Review Article

A model for the role of the proline-linked pentose-phosphate pathway in phenolic phytochemical bio-synthesis and mechanism of action for human health and environmental applications*

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**This manuscript is dedicated to Huang Soo Sien for her support, friendship, dignity and wisdom.*

The combination of immunodeficiency, inflammatory process and nutritional status that is characteristic of infective and food-borne illness is more evident in chronic diet- and environment-influenced chronic diseases such as diabetes, obesity, cardiovascular disease, cancer, arthritis and neuro-degeneration diseases. These chronic diseases tend to be oxidation-linked and may manifest in communities around the world, irrespective of income. In addressing the challenges of the above diseases, a significant role for dietary phytochemicals is emerging. Phytochemicals are required from a spectrum of food for at least their antioxidant role, if not for other properties, to protect tissues from activities that manifest themselves into what we call chronic disease. Among the diverse groups of phytochemicals, phenolic antioxidants and antimicrobials from food plants are being targeted for designed dietary intervention to manage major oxidation-linked diseases such as diabetes, cardiovascular diseases, arthritis, cognition diseases and cancer. Foods containing phenolic phytochemicals are also being targeted to manage bacterial infections associated with chronic diseases such as peptic ulcer, urinary tract infections, dental caries and food-borne bacterial infections. Plants produce phenolic metabolites as a part of growth, developmental and stress adaptation response. These stress and developmental responses are being harnessed to design consistent phytochemical profiles for safety and clinical relevancy using novel tissue culture and bioprocessing technologies. The biochemical strategy for harnessing phenolic phytochemicals for human health and wellness is based on the hypothesis that phenolic metabolites in plants are efficiently produced through an alternative mode of metabolism linking proline synthesis with pentose-phosphate pathway. In this model, stress-induced proline biosynthesis is coupled to pentose-phosphate pathway, driving the synthesis of $NADPH_2$ and sugar phosphates for anabolic pathways, including phenolic and antioxidant response pathways, while simultaneously providing reducing equivalents needed for mitochondrial oxidative phosphorylation in the form of proline as an alternative to NADH from Krebs/TCA cycle. Based on this model, tissue culture techniques and elicitation concepts have been used to stimulate phenolic metabolites with an antioxidant response in germinating seeds, sprouts and clonal lines of dietary plants. From our initial investigations, a model has been proposed in which the proline-linked pentose-phosphate pathway is suggested to be critical for modulating protective antioxidant response pathways in diverse biological systems, including biochemical and cellular pathways important for human health. The proposed proline-linked pentose-phosphate pathway model provides a mechanism for understanding the mode of action of phenolic phytochemicals in modulating antioxidant pathways and provides avenues by which dietary approaches may manage oxidation-linked chronic and infectious diseases. The model also has implications for the development of antimicrobial phenolic phytochemicals against bacterial pathogens in an era of increasing antibiotic resistance. Further, this model also has relevance for improving fungal and yeast-based food bioprocessing for designing functional foods and for environmental bioremediation using plant and microbial systems, as well as for improving agricultural and food systems in harsh environments.

Key Words: antioxidant response, antimicrobials, chronic disease, environmental applications, human health, oxidation-linked disease, oxidative phosphorylation, phenolic antioxidants, phytochemicals, proline-linked pentose phosphate pathway.

Introduction to food and nutrition issues and relevance of phenolic antioxidants

Increasing efforts are being made world wide to address both persistent nutritional deprivation in economically disadvantaged communities and the increase of chronic oxidation-linked diseases (i.e., abdominal obesity, diabetes, cardiovascular disease (CVD), certain cancers, osteoporosis, arthritis and inflammatory diseases) in all communities that have reached caloric and protein sufficiency. The combination of immunodeficiency, inflammatory process and nutritional status that is characteristic of infective and food-borne illness is more evident in chronic diet- and environment-influenced diseases. Therefore such diseases

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in fact may be considered as 'eco-diseases' with environmental and behavioural contributors such as physical inactivity as opposed to nutritionally-dependent 'econutritional diseases'.[1] These chronic diseases are oxidation-linked and manifest in communities around the world, irrespective of income or age. In addressing the above challenges, a significant role for phytochemicals in the diet is emerging. Dietary phytochemicals are required for, at least, their antioxidant role, if not other properties to protect tissues from chronic disease.[2]

Food plants are excellent sources of phenolic phytochemicals, especially as antioxidants. While phenolic antioxidants from dietary sources have a history of use in food preservation, many increasingly have therapeutic and disease prevention applications.[3-6] Therefore, understanding the nutritional and therapeutic role of dietary phytochemicals and particularly phenolic antioxidants is an important scientific agenda for Food Science and Nutrition, now and well into the foreseeable future.[7] This role is becoming very significant at a time when the importance of phytochemicals in the prevention of oxidation-linked chronic diseases is gaining rapid recognition globally. Disease prevention and management through the diet can be considered an effective tool to improve health and/or reduce the increasing health-care costs for these oxidation-linked chronic diseases, especially in low-income countries.

