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Invited review

Biotechnology to harness the benefits of dietary phenolics; focus on *Lamiaceae*

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Phytochemicals from herbs and fermented legumes are excellent dietary sources of phenolic metabolites. These phenolics have importance not only as food preservatives but increasingly have therapeutic and pharmaceutical applications.

The long-term research objecitves of the food biotechnology program at the University of Massachusetts are to elucidate the molecular and physiological mechanisms associated with synthesis of important health-related, therapeutic phenolic metabolites in food-related plants and fermented plant foods. Current efforts focus on elucidation of the role of the proline-linked pentose phosphate pathway in regulating the synthesis of anti-inflammatory compound, rosmarinic acid (RA).

Specific aims of the current research efforts are: (i) To develop novel tissue culture-based selection techniques to isolate high RA-producing, shoot-based clonal lines from genetically heterogeneous, cross-pollinating species in the family Lamiaceae; (ii) To target genetically uniform, regenerated shoot-based clonal lines for: (a) preliminary characterization of key enzymes associated with the pentose phosphate pathway and linked to RA synthesis; (b) development of genetic transformation techniques for subsequent engineering of metabolic pathways associated with RA synthesis.

These research objectives have substantial implications for harnessing the genetic and biochemical potential of genetically heterogeneous, food-related medicinal plant species. The success of this research also provides novel methods and strategies to gain access to metabolic pathways of pharmaceutically important metabolites from ginger, curcuma, chili peppers, melon or other food-related species with novel phenolics.

Key words: Biotechnology, medicinal plants, phenolics, phytochemicals, phytopharmaceuticals, *Lamiaceae*, rosmarinic acid, proline, pentose phosphate pathway, ginger, curcurma, chili pepper, melon

Phenolic phytopharmaceuticals from Lamiaceae

Higher plants are very important sources of secondary metabolites which have therapeutic and pharmaceutical applications. These secondary metabolites are species specific and can be termed phytopharmaceuticals. These metabolites, without the knowledge of the specific physio-logically active components, have been a part of human health for over many centuries and across all cultures. Despite excellent advances in synthetic organic chemistry, plants are still the sole source of about 25% of prescribed medicines[1]. Some economically important plant-derived drugs obtained from plants are provided in Table 1[2-4].

Among various phytopharmaceuticals, plant phenolics are an important group of secondary metabolites having diverse food processing, dietary and medicinal applications. Examples of plant phenolics that are used or have potential as food preservatives and pharmaceuticals are provided in Table 2.

Species belonging to the family Lamiaceae (Labiatae) are important sources of phenolic-type food preservatives and pharmaceuticals. Therapeutic and food processing use of these species can be observed in several countries. Examples are provided in Table 3.

Genetic heterogeneity in family Lamiaceae

The major problem in the use of phytopharmaceuticals from the family Lamiaceae is the plant to plant variability of specific metabolites due to genetic heterogeneity common to all species in this family. Much of this genetic hetero-geneity is due to gynodioecy, resulting in breeding character being influenced by natural cross-pollination[5]. Floral diversity and bee pollination also contribute to high cross-pollination. This gives rise to substantial variability in active ingredient levels and quality[6,7]. Therefore, the biochemical characterization of pathways and genetic access to specific metabolites in all species in Lamiaceae is difficult. Each plant within a given sample extract originates from a different heterozygous seed. Also, this makes breeding of elite varieties targeting enhancement of specific metabolites very challenging. Current genetic improvements have been limited to random selection and in some cases vegetative propagation[3].

Rosmarinic acid and medical applications

Rosmarinic acid is an important caffeoyl ester (phenolic depside) with proven medicinal properties and well characterized physiological functions. Rosmarinic acid is found in substantial quantities in several species in the family Lamiaceae with medicinal uses. *Salvia lavandulifolia* is used as choleretic, antiseptic, astringent and hypoglycemic drug in southern Europe and contains high quantities of RA[8]. Rosmarinic acid-containing *Ocimum santctum* (holy basil) is widely used to reduce fevers and against gastrointestinal disease in India. In Mexico, high RA-containing *Hyptis verticillata* is widely used by Mixtec Indians against gastrointestinal disorders and skin infections[9]. In Indonesia and several other parts of the southeast Asia RA-containing

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Orthosiphon aristatus is known for its diuretic properties and is also used against bacterial infections and inflammations of the urinary system[10]. *Salvia cavaleriei*, a high RA-containing species is used in China for treatment of dysentery, boils and injuries[11]. Rosmarinic acid-containing plant extracts also have excellent potential as antioxidants for food preservation[12-14].

Table 1. Economically important plant-derived metabolites.

Species	Metabolites	Use/Potential Use
Artemesia annua	artemesinin	antimalarial
Atropa belladonna	atropine/ hyoscyamine	parasympatholytic
Buddleja globosa	verbascoside	antihepatotoxic
Catharanthus roseus	vincristine	anticancer
Chondodendron tomentosum	d-tubcurarine	muscle relaxant
Coleus blumei	rosmarinic acid	anti-inflammatory
Colchicum autumnale	colchicine	antigout
Digitalis purpurea	digitoxin/digoxin	cardiotonic glycoside
Dioscorea spp	diosgenin	oral contraceptive
Forsythia suspensa	suspensaside	antiasthmatic
Lithospermum spp.	lithospermic acid	antigonadotropic
Origanum vulgare	galangin	antimutagen
Papaver somniferum	morphine	analgesics
Pilocarpus jaborandi	imidazole alkaloids	glaucoma
Rauwolfia serpentina	reserpine	antihypertensive
Rheum officinale	anthrones	cathartic
Salvia multiorrhiza	salvianolic acid A	antiulcer
Thymus vulgaris	thymol	anticaries/ antibacterial
Zingiber cassumunar	curcuminoids	anti-inflammatory

Table 2. Important plant phenolics and pharmaceutical potential.

