Promega) at 37 ^{0}C for 30 mins.

DNA flow cytometric analysis (FACSCaliber, Becton Dickinson, Newyork, NJ) was performed with an excitation at 488 nm and an emission at 630 nm.

HPLC quantification of SDG and enterolactone in cell culture medium

HPLC-UV quantification of SDG and enterolactone was done according to previous methods (1, 24) with slight modification. Generally, cell culture medium containing remaining lignans was separated from cancer cells that had been treated with 0-40 μM SDG or enterolactone by centrifugation at 1000 X g for 10 minutes. SDG and enterolactone were separated by HPLC chromatography on a C18 column (5 μm, 250 X 4.6 mm, Alltech), eluted with a 5% acetonitrile in pH 2.8, 0.01 mM phosphate buffer (solvent A) over 100% acetonitrile (solvent B) at a flow rate of 1 mL/ml. A gradient run of 0-10 minutes, 100% solvent A, 10-30 minutes, 0-100% solvent B and finally 30-40 minutes, 100% solvent B was determined as optimum. Peaks were detected by monitoring absorbance at 283nm. The enterolactone and SDG peak was identified according to both retention time and spectrum by comparison with a standard commercial SDG and enterolactone. A linear HPLC calibration curve for both lignans were obtained for the concentrations 0-100 μM.

HPLC analysis of intracellular extracts of cancer cells treated with SDG and enterolactone

SW480 cells treated with 40 μM SDG or enterolactone for 48 hrs were harvested by detaching with trypsin-EDTA as previously described (9) and centrifuged at 1000 X g for 10 minutes. The supernatant was discarded, and the pellet was washed with 0.01 M phosphate buffer saline solution, pH 7.4. The collected cells were then re-suspended in washing buffer, and sonicated under an ice bucket, followed by three 1:1 diethyl ether extractions. The upper organic

phase containing the lignans was obtained and evaporated to dryness by passing a stream of nitrogen gas through it. The resulting residue was dissolved in 100% methanol and subjected to HPLC using the method described above.

Mass spectrometry analysis of intracellular extracts of cancer cells treated with SDG and enterolactone

SW480 cells treated with 40 μM SDG or enterolactone for 48 hr were harvested by detaching with trypsin-EDTA as previously described (9) and centrifuged at 1000 X g for 10 minutes. The supernatant was discarded and the pellet washed and re-suspended in 0.01 M phosphate buffer saline solution, pH 7.4. The resulting solution was homogenized under an ice bucket followed by three 1:1 diethyl ether extractions. The upper organic phase containing the lignans was evaporated to dryness in a water bath set at 350C under high pressure conditions. The residue remaining at the bottom of the flask was re-dissolved in 100% methanol and ultra-centrifuged at 40,000 X g for 30 minutes. HPLC-MS/ESI analysis was performed with a Finnigan LCQ Deca XP mass spectrometer (Thermo Finnigan, Dreieich, Germany) coupled to an Agilent (Agilent, Waldbronn, Germany) 1100 series HPLC system. Separations were achieved with a synergi (Berlin, Germany) RP C18 column (250 .2 mm i.d., 5 μm) using acetonitrile:water (containing 0.1% formic acid) for elution in a gradient from 7:3 to 9.5:0.5 in 3 min, followed by isocratic elution with 0.5:9.5 between 3 and 21 min, and finally isocratic elution with acetonitrile 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. A MS software (Applied Biosystem ESI software, CA) was used to differentiate between peaks resulting from treatment effect and background noise peaks. Only mass/charge ratio [M/Z]+ of peaks resulting from the treatment are shown.

Statistical analysis

All data was analyzed by the SAS statistical system, version 8.2. The cell number, cell cycle and HPLC quantification were analyzed by the 1-way ANOVA protocol using the general linear model procedure followed by Fisher's protected least square difference. For figure 2.1 and figure 2.3, the variable was the lignan amount at 0-40 μM. The relationship between lignan concentrations remaining in cell culture medium and the original lignan amounts was determined by a two-tailed t test. A probability ≤ 0.01 was considered significant as represented by the asterisk.

Results

Cell growth inhibition

Human cancer cells treated with either SDG or enterolactone showed a growth inhibitory effect. The treatment of SW480 cells with SDG and enterolactone at 0-40 μM resulted in dose and time dependent decrease in cell number compared to vehicle control (Figure 2.1). The inhibition increased with increasing concentration of SDG or enterolactone.

Cytotoxicity

Cell viability was generally >80% in adherent cells, and the treated cells did not differ from the vehicle controls (Figure 2.2).

S-phase cell cycle arrest at 24 and 48 hrs

The treatment of SW480 cells with either SDG or enterolactone induced cell cycle arrest at S-phase in a dose and time dependent manner (Figure 2.3). DNA flow cytometry profiles indicated that S-phase was significantly increased with SDG and enterolactone treatment. As the

percentage of cells in S-phase increased, the percentage of cells at both G1 and G2/M phases decreased correspondingly (data not shown).

HPLC chromatogram of SDG and enterolactone in medium

HPLC showed that SDG and enterolactone are depleted in the cell culture medium over time. A HPLC chromatogram for reference standards; SDG, enterolactone and enterodiol is presented in Figure 2.4A. The order of lignan elution and retention time was SDG, enterodiol and enterolactone at 20.5, 25.4 and 28.6 mins respectively. The HPLC chromatogram of medium treated with either SDG and enterolactone alone are presented in Figure 2.4B and 2.4C respectively.

HPLC quantification of SDG and enterolactone in medium with and without cells for stability analysis

The quantification of SDG and enterolactone in medium with cells was done with 0-40 μM SDG and enterolactone respectively for 48 hrs as shown in Figure 2.5. The data was used to calculate stability of the lignans in medium. Stability was investigated further with 40 μM SDG vs. enterolactone for 48 hrs without SW480 cell usage.

HPLC chromatogram of SDG and enterolactone in intracellular cancer cell extracts

HPLC chromatograms for SDG, enterodiol, enterolactone and internal standard, flavone is presented in Figure 2.6. The chromatogram for commercial SDG standard is shown by Figure 2.6A. HPLC analysis of intracellular extracts of cancer cells detected enterolactone levels in enterolactone treated cells as shown in Figure 2.6B. However, SDG was not detected in intracellular extracts of cancer cells treated with SDG as shown in Figure 2.6C.

HPLC-MS/ESI analysis of intracellular extracts of cancer cells treated with enterolactone and SDG

A HPLC-MS/ESI chromatogram showing the fragmentation pattern of commercial lignan standard, enterolactone is shown in Figure 2.7A. The major ion had a mass/charge ratio $[M/Z]^+$ of 298.5 and can be identified as enterolactone. The intracellular extract chromatogram of cells treated with 40 μM enterolactone is shown in Figure 2.7B. Peak with $[M/Z]^+$ of 298.47 confirms the intracellular presence of enterolactone in enterolactone treated cancer cells. Peak with $[M/Z]^+$ 217.98 is a contaminant plastic peak associated with the column used and [M/Z]+ 857.03 was determined to be a true fragmentation peak from the enterolactone treatment. HPLC-MS/ESI chromatogram of commercial lignan standard SDG is presented in Figure 2.8A. The major ion peak has a $[M/Z]^+$ ratio of 704.09 and can be suggested as SDG combined with a molecule of water $[M+H2O]^+$. The commercial standard SDG has molecular mass of 687 and is shown in $[M/Z]^+$ of 687.02. The intracellular extract chromatogram of cells treated with 40 μM SDG is also shown in Figure 2.7B. Peaks with $[M/Z]^+$ of 341.31, 398.0, 472.12 and 917.09 were identified as authentic new peaks that were absent in the HPLC-MS/ESI chromatogram of intracellular extract of vehicle control (data not shown).

Discussion

Although it is known that SDG undergoes bacterial fermentation to generate lignan metabolites that have cancer preventive activity, little information is available about the potential cancer preventive property of SDG. In this present study, we show that SDG inhibits colon cancer cell growth by inducing S-phase cell cycle arrest in a dose and time dependent manner, a characteristic previously associated mostly through its metabolite, enterolactone. In addition, not only does SDG inhibit colon cancer growth but it is also more stable (95%) than enterolactone

(57%) in cell culture medium. Intracellular extraction of lignans to detect for their bioavailability through HPLC-MS/ESI confirmed the presence of enterolactone in enterolactone treated cells and potential metabolite peaks in SDG treated cells.

We used human cancer cell line SW480 for our studies because previous studies had indicated that this cell line is sensitive to enterolactone and enterodiol (9)

In this study, we assessed the effect of different doses of commercially available SDG on human colon cancer cell growth. It was found that cancer cell growth was significantly inhibited by SDG treatment. Increasing SDG dosage (0-40 μM) did not kill the cancer cells as cell viability was not affected but instead resulted in S-phase cell cycle arrest as measured by DNA flow cytometry analysis.

Stability analysis of lignans through quantification with HPLC showed that SDG is almost twice stable (95 %) in medium compared to enterolactone (57%). Structure stability of lignans may be determined by the nature of chemical forces involved in the bonding of the inherent structure and by the attached chemical groups. SDG is bulky with two glucose bonds attached to its structure and therefore has higher entropy than enterolactone making it more kinetically stable. In addition, the bulky glucose moieties in the SDG structure may prevent attack from possible electrophiles in cell culture medium. In addition, the presence of methoxy groups attached to outer carbon benzene rings in the SDG structure allow for increased electron delocalization and hence more stability in the inherent SDG structure (19). This may also explain why SDG remains stable in bread products during baking (20). On the other hand, the reactive lactone group on enterolactone tends to make it unstable as has been shown seen in antibacterial studies using lactone functional groups (21, 22). In addition, the accessibility of enterolactone functional groups to possible attacks by potential electrophiles is high. Although,

ChemDraw ultra software (23) predicts a negative overall enthalpy on the enterolactone structure, the lactone functional group and the openness of the enterolactone structure tend to overshadow this positive effect towards increased enterolactone instability. However, we did not detect any new breakdown peaks in the HPLC stability chromatogram of enterolactone compared to SDG (Figure 2.4).

Intracellular level studies detected enterolactone in intracellular extracts of cancer cells treated with enterolactone but four (4) unknown peaks were detected in SDG treated cells. HPLC-MS/ESI confirmed enterolactone as a molecular ion with a mass/charge ratio ($[M/Z]^+$ of 298. A new novel unknown peak with the $[M/Z]^+$ 857 was also identified in enterolactone treated cells. Further fragmentation ion analysis, indicated that $[M/Z]^+$ 857 was a true peak probably for a new metabolite compound. We suggest here that $[M/Z]^+$ 857 is probably a high molecular weight compound obtained through a 5' 5' coupling reaction or through conjugation with other compounds. On the other hand, HPLC-MS/ESI chromatogram of cancer extracts treated with SDG showed $[M/Z]^+$ 341.31, $[M/Z]^+$ 398, $[M/Z]^+$ 472.12. The $[M/Z]^+$ 341.31, $[M/Z]^+$ 398, and $[M/Z]^+$ 472.12 are suggested to be products of SDG breakdown within the cell since their $[M/Z]^+$ are less than the SDG $[M/Z]^+$ of 687.02. A high molecular weight peak, $[M/Z]^+$ 917.09 was also detected which probably represents a peak formed through a SDG 5'5' coupling reaction occurring intracellular within cancer cells.

