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Allan. K. Ayella
Department of Biology, McPherson College, McPherson, KS 67460

Weiqun Wang
Department of Human Nutrition, Kansas State University, Manhattan, KS 66506

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Lignan Biosynthesis Enhancement in Transgenic Wheat*

Allan. K. Ayella[1], Weiqun Wang[2]

[1] Department of Biology, McPherson College, McPherson, KS 67460, [2]Department of Human Nutrition, Kansas State University, Manhattan, KS 66506

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Abstract

Phytochemical 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, in which phenylalanine ammonia lyase (PAL) catalyzes the initial step of the secondary metabolism and pinoresinol laciresinol reductases (PLR) accelerates the final steps of biosynthesis of lignans. Lignans not only present abundantly in flaxseed but also in various grains such as wheat. Epidemiological and clinical studies have shown a correlation between the consumption of lignan-rich whole grain products and a reduced cancer risk. In animal models, lignans have been shown to have cancer preventive activities. Our previous studies demonstrated that the contents in lignans in various wheat cultivars were significantly associated with anti-tumor activities in APC^{min} mice. This review article covers updated aspect of lignans with emphasis on our recent lignan biosynthesis enhancement via transgenic engineering in wheat by over-expressing PLR gene. To develop a stably expression and more efficient transformation, a future study in genetically engineered wheat with pathway transformation by over-expressing both the PAL and PLR enzymes will be further discussed.

1.0 Introduction

A number of reports have recently been published that looked at the importance of enhancing health important phytochemicals in plants (1-2). Consistent consumption of phytochemicals may help prevent cardiovascular disease and cancer through various mechanisms including anti-oxidation, anti-proliferation, cell cycle arrest, anti-estrogen,

and/or apoptosis induction, etc. as shown in epidemiological and clinical studies (3-4). Of utmost importance in our study are the phytochemicals classified as lignans. Phytochemical lignans are phenylpropane dimers linked by β-β bonds with a 1, 4 diarylbutane structure. In animal models and cell culture studies, lignans have cancer preventive activities (5). Our previous study also demonstrated that the contents in lignans of various wheat cultivars were significantly associated with anti-tumor activities in APC^{min} mice (6). In cell cultures, treatment of human colon cancer SW480 cells with lignan metabolites, enterolactone and enterodiol, results in S-phase cell cycle arrest in a dose- and time- dependent manner (6). To therefore increase the levels of these health important phytochemicals, genetic manipulation of the key enzyme(s) that control the lignan biosynthesis pathway seems a necessary approach.

Many authors have published in support of genetic manipulation as a way of enhancing phytochemical biosynthesis in plants (7-11). Noticeably, all these approaches emphasize the over-expression of a branch stage enzyme within the phenylpropanoid pathway. Some studies focus on the initial branch stage enzymes such as phenylalanine ammonia lyase (PAL) (12-13) while others on the final stage enzymes such as pinoresinol laciresinol reductases (PLR) (14-15). Whichever approach taken, significant changes in levels of phytochemicals have been reported in the resultant transgenic lines. Especially, a study conducted by us showed that genetic transformation with the PLR gene results into significantly high levels of secoisolariciresinol diglucoside (SDG) in transgenic wheat plants (16).

In addition to manipulation of individual enzymes, some authors have also tried a pathway transformation technique. Pathway transformation is a multi-faced approach

involving the transformation of one or more enzymes within a specific pathway. Pathway transformation has already been used for the phenylpropanoid pathway in plants. Schijlen et al (2007) genetically transformed the stilbene synthase, chalcone synthase, chalcone reductase and chalcone isomerase genes into tomato plants for purposes of increased biosynthesis of phytochemical flavonoids (17). Lorenc-Kukua et al (2005) transformed the chalcone synthase, chalcone isomerase and dihydroflavone reductase genes into flax plants for increased biosynthesis of anti-oxidants (18). Pathway transformation takes advantage of the fact that phytochemical biosynthesis has various control steps and therefore manipulation of more than one enzyme within the same pathway is likely to generate more of the targeted end products. In this review, we discuss a potential way of doing a pathway transformation technique for the wheat phenylpropanoid pathway by over-expressing both PLR and PAL genes. We present some of our earlier data on genetic manipulation of the PLR gene and look at the possibility of using these wheat transgenic sub-lines together with a new transgenic construct containing the PAL gene genetically expressed under a ubiquitous promoter.