Species	Metabolite	Use/Potential	Ref.
Curcuma longa	curcumin	cancer chemo-preventive	15
Curcuma mangga	curcumin	antioxidant	16
Digitalis purpurea	purpureaside	Immuno-suppressive	4
Glycine max	isoflavonoids	cancer chemo-preventive	17
Lithospermum sp.	lithospermic	antigonodo-acid tropic	18
Origanum vulgare	galanigin	antimutagen	19
Pimpinella anisum	anethole	antifungal	20
Plantago asiatica	Hellicoside	antiasthmatic	4
Rosmarinus officinalis	rosmarinic acid	anti-inflammatory	21
Salvia multiorrhiza	salvianolic acid	antiulcer	4
Thymus vulgaris	thymol	anticaries	22
Vitis vinifera	flavonoids	prevents cardio-vascular problems	23
Zingiber cassumunar	curcuminoids	antioxidants	24
Zingiber officinale	gingereonone A	antifungal	4

Pharmacological functions of rosmarinic acid

The pharmacological effect of RA through inhibition of several complement-dependent inflammatory processes has been clearly proven[21]. Therefore, it has tremendous potential as a therapeutic agent for control of complement activation diseases[21,29]. Rosmarinic acid has been reported to have effects on both the classical C3-convertase and on the cobra venom factor-induced alternative convertase pathway[21]. Other *in vivo* studies show that RA inhibits several complement-dependent inflammatory processes including paw edema induced by cobra venom factor and ovalbumin/anti-ovalbumin-mediated passive cutaneous anaphylaxis[29]. It also inhibits prostacyclin synthesis induced by complement activation[30,31]. It is also known to have complement-independent effects such as scavenging of oxygen free radicals[32] and inhibition of elastase. The relative safety of RA in relation to other methods of complement depletion is well documented[32]. Among other actions of RA are antithyrotropic activity in tests with human thyroid membrane preparations, inhibition of complement dependent components of endotoxin shock in rabbits and the ability to react rapidly to viral coat proteins and so inactivate the virus[25,29]. Rosmarinic acid also inhibits Forskolin-induced activation of adenylate cyclase in cultured rat thyroid cells[33].

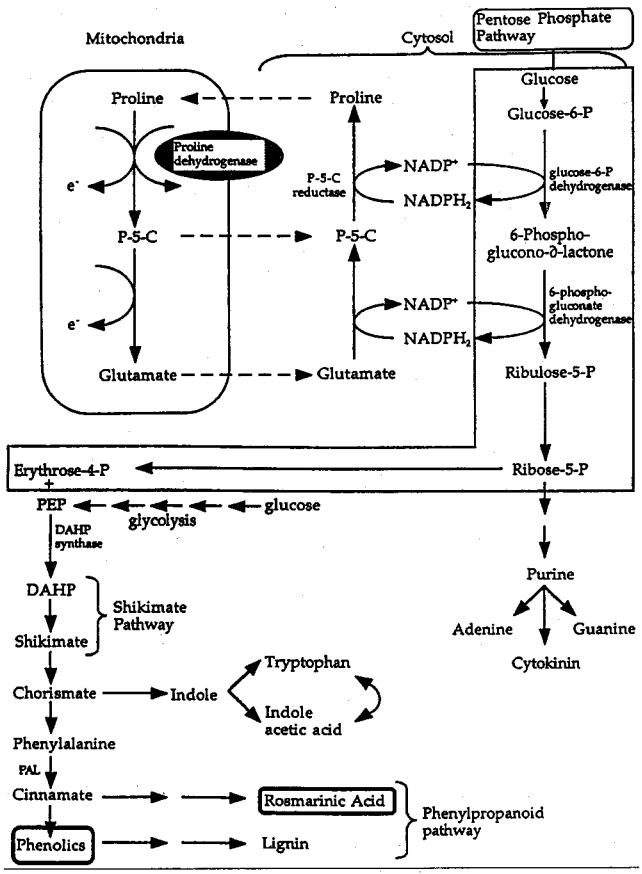
Table 3. Examples of species in Lamiaceae used as medicine and food preservatives.

Species	Key Metabolite	Use	Ref.
Hyptis verticillata	rosmarinic acid	gastro- intestinal disorders	9
Lavandula spp.	rosmarinic acid	anti-inflammatory	25
Lithospermum erythrorhizon	rosmarinic acid	anti-inflammatory -	26
	lithospermic acid	antigonodotropic	18
Origanum vulgare	galangin	antimutagen	19
	rosmarinic acid	anti-inflammatory	27
Orthosiphon aristatus	rosmarinic acid	diuretic and anti-inflammatory	10
Rosmarinus officinalis	rosmarinic acid	anti-inflammatory	21
Salvia multiorrhiza	salvianolic acid	antiulcer	4
Salvia officinalis	rosmarinic acid	antioxidant	12
Thymus vulgaris	thymol	antioxidant	28
Thymus vulgaris	Thymol, carvacrol	anticaries	22