In conclusion, SDG had significant anticancer effects in inhibition of human colon cancer cell growth as seen with S-phase cell cycle arrest in a dose and time dependent manner. HPLC stability analysis of the cell culture medium treated with SDG or enterolactone indicated that SDG was more stable than enterolactone. Bioavailability studies revealed that enterolactone is

bioavailable in enterolactone treated cells while the unknown metabolite peaks in SDG treated cells could be associated with the cancer preventative impact in these treatments

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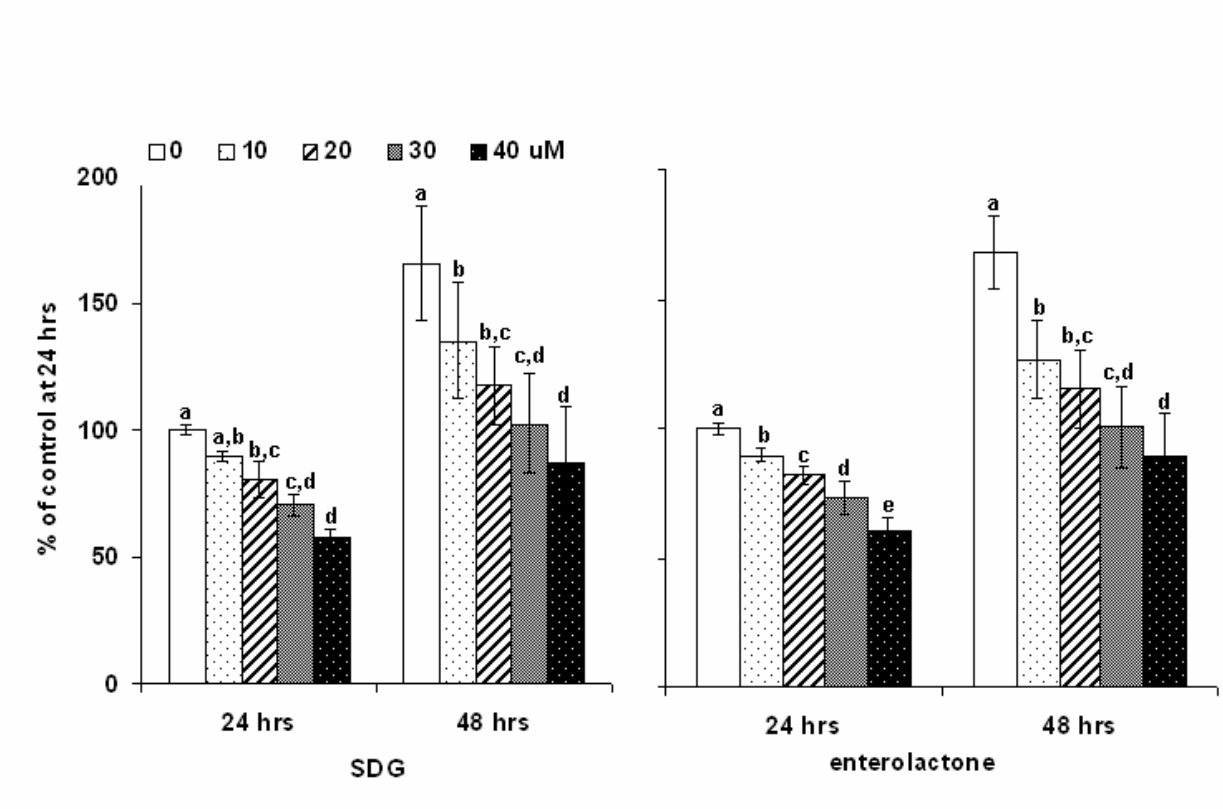

Figure 2:1 Comparison of SDG vs. enterolactone in SW480 cells for cancer growth inhibition analysis

Cells were cultured with either 0-40 μM SDG or enterolactone for 24 and 48 hrs. Cell count was performed with a hemacytometer. Percentage of cells at each treatment was compared to vehicle control and is represented as ± SD from 7-11 independent experiments. Means within a treatment without a common letter differ, $p \leq 0.05$

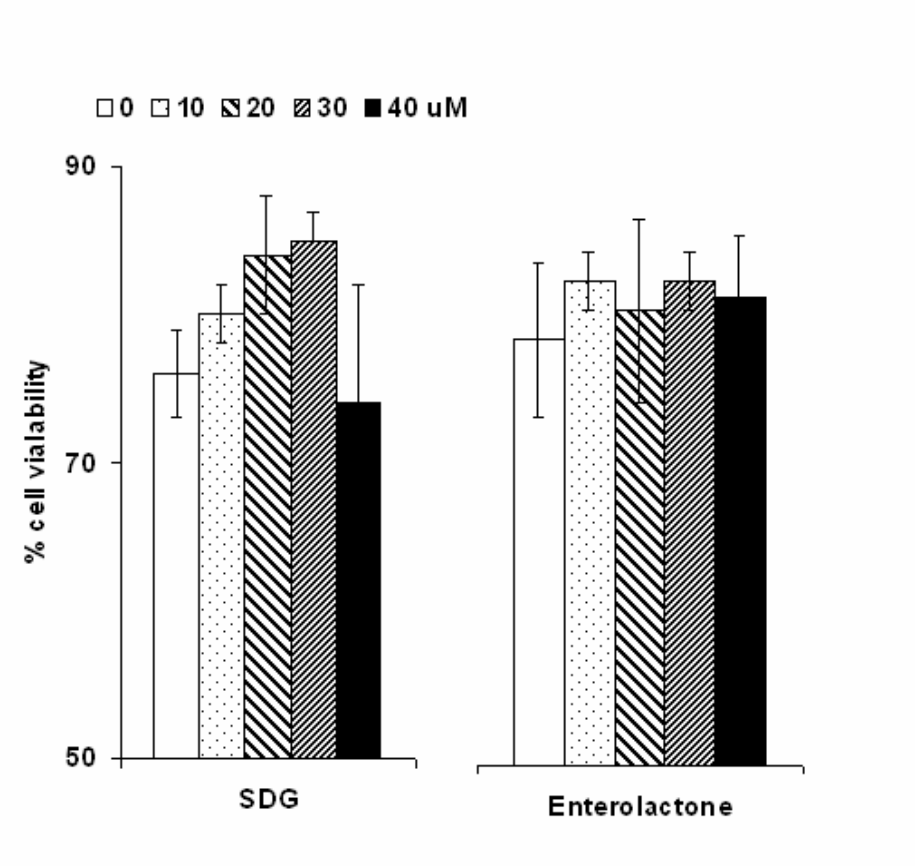

Figure 2:2 Cell vialability analysis

Cell vialability analysis to detect for total number of live cells in cancer growth inhibition analysis of SW480 cells treated with 0-40 μM concentrations of SDG vs. enterolactone measured by trypan blue staining. No significant difference was observed between treatments.

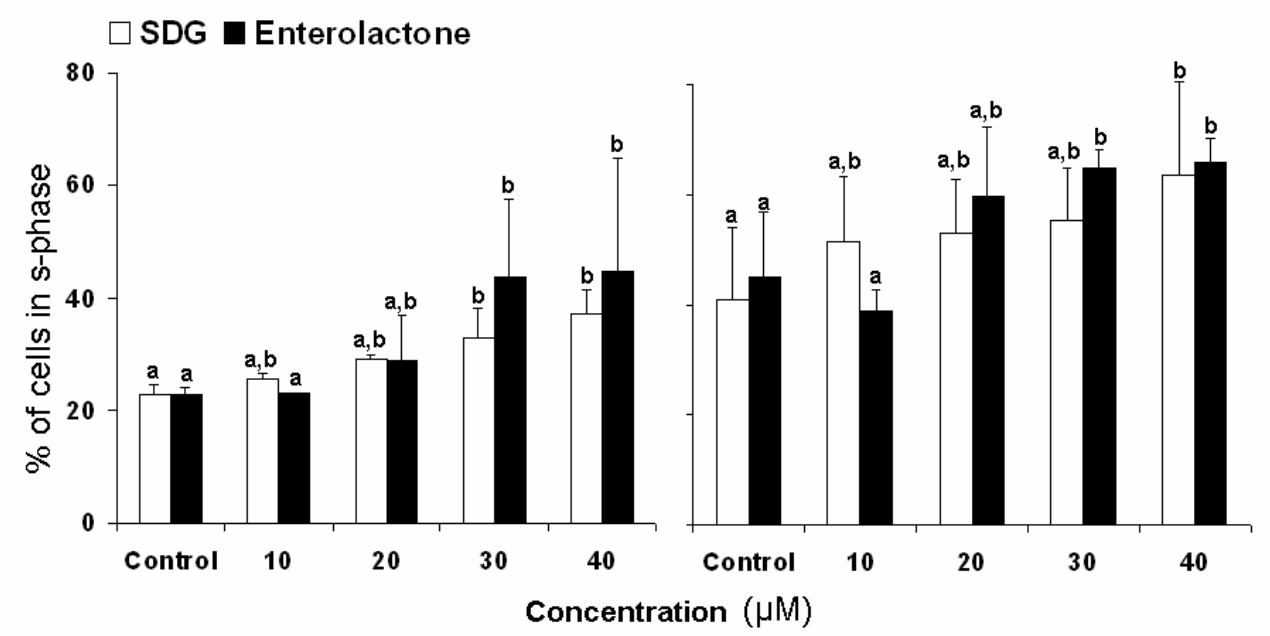

Figure 2:3 Comparison of SDG vs. enterolactone in SW480 cells in S-phase cell cycle analysis

Cells were cultured with either 0-40 μM SDG or enterolactone for 24 and 48 hrs and cell cycle change was measured through use of DNA flow cytometry. Percentage of cells in S-phase was compared to vehicle control and is represented as ± SD from 5-7 independent experiments for SDG treated cells and 3-4 independent experiments for enterolactone treated cells. Means within a treatment without a common letter differ, $P \leq 0.05$.

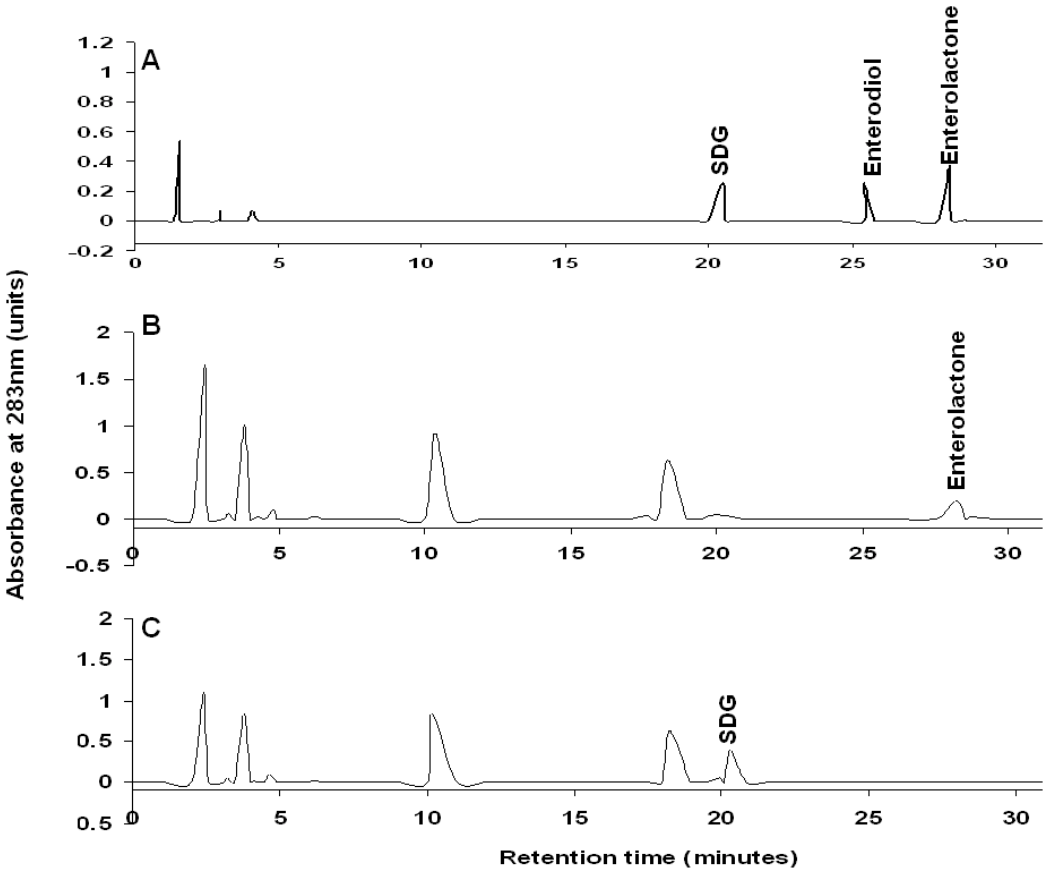

Figure 2:4 HPLC chromatogram of lignans treated in cell culture medium

Representative HPLC chromatograms of commercial lignan standards: SDG, enterodiol and enterolactone (A). Cells were treated with 40 μM of either SDG or enterolactone, centrifuged to obtain a supernatant that was run on HPLC to detect for remaining enterolactone (B) vs. for remaining SDG (C).