2.0 Enzymatic roles of PLR and PAL genes in lignan biosynthesis

In our recent publication, we looked at the possible enzymatic role of the PLR gene in wheat (16). PLR belongs to a group of enzymes classified as aromatic alcohol reductases together with phenylcouraman and isoflavone reductases (19). Most reactions catalyzed by this class of enzymes are usually first order reactions. PLR catalyses the final two steps in the biosynthesis of lignans through conversation of pinoresinol to lariciresinol and then to secoisolaciresinol. This catalysis requires reducing equivalents in

terms of NADPH . Since NADHP is relatively available in plants, the availability of pinoresinol in sufficient amounts appears critical for the reaction to proceed in the first order and in the forward direction. Increasing the enzymatic activity of PLR would therefore serve to form more secoisolaciresinol. To this effect, we genetically engineered the *Forsthyia intermedia* PLR gene behind the maize ubiquitin promoter and successively obtained transgenic wheat plants with increased genetic expression of PLR transcripts as measured by real-time PCR (16). Analysis of the SDG amounts by HPLC-MS method showed significant increase in one of the transgenic wheat sub-lines over the control. As shown in the Figure 1, SDG contents are up-and-down in various wheat cultivars with an average level at 53 ± 19.8 μg/g in Wheat '*Fielder'*. We transformed wheat '*Fielder*' with the *Forsythia intermedia* PLR gene and successfully obtained one of the transformants, i.e., #4970 I5, that contained SDG levels as high as 117.9 ± 4.5 μg/g.

Phenylalanine ammonia lyase (PAL) (E.C. 4.3.1.5) is an enzyme that is involved in the first step of the phenylpropanoid pathway. PAL catalyzes the oxidative deamination of L-phenylalanine to form trans-cinnamic acid and an ammonia ion (20). In addition to using phenylalanine, PAL may also use L-tyrosine to yield p-couramic acid (Figure 2). Maize PAL has been shown to have both phenylalanine ammonia lyase and tyrosine ammonia lyase activites (21). Other plant PALs also catalyze similar reactions (22). Like PLR, PAL catalyzes reactions in the first order. Unlike PLR, PAL does not require any cofactors for its catalysis. However, the amounts of phenylalanine available may be considered as a limiting factor in the enzymatic action of PAL. This is because phenylalanine is an essential aromatic amino acid that is required in protein, carbohydrate and lipid metabolism (23). Increasing the levels of PAL will therefore mean channeling

phenylalanine into the phenylpropanoid pathway, which may create a metabolic sink (24). In addition to that once within the phenylpropanoid pathway, phenylalanine is assimilated into different major end products such as flavonoids and lignans as illustrated in Figure 2. Therefore, manipulation of PAL gene is likely to have an overall drastic consequence on all key products of the phenylpropanoid pathway.

3.0 Previous genetic manipulation on PAL and PLR genes and their consequences

Table 1 summarizes all the current known studies on genetic manipulation of either PAL or PLR gene. Most of studies use either the PAL or PLR gene but not both. Sewalt et al (1997) genetically engineered both bean PAL gene and cinnamate-4-hydroxylase enzyme in transgenic tobacco plants and showed an increase in ratio of monolignol precursors necessary for lignin biosynthesis (25). Sewalt's study is unique in that different phenotypes with increased and suppressed levels of PAL were generated. In addition, most of the other studies summarized in Table 1 showed an increase in levels of the immediate product catalyzed by either PAL, i.e., p-couramic acid or PLR, i.e., SDG.