Production of rosmarinic acid

Rosmarinic acid has been targeted for production using undifferentiated cell suspension cultures of several species[10,25,34-38]. The main purpose of cell suspension production of RA is the potential for large-scale production in bioreactors[1,39]. Although large-scale production in bioreactors is feasible for RA, undifferentiated cell suspensions are not practical for metabolites produced in differentiated structures (e.g. anethole in seeds, curcumin in rhizomes and thymol in glandular cells of leaves). An additional disadvantage of undifferentiated callus-based suspension cultures is that the DNA is more error prone and therefore cell lines are genetically unstable[40]. Even if problems of genetic stability and differentiation-linked metabolite production are solved, bioreactor-based production requires high initial operating costs and is not feasible in regions with poor industrial infrastructure. Gaining access to phenolic phytopharmaceuticals like rosmarinic acid for all people at low cost can be best achieved by improving plant varieties for specific metabolites using modern biotechnology. Such improved, elite varieties can be incorporated into existing agricultural practices and systems. With the above perspectives, the strategies outlined in this paper are to isolate high RA-producing genetically uniform, shoot-based clonal lines of several species belonging to the family Lamiaceae using tissue culture techniques. These species are known to produce RA and are used in several parts of the globe either as food additives, preservatives or medicines. The species chosen for this study are: (i) *Rosmarinus officinalis* (rosemary), (ii) *Ocimum sanctum* (holy basil), (iii) *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme). The high RA-producing, elite, shoot-based clonal lines will be screened based on resistance to *Pseudomonas* sp.[41-43]. The rationale for such a selection strategy is outlined in Figure 1 and described below.

Figure 1. Proline-linked pentose phosphate pathway.



Selected elite, shoot-based clonal lines will be used to gain access to pathways important for biosynthesis of RA and for developing genetic transformation techniques. This will be used for subsequent engineering of RA biosynthesis using modern molecular biology techniques. Such a model for isolating genetically uniform, elite, shoot-based clonal lines from a heterogeneous and unknown genetic background can be extended for the metabolic engineering of other phytopharmaceuticals.

Pathways associated with rosmarinic acid biosynthesis

It has been shown that two aromatic amino acids phenylalanine and tyrosine are precursors of RA biosynthesis[44]. Using radioactive phenylalanine and tyrosine it was established that they are incorporated into caffeic acid and 3,4-dihydroxyphenyllactic acid moieties, respectively[44]. Steps in RA biosynthesis originating from phenylalanine and tyrosine have been characterized (Figure 2)[10,38,45-47]. In several cell cultures, the activity of phenylalanine ammonia-lyase was correlated to RA[10,47]. Using *Anchusa officinalis* cell suspension cultures it was reported that tyrosine aminotransferase catalyzes the first step of the transformation of tyrosine to 3,4-dihydroxyphenyllactic acid. Several isoforms of tyrosine aminotransferase were found to be active in cell suspension cultures of *Anchusa officinalis*[46,47]. Prephenate aminotransferase in *Anchusa* cell suspension cultures was found to be important, and its activity was affected by 3,4-dihydroxyphenyllactic acid[48]. Other enzymes of late steps in the RA biosynthesis pathway like hydroxyphenylpyruvate reductase and RA synthase were isolated and characterized in cell cultures of *Coleus blumei*[49-51].

Recently, microsomal hydroxylase activities that introduce hydroxyl groups at position 3 and 3' of the aromatic rings of ester 4-coumaroyl-4'-hydroxy-phenyl-lactate to give rise to RA were isolated[38] . This led to the proposed complete biosynthetic pathway for RA biosynthesis originating from phenylalanine and tyrosine (Figure 2).

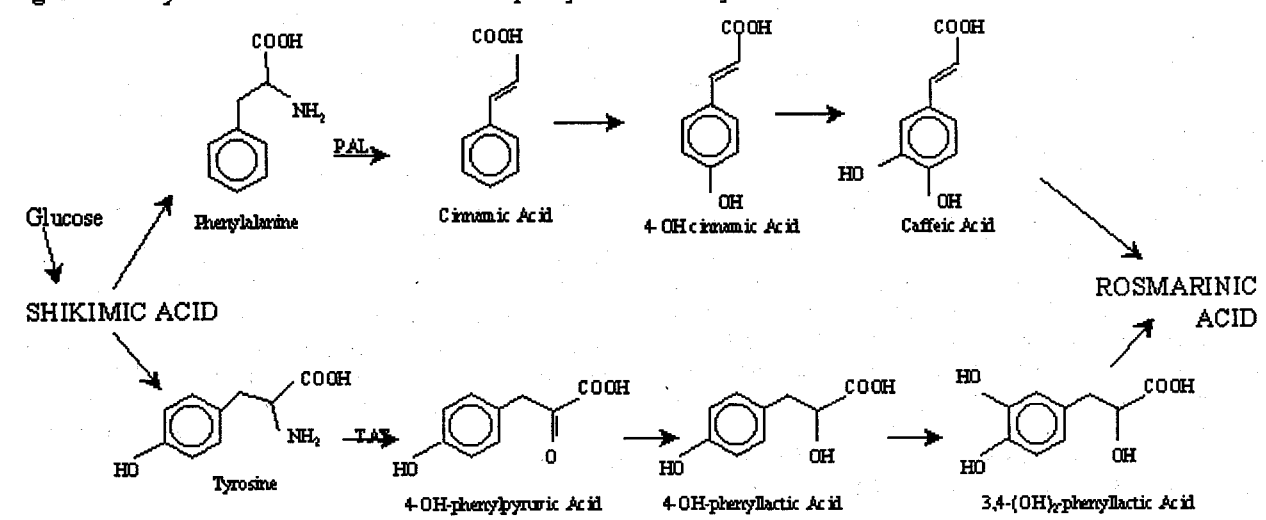
The above reports point to good success in understanding RA biosynthesis from phenylalanine and tyrosine using various cell suspension cultures. However, several major issues have to be addressed to gain access and control the interacting metabolic fluxes critical to RA biosynthesis and for subsequent metabolic engineering. The major gaps in understanding and significant questions are: (i) What is the role of primary metabolism, particularly the pentose phosphate pathway?; (ii) How is the pentose phosphate pathway regulated during RA synthesis?; (iii) What is the role of light in regulating RA biosynthesis?; (iv) How can the problems associated with genetic instability of undifferentiated callus cultures be resolved?; (v) How can the understanding of metabolic pathways and subsequent engineering of efficient RA biosynthesis be used to develop elite varieties for traditional and contemporary agricultural production systems?