Phenolic phytochemicals have been associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals.[8-11] Recent studies have indicated a role for phenolics from food plants in human health and, in particular cancer.[10,12] Phenolic phytochemicals (i.e phenylpropanoids) serve as effective antioxidants due to their ability to donate hydrogens from hydroxyl groups positioned along the aromatic ring to terminate free radical oxidation of lipids and other biomolecules.[13] Phenolic antioxidants, therefore, short-circuit a destructive chain reaction that ultimately degrades cellular membranes.

Plant phenolics are an important sub-group of secondary metabolites, which have diverse functional and medicinal applications. Examples of phenolics that are used as antioxidant and anti-inflammatory compounds are curcumin from *Curcuma longa,*[14-16] *Curcuma mannga,*[17] and *Zingiber cassumunar,*[18] and rosmarinic acid from *Rosmarinus officinalis.*[6,19] Examples of phenolics with cancer chemopreventive potential are curcumin from *Curcuma longa*1,[4,20-23] isoflavonoids from *Glycine max*[24-26] and galanigin from *Origanum vulgare.*[27] Other examples of plant phenolics with medicinal uses include lithospermic acid from *Lithospermum* sp. as anti-gonodotropic agent,[28] salvianolic acid from *Salvia miltiorrhiza* as an antiulcer agent,[29] proanthocyanidins from cranberry to combat urinary tract infections,[30,31] thymol from *Thymus vulgaris* for anti-caries,[32] and anethole from *Pimpinella anisum* as an antifungal agent.[33]

Important plant diphenyl metabolites targeted for enhanced production against oxidation-linked disease are rosmarinic acid, resveratrol, ellagic acid and curcumin. Other phenolic phytochemicals also targeted are flavonoids, quercetin, myrcetin, scopoletin and isoflavonoids. Among simple phenolics, there is major interest in the over-expression of L-tyrosine and L-DOPA from legumes in a high-phenolic antioxidant background.[34,35] Rosmarinic acid has been targeted from clonal herbs[3] for its anti-inflammatory and antioxidant properties.[19,36,37] Resveratrol has shown antioxidant and cancer chemopreventive properties[38,39] and its over-production has been targeted from several fruits using solid-state bioprocessing.[40,41] Ellagic acid has been targeted for antioxidant and cancer chemopreventive properties[42,43] and has been similarly targeted via solid-state bioprocessing from fruits and fruit processing byproducts.[40] As extensive studies have shown cancer chemopreventive and antioxidant properties for *Curcuma longa* and its major active compound, curcumin,[15,20,44] and its elicitor and physical stress-mediated over expression of curcumin is being investigated.

The emergence of dietary and medicinal applications for phenolic phytochemicals, harnessing their antioxidant and antimicrobial properties, in human health and wellness is not altogether surprising. As stress damage on the cellular level appears similar among eukrayotes, it is logical to suspect that there may be similarities in the mechanism for cellular stress mediation between eukaryotic species. Plant adaptation to biotic and abiotic stress involves the stimulation of protective secondary metabolite pathways[45-47] that results in the biosynthesis of phenolic antioxidants. Studies indicate that plants exposed to ozone responded with increased transcript levels of enzymes in the phenylpropanoid and lignin pathways.[48] Increase in plant thermo-tolerance is related to the accumulation of phenolic metabolites and heat shock proteins that act as chaperones during hyperthermia.[49] Phenolics and specific phenolic-like salicyclic acid levels increase in response to infection, acting as defence compounds or to serve as precursors for the synthesis of lignin, suberin and other polyphenolic barriers.[50] Antimicrobial phenolics called phytoalexins are synthesized around the site of infection during pathogen attack and, along with other simple phenolic metabolites, are believed to be part of a signalling process that results in systemic acquired resistance.[45-47] Many phenylpropanoid compounds such as flavonoids, isoflavonoids, anthocyanins and polyphenols are induced in response to wounding,[51] nutritional stress,[52] cold stress[53] and high-visible light.[54] UV irradiation induces light-absorbing flavonoids and sinapate esters in *Arabidopsis* to block radiation and protect DNA from dimerization and/or cleavage.[55] In general, the initiation of the stress response arises from certain changes in the intracellular medium[56] that transmit the stress-induced signal to cellular modulating systems and results in changes in cytosolic calcium levels, proton potential as a long-distance signal,[57] and low-molecular weight proteins.[58] Stress can also initiate free radical generating processes and shift the cellular equilibrium towards lipid peroxidation.[59] It is believed that the shift in prooxidant-antioxidant equilibrium is a primary non-specific event in the development of the general stress response.[60] Therefore, protective phenolic antioxidants involved in such secondary metabolite-linked stress responses in food plant species can be targeted as a source of therapeutic and disease-preventing functional ingredients. This is especially applicable to: 1) oxidation