Most scientists preferred agrobacterium transformation as a method of choice for genetic transformation compared to particle-gun bombardment (Table 1). Infact, the only study that has so far used particle-gun bombardment is ours (16). For future study, we propose to use particle bombardment because of the already established and optimized conditions. Besides using individual genes, some studies have focused on use of transcription factors as promoters for enhancing lignans biosynthesis in plants. We plan

to use the ubiquitin promoter that targets for expression of both PAL and PLR genes in all the tissues.

Transcriptional factor modification of the key genes involved in the phenylpropanoid pathway may be a future major approach for use in enhancing lignans in transgenic plants. Unlike insertion or modification of individual enzymes, transcriptional modification may provide the added advantage of being able to use master transcription factors inserted into targeted plant systems that function to enhance not just lignan biosynthesis but all phenylpropanoid biosynthesis (29). The MYB-protein family of transcription factors is the only currently well known transcription factors that function to enhance lignan biosynthesis in transgenic plants (30). We anticipate discovery of more transcription factors in the near future.

4.0 Plant PAL gene families and structural organization

PAL gene has been isolated and characterized in various plants, which showed a multi-gene family with 2-40 members (Table 2). Selection of a special PAL gene member for usage in lignan enhancing experiments, therefore, might depend on completeness of the targeted PAL gene sequence and the relationship between the plants from which the gene is abundant and key controller for corresponded secondary metabolism biosynthesis. In cases where one has to be chosen over the other, completeness of a gene sequence would hold preference to the plant family gene relationship.

The structural organization of the Arabidopsis PAL gene has recently been described (31). The 3.1 kb fragment of the Arabidopsis PAL gene was sub-cloned from a genomic library (32) and shown to consist of a 1.8 kb promoter region and 0.7 kb coding

region. Deletion of the proximal 5' promoter region to -290 indicated that this region was sufficient to allow for PAL expression in all tissues while the proximal to –540 was responsive to environmental stress. Region –1816 to –290 had the negative and positive elements. However, the coding region has a 448bp intron with AG/GT and AG/AT consensus boundaries similarly found in other PAL genes (31). Cochrane et al (2004) isolated the 4 Arabidopsis PAL isoforms and analyzed their 5'3' UTR regions and ORF using clustalW analysis (40). He found that there was a 2+2 pattern similarity in the PAL isoforms with *AtPal* 1 and 2 having similar amino-acid similarity compared with *AtPal* 3 and 4. In fact, limited information is still available on the wheat PAL gene. The grain gene database currently has 130 hits on isolated expressed sequence tags (ESTs) for the wheat PAL gene (41). Choosing one of those EST clones to use for genetic transformation will provide us with a source for the PAL gene. On the other hand, more completely characterized and fully expressible PAL genes from other plants have been available as shown in Table 2.

4.0 Conclusion

It is obvious that both PLR and PAL genes play significant roles in lignan biosynthesis in wheat. Generation of transgenic wheat lines with enhanced lignan levels and particularly SDG biosynthesis may support current efforts in reduction of cardiovascular and cancer disease risks through consumption of healthy foods.

It is obvious, however, that there still remain many questions that may have been answered. Among the key questions is whether our current transgenic wheat sub-lines with enhanced SDG levels can be stably expressed in subsequent generations. As

discussed in our previous publication (16), it is critical to distinguish our PLR transgenic wheat lines between homozygous and heterozygous for additional PAL transformation. It is also not entirely clear how many copies of the endogenous PLR and PAL genes that may exist in wheat. How are these genes structurally organized in wheat? Can their endogenous promoter regions be modified for enhanced lignan biosynthesis through the use of transcriptional factors? Nevertheless, the new wheat line with significantly higher SDG contents obtained from our recent study may suggest a promising future in value-added health benefits for whole grains by genetic manipulation means.