Model and rationale for current research

1) Selection of high RA-producing, shoot-based clonal lines

This paper focusses on techniques to isolate genetically uniform, high rosmarinic acid-containing, shoot-based clonal lines for metabolic pathway analysis and for developing gene

Figure 2. Biosynthesis of rosmarinic acid from phenylalanine and tyrosine.



transfer techniques for subsequent metabolic engineering. This will help to develop excellent experimental systems and directions to fill gaps in knowledge of RA biosynthesis using several species used for food and medicinal applications. The basic strategy involves the isolation of genetically uniform, high RA-producing, shoot-based clonal lines from a heterogeneous genetic background. This heterogeneity is found in all species in the family Lamiaceae and since the breeding character is influenced by natural cross-pollination[5] . The use of genetically uniform, shoot-based clonal lines envisioned in this paper have the following advantages: (i) Shoot clones are genetically more stable than undifferentiated callus cultures; (ii) Shoot clones allow the characterization of light-regulated pathways associated with RA synthesis; (iii) Shoot clones can easily be targeted for large-scale greenhouse production of elite clonal lines or for incorporating into plant breeding programs to develop superior RA-producing seed varieties; (iv) Shoot clones targeted for genetic engineering of metabolic pathways can be easily regenerated to whole plants for incorporation into plant variety improvement programs.

2) Role of proline-linked pentose phosphate pathway

High RA-producing, shoot-based clonal lines originating from a single heterozygous seed among a heterogeneous bulk-seed population will be selected based on tolerance to a novel *Pseudomonas* sp. isolated from oregano. This strategy for selection of high RA clonal lines is based on the model that proline-linked pentose phosphate pathway is critical for driving metabolic flux (erythrose-4-phosphate) towards shikimate and phenylpropanoid pathways (Figure 1). Any clonal line with a deregulated proline synthesis pathway should have an overexpressed pentose phosphate pathway which allows excess metabolic flux to drive shikimate and phenylpropanoid pathway towards RA synthesis. Such proline-overexpressing clonal line should be tolerant to *Pseudomonas* sp. If the metabolic flux to RA is overexpressed it is likely to be stimulated in response to *Pseudomonas* sp. Therefore such a clonal line is likely to be tolerant to *Pseudomonas* sp. Such a clonal line should also have high proline and proline oxidation (proline dehydrogenase) and high RA content in response to *Pseudomonas* sp. In addition, in the presence of *Pseudomonas* sp. increased activity of key enzymes glucose-6-phosphate dehydrogenase (pentose phosphate pathway), pyrroline-5-carboxylate reductase (proline synthesis pathway), proline dehydrogenase (proline oxidation pathway), 3-deoxy-D-arabino heptulosonate-7-phosphate synthase (shikimate pathway) and phenylalanine ammonia-lyase (phenylpropanoid pathway) should be observed. The rationale for this model is based on the role of the pentose phosphate pathway in driving ribose-5-phosphate towards purine metabolism in cancer cells[52], differentiating animal tissues[53] and plant tissues[54]. The hypothesis of this model is that the same metabolic flux from overexpression of proline-linked pentose phosphate pathway regulates the interconversion of ribose-5-phosphate to erythrose-4-phosphate driving the shikimate pathway. Shikimate pathway flux is critical for both auxin and phenylpropanoid biosynthesis, including RA. This hypothesis has been strengthened by preliminary results that in several oregano clonal lines RA biosynthesis was significantly stimulated by *Pseudomonas* sp. and responded to the bacterium by increasing both RA and proline biosynthesis[42,55]. High RA-producing clonal lines selected based on the model will be targeted for preliminary characterization of key enzymes mentioned above. Such genetically uniform clonal lines will also be targeted for developing gene transformation techniques using *Agrobacterium* or particle gun bombardment. The model will provide access to critical interlinking metabolic pathways associated with RA biosynthesis. This will allow more detailed analysis that will lead to metabolic engineering for efficient RA biosynthesis. This strategy for RA biosynthesis can be the foundation for metabolic engineering of other phytopharmaceuticals from cross-pollinating, heterogeneous species.

Regulation of the pentose phosphate pathway in plants

The pentose phosphate pathway is the alternate route for breakdown of carbohydrates[56]. The important functions of this pathway are to generate NADPH for use in biosynthetic (anabolic) reactions and to provide ribose-5-phosphate for nucleotide synthesis and erythose-4-phosphate for shikimate pathway syntheses[56,57]. Therefore, it is also critical for the synthesis of phenylpropanoid pathway metabolites, including RA.

Up to 60% of the dry weight in some plant tissues can consist of metabolites derived from the shikimate pathway[58,59]. Activity of the distinct isozymes of 3-deoxy-arabino heptulosonate-7-phosphate (DAHP) synthase in the shikimate pathway are dependent on metabolic flux from erythrose-4-phosphate[60-64]. This large flux of erythrose-4-phosphate cannot be exported from the Calvin cycle without rapid depletion[59,65]. This has led to the suggestion that the oxidative pentose phosphate pathway must operate to provide the erythose-4-phosphate for the shikimate pathway[59]. This may be particularly important during plant response to micro-organisms, when metabolites derived from the shikimate pathway increase substantially[64].