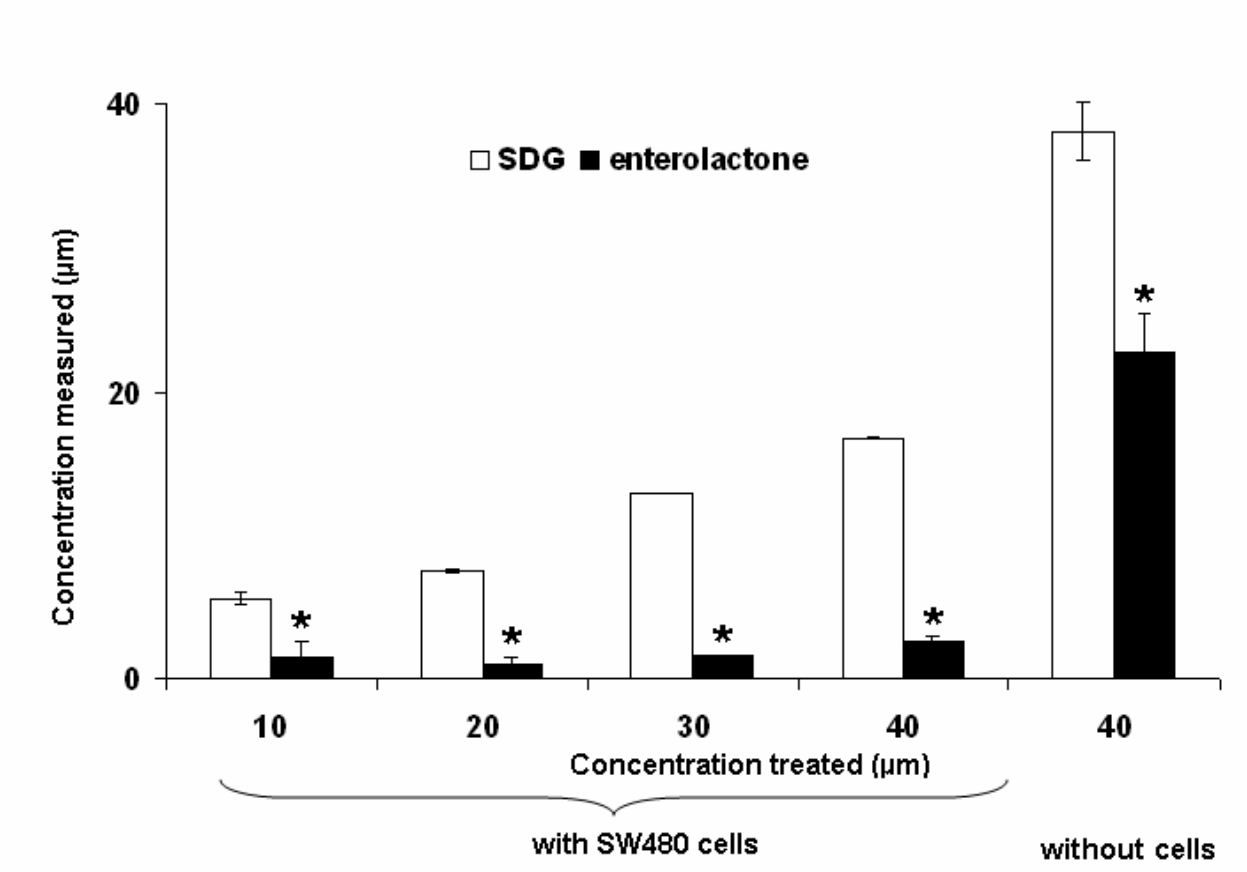

Figure 2:5 Stability analysis of SDG vs. enterolactone in medium with and without SW480 cells

SDG and enterolactone were treated 0-40 µM in SW480 cells for 48 hrs and the concentration of lignans remaining quantified by HPLC. Stability analysis was also done without SW480 cells at 40 µM SDG or enterolactone for 48 hrs. Stability of SDG vs. enterolactone is represented as ± SD from 3-4 independent experiments. Means within a treatment represented by a asterisk differ, $P \leq 0.01$

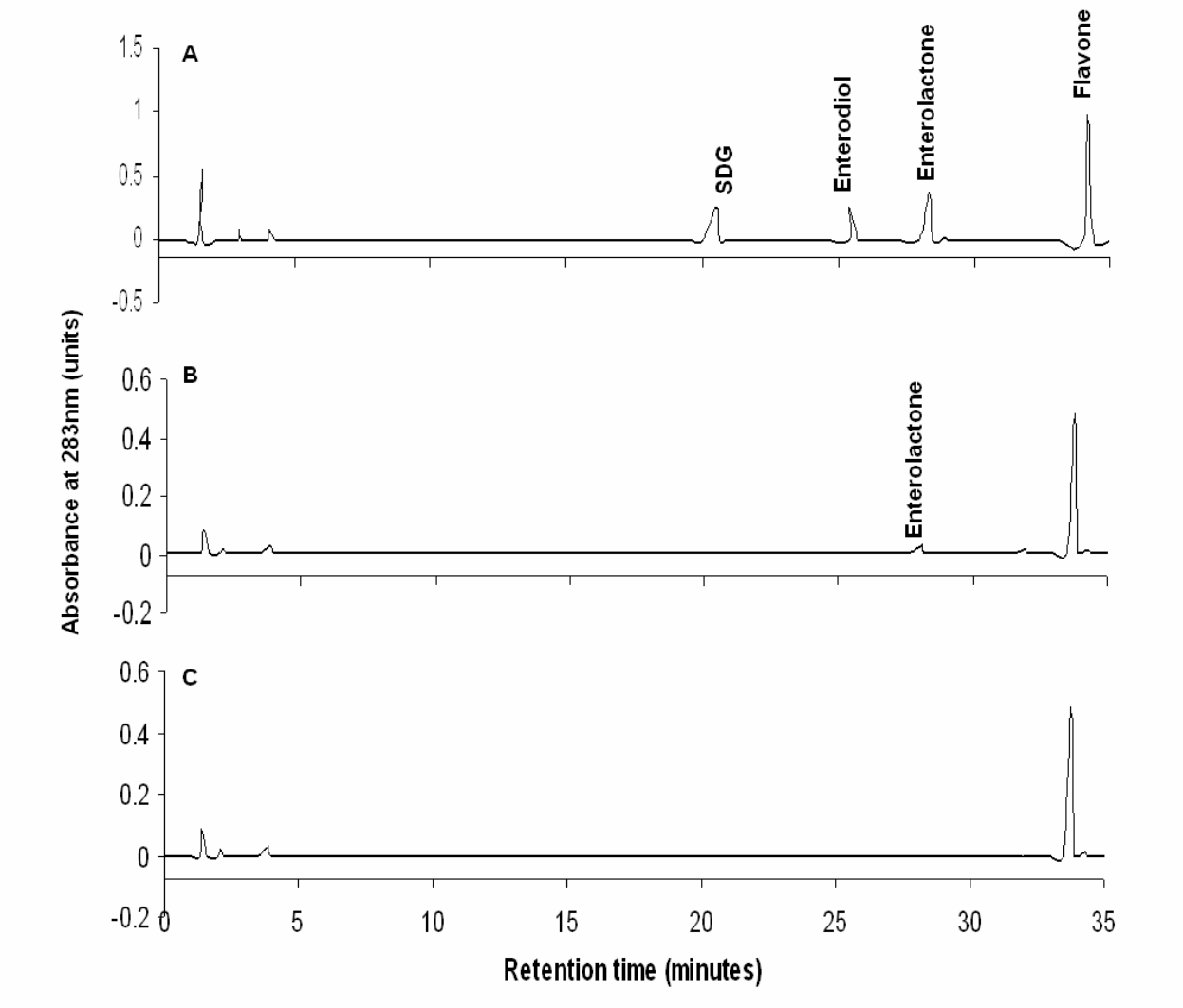

Figure 2:6 HPLC chromatogram of lignans from intracellular extractions from cancer cells

Representative HPLC chromatograms of commercial lignan standards: SDG, enterodiol, enterolactone and internal standard, flavone (A). Cells were treated with 40 μM of either SDG or enterolactone, centrifuged to obtain a pellet containing cancer cells. Cancer cells were then extracted for intracellular lignans as described in the material and methods. HPLC chromatograms for intracellular detection of enterolactone (B) and SDG (C) are presented.

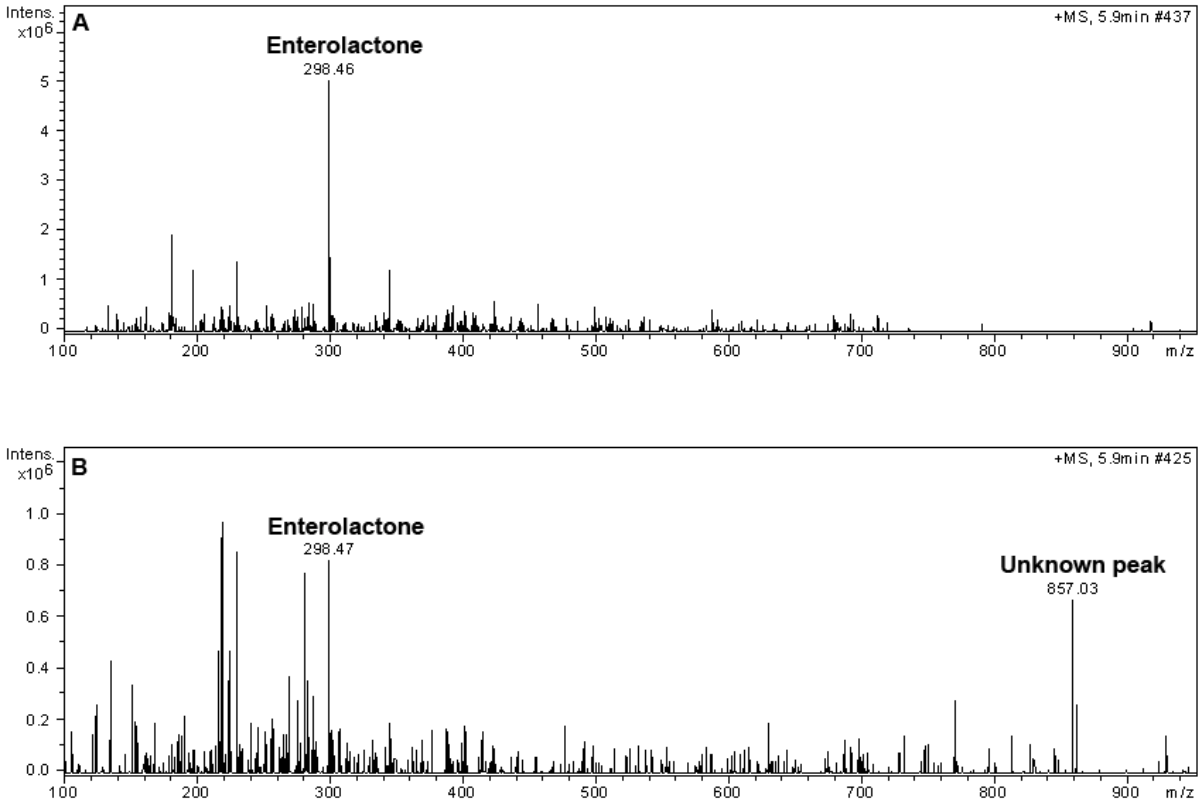

Figure 2:7 HPLC-MS/ESI chromatograms of lignans after intracellular extractions with enterolactone

Representative HPLC-ESI/MS chromatograms of commercial lignan standard, enterolactone is shown (A). The major peak is $[M/Z]^+$ 298.5 representing molecular mass of enterolactone. A HPLC-ESI/MS chromatogram of intracellular extract from cancer cells treated with enterolactone is shown (B). The major peaks $[M/Z]^+$ 217.98 is a plastic contaminant, $[M/Z]^+$ 298.47 confirms intracellular bioavailability of enterolactone, and $[M/Z]^+$ 857.03 is a unknown peak from enterolactone treatment.

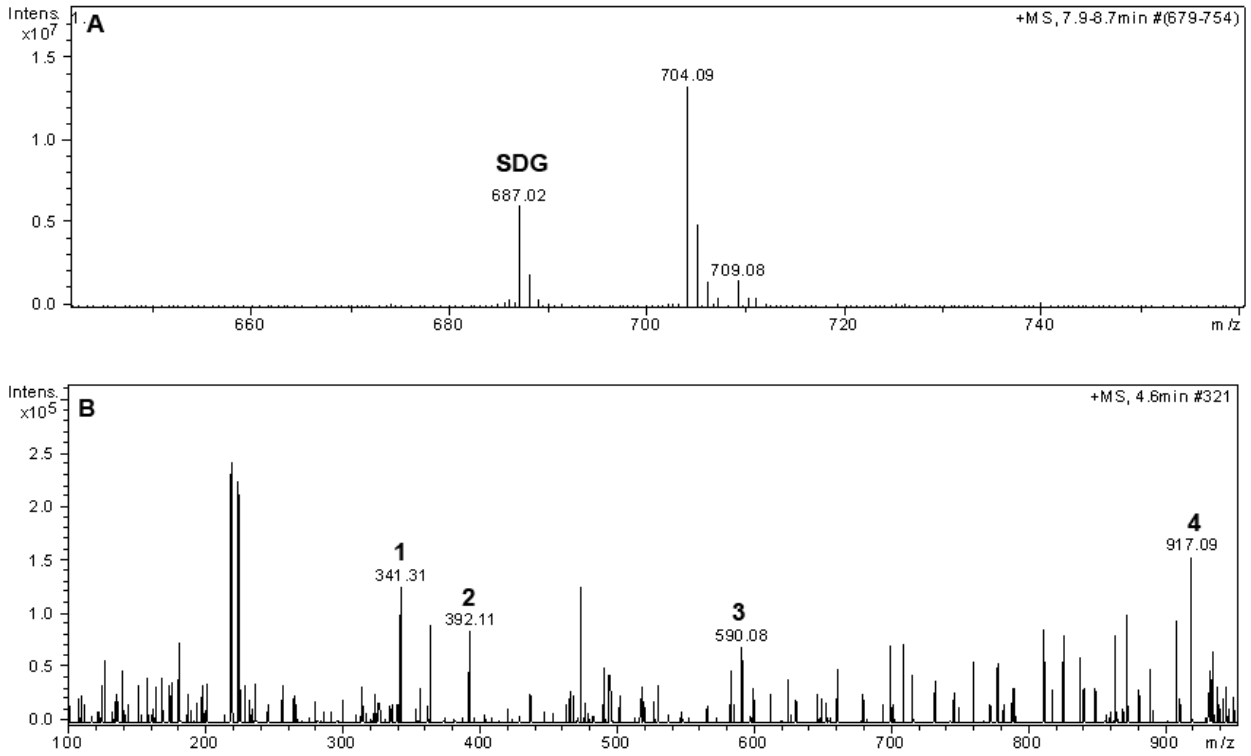

Figure 2:8 HPLC MS/ESI chromatograms of intracellular extracts of cancer cells treated with SDG

Representative HPLC-MS/ESI chromatograms of commercial lignan standard SDG with $[M/Z]^+$ 687.02 and SDG $[M+ H20]^+$ 704.09 (A). Unknown peaks (peak 1 to peak 4) with $[M/Z]^+$ of 341.31, 398.0, 472.12 and 917.09 were detected after intracellular extraction of cancer cells treated with SDG.