5.0 References

[1] de Kok, T.M., van Breda, S.G., and Manson, M.M. Mechanisms of combined action of different chemopreventive dietary compounds: a review. Eur J Nutr., 2008, 47, Suppl 2, 51-59.

[2] Martinez-Ballesta C.M., Lopez-Perez, L., Hernandez, M., Lopez-Berenguer, C., Fernandez-Garcia, N., and Carvajal, M. Agricultural practices for enhanced human health. Phytochemistry, 2008, 7, 2, 251-260.

[3] Adlercreutz, H. Phytoestrogens and breast cancer. J. Steroid Biochem. Mol. Biol. 2002, 83, 113–118.

[4] Webb, A. L.; McCullough,M.L. Dietary lignans: potential role in cancer prevention. Nutr. Cancer 2005, 51, 117–131.

[5] Weiqun, W., Ayella, A., Jiang, Y., Ouyang, P., and Qu, H. Wheat lignans: Promising cancer preventive agents. In Wheat Antioxidants, Edited by Liangli Yu, John Wiley and sons, 2008, 264-272

[6] Qu, H.; Madl, R. L.; Takemoto, D. J.; Baybutt, R. C.;Wang,W. Lignans are involved in the anti-tumor activity of wheat bran in colon cancer SW480 cells. J. Nutr. 2005, 135, 598–602.

[7] Siebert, M., Sommer, S.-M., Li, Z.W., Severin, K and Hiede, L. Genetic engineering of plant secondary metabolism. Accumulation of 4-hydroxybenzoate glucosides results from the expression of the bacterial ubiC gene in tobacco, Plant Physiol, 1996, 112, 811-819.

[8] Seo, J-W., Jeong, J-H., Shin, C-G., Lo, C-S., Han, S.S., Yu, K-W., et al., Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation, Phytochemistry 2005, 26, 869-877.

[9] Hain, R., Bieseler, B., Kindl, H., Schroder, G., and Stocker, G., Expression of a stilbene synthase gene in *Nicotiana tabacum* results in synthesis of the phytoalexin resveratrol. Plant Mol Biol 1990, 15, 325-335.

[10] Robbins, M.P., and Morris, P. Metabolic engineering of condensed tannins and other phenolic pathways in forage and fodder crops. In: Verporte, R ., and Alfermann, AW (eds) Metabolic engineering of plant secondary metabolism (pp. 165-177) Kluwer Academic Press, Dordrecht

[11] Fettig, S., and Hess, Dieter., Expression of a chimeric stilbene synthase gene in transgenic wheat lines. Transgenic Research, 1999, 8, 179-189

[12] Pallas, J.A., Paiva, N.L., Lamb, C., and Dixon, R.A Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. Plant J, 1996, 10, 281-293

[13] Ma, L.Q., Gao, D.Y., Wang, Y.N., Wang, H.H., Zhang, J.X., Pang, X.B., et al Effects of overexpression of endogenous phenylalanine ammonia-lyase (PALrs1) on accumulation of salidroside in Rhodiola sachalinensis. Plant Biology (Stuttg), 2008, 10, 323-333

[14] Hano, C., Martin, I., Fliniaux, O., Legrand, B., et al., Pinoresinol-lariciresinol reductases gene expression and secoisolariciresinol diglucoside accumulation in developing flax (*Linum usitatissimum*) seeds. Planta 2006, 224, 1291-1301.

[15] Hemmati., S., Schmidt, T.J., and Fuss, E. (+)-Pinoresinol/(-)-lariciresinol reductase from Linum perenne Himmelszelt involved in the biosynthesis of justicidin B. FEBS Letters, 2007, 581, 603-610.

[16] Ayella A.K., Trick, HN., and Wang, W. Enhancing lignan biosynthesis by over-expressing pinoresinol lariciresinol reductase in transgenic wheat. Mol Nutr. Food Res., 2007, 51, 1518-1526.