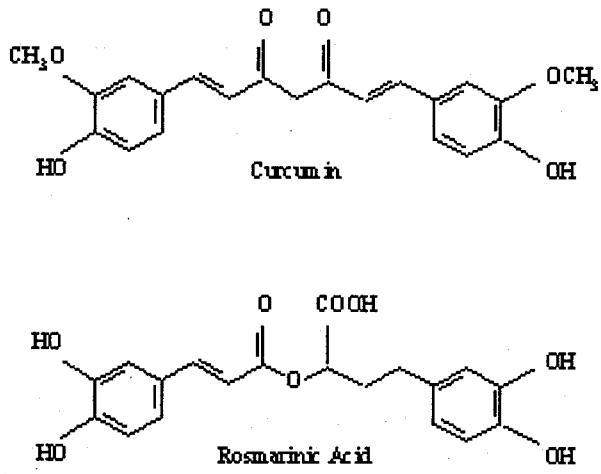
Glucose-6-phosphate dehydrogenase catalyses the first committed and rate limiting step of the pentose phosphate pathway[56,57]. This enzyme is strongly inhibited by NADPH and is known to be affected by NADPH/NADP ratio, pH, Mg^{+2} and substrate concentration in the chloroplast[56,66,67]. An increased requirement for NADPH during anabolic reactions would result in lowering of NADPH/NADP ratio that would deregulate glucose-6-phosphate dehydrogenase and increase metabolic flux through the pentose phosphate pathway (e.g. erythrose-4-phosphate for shikimate pathway)[56]. The opposite would be true when NADPH/NADP ratio is high and metabolic flux to anabolic reactions would be reduced by inhibition of glucose-6-phosphate dehydrogenase.

In plants, glucose-6-phosphate dehydrogenase is known to exist both in the cytosol and plastids[57]. To avoid futile cycles with photosynthetic carbon fixation, the pentose phosphate pathway in the chloroplast is known to operate at night[57]. The cytosolic isoform is regulated by metabolites and the chloroplastic isoform is known to be regulated by covalent redox modification in light[57,68,69]. A confirmation of these different modes of regulation is made difficult due to purification problems associated with the chloroplastic form[70] but not the cytosolic form[69]. Recent success in isolating cDNA clones for both forms, cytosolic[71] and plastidic[57] could open the door to more detailed study about the mode of regulation of the two isoforms.

Unlike animal systems, no clear evidence exists to show that the pentose phosphate pathway is activated and regulated by depletion of NADPH required for proline synthesis (Figure 1). The creation of such a proline-linked redox cycle is well established in animal models[52,72,73] and some indirect evidence exists in plants[54]. The stimulation of a proline-linked redox cycle in animal systems not only drives the pentose phosphate pathway but in addition proline may serve as a reductant replacing NADH as the hydrogen donor for oxidative phosphorylation in the mitochondria[52,72]. Empirical indicators

that lead to the hypothesis and model outlined in this paper suggest that a proline-linked pentose phosphate pathway through depletion of NADPH for proline synthesis, may help drive metabolic flux towards the shikimate pathway and RA biosynthesis in response to *Pseudomonas* sp. These empirical evidences are outlined in the section on Preliminary Studies (below). Molecular investigation of this unique proline link to pentose phosphate pathway in relation to pharmaceutically important phenolic metabolites like RA and curcumin (Figure 3) are the long term goals based on the research directions described in this paper. This unique link will be targeted for detailed pathway analysis and metabolic engineering following the selection and access to elite clones, preliminary pathway analysis and optimization of gene transfer techniques.

Figure 3. Structure of (a) curcumin (b) rosmarinic acid.



Preliminary studies

(1) Tissue culture-based clonal propagation of species in Lamiaceae

As stated previously, species in the family Lamiaceae are open-pollinated and therefore genetically highly heterogeneous[5]. Therefore, every plant from a bulk seed population should have variability in the mode of regulation of RA biosynthesis. Therefore, genetically uniform clonal lines originating from a single heterozygous seed are critical for studying RA biosynthesis and its eventual engineering for overproduction. Genetically uniform clonal lines were generated via *in vitro* tissue culture techniques. This was initially attempted in oregano (*Origanum vulgare*). Multiple shoots from an apex explant were induced in benzylaminopurine-containing growth medium . This mode of shoot organogenesis is without an intervening callus phase and was induced through axillary bud proliferation. The rationale for the avoidance of callus phase was to avoid the genetic instability associated with undifferentiated callus tissue[40]. Using benzylaminopurine-induced axillary bud proliferation, multiple clonal shoots were generated from single seedlings. Axillary bud proliferation results in two shoots along each node in the growing shoot axis. About 10-12 shoots were induced per apex explant. These clonal shoots were multiplied further or regenerated to whole plants to assemble a clonal line originating from a single seed[74]. Such shoot-based clonal lines will be used for investigating the regulation of RA biosynthesis.

An accidental seed-borne contaminant in clonal line 0-4 has led to novel methods to select high and low RA clonal lines. The contaminant, identified as *Pseudomonas* sp.[74] induced higher levels of chlorophyll, phenolics and reduced water content in several clonal lines tested[74]. These *Pseudomonas* sp-induced physiological modifications helped oregano clones to adapt to outside environmental conditions without as much acclimation as normally required for *in vitro* tissue culture generated clonal plants. Other studies also confirmed that only polysaccharide-producing *Pseudomonas* strains induced the increased phenolics and reduced hyperhydration effects[75]. Furthermore, the polysaccharide was purified, partially characterized, and treatment of shoot clones resulted in enhanced acclimation of plants to outside environmental conditions without increased total phenolics[76]. Since polysaccharide-treated shoots were rigid, it was theorized that phenolic flux ended up as lignin[76].