CHAPTER 3 - ENHANCING LIGNAN BIOSYNTHESIS BY OVER EXPRESSION OF FORSTHYSIA PINORESINOL LARICIRESINOL REDUCTASE IN TRANSGENIC WHEAT

Enhancing Lignan Biosynthesis by Over expression Forsthysia Pinoresinol Lariciresinol Reductase in Transgenic Wheat

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[3] The abbreviations used are; PCR, polymerase chain reaction; PLR, pinoresinol lariciresinol reductase; SDG, secoisolariciresinol diglucoside; UBI, ubiquitin; HPLC, High performance liquid chromatography

Summary

Lignans are phytochemicals that have been shown to have cancer preventive activities in animal models. Our previous studies demonstrated that the contents of lignans in various wheat cultivars were significantly associated with anti-tumor activities. Phytochemical lignans are biosynthesized via the phenylpropanoid pathway, in which pinoresinol lariciresinol reductase (PLR) catalyzes the last steps specifically for lignan production. To enhance lignan biosynthesis, we transformed wheat cultivars (*'Bobwhite', 'Madison', and 'Fielder'* respectively) with the *Forsythia intermedia* PLR gene under the regulatory control of the maize ubiquitin promoter. Of 217 putative transgenic wheat lines, we successfully obtained 7 transformants with the inserted ubiquitin PLR gene as screened by PCR. Southern blot analysis further demonstrated that different copies of the PLR gene were carried in these wheat genomes. Furthermore, real-time PCR indicated 109-117% over-expression of PLR gene over the control in 3 positive transformants at the T_0 generation. In addition, the levels of secoisolariciresinol diglucoside, a prominent lignan whose biosynthesis is catalyzed by PLR as determined by HPLC, were found to be significantly elevated in one of the three positive transgenic plants. To the best of our knowledge, this is the first study that elevated lignan levels in transgenic wheat has been successfully achieved through genetic engineering of the over expressed lignan biosynthesis enzyme.

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 *forsthysia*, lignans occur in the fruits and stems [6]. Also in eudicots, lignans are formed through dimerization of alcohols in the phenylpropanoid pathway through skeletal rearrangements and oxygen incorporation [3] while in monocots, literature review on the phenylpropanoid pathway is scarce.

The biological importance of lignans has also recently been 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 in breast [12], prostate [13], and colon cancers [14, 15]. A recent study done in rats showed that exposure of 10% flax seed or secoisolariciresinol diglucoside (SDG) during suckling suppressed DMBA-induced mammary tumorigenesis [16]. Studies in animals are supported by *in vitro* cell culture results that show that lignan metabolites reduce growth and metastasis of breast cancer cells [17]. Furthermore, in colon cancer cells, lignan metabolites have been shown to reduce cell growth by our previous publication [18]. In both studies, cancer growth inhibition could be correlated to the amount of lignans used. More specifically, the correlation between cancer cell growth inhibition and anti-tumor activity was found with the lignan, SDG as in our previous study ($r = 0.73$, $p < 0.02$) [18]. Higher SDG amounts may therefore mean higher anti-tumor activity. As a result, increase of the SDG levels in wheat plants may provide more lignans for cancer preventive effects.

Cancer preventive SDG in wheat may therefore be increased through genetic manipulation methods as shown by others [19, 20]. Genetic manipulation of the phenylpropanoid pathway through targeting of enzymes that catalyze entry of first and last lignans into this pathway may present a novel approach for enhanced lignan biosynthesis. One of the key control

enzymes, pinoresinol lariciresinol reductases (PLR) which catalyzes the last steps of the lignan biosynthesis pathway can be genetically engineered to increase lignans [21]. To do this, we may use any of the PLR genes already isolated from woody plants (3, 22, 23], and also most recently from flax seeds [24]. In wheat, the full-length PLR gene is yet to be isolated.

The purpose of this study was therefore to enhance SDG biosynthesis in transgenic wheat plants by genetic transformation of wheat with the *F. intermedia* PLR cDNA. To the best of our knowledge, this is the first study to apply genetic engineering of wheat for lignan biosynthesis enhancement.

Materials and methods

Plasmid construction of Ubi PLR

We obtained the *F. intermedia* PLR cDNA as a kind gift from Dr. Lewis Norman of Washingston State University. The PLR cDNA was initially cloned into a pGem Teasy vector (Promega, Madison, WI) and then subsequently PCR modified by primers *BgL 1* F and *BgL 1* R for insertion of *BgL* 1 sites at the 3' and 5' end (Table 3.1). The PLR gene was then placed under the control of the maize ubiquitin promoter, pAHC 17 [25]. The new generated plasmid designated as 'Ubi PLR' was used for co-transformation purposes with the bar gene, pAHC 20.

Co-transformation and tissue culture of wheat with Ubi-PLR

Vector plasmids, Ubi PLR and the selectable marker gene, pAHC 20 [25] (plasmid for the bar resistance gene) were co-bombarded into embryogenic calli of wheat (cultivars *'Bobwhite', 'Madison' and 'Fielder'*). The method of co-transformation and selection of transgenic events have been detailed by Anand et al 2003 [26]. Briefly, premature seeds were surface sterilized with 20% sodium hypochlorite and 0.02% TWEEN-20 and embryos aseptically

excised on CM4 medium to initiate somatic embryo formation. Somatic embryos that proliferated on CM4+ osmoticum (0.2 M mannitol, 0.2 M sorbitol) were co-bombarded with the pAHC 20 and Ubi PLR plasmid by use of the particle flow gun. For co-transformation, plasmid DNA at a ratio of pAHC 20 to Ubi PLR of 1:1 was precipitated onto tungsten particles and applied on a swinney filter holder on the particle inflow gun. Helium gas with a 60 pound per square inch microburst was then propelled towards the target embryo. Selection and regeneration of transgenic plants were then done as described before [27] with slight modifications and here is a brief summary. Sixteen hours (16) after co-bombardment, selection for transformed tissue was on CM4 medium containing 5 mg l-1 glufosinate. Sub-culturing was done after 2 weeks to medium strength of 10 mg l-1 glufosinate. After 10-15 weeks, growing clumps transferred to shoot production medium, MSP containing MS [28] with glufosinate selection until green shoots were observed. The cultures were then transferred to elongation and rooting medium (MSE) containing 5 mg l-1 glufosinate and cultured 2-3 weeks. Healthy looking plantlets obtained were transferred to soil and grown in environmentally green house.

Leaf painting assay for confirmation of the selectable marker, bar

To examine the expression of the selectable bar resistance gene in the transgenic plants, leaf painting was done as previously described [26] and here is a brief summary. Freshly prepared solution of herbicide, liberty (0.2% v/v) was applied on the second/third youngest leaf using a cotton plug and marked off with a marker pen. Visual observations were recorded 7-10 days after painting. Positive lines with resistant green leaves were used for PCR analysis

Polymerase chain reactions (PCR) for the bar, PLR and Ubi-PLR gene

For all the three primer sets, PCR analysis was done with genomic DNA extracted from leaves of transgenic wheat plants using the phenol chloroform extraction method [29, 30]. In brief, 100-500 ng of genomic DNA from transgenic plants was screened by primer sets; Bar AB F and Bar AB R, PLR F and PLR R, and Ubi PLR F and Ubi PLR R (Table 3.1) in a PTC-220 thermal Cycler (Hybaid Limited, Hastings, UK). Samples were denatured, annealed and extended at 94^0C, 58-60 ^{0}C, and 72^0C for 1 minute, 30 sec and 45 sec, respectively for 35 cycles. PCR products were visualized through 1.8% agarose gel electrophoresis. Only transformants that tested positive with the three primer sets positive were used for confirmation of transgenic success.

Southern blot analysis of positive transgenic wheat lines for detection of integration of the Ubi-PLR gene combination

About 25 µg of isolated genomic DNA isolated was fully digested with a single enzyme cutter, *Bam HI* and separated by electrophoresis in 0.8% agarose (30V) in 12-14 hrs. Genomic DNA was denatured and transferred onto Hybond N+ membrane using a alkaline transfer procedure described by Amersham (Amersham, Piscataway, NJ). The detection of introduced DNA was performed according to established protocol. Briefly, the nylon membrane was hybridized for 24 hrs with the 1.2kb ^{32}P-dCTP labeled *F .intermedia* PLR gene. After hybridization, blotted membrane was exposed in a phosphor imager cassette and developed using the Scan Quant software (Molecular Dynamics Inc., Sunnyvale, CA)

Isolation of the partial Wheat PLR sequence

To isolate the partial wheat PLR sequence, wheat genomic DNA was extracted as described above from wild wheat type (cultivar '*Fielder*'). Primer sets (PLR F and PLR R) were used to PCR amplify the wheat PLR fragment (same PCR conditions as above for PLR primers). The PCR products obtained were purified using the montage DNA PCR purification kit (Millipore Corporation, Bedford, MA) and inserted into the multiple cloning site of the pGem Teasy vector (Promega, Madison, WI) before sequencing with pGem Teasy vector primers. The sequences obtained from the vectors were then compared to *Forsthysia* PLR sequence as determined by national centre for biotechnology sequence comparison software (NCBI).

Relative real time PCR quantification of Ubi-PLR expression in positive transgenic wheat plants

To quantify for levels of *F. intermedia* PLR expression in different positive transgenic plants, total RNA was isolated from young leaf tissue by use of the total RNA isolation kit (Promega, Madison WI). Total RNA amounts were determined by use of the nanodrop (Nanodrop Technologies, Wilmington, DE) through measuring UV absorption at 260. The ratio of 230/260 was used determine mRNA purity. First strand cDNA synthesis was performed according manufacturer's instructions using 1 µg of total RNA with AMV reverse transcriptase (Promega, Madison, WI). The samples were diluted to an equivalent volume and an equivalent amount used for real time PCR analysis. The primer set, PLR RT F and the PLR RT R, (Table. 3.1) was used to amplify a 99bp fragment of the *F. intermedia* PLR gene using the SyBr green master mix protocol as suggested by the manufacturer (Bio-rad Laboratories, Hercules, CA). Real time PCR was performed with a iCycler thermal cycler (Bio-rad Laboratories, Hercules, CA). Ten to eighty pg of Ubi PLR was amplified to determine the efficiency of the *F. intermedia*

PLR RT primers. PCR products from the cDNA were then qualified with the iCycler Bio-rad software (Bio-rad Laboratories, Hercules, CA). The experiment was repeated three times and the results plotted as relative quantification in log CT units.

SDG identification and quantification in transgenic wheat

Sample extracts from transgenic wheat T_2 seeds were quantified for SDG levels by HPLC analysis. Briefly, transgenic and non transgenic seeds (10-30) equivalent to 0.2-0.5 mg were ground and defatted using hexane and dried in the hood overnight. Defatted whole extracts were then homogenized under cold conditions through addition of liquid nitrogen. Subsequently, the supernatant after centrifugation was extracted for lignans with diethyl ether before evaporation to obtain a residue that was re-dissolved in 100% methanol. The dissolved residue was then subjected to HPLC with 5 mM flavone as an internal standard. HPLC-UV was done according to previous methods (34) with slight modification. Generally, extracts were subjected to HPLC chromatography on a C18 column (5 μm, 250 X 4.6 mm, Alltech), eluted with a 5% acetonitrile in pH 2.8, 0.01 mM phosphate buffer (solvent A) over 100% acetonitrile (solvent B) at a flow rate of 1 ml/min. A gradient run of 0-10 minutes in 100% solvent A, 10-30 minutes in 0-100% solvent B and finally 30-40 minutes in 100% solvent B was determined as optimum. The SDG peak was detected by monitoring absorbance at 280nm and identified by both retention time and mass spectrum comparison with a standard commercial SDG.

Statistical analysis

All data was analyzed by the SAS statistical software, version 8.2. The real time PCR determination of *F. intermedia* PLR expression levels and HPLC quantification of SDG were

analyzed by the one ANOVA protocol using the general linear model procedure followed by Fisher's protected least square difference. A probability of ≤ 0.05 was considered significant.

Results

Transgenic wheat plants

After embryogenic tissue culture selection and regeneration with the shoot production medium, 217 liberty painting positive wheat plants were obtained. PCR screen indicated twenty four putative transformants to be *bar* and PLR gene positive as represented (Figure. 3.3A and 3.3B). Out of these, 3 from wheat cultivar ('*Fielder*') were positive for the Ubi PLR gene by the PCR screen (Figure. 3.3C).