[17] Schijlen, E., Ric de vos, C.H., Jonker, H., van den Broeck, H., Molthoff, J., van Tunen, A., et al., Pathway engineering for healthy phytochemicals leading to production of novel flavonoids in tomato fruit. Plant Biotechnology Journal, 2006, 4, 433-444.

[18] Lorenc-Kukula, K., Amarowicz, R., Oszmianski, J., Doermann, P., Starzycki, M., Skala, J., et al., Pleiotropic effect of phenolic compounds content increases in transgenic flax plant. J. Agric. Food Chem., 2005, 53 (9), 3685-3692.

[19] Min, T., Kasahara, H., Bedgar, D.L., Youn, B., Lawrence, P.K., Gang, D.R., et al ., Crystal structures of pinoresinol-lariciresinol and phenylcoumaran benzylic ether reductases and their relationship to isoflavone reductases. J.Biol.Chem., 2003, 278**,** 50714-50723.

[20] Hahlbrock, K., and Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol., 40, 347-369.

[21] Rosler, J., Krekel, F., Amrhein, N., and Schmid, J. Maize Phenylalanine Ammonia-Lyase activity. Plant Physiol. 1997, 113, 175-179.

[22] Jangaard, N.O. The characterization of phenylalanine ammonia-lyase from several plant species. Phytochemistry, 1974, 13, 1765-1768.

[23] Hahlbrock, K., and Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol. 1989, 40, 347-369.

[24] Yao, K., De Luca, V., and Brisson, N. Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to phytophthora infestans. The plant cell, 1995, 7, 1787-1799.

[25] Sewalt, V.J.H., Ni, W., Blount, J.W., Jung, H.C., Masoud, S.A., Howle, P.A., Lamb, C., et al. Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-Phenylalanine ammonia-lyase or cinnamate 4-hydroxylase, Plant Physiol., 1997, 115, 41-50

[26] Magiri, N.E., Lomo, P., Guo, Z., and Debao, Li. Genetic engineering of rice using antisense strategy suppressed PAL activity in 7th Biochemistry society of Kenya (BSK) conference proceedings, 2002

[27] Howles, P.A., Paiva, N.L., Sewalt, V.J.H., Elkind, N.L., Lamb, C.J., and Dixon, R.A. Over-expression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. Plant Physiol., 1996, 112, 1617-1624

[28] Liao,Y.C., Li,H.P., Kreuzaler,F., Reisener,H.J. and Fischer,R. (Unpublished data) in Gene Bank

[29] Pattanaik, C.H., Xie, C.H., and Yuan, L. Creation of master transcription factors to regulate plant metabolic pathways. Acta Hort., 2007, 756, 109-118

[30] Sablowski, R.W., Moyano, E., Culianez-Macia, F.A., Schuch, W., Martin, C., and Bevan, M. A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO J, 1994, 13, 1, 128-137

[31] Ohl, S., Hedrick, A.S., Chory, J., and Lamb, J.C. Functional properties of a phenylalanine ammonia-lyase promoter from Arabidopsis, The plant cell, 1990, 2, 837-848

[32] Chang, C., and Meyerowitz, E.M. Molecular cloning and DNA sequence of the Arabidopsis thaliana alcohol dehydrogenase gene. Proc. Natl. Acad. Sci. USA, 1986, 83, 1408-1412.

[33] Qun, Z., Dabi, T., Beeche, A., Yamamoto, R., Lawton, A.M., and Lamb, C. Cloning and Properties of a rice gene encoding phenyalanine ammonia-lyase. Plant. Mol. Biol., 1995, 29, 535-550

[34] Cramer, L.C., Edwards, K., Dron, M., Liang, X., Dildine, S.L., Bolwell, G.P., et al., Phenylalanine ammonia lyase gene organization and structure. Plant Mol. Biol., 1989, 12, 367-383.