(2) Selection of high RA clonal lines using Pseudomonas sp.

Pseudomonas sp-induced enhancement of acclimation of tissue cultured plants resulted in stimulation of phenolic flux[74]. Since each clonal line was isolated from a heterogeneous background it is likely that different responses to *Pseudomonas* sp.should exist among different clonal lines. This should allow selection of clonal lines with diverse phenolics synthesizing abilities which may be directly related to their resistance to *Pseudomonas* sp. Other studies have shown that the ability to produce secondary metabolites in response to microbial interactions is correlated to increased resistance and survival of plants[77,78]. It has also been shown that RA synthesis can be stimulated by microbial elicitors like yeast extract[10,47] and that RA has antibacterial activity[9]. Based on these observations, polysaccharide-producing *Pseudomonas* sp. strain F was used to isolate high RA-producing clonal lines of oregano (*Origanum vulgare*)[42], rosemary (*Rosmarinus officinalis*)[43] and thyme (*Thymus vulgaris*)[41,79].

(3) Selection of high RA-producing clonal lines of oregano[42]

Using shoot-based clonal lines generated by axillary bud proliferation, resistance to *Pseudomonas* sp. and RA levels were measured (Table 4). It was clear that clonal lines tolerant to *Pseudomonas* sp. also had enhanced stimulation of RA synthesis. From this study 0-4 and 0M-1 were grouped as responsive clonal lines. Clonal lines 0M-8 and 0-5 were inhibited clonal lines. Clonal lines 0-4 and 0-5 are being used for further studies to determine if a proline-linked pentose phosphate pathway is associated with enhanced *Pseudomonas* tolerance and RA biosynthesis.

Table 4. *Pseudomonas* tolerance and RA synthesis in oregano clonal lines.

Clonal lines	*Pseudomonas* tolerance	RA (mg/g FW)	± SD
0-1 Control	Moderate (++)	1.6	(0.7)
0-1 Inoculated		3.3	(0.2)
0-4 Control	Moderate (+++)	1.6	(0.8)
0-4 Inoculated		4.2	(0.2)
0M-1 Control	High (++++)	2.8	(0.3)
0M-1 Inoculated		5.7	(0.8)
0M-8 Control	Poor (+)	2.9	(0.4)
0M-8 Inoculated		3.1	(0.3)
0-5 Control	Poor (+)	1.8	(0.8)
0-5 Inoculated		2.7	(0.9)

*Note: RA was measured 30 days after inoculation. Modified from ref 42. (Control refers to uninoculated shoots for each clonal line).

(4) Selection of high total phenolics and thymol-producing clonal lines of thyme[41]

Since oregano is closely related to thyme it is possible that the *Pseudomonas* strain F could be used to isolate high and low

phenolics clonal lines of thyme. This study analyzed 10 clonal lines of thyme (Table 5; includes 5 examples). These clonal lines were generated via benzylaminopurine-induced multiple shoot organogenesis through adventitious bud proliferation (multiple buds from a single node). This resulted in isolation of high (T-12), medium (T-16G) and low phenolic clonal lines for further investigations. The high phenolic clonal line also had high thymol content.

(5) Rosmarinic acid synthesis in high and low phenolics-producing thyme clonal lines in response to Pseudomonas sp.[79]

Biosynthesis of RA in response to mucoid, partially mucoid and non-mucoid *Pseudomonas* sp. was investigated in high (T-12), medium (T-16G) and low (M-3) phenolics-producing clonal lines. This study provided insights, that in addition to RA stimulation, that the metabolic flux to lignin could be critical for thyme tolerance to *Pseudomonas* sp. The high RA-producing clonal line T-12 was highly tolerant to *Pseudomonas* sp. and mechanically rigid (indicating the possibility of higher lignification). This clonal line consistently produced high RA over a 30 day growth period in response to *Pseudomonas* sp.

This investigation also led to a thyme clonal line T-16G-non mucoid *Pseudomonas* sp. strain NMA combination that may favor metabolic flux to RA rather than lignin. This clonal line, T-16G was moderately tolerant to mucoid *Pseudomonas* sp. strain M4 and appeared partially rigid and inhibited.

Table 5. Total phenolic and thymol content of clonal lines of thyme and corresponding *Pseudomonas* tolerance.

Clonal Line	Total Phenolics (mg/g FW) ± SD	Pseudomonas Tolerance	Thymol (µg/g FW) ± SD
M-3 (control)	1.5 (0.2)	+	75 (10)
M-3 (inoculated)	2.0 (0.5)		
T-35 (control)	1.6 (0.4)	+	55 (10)
T-35 (inoculated)	2.2 (0.8)		
M-4 (control)	2.2 (0.2	+	10 (5)
M-4 (inoculated)	2.2 (0.4)		
KM-40 (control)	1.3 (0.7)	++	125 (20)
KM-40 (inoculated)	3.2 (0.5)		
T-16G (control)	2.3 (0.2)	+++	150 (15)
T-16G (inoculated)	3.7 (0.6)		
T-12 (control)	2.7 (1.0)	++++	155 (15)
T-12 (inoculated)	4.8 (0.3)		

*Note: Phenolics were measured 25 days after inoculation and thymol was only measured in control after 60 days. Modified from ref 41.