disease-linked diet problems (high glycaemic index and saturated fats) and 2) environment-influenced (physical, chemical and biological) chronic disease problems.

Role of proline in plants

Proline biosynthesis is a common stress response in plants, especially to water and salinity stress.[61-65] This amino acid accumulates in saline-tolerant halophilic plants,[66] under water stress,[67] in dessicating pollen,[68] plant cell culture under water stress,[69] salt stress,[70,71] during senescence,[72] in response to abscisic acid,[73] proline analogue,[74] dehydration,[75] freezing tolerance,[76] general osmotic tolerance over-expressing proline synthesis pathway[77] and frost tolerance.[78] Proline is suggested to protect membranes and proteins from the various imposed stress conditions mentioned above.[79,80] Proline may also act as an antioxidant through hydroxyl radical scavenging activity.[81] In microorganisms, especially bacteria, proline over-expression can increase osmo-tolerance.[82-84]

Proline is also suggested to have roles in plants in addition to stress tolerance. Proline synthesis is known to be stimulated during senescence.[72] Further, it has been implicated in energy transfer and regulation of purine synthesis in nitrogen fixing root nodules in soybean.[85,86] It has been linked to development-linked rhizogenesis associated with drought[87] and thermogenic response in inflorescence of Voodoo lily.[88] It has also been associated with non-stress related developmental conditions. In the fava bean, the ratio of free and bound proline content was very high during early pod development and decreased during late stages at maturity.[89] The very high proline levels were not a consequence of water stress. It was suggested that free proline has a characteristic function in early development.[89] Previous studies in soybean[90] and peanut[91] have indicated that accumulation of proline was not associated with water stress. A function of proline in formation of flowers has also been suggested.[92]

Proline in plant tissue culture

Proline alone or in combination with other amino acids is known to stimulate somatic embryogenesis in plants. Proline in combination with serine, stimulated somatic embryogenesis in orchard grass.[93] Proline alone stimulated *in vitro* somatic embryogenesis in maize[94] and carrot.[95] Proline and glutamine stimulated induction of embryogenic callus in a grass species *Agrostis alba.*[96] Further, the proline analogue, thioproline was used to screen highly embryogenic cell lines from single seed origin in a heterogeneous genetic background of *Agrostis alba.*[97] Proline has been used to stimulate cytokinin-induced *in vitro* shoot organogenesis in melon[98] and cucumber,[99] as well as auxin-induced somatic embryogenesis on alfalfa.[100] Proline, proline analogue and proline precursor, ornithine were effectively used to stimulate cytokinin-induced shoot organogenesis in melon.[101] *In vitro* shoot organogenesis was also stimulated using proline and glutamic acid-enriched fish protein hydrolysates in combination with proline analogues.[102] It was clear from the melon study that the extent of shoot organogenesis was correlated to increased proline content in such differentiated tissues.[101,102]

An interesting alternative role for proline in stress-induced phenolic synthesis has been proposed.[3] This concept is based on the original model on the role of proline and pyrroline-5-carboxylate (P5C) in regulating redox and hydride ion-mediated stimulation of pentose-phosphate pathway, which in turn modulates purine metabolism in animal cells.[103] In this model,[103] stimulation of proline synthesis regulates the ratios of $NADP^+$/ $NADPH_2$ and sugar phosphates (that are required for purine metabolism) through the stimulation of the $NADPH_2$-producing committed steps of the pentose-phosphate pathway. Further, it was proposed that rapid catabolism of proline during recovery from stress may provide redox equivalents for mitochondrial oxidative phosphorylation and therefore it is an alternative route to ATP synthesis.[3] Extending from the model proposed in animal systems by Phang[103] the role of proline-linked pentose phosphate pathway in stimulating phenolic metabolites in plants was proposed by Shetty[3] (Fig. 1).