[35] Logemann, E., Parniske, M., and Hahlbrock, K. Modes of expression and common structural features the complete phenyalanine ammonia-lyase gene family in parsley. Proc. Natl. Acad. Sci., 1989, 92, 5905-5909.

[36] Subramaniam, R., Reinold, S., Molitor, E., and Douglas, C.J. Structure, inheritance and expression of hybrid poplar (Populus trichocarpa x populus deltoids) phenylalanine ammonia-lyase gene. Plant Physiol, 1993, 102, 71-83.

[37] Joos, H.J., and Hahlbrock, K. Phenylalanine ammonia lyase in potato (Solanum tuberosum L.) Eur. J. Biochem., 1992, 204, 621-629.

[38] Frank R.L., and Vodkin L.O. Sequence and structure of a phenylalanine ammonia-lyase gene from Glycine max. DNA Seq. 1991, 5, 335–346.

[39] Gowri, G., Paiva, N.L., and Dixon, R.A. Stress responses in alfalfa (Medicago sativa L.) 12. Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell cultures and developing plants. Plant Mol Biol., 1991, 17, 3, 415–429.

[40] Cochrane, C.F., Davin, B.L., and Lewis, N. The Arabidopsis phenylalanine ammonia lyase gene family: kinectic characterization of the four PAL isoforms. Phytochemistry, 65, 1557-1564

[41] http://wheat.pw.usda.gov/wEST/

Table 1: Reported genetic transformation studies on either PLR or PAL gene

Target Plant	Enzyme	Promoter	Transformation method	Target Impact	Reference
Rhodiola	PAL	35S	Agrobacterium	Increased P-couramic acid	13
Flax	PLR	Gus-intron	Hypocotyls with Agrobacterium	Increased SDG	14
Wheat	PLR	Maize ubiquitin	Particle bombardment	Increased SDG	16
Tobacco	PAL and Cinnamate -4-hydroxylase	None	Antisense and sense suppression	Increased syrinyl/guaiacyl ratio	25
Rice	PAL	35S	Antisense suppression	Decreased PAL activity	26
Tobacco	PAL	35S	Agrobacterium	Increased quercetin	27
Tobacco	PAL	Bean PAL promoter and 35S enhancer sequences	Agrobacterium	Increased chlorogenic acid and 4-Coumaric acid	28

Table 2: Identified and characterized plant PAL gene as selected as representative examples

Plant	Gene size (bp) and type	Number of multi-gene family	Reference
Wheat	1419, DNA	Not Known	28
Rice	2130, ORF	4	33
Bean	2136 &2130, ORF	2	34
Parsley	Not given	4	35
Popular	2400, cDNA	2	36
Potato	1200, DNA	40	37
Soybean	2142	2	38
Alfalfa	2175	Not known	39
Arabidopsis	2085-2178	4	40

Figure Legends

Figure 1: Quantification of SDG contents in normal wheat cultivars vs. transgenic wheat seeds at T_2 from various sub-lines. SDG contents were determined by HPLC-MS method as outlined in our previous publication (16).Up Panel: SDG contents in three wheat cultivars including *'Fielder'*. Bottom panel: SDG contents in various transgenic wheat clones at T_2 vs. non-transgenic *'Fielder'* controls. Results are means ± SD, n = 3.

Figure 2: Schematic of the initial enzymatic reactions catalyzed by phenylalanine ammonia lyase (PAL). The major products formed may be impacted for selective phenylpropanoid pathway derivatives depending on following active enzymes used.