(6) Selection of high RA-producing clonal lines of rosemary using Pseudomonas sp.[43]

Using the *Pseudomonas* sp.isolated from oregano, high RA-producing clonal lines of rosemary were isolated. Unlike oregano, multiple shoot organogenesis was induced via adventitious bud proliferation (multiple shoots from the same node). About 3-10 shoot were induced/explant depending on the clonal line. In this study 5 classes of clonal lines with different abilities to produce RA were isolated . Clonal line R-1 was clearly low RA-producing and was inhibited by *Pseudomonas* inoculation[43]. Clonal line R-7, consistently produced high RA in response to *Pseudomonas* sp. and RA production could be sustained over an extended 60 day growth period. Similarly, clonal lines R-16 and R-35 produced high RA, but unlike R-7 mechanical rigidity was reduced which may indicate reduced lignification. Clonal line R-15 was the most interesting clonal line in that it responds to *Pseudomonas* sp. by producing high RA on day 25 but does not survive over a 60 day growth period due to excess phenolics. This could be an interesting clonal line to study what role glutathione-S-transferase has in controlling RA synthesis and *Pseudomonas* tolerance.

(7) Role of proline metabolism in regulating RA biosynthesis

Rationale: Proline biosynthesis is known to be activated during drought and frost tolerance[80,81]. It is also known that glucose-6-phosphate dehydrogenase[82], DAHP synthase[83,84] and phenylalanine ammonia-lyase[78,85] are activated during microbial interaction. Utilization of NADPH for proline synthesis during microbial interaction may reduce NADPH/$NADP^+$ ratio which should activate glucose-6-phosphate dehydrogenase[56,67]. Therefore, deregulation of pentose phosphate pathway by microbial-induced proline synthesis may provide the excess erythrose-4-phosphate for shikimate and therefore the phenylpropanoid pathway. At the same time proline could serve as a reductant (instead of NADH) for oxidative phosphorylation (ATP synthesis) in the mitochondria[52,73].

Table 6: Rosmarinic acid and proline levels of oregano clonal lines 0-4 and 0-5 in response to *Pseudomonas* sp

Clonal Lines	RA(mg/g FW) ± SD	Proline ± SD (µmol/g FW)
0-4 Control 30d	1.7 (0.1)	21.25 (7.3)
0-4 Inoculated 30d	3.9 (0.5)	36.37 (6.6)
0-5 Control 30d	2.8 (1.3)	14.82 (4.7)
0-5 Inoculated 30d	2.2 (0.7)	14.93 (9.2)
0-4 Control 41d	3.3 (0.6)	9.34 (0.8)
0-4 Inoculated 41d	7.1 (1.4)	26.64 (4.2)

*d-denotes days after inoculation.

Preliminary results[55] indicate that *Pseudomonas*-induced stimulation of RA biosynthesis is positively correlated with proline synthesis in *Pseudomonas* tolerant clonal line 0-4. This correlation is absent in *Pseudomonas*-inhibited 0-5 clonal line (Table 6).

(8) Polymerase chain reaction for identification of clonal lines

Polymerase chain reaction (PCR) was used to identify various clonal lines of oregano[86]. Two methods were used in our preliminary studies. In the first method a pair of consensus tRNA gene primers facing outward from tRNA genes were used to amplify DNA[87]. The PCR fingerprints developed from these primers are mainly derived from regions between closely linked tRNA. Genomic fingerprints for tRNA are largely conserved within species and any small variations should help classification of clonal lines in highly heterogeneous, cross-pollinating family like Lamiaceae. The two primers used were:

P#1: 5'AGTCCGTGCTCTAACCAAC 3';

P#2:5'GGGGGTTCGAATTCCCGCCGGC 3'.

PCR amplification was done in total volume of 25 µl using standard reagents[87]. The amplification conditions were: 94°C for 1 min to denature, 50°C for 1 min for annealing of primer and 72°C for 2 min for primer extension. The PCR amplified products were separated on agarose gel and photographed by Poloroid camera by placing the gel on a UV transilluminator. Use of above tRNA primers allowed clear separation of all clonal lines of oregano reported in this paper[86].

A second method used Operon 10-mer kits (Operon Tech; CA) containing 10-base oligonucleotide primers used in genetic mapping[88]. The arbitrary primers have been used

widely for genome mapping in plants, microorganisms and animals. A 10-mer (OP10-10) primer with the sequence: 5' GGTCTACACC 3' allowed separation of 0-series oregano clonal lines from Kentucky gene pool vs M-series from Connecticut. Identification of clonal lines is now being attempted using a combination of arbitrary primers (Operon Tech, CA) with known consensus primers for the sequenced genes of key enzymes targeted in this proposal. This would allow separation of clonal lines based on specific variations in genes of interest for metabolic engineering. This will also provide the foundation to develop methods like RT-PCR to isolate cDNA of specific key enzymes from high RA and low RA-stimulated clonal lines.

(9) Genetic transformation using Agrobacterium

Preliminary studies indicate that species in the family Lamiaceae were susceptible to *Agrobacterium*. *Agrobacterium rhizogenes*-induced root and callus cultures have been developed from several clonal lines of rosemary, oregano and thyme. Some clonal lines in all the species were not susceptible. Such non-susceptible clonal lines were made susceptible when proline was added to the medium during the co-culture. This increased susceptibility may be linked to stimulation of total phenolics. Phenolics such as acetosyringone are essential for *Agrobacterium vir* gene expression, which in turn activates the transfer of T-DNA into the plant genome[89-91]. Currently the susceptibility of various oregano clonal lines to wild strains of *Agrobacterium tumefacians* is being probed. This will lay the foundation for transfer of marker genes coding for antibiotic resistance and β-glucuronidase. The strategies to improve *Agrobacterium*-mediated transformation were based on previous studies in melon[92]. These strategies were:

(i) improvement of multiple shoot organogenesis via adventitious buds, which allows maximum multiple shoot forming zone to be exposed to *Agrobacterium*; (ii) modulation of potential host factors like proline and phenolic metabolites during co-culture of explant and *Agrobacterium*; (iii) transformation at undifferentiated callus stage with subsequent regeneration of whole plants. In addition, the potential use of particle gun bombardment for genetic transformation (after development of efficient regeneration system) will be probed.