Detection, expression and integration of the selectable marker bar and Ubi PLR

The selectable marker, bar was detected by both the leaf painting assay and PCR (Fig. 3.3A). Leaf painting performed on T_0, T_1 and T_2 leaves of transgenic wheat identified putative transformants after 7-10 days. Putative transformants showed tolerance to the herbicide liberty, but null segregates showed leaf yellowing in the same period. PCR analysis for the 'bar' gene confirmed presence of the 'bar' resistance gene in some of the putative transformants (24/217 transformants. PCR analysis also detected the 'PLR' gene and 'Ubi-PLR' genes in the bar positive plants.

For southern blot integration of the PLR gene in T_0 leaves of transgenic wheat lines, we used T_0 plant 4995 as the negative transgenic control because it was PCR positive for the 'bar' gene but not for the 'Ubi-PLR' gene as seen in figure 3.3A and 3.3C respectively. In addition to plant 4995, we also found out that plant 4907 and 5010 that showed presence of 'PLR' as the wild type plant (Fig. 3.3B-most extreme right lane) did not show presence of 'Ubi-PLR' (Figure

3.3C) perhaps suggesting to us that we would have detected the indigenous wheat PLR gene in these 2 transgenic plants. We later determined this to be true for the 24 positive transgenic plants tested (Figure 3.3C). Southern integration patterns of transgenic lines; 4995, 4907 and 5010 helped confirm the presence of the indigenous wheat PLR gene since they had two integrations bands similar to the wild type plant (Figure 3.4). On the other hand, southern integration patterns of 3 positive transgenic lines showed more than 2 integrated bands of PLR with the single enzyme cutter i.e. *BamH1*. T_0 plants 4962 and 4909 had similar 3 major hybridization bands while T_0 plant 4970 with 5 major hybridization bands.

Real time PCR quantification of the PLR gene in transgenic plants

Quantitative real-time PCR was used to quantify for the expression of the F. intermedia PLR gene (Figure 3.5). Quantification of the relative differences in transgene expression of PLR using real time PCR revealed that plant 4970 and 4962 had significantly higher levels (~ 6.2 relative units) than the transgenic control plant 4995 (~ 5.4 relative units).

Detection and quantification of SDG in transgenic wheat T2 seeds

We then determined the SDG amount in transgenic wheat T_2 seeds through HPLC as a measure of the functionality of transformation process (Figure. 3.6). The HPLC SDG peak confirmed by mass spectrometry had a mass to charge ratio of 688 similar to that of commercial SDG. T0 plant 5010 whose 2nd generation seeds are T_2 5010 A2 (A2 - The A represents the 1st generation letter annotation used for differentiating between individual transgenic lines in the same transformation event while the 2 represents the 2nd generation numeral annotation used for individual transgenic lines) is used as the negative transgenic control plant because earlier results indicated that it had similar hybridization patterns to the transgenic control plant, 4995. It was

therefore not surprising to find that T_2 5010 A2 seeds had the lowest amount of SDG. The SDG amount was highest in the T_2 wheat seeds of plant 4970 I5 (Figure. 3.6C), which are 2nd generation seeds from T_0 plant 4970. The seeds of T_2 4970 I5 came from T_0 plant 4970 whose leaf extracts had the highest levels of the PLR transcript as shown earlier (Figure 3.5). T_0 plants 4909, 4970, 4970 whose 2nd seed generations are T_2 seeds 4909 E5, 4970 B1 and 4970 A3, respectively were also relatively high in SDG amount but not significantly different from transgenic control plant 5010. The overall percentage increase in SDG amount varied was about 3-fold compared to transgenic control or the wild type non-transgenic control.

Discussion

Of the 217 transgenic wheat plants obtained after co-bombardment with the constructed Ubi PLR plasmid, 3 shown here were positive for 'bar', 'PLR' genes and Ubi-PLR gene combination. Putative transgenic plants and control lines showed similar morphological characteristics. The southern blot indicated integration of one (1) or more additional 'copies' of PLR in plants that were genetically positive. Relative real time PCR quantification showed an increase in relative expression of PLR from 5.4 to 6.2 units, a value equivalent to 109-117% PLR over-expression over the control. Quantification of the PLR biosynthesis product, SDG showed a 3 -fold increase in lignan amount compared to the non transgenic control.

Putative transformants that survived the selection process, were screened for the presence of the 'bar' gene by leaf painting and then PCR analysis. Plants whose leaves survived painting with the herbicide liberty and showed positive PCR bands for the 'bar' gene were considered as successful transgenic lines for next analysis. Escapes were considered as plants that survived the selection process but were in fact not transformed. Such plants were liberty painting positive but 'bar' PCR screen negative. Confirmation by PCR analysis of the 'PLR' gene identified 24

positive transgenic plants out of 217 transformants for both the 'bar' and 'PLR' genes. PCR analysis of the 'PLR' gene turned out to indicate that all plants were positive for the gene regardless of its background including the wild type cultivar, *fielder*. PCR analysis of the Ubi PLR gene combination on the other hand detected plants that had Ubi PLR gene combination. In some positive transgenic plants, we detected the 'bar' and 'PLR' gene but not Ubi-PLR. Those plants such as 4995, 4907 and 5010 suggested to us that the detected 'PLR' gene could have come from another source i.e. the indigenous wheat 'PLR' gene. This was later proved when we isolated and sequenced the partial sequence of the wheat PLR gene and found that it to share 98% sequence similarity to the *F. intermedia* PLR gene sequence (Figure 3.5).

To determine the integration pattern of Ubi PLR gene combination in the wheat genome, We performed a southern blot where we used a single site enzyme cutter, *BamH1* which cuts the PLR gene at the 47th position. We also used the same enzyme for wheat genomic DNA digestion. When the southern blot was probed with the 1.2 kb gene fragment of the *F. intermedia* PLR, different numbers of integrated hybridization bands were observed. Surprising, all transgenic wheat lines including the transgenic control plant 4995 detected the presence of an integrated hybridization band at the ~1.2 kb position. This band was also present in the wild type wheat *fielder* cultivar. This further confirmed our initial PCR experiments above which were suggestive of the indigenously expressed wheat 'PLR' gene in wild type control.

In addition to that, differences in number and patterns of other hybridization bands [5 different bands in 4970, 2 in 4909& 4962 and 1 in 4995] were observed and these suggested that these plants may have arose from different transformation events. Different transformation events will produce plants with different copy numbers of the PLR gene mainly because of the unpredictability of the particle inflow gun [31]. *BamHI* cuts the Ubi PLR gene combination at

the 47th base position from the 5' end of PLR gene in the gene combination (Figure 3.4) and therefore may create two integration bands, one very strong (from the longer length fragment, < 1.2 kb) and another, weak one (from the fragment length fragment, observed close to the < 0.047 kb). For negative plants, the second bar will be weak as seen with 4995, 4907 and 5010 as also seen with the wild type band because of their single PLR gene copy while for positive transgenic plants, the second band will be stronger if there is an additional longer PLR gene fragment as observed with transgenic plants 4962 and 4909. Plant 4970, on the other hand showed five major hybridization bands probably indicating integration of more PLR genes.

Also, other authors have suggested that co-transformation experiments with the 'bar' gene could result into multiple copies of the unselected gene [32]. However, it is not clear whether high copy number may translate into higher transcript level of the PLR gene.

To quantify for relative PLR expression, we used a real time PCR technique to estimate for expression levels of 'PLR' transcripts in transgenic wheat leaves. Relative real time PCR expression indicated a variation in expression from 5.4 to 6.2 units (Figure 3.6). This variation was equivalent to a 109-117% over-expression of PLR over the transgenic control. The variation in expression would have resulted from the difference in expression of PLR between different transformation events. The variation could have also resulted from difference in expression of 'PLR'. The variation in difference in expression of 'PLR' can be evidenced through southern blot data which indicates that different transgenic wheat lines had different number of 'PLR' hybridization bands probably due to difference in copy numbers of PLR being expressed. Lower 'PLR' copy numbers could mean lower PLR relative expression. However, further analysis indicated that transgenic plant 4970 which had 5 hybridization bands (indicative of 5 'PLR' copies) had the similar expression level as plant 4909 with only 2 hybridization bands hence

suggesting difference in number of integration bands was not sufficient to explain for variation in expression levels. Perhaps gene silencing, biological variation or segregation of the 'PLR' gene would have occupied in the transgenic plants with high copy number of PLR such as plant 4970. The later phenomenon usually occurs in genes whose expression is under the control of the ubiquitin promoter [33].

However, stability analysis of the 'PLR' gene in T_2 seeds did not detect the PLR PCR amplicon perhaps suggesting that the experiments above need to be verified. In addition, another possible reason for failure to detect for PLR in T_2 seeds could be, because the homozygosity of the PLR gene was not established. Seeds used for SDG quantification could have come were from heterozygous lines and therefore when PCR analysis is performed on these (as per mendelian genetics ratio of inheritance of genes assuming independent assortment; 3:1, dominant to recessive inheritance), no products will be detected if the seeds were obtained from the recessive line. In addition, gene silencing at later generations rather than the T_0 generation is usual in transgenic wheat plants [34, 35]. Also, the PLR integration bands observed in the T_0 plants could have resulted from transient expression.

Finally, we determined the levels of SDG, and found that transgenic wheat plants showing the highest level of PLR expression had the highest amount of SDG. Comparison with the wild type control plant suggested that the levels of SDG were ~ 3 fold significantly higher in positive transgenic wheat line, 4970I5 that over-expressed PLR. The elevated levels of SDG therefore could have occurred from increased first generation synthesis of SDG. Elevated SDG levels also mean increased anti-tumor activity from ~ 30% to 60% as seen in previous study from the Wang Lab [26 chapter 2).

This is the first report that shows the genetic transformation of wheat for the purposes of increased accumulation of SDG. Stable expression of the *F. intermedia* PLR gene probably through use of a separate promoter or different transformation method is suggested as an alternative for SDG manipulation in wheat plants. Targeting other enzymes such as phenylalanine ammonia lyase (PAL) by pathway transformation may provide an opportunity for manipulation of more than one gene involved in lignan biosynthesis for enhanced lignan levels. Isolation of the full-length wheat PLR gene is a scientific pursuit for future genetic transformation purposes.

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Table 3.1 PCR primers used for plasmid construction and analysis of transformants

Primer set	Primer sequence	Position in DNA	Amplicon size (bp)
PLR Bg1 L F:	5’ AGG AGG ATC TGA ATT CGC CAC GAG 3’	Insert	N/A
R:	5’ AGG AAG ATC AGC AAA ATT CAA CAC TAT TAT T 3’	Insert	
PLR F:	5’ TCG TAG ACG TAG TAA TCA GCG CCA 3’	260	539
R:	5’ TCG AGC TCT TTC ACG GAG GCT AAA 3’	799	
Ubi PLR F:	5’ GAT GCT CAC CCT GTT GTT TGG TGG TGT 3’	1974	583
R:	5’ AGG AAG ATC AGC AAA ATT CAA CAC TAT TAT T 3’	2557	
Bar AB F:	5’ CCT GCC TTC ATA CGC TAT TTA TTT 3’	1958	600
R:	5’ CTT CAG CAG GTG GGT GTA GAG CGT G 3’	14	
PLR RT F:	5’ ATC CAA GAA CCC TCA ACA AGC TGG TGT 3’	110	99
R:	5’ TCC CAT GTC TGA ACA ATT CTC 3’	209	

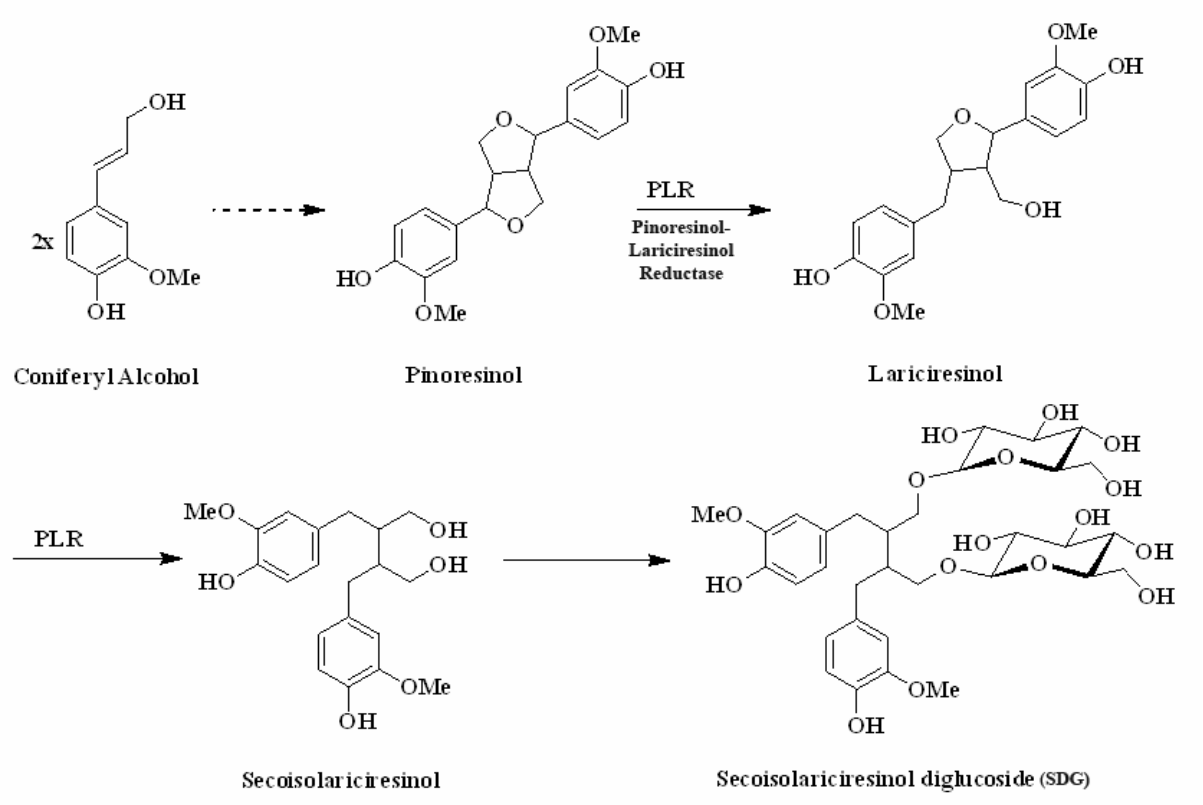

Figure 3:1 The phenylpropanoid pathway showing the final enzymatic stages of lignan formation catalyzed by pinoresinol lariciresinol reductase

The phenylpropanoid pathway showing the enzymatic stages of lignan formation catalyzed by other enzymes and pinoresinol lariciresinol reductase. Pinoresinol lariciresinol reductase catalyses the last critical final stages of the pathway for SDG biosynthesis.