Proline-linked phenolic synthesis in herb clonal shoot culture systems

The hypothesis that synthesis of plant phenolic metabolites is linked to the proline-linked pentose-phosphate pathway[3] (Fig. 1) was developed based on the role of the proline-linked pentose-phosphate pathway in regulation of purine metabolism in mammalian systems.[103] Proline is synthesized by a series of reduction reactions from glutamate. In this sequence, P5C and proline, known to be metabolic regulators, function as a redox couple.[103,104] During respiration, oxidation reactions produce hydride ions, which augment reduction of P5C to proline in the cytosol. Proline can then enter mitochondria through proline dehydrogenase[105] and support oxidative phosphorylation (alternative to NADH from Krebs/TCA cycle). This is important because shunting the TCA cycle towards proline synthesis likely deregulates normal NADH synthesis. The reduction of P5C in the cytosol provides NADP+, which is the co-factor for glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that catalyses the rate-limiting step of the pentose-phosphate pathway. Proline synthesis is therefore hypothesized and has been partly shown to both regulate and stimulate pentose-phosphate pathway activity in erythrocytes[106] and cultured fibroblasts[107] when P5C is converted to proline. This was shown to stimulate purine metabolism via ribose-5-phosphate, which affects cellular physiology and therefore function.[103,108]

From the above insights Shetty[3] proposed a model that proline-linked pentose-phosphate pathway could stimulate shikimate and phenylpropanoid pathways and hypothesized that stress-linked modulation of this pathway lead to the stimulation of phenolic phytochemicals[3] (Fig. 1). Using this model, proline, proline precursors, and proline analogues were effectively utilized to stimulate total phenolic content and a specific phenolic metabolite, rosmarinic acid.[109,110] Further, it was shown that proline, proline precursors, and proline analogues stimulated somatic embryogenesis in anise,