Some of the strategies that were successful in melon[92] were used to develop *Agrobacterium tumefacians*-mediated transformation in *Pimpinella anisum* (anise-family: Umbelliferae)[86]. Prior to this success it was extremely difficult to genetically transform anise using *Agrobacter-ium*. The transformation efficiency for anise in several experiments were in the range of 10 to 20%[86]. The keys to success were: (i) initial dedifferentiation of root explants to produce callus tissue using napthalene acetic acid; (ii) regeneration of undifferentiated callus to form maximum number of shoot using benzyladenine (25-30 shoots/callus explant); (iii) use of proline during the co-culture of callus and *Agrobacterium* increased transformation efficiency; (iv) the potential problem of genetic instability through prolonged subculture of callus culture was overcome by clonally multiplying transformed shoots via direct shoot organogenesis without an intermediate callus phase like in melon[93] and oregano[74]. Several transformed plants carrying markers for antibiotics (kanamycin and hygromycin) and β-glucouronidase (GUS-stains blue when aglycone is released) have been isolated[86].

Since callus-based plant regeneration is feasible in oregano[94], the strategy developed for anise could be adopted for all key RA-producing clonal lines in all species mentioned in this paper. A successful genetic trans-formation strategy will be critical for subsequent metabolic engineering after key biochemical links and genes for RA biosynthesis are characterized.

Future goals

Future goals will involve purification and characterization of key enzymes and isolation of corresponding cDNA. Based on preliminary results there is some empirical evidence for the link between proline biosynthesis, oxidation and stimulation of glucose-6-phosphate dehydrogenase. Following the studies envisioned in this paper, it would be only logical to initially target complete characterization of glucose-6-phosphate dehydrogenase and proline dehydrogenase (proline oxidation) at the enzyme and gene level. Some work in this direction is planned for the immediate future. This will evolve from the work on polymerase chain reaction for clonal identification and on enzyme characterization. Purification of cytosolic glucose-6-phosphate dehydrogenase and isolation of corresponding cDNA will be pursued based on methods for potato[57,71]. Mitochondrial membrane associated proline dehydrogenase will also be the target for purification based on Rayapati and Stewart[95] and isolation of cDNA will be pursued based on recent success in Arabidopsis[96]. This would pave the way for: (i) characterization of promoters through isolation of genomic clones using cDNA probes; (ii) promoter-GUS fusions and analysis of expression in transgenic model plants like tobacco; (iii) overexpression and antisense technology for metabolic engineering of RA biosynthesis.

Conclusions

The above research goals have substantial implications for harnessing the genetic and biochemical potential of genetically heterogeneous, food-related medicinal plant species. Metabolic engineering to improve elite clonal lines can be subsequently targeted using modern biotechnology. Elite transgenic clonal lines can then be incorporated into commercial production systems linked to current agri-cultural/ horticultural practices. This ensures global access of important plant-based metabolites for food and pharmaceutical applications. The success of this project will result in techniques to generate metabolically diverse genetic stocks of several clonal lines that are amenable to gene transfer techniques. This research will also help to gain preliminary insights into how primary metabolism (proline-linked pentose phosphate pathway) may influence RA biosynthesis. This should pave the way for more detailed analysis of key enzymes and genes for subsequent metabolic engineering. The success of this research also provides novel methods and strategies to gain access to metabolic pathways of food-related, pharmaceutically important metabolites like curcumin, salvianolic acid, gingernone, capsaicin, salicylic acid or any newly discovered phenolic metabolites.

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Biotechnology to harness the benefits of dietary phenolics; focus on *Lamiaceae*

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利用生物工程技術來加强食物類酚化物的效益; 重點研究唇科類植物

摘要

來源草藥和發酵豆類的植物化學物質是最佳的食源性酚類代謝物. 酚類化合物的重要性不僅在于對食品的防腐性能, 它在藥物治療方面的應用也日漸增加.

馬薩諸塞大學食品生物工程的長遠研究課題的重點是探討食物性植物和植物發酵食品中對醫藥和健康有益的酚化物及其在生物合成中的分子學和生理學機制. 目前的主攻方向是闡明連接脯氨酸的磷酸戊糖途徑在調節抗炎物, 迷迭香酸〔RA〕合成中的作用: 〔I〕建立全新的組織培養技術從唇科類交叉授粉分離出迷迭香酸〔RA〕生產量高的异源性克隆芽株; 〔II〕集中在具有同一的遺傳性并且能重復生產的克隆芽株: (a)首先應有〔RA〕合成相關的磷酸戊糖途徑的關鍵酶; (b)其次是建立能利用遺傳轉化技術來生產與〔RA〕合成相關的代謝途徑.

上述研究課題對促進食物有關的异源遺傳性藥性植物的遺傳和生物化學性能具有實際的利用價值. 這一研究的成功還將會爲我們提供嶄新的方法和策略去探討生姜, 黃瓜, 辣椒, 瓜類或其它含新的酚類化合物食物調料中具有的重要藥物代謝物的代謝途徑.

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