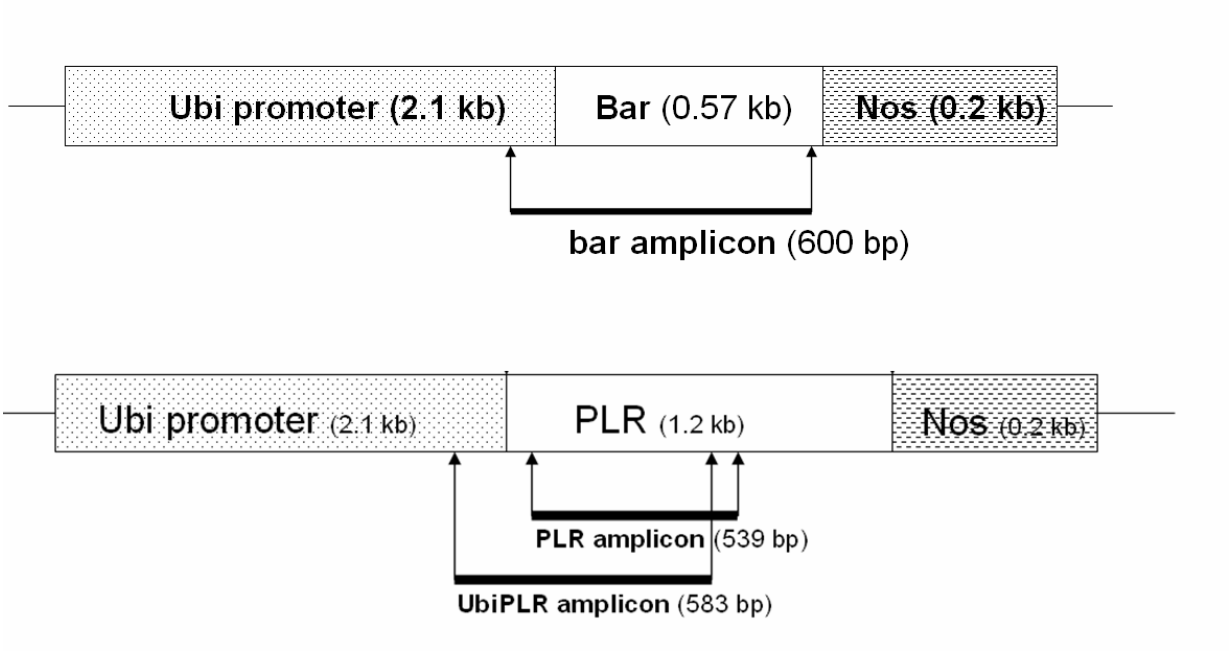

Figure 3:2 A schematic representative of the 'bar' and Ubi PLR plasmids showing locations of expected amplicon sizes

'Bar' plasmid has the ubiquitin promoter and the nos terminator. 'Ubi PLR' contains the ubiquitin promoter, the PLR gene insert and the nos terminator segments.

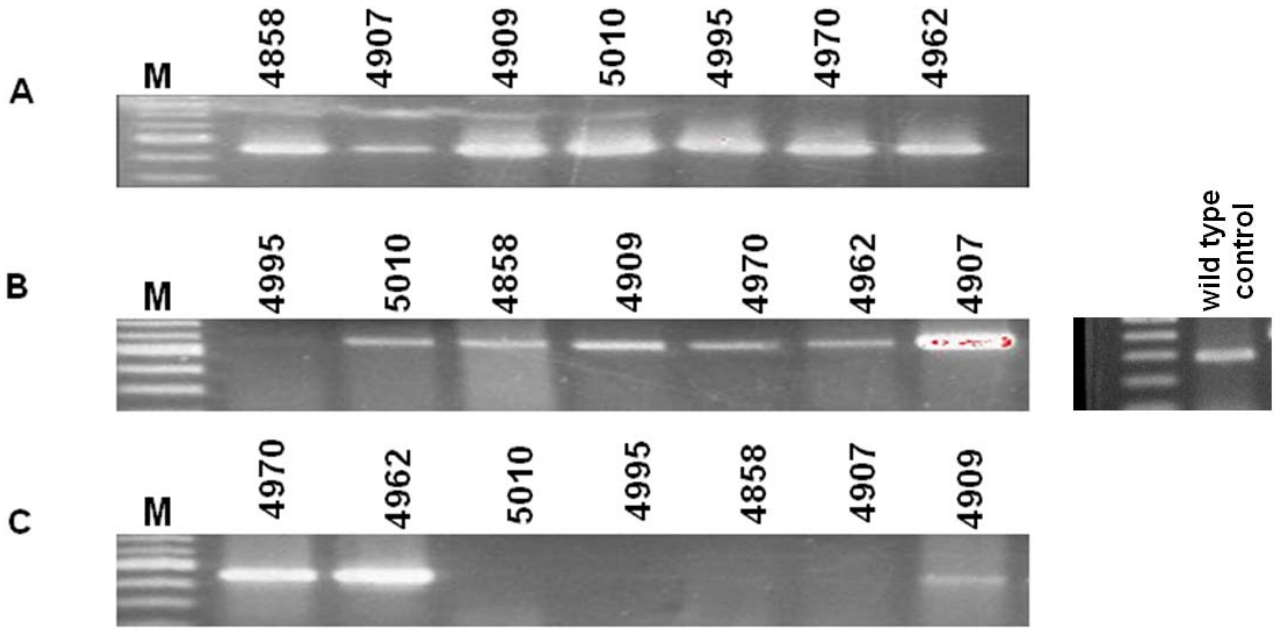

Figure 3:3 PCR screening for transgenic wheat plants run on 1.8% agarose gel electrophoresis

The first lane on the far left on each gel strip, A to C is M for the molecular weight marker used. The next seven lanes are representative of different transgenic plants tested. A is the representative strip of a 539bp *forsthysia* PLR amplicon. B is a 600bp bar gene amplicon. Also shown in B, the PLR amplicon obtained from PCR analysis of wild type wheat cultivar. C is a 583bp Ubi PLR amplicon.

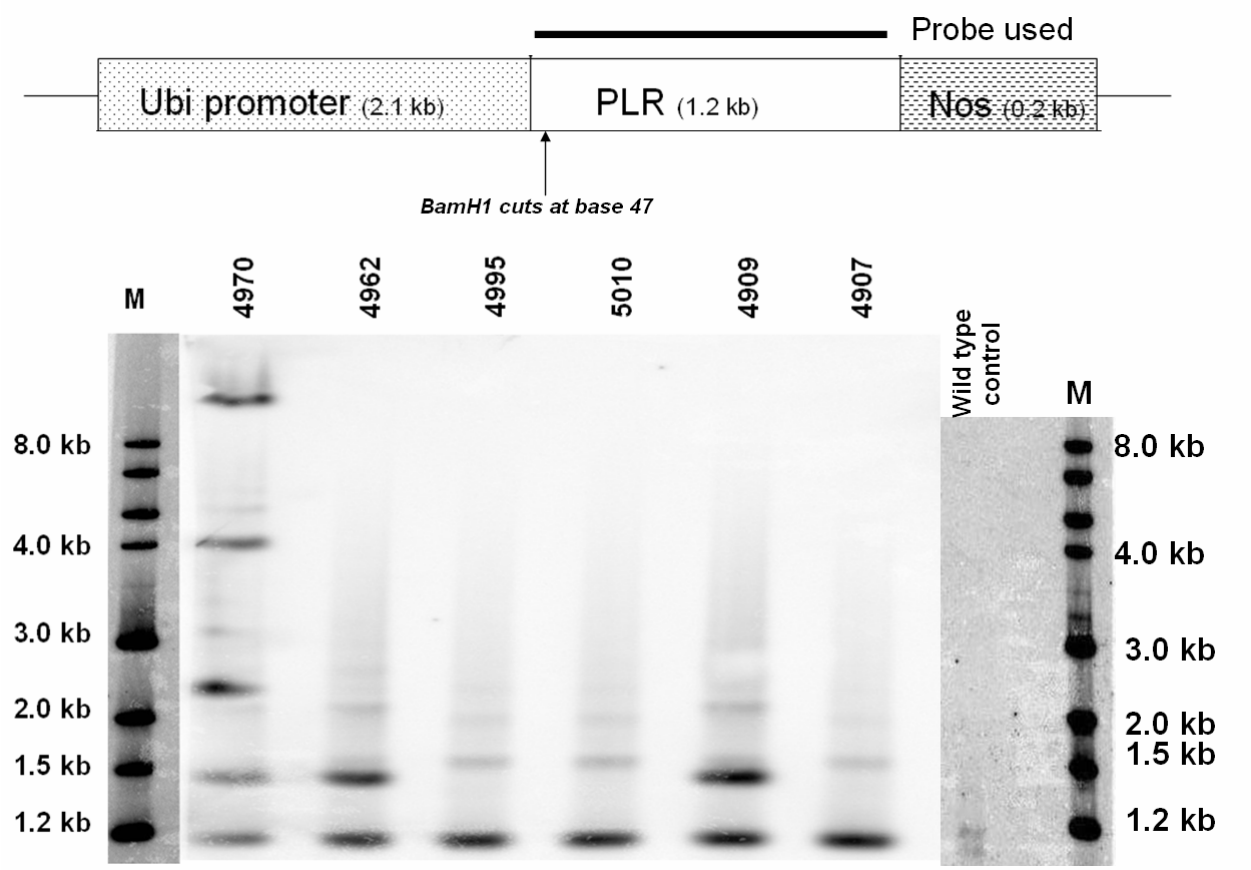

Figure 3:4 Southern blot analysis showing integration patterns of different positive transgenic plants.

BamH1 was used to cut genomic DNA and the probe used was 1.2 kb *forsthysia* PLR probe. Transgenic T_0 plant 4970 has 5 hybridization bands, while 4962 and 4909 have three major hybridization bands. Transgenic negative plant 4995, 5010 and 4907 showed two major hybridization bands. The PLR bands resulting from running the wild type control DNA are shown on the right end blot, together with the molecular weight markers, M.

1TCGAAGCCNCCAAATTCTTCTTCAACTCAAGCTTGTTGAAGCTATTAAAGG

52GCTGGAAATGTCAAGAGNNTTTTACCATCTGAGTTTGGAATGGATCCTGCAAA

123ATTTATGGATACGGCCATGGAACCCGGAAAGGTAGCACTTGATGAGAAGATG

156GTGGTAAGGAAAGCAATTGAAAAGGCTGGGATTCCTTTCACATATGTCTCTGC

209AAATTGCTTTGCTGGTTATTTCTTGGGAGGTCTCTGTCAATTTGGAAAATTCTT

263CCTTCTAGAGATTTTGTCATTATACATGGAGATGGTAACAAAAAAGCAATATAT

318AACAATGAAGATGATATAGCAACTTATGCCATCAAAACAATTAATGATCCAAGA

372ACCCTCAACAAGACAATCTACATTAGTCCTCCAAAAAACATCCTTTCACAAAAA

429AGTTGTTCAGACATGGGAGAAGCTTATTGGAAAGAACTGCAGAAAATTACACT

484CTCGAAGGAAGATTTTTTAGCCTCCGTGAAAGAGCTCGA

Figure 3:5 The partial sequence of the isolated wheat PLR gene (~ 50% length of the *Forsthysia* 1.2 kb PLR gene).