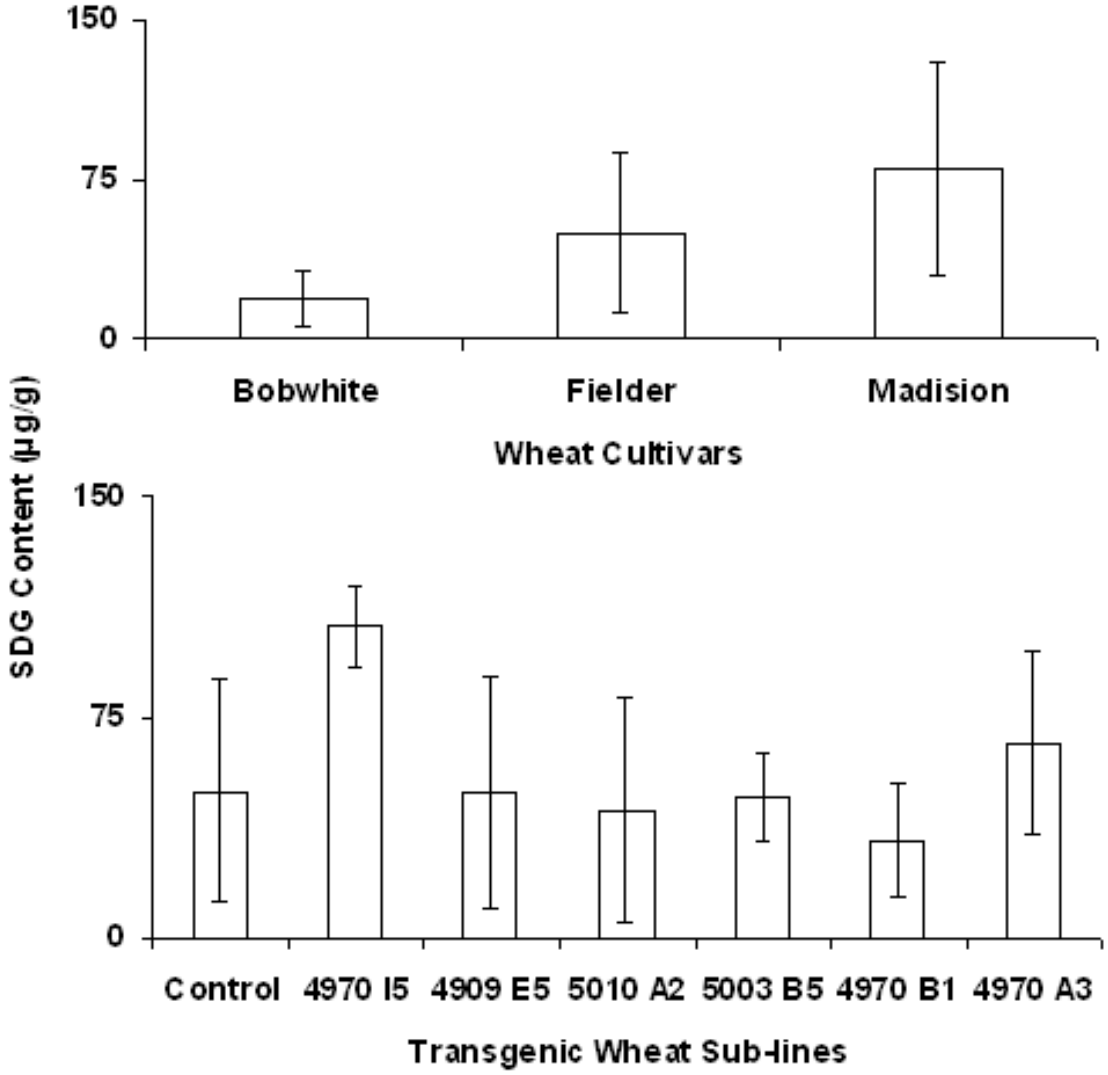
150
75
0
Bobwhite
Fielder
Madision
Wheat Cultivars
SDG Content (µg/g)
150
75
0
Control
4970 I5
4909 E5
5010 A2
5003 B5
4970 B1
4970 A3
Transgenic Wheat Sub-lines