Comparison of the partial sequence of the wheat PLR gene through the NCBI database indicated 98% sequence similarity to the Forsthysia PLR gene.

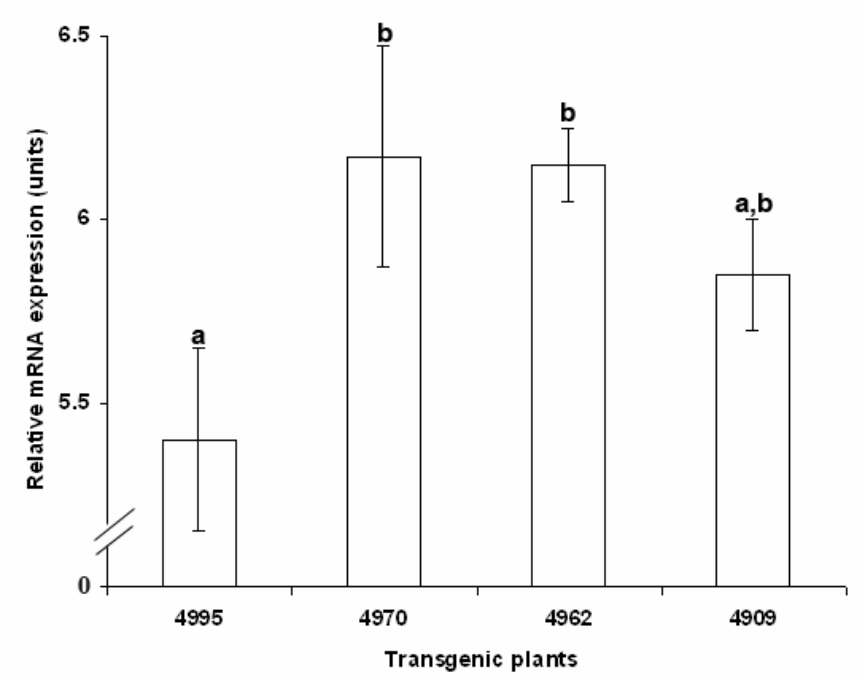

Figure 3:6 The quantification of PLR transcript levels in transgenic wheat lines, T_0 generation plants, 4995, 4962, 4970 and 4909

Relative PLR mRNA expression was obtained in log CT units (y-axis) in 3-4 independent samples. Relative PLR expression is represented as ± SD from 3-4 independent experiments. Means within a treatment without a common letter differ, $P \leq 0.05$.

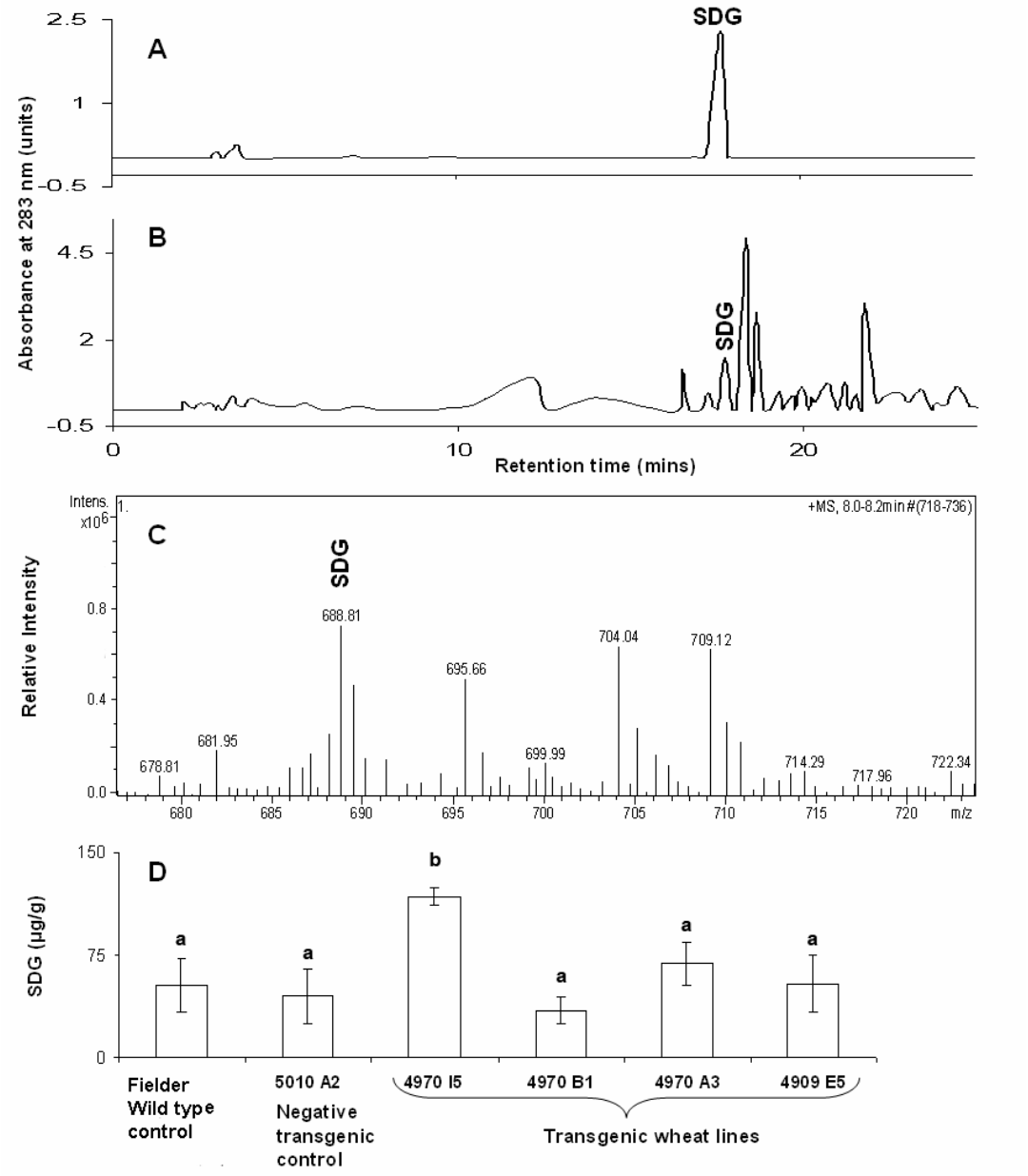

Figure 3:7 HPLC analysis and quantification of SDG in transgenic wheat

Representative HPLC chromatograph of commercial SDG (A) and of SDG extract from transgenic wheat seeds (B). Representative HPLC MS/ESI of SDG peak in B identified as peak with M/Z+ [688] (C). The quantification of SDG in transgenic wheat plants were compared to both the non transgenic control (fielder) and the negative transgenic control (plant 5010A2) (D).

SDG amount is represented as ± SD from 3-5 independent experiments. Means within a treatment without a common letter differ, $P \leq 0.05$.

Appendix A - Detailed methods for Chapter 2

Cell culture and treatment

The human colon cancer cells, SW480, were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis MO). The cells were cultured in either 6-well plates or 200 ml volumetric flasks at 37 ^{0}C in 5% CO_2 until 60-80% confluence. At this confluence, cells were treated with SDG and enterolactone, at 0-40 μM for 24 and 48 hrs, at which time the cells had reached <100 % confluence. To perform treatment, SDG (99% purity) and enterolactone was purchased from ChromaDex (Irvine, CA) and/ or Sigma-Aldrich (St Louis, Mo) respectively, dissolved in DMSO to make a final concentration of 20 mM which served as the stock solution for making other concentrations. To make 1000 ml with a final concentration of 40 μM, 2 ul of 20 mM was dissolved in 10 ml of medium, vigoursly shaken on table top mixer and added to 990 ml of medium. The same method was also used for making other concentrations of SDG and enterolactone. After treatment cells were detached and counted by hemacytometer as our previously reported. To detach cells, the medium used was first poured, then a aliquot of trypsin/EDTA solution, was carefully added to 6-well plate containing the cells, put back into the incubator, and allowed to incubate for 5-10 minutes. Detached cells obtained were transferred into a centrifuge tube for counting. To count, cells were stained in a 1:1 ratio with trypan blue and an aliquot transferred to a glass slide and

put under a focal microscope. The heamocytometer placed next to the microscope was then pressed with each visual count of cells.

Cytotoxicity Assay

The cell viability was measured in adherent cells by trypan blue staining. The viable cell numbers in treated cells were compared with that in vehicle controls. To measure cell viability, only live cells were counted as outlined in the cell culture and treatment method above. Live were not stained with the trypan blue dye and therefore were clear under the microscope.

Cell cycle analysis

To perform cell cycle analysis, cells were obtained after treatment as outlined in the cell culture methods and treatment above, except that a cell scrapper was used to detach cells instead of the trypsin/EDTA solution. Cells obtained were fixed in an aliquot of ethanol, centrifuged at 1500 X g for 10 minutes and the supernatant poured out. The pellet was resuspended in phosphate buffer saline solution, pH 7.4, containing 20 g/L propidium iodide and 5000 U/L of RNase (Promega, Madison WI) and incubated at 37 ^{0}C for 30 min on a water bath. DNA flow cytometric analysis (FACSCaliber, Becton Dickinson, Newyork, NJ) was performed by blanking the flow cytometer with a phosphate saline solution before measurement of either SDG or enterolactone treated cancer cells. The excitation wavelength was at 488 nm and emission at 630 nm. The obtained information was further analyzed with the cytometer software to calculate percentages of cells in different cell cycle phases.

HPLC quantification of SDG and enterolactone in cell culture medium

HPLC-UV quantification of SDG and enterolactone was done according to previous methods with slight modification. Generally, cell culture medium containing remaining lignans was separated from cancer cells that had been treated with either 0-40 μM SDG or enterolactone by centrifugation at 1000 X g for 10 minutes. An aliquot of the supernatant was transferred to HPLC vials and run on a HPLC column whose baseline had been stabilized for at least 30 minutes with 100% solvent A. SDG and enterolactone were separated by HPLC chromatography on a C18 column (5 μm, 250 X 4.6 mm, Alltech), eluted with a 5% acetonitrile in pH 2.8, 0.01 mM phosphate buffer (solvent A) over 100% acetonitrile (solvent B) at a flow rate of 1 mL/ml. A gradient run of 0-10 minutes, 100% solvent A, 10-30 minutes, 0-100% solvent B and finally 30-40 minutes, 100% solvent B was determined as optimum. Peaks were detected by monitoring absorbance at 283nm. The enterolactone and SDG peak was identified according to both retention time and spectrum by comparison with a standard commercial SDG and enterolactone. A linear HPLC calibration curve for both lignans were obtained for the concentrations 0-100 μM.

HPLC quantification of SDG and enterolactone in cancer cell extracts

SW480 cells treated with 40 μM SDG or enterolactone for 48 hrs were harvested by detaching with trypsin-EDTA as previously described and centrifuged at 1000 X g for 10 minutes. The supernatant was discarded, and the pellet was washed with 0.01 M phosphate buffer saline solution, pH 7.4. The collected cells were then resuspended in washing buffer, and sonicated under an ice bucket for 3 minutes. The extract was then subjected to lignan extraction with three consecutive 1:1 diethyl ether extractions. The upper organic phase containing the

lignans was obtained and evaporated to dryness by passing a stream of nitrogen gas through it. The resulting residue was dissolved in 100% methanol and subjected to HPLC using the method described above.

Mass spectrometry analysis of intracellular extracts of cancer cells treated with SDG and enterolactone

SW480 cells treated with 40 μM SDG or enterolactone for 48 hrs were harvested by detaching with trypsin-EDTA as previously described and centrifuged at 1000 X g for 10 minutes. The supernatant was discarded and the pellet washed and resuspended in 0.01 M phosphate buffer saline solution, pH 7.4. The resulting solution was homogenized under for 3 minutes at high power under ice bucket. Nitrogen gas was then passed through the homogenate to avoid reduction conditions. The homogenate was then subjected to three 1:1 diethyl ether extractions. The upper organic phase containing the lignans was evaporated to dryness in a water bath set at 35 ^{0}C under high pressure conditions. The residue remaining at the bottom of the flask was re-dissolved in 100% methanol and ultra-centrifuged at 40,000 X g for 30 minutes. HPLC-MS/ESI analysis was performed with a Finnigan LCQ Deca XP mass spectrometer (Thermo Finnigan, Dreieich, Germany) coupled to an Agilent (Agilent, Waldbronn, Germany) 1100 series HPLC system. The base line was stabilized with 100% solvent A for 3 minutes. Separations were achieved with a synergi (Berlin, Germany) RP C18 column (250 .2 mm i.d., 5 μm) using acetonitrile:water (containing 0.1% formic acid) for elution in a gradient from 7:3 to 9.5:0.5 in 3 min, followed by isocratic elution with 0.5:9.5 between 3 and 21 min, and finally isocratic elution with acetonitrile 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. A MS software (Applied Biosystem ESI software, CA) was used to differentiate between peaks resulting from treatment

effect and background noise peaks. Only mass/charge ratio [M/Z]+ of peaks resulting from the treatment are shown.