Figure 1

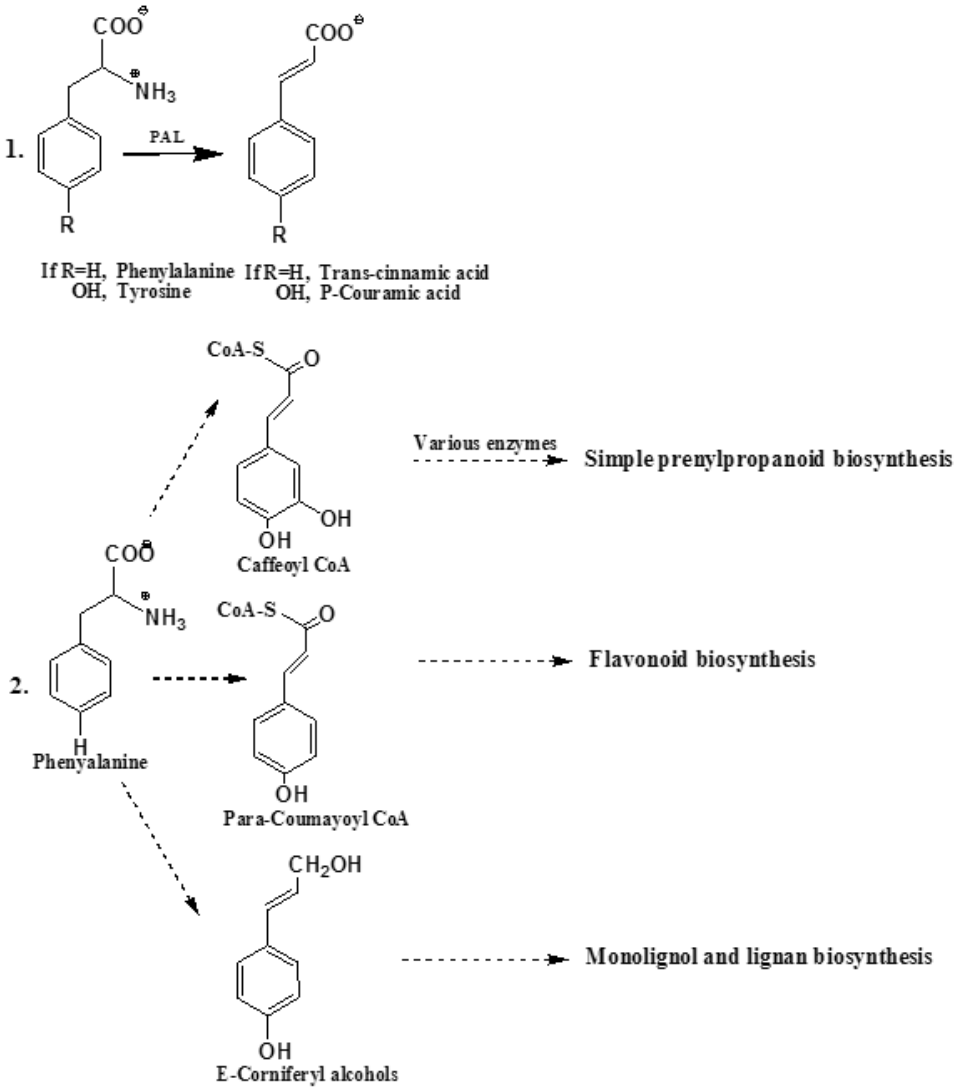
1.
PAL
If R=H, Phenylalanine
OH, Tyrosine
If R=H, Trans-cinnamic acid
OH, P-Couramic acid
2.
Phenyalanine
Caffeoyl CoA
Para-Coumayoyl CoA
E-Corniferyl alcohols
Various enzymes
Simple prenylpropanoid biosynthesis
Flavonoid biosynthesis
Monolignol and lignan biosynthesis

Figure 2