Appendix B - Detailed methods for Chapter 3

Plasmid construction of Ubi PLR

A cDNA clone for PLR-Fi1 GenBank accession number U81158 encoding (+)pinoresinol-(+)lariciresinol reductase from *Forthysia intermedia* was obtained as a gift from Dr Lewis Norman of Washingston State University.

Primers sets (PRL BgL 1 F and PRL-BgL 1R) (Table 1) were designed according to the Primer 3 software to insert BgL 1 sites at the 3' and 5' end of the PLR gene. Polymerase chain reaction (PCR) was performed using a high fecundity Taq polymerase (Bioline, Randolph MA) to reduce wrong sequence insertions during PCR amplication. The obtained 1.2 kb fragment was purified using a montage DNA gel extraction kit (Millipore Corporation, Bedford, MA) and inserted into a pGEM® T Easy Vector (Promega, Madison, WI). The sequence of the purified PLR gene product was determined using pGEM® T Easy primer sets at the gene sequencing facility, plant pathology department Kansas State University. The PLR gene was then digested from the plasmid by use of restriction enzyme *BgL 1*. A plasmid (pAHC 17) that contains the ubiquitin gene and the nopathaline Synthetase terminator (nos) was selected and digested at the multiple cloning sites with *BamH1* (compatible ends with *BgL 1*). The purified PLR gene was then ligated together with the cut pAHC 17 plasmid. To ligate, 15 µl of pAHC 17 (100 ng/µl), 25 µl of PLR cut plasmid (79 ng/µl), 8 µl of rapid T4 DNA ligase buffer (Promega, Madison WI), and 2 µl of T4 DNA ligase (Promega, Madison WI) was set to incubate at 4 ^{0}C overnight. The reaction mix was transformed into JM109 component cells (Promega, Madison WI) and selected with ampicillin resistance. Positive clones obtained, were grown in Luria broth (LB) medium and

plasmid DNA isolated using the QIAprep Miniprep Kit (Qiagen, Hilden, Germany). Restriction digestion with *Pst I, Ecor I and Bam HI* were used to confirm correct directional insertions.

Selected clones were then, sent for sequencing with both primer sets 'PLR F and PLR R' and primer sets 'Ubi PLR F and Ubi PLR R' which targets the internal sequence of the ubiquitin PLR construct. The resulting vector plasmid with both the ubiquitin promoter and the PLR gene was designated as Ubi PLR.

Co-transformation and tissue culture of wheat with Ubi PLR

Vector plasmids, Ubi PLR and the selectable marker gene, pAHC 20 [25] (plasmid for the bar resistance gene) were co-bombarded into embryogenic calli of wheat (cultivars '*Bobwhite', 'Madison' and 'Fielder'*). The method of co-transformation and selection of transgenic events was based on earlier methods used in the laboratory. Premature seeds were surface sterilized with 20% sodium hypochlorite and 0.02% TWEEN-20 and embryos aseptically excised on CM4+ osmoticum (0.2 M mannitol, 0.2 M sorbitol) to initiate somatic embryo formation. Somatic embryos that proliferated CM4+ osmoticum were co-bombarded with the pAHC 20 and Ubi PLR plasmid by use of the particle flow gun. For each transformation event, 50 mg of tungsten particles were sterilized in a 1.5 ml microcentrifuge tube with 500 µl of 95% ethanol for 20 minutes, washed five times with sterile water and resuspended in 500 µl of sterile de-ionized water. All transformation was co-bombarded with a pAHC 20 plasmid that encodes for herbicide resistance as a selectable marker. The ratio of DNA to selectable marker was 1:1. Plasmid DNA used prepared at a concentration of 1 µg/µl and a total of 5 µl of plasmid DNA was mixed with 25 µl of the re-suspended tungsten particles and allowed to incubate at room temperature for 1 minute. Next, plasmid DNA was precipitated onto tungsten particles by adding 25 µl of 2.5 M CaCl2 and 10 µl of 100 mM spermidine and then incubated on ice for 4 minutes.

Following incubation, 50 µl of the supernatant was discarded and 15 µl of DNA-coated particles used for bombardment. For bombardment, 2 µl aliquot of DNA-coated particles was applied on a swinney filter holder which was secured to a leur-lock adapter located at the top of the PIG chamber. The arranged embryogenic clusters were covered with a 500 um mesh baffle and placed in the PIG chamber 15 cm below the filter holder. A vaccum of approximately 94 kPa was applied to the chamber and the DNA coated particles were propelled towards the target embryo with 60 pounds per square inch microburst of helium gas. After transformation, transformed plants were selected through media containing herbicide of varying strength according to published protocols

Sixteen hours (16) after co-bombardment, selection for transformed tissue was on CM4 medium containing 5 mg l-1 glufosinate. Sub-culturing was done after 2 weeks to medium strength of 10 mg l-1 glufosinate. After 10-15 weeks, growing clumps transferred to shoot production medium, MSP containing MS with glufosinate selection until green shoots were observed. The cultures were then transferred to elongation and rooting medium (MSE) containing 5 mg l-1 glufosinate and cultured 2-3 weeks. Healthy looking plantlets obtained were transferred to soil and grown in environmentally green house.

Leaf painting assay for confirmation of the selectable marker, bar

To examine the expression of the selectable bar resistance gene in the transgenic plants, leaf painting was done as previously described. Leaves from each tiller to be tested were marked with a sharpie 2/3 from the leaf base. Using cotton as the brush, freshly prepared solution of herbicide, liberty (0.2% v/v) was applied on the topside of the leaf. Visual observations were recorded 7-10 days after painting and liberty sensitive tillers were clipped off.

Polymerase chain reactions (PCR) for the bar, PLR and Ubi PLR gene combination

For all the three primer sets, PCR analysis was done with genomic DNA extracted from leaves of transgenic wheat plants using the phenol chloroform extraction method. To extract DNA, 100mg of leaf tissue was grinded in microcentrifuge tube or porcelain pots under liquid nitrogen. Grinded tissue was then subjected to the 500 ul of extraction buffer (Glycine-SDS) and 500 ul phenol: choloform: IAA (25:24:1) and mixed on a vertical shaker. The extract was then centrifuged for 15 minutes at 4,000 x g and the supernatant subjected to second extraction with 500ul chloroform: IAA (24:1). The second extract was mixed for 10 minutes, centrifuged at 4000 x g and the supernatant transferred to a fresh tube. Sodium acetate (3 M), 1/10 volume and isopropanol, I volume of supernatant volume was then used to pellet the DNA. Pelleted DNA was washed in 70% ethanol and let dry at room temperature for 10-60 minutes. The pellet was subsequently dissolved in 10 mM Tris-HCI buffer under a water-bath set at 65 ^{0}C for 10 minutes. RNase (1.0 ul) was then added to the DNA sample and DNA amount determined through measuring absorbance at 260 nm with the nanodrop (Nanodrop Technologies, Wilmington, DE). To perform PCR, 100-500 ng of genomic DNA from transgenic plants was screened by primer sets; Bar AB F and Bar AB R, PLR F and PLR R, and Ubi PLR F and Ubi PLR R (Table 1) in a PTC-220 thermal Cycler (Hybaid Limited, Hastings, UK). Samples were denatured, annealed and extended at 94^{0}C, 58-60 ^{0}C, and 72 ^{0}C for 1 minute, 30 sec and 45 sec, respectively for 35 cycles and a final extension at 72 ^{0}C . PCR products were visualized through 1.8% agarose gel electrophoresis. Positive transformants were subjected to southern blot analysis to confirm integration of the PLR gene

Southern blot analysis for detection of integration of the PLR gene

Genomic DNA was isolated as described above, digested with a single enzyme cutter, *Bam HI* and separated by electrophoresis in 0.8% agarose (30V) over 14-24 hrs. At the same time, the probe to be used was obtained by restriction enzyme digestion of the pGem Teasy plasmid with *EcoR 1* to obtain the 1.2 kb PLR gene probe. After electrophoresis, the gel was stained with fresh ethidium bromide (EtBr) for 10 minutes, rinsed in 2 volumes of double distilled H_20 and depurinated with 2 volumes of 0.25N HCl until the bromophenol blue dye marker turned yellow. DNA in the gel was denatured twice with 0.4M NaOH for 10 minutes. A Hybond N+ membrane soaked in the same concentration of NaOH was used for DNA transfer by an alkaline transfer procedure according to manufacturer's instructions (Amersham, Piscataway, NJ). Alkaline transfer was performed overnight at room temperature. To detect for introduced PLR DNA within the wheat genome, the nylon membrane was hybridized for 12-24 hrs with the 1.2kb ^{32}P-dCTP labeled *F .intermedia* PLR gene probe. After hybridization, the blotted membrane was washed in phosphate buffer solution containing 20% SDS for 20-40 minutes twice and again with the same phosphate buffer solution containing ¼ SDS amount of the first solution. The membrane was then exposed in a phosphor image cassette and developed using the Scan Quant software (Molecular Dynamics Inc., Sunnyvale, CA).

Isolation of partial wheat PLR sequence

Wheat genomic DNA was extracted as described above from non-transgenic wheat (*cultivar 'Fielder'*). Primer sets (PLR F and PLR R) were used to PCR amplify the 539bp PLR fragment from wheat (same PCR conditions as above for PLR primers). The PCR products were purified using the montage DNA PCR purification kit (Millipore Corporation, Bedford, MA), inserted into the cloning site of the pGem Teasy vector (Promega, Madison, WI) through ligation

as described above. To increase plasmid DNA, bacterial transformation was performed using JM109 competent cells as described by manufacturer's instructions (Promega, Madison, WI). Positive clones with the inserted PLR gene fragment from wheat were confirmed through PCR analysis with the PLR primers as described above and subjected to sequencing at the Kansas State University, gene sequencing facility with pGem Teasy primers (Promega, Madison, WI). The sequences obtained from the positive clones were then compared to *Forsthysia* PLR sequence by use of the national centre for biotechnology sequence comparison software (NCBI).

Relative real time PCR quantification of Ubi PLR expression in transgenic wheat lines

To quantify for levels of *F. intermedia* PLR expression in different positive transgenic plants, total RNA was isolated from young leaf tissue by use of the total RNA isolation kit according to manufacturer's instructions (Promega, Madison WI). Briefly, samples were homogenized with the RNA lysis buffer®, transferred to a fresh tube and RNA dilution buffer® added and centrifuged to obtain supernatant. The supernatant was mixed with 95% ethanol and transferred to a spin column containing cellulose for attachment of poly + (A) RNA. Dnase activity was stopped with the Dnase stop solution® and the column washed with the RNA wash solution® before elution with nuclease free water. Total RNA was then qualified by use of the nanodrop (Nanodrop Technologies, Wilmington, DE) through measuring UV absorption at 260nm. The ratio of wavelength 230/260 was used to determine for RNA purity. First strand cDNA synthesis was performed using 1 µg of total RNA and random hexamer primers with AMV reverse transcriptase® according to manufacturer's conditions (Promega, Madison, WI). The samples were then diluted to an equivalent volume and an equivalent amount used for real time PCR analysis. The primer set, PLR RT F and the PLR RT R, (Table. 1) were used to

amplify a 99bp fragment of the *F. intermedia* PLR gene using the Sybr green PCR master-mix® (Bio-rad Laboratories, Hercules, CA). Real time PCR was performed according to manufacturers instructions with the PCR mastermix® in the iCycler thermal cycler (Bio-rad Laboratories, Hercules, CA). Ten to eighty pg of Ubi PLR was initially amplified to make sure that the efficiency of the F. intermedia PLR RT primers was 100%. PCR products from the cDNA were then qualified with the iCycler Bio-rad software (Bio-rad Laboratories, Hercules, CA). The experiment was repeated three times and the results plotted as relative quantification in log CT units.

SDG identification and quantification in transgenic wheat seeds

Sample extracts from transgenic wheat T2 seeds were quantified for SDG levels by HPLC analysis. Transgenic and non transgenic seeds (10-30) equivalent to 0.2-0.5 mg were ground using magic mill II (Nutrition life styles®, Gilmer, TX) and defatted using hexane before drying in the hood overnight. Flavone was added to the extraction solution (methanol: acetone: water, ratio 1:5:4) to make a final concentration of 5 mM. Extraction buffer was then added to defatted whole extracts which were homogenized for 2 minutes under cold conditions through addition of liquid nitrogen. The homogenate was subjected to centrifugation at 4000 x g for 15 minutes and the supernatant obtained was kept under reducing conditions through passage of liquid nitrogen. SDG was then extracted with 3 diethyl ether extractions as in study 1. The residue obtained after evaporation of the diethyl ether was dissolved in 100% methanol and subjected to a HPLC and HPLC-MS/ESI protocol as described in study 1. Quantification was done by comparing peak area ratio of a known amount of commercial SDG run on the HPLC with the internal standard and the peak area ratio of the SDG obtained from individual transgenic/wild type wheat plant lignan extracts.