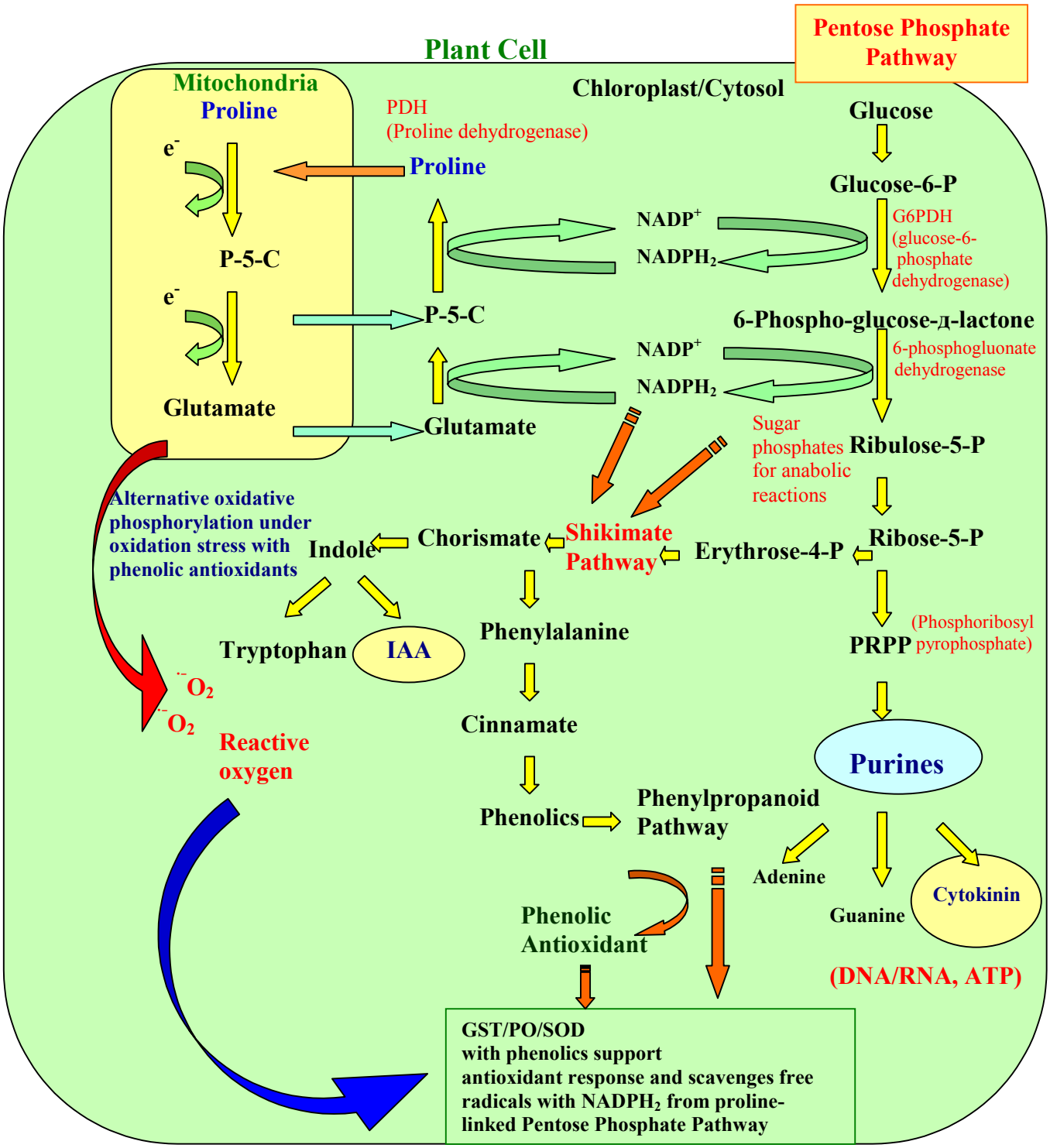

Figure 1. Original proposed model (Shetty, 1997) for the role of proline-linked pentose phosphate pathway in regulating phenolic biosynthesis. (Abbreviations: P5C;pyrroline-5-carboxylate, IAA; indole acetic acid, GST; Glutathione-s-transferase, PO;peroxidase, SOD; superoxide dismutase).

which correlated with increased phenolic content.[111] It was also established that during *Pseudomonas*-mediated stimulation of total phenolic and rosmarinic acid (RA), proline content was stimulated in oregano clonal shoot cultures.[112] Therefore, it was proposed that $NADPH_2$ demand for proline synthesis during response to microbial interaction and proline analogue treatment[3] may reduce the cytosolic $NADPH_2/NADP^+$ ratio, which should activate G6PDH.[113,114] Therefore, deregulation of the pentose-phosphate pathway by proline analogue- and microbial-induced proline synthesis may provide the excess erythrose-4-phosphate (E4P) for shikimate and, therefore, the phenylpropanoid pathways. At the same time, proline and P5C could serve as superior reducing equivalents (RE), alternative to NADH (from Krebs/TCA cycle) to support increased oxidative phosphorylation (ATP synthesis) in the mitochondria during the stress response.[103,104]

The proline analogue, azetidine-2-carboxylate (A2C), is an inhibitor of proline dehydrogenase.[74] It is also known to inhibit differentiation in Leydig cells of rat fetal testis, which can be overcome by exogenous proline addition.[115] Another analogue, hydroxyproline, is a competitive

inhibitor of proline for incorporation into proteins. According to the model of Shetty,[3] either analogue at low levels should deregulate proline synthesis from feedback inhibition and stimulate proline synthesis.[3] This would then allow the proline-linked pentose-phosphate pathway to be activated for $NADPH_2$ synthesis, and concomitantly drive metabolic flux towards E4P for biosynthesis of shikimate and phenylpropanoid meta-bolites, including RA. Proline could also serve as a RE for ATP synthesis via mitochondrial membrane-associated proline dehydrogenase.[105]

High RA-producing, shoot-based clonal lines originating from a single heterozygous seed among a heterogeneous bulk-seed population of lavender, spearmint and thyme have been screened and isolated based on tolerance to the proline analogue, A2C and a novel *Pseudomonas* sp. isolated from oregano.[116-118] This strategy for screening and selection of high RA clonal lines is also based on the model that proline-linked pentose phosphate pathway is critical for driving metabolic flux (i.e E4P) towards shikimate and phenylpropanoid pathways (Fig. 1). Any clonal line with a deregulated proline synthesis pathway should have an over-expressed pentose phosphate pathway which allows excess metabolic flux to drive shikimate and phenylpropanoid pathway towards total phenolic and RA synthesis. Similarly, such proline over-expressing clonal lines should be more tolerant to proline analogue, A2C. If the metabolic flux to RA is over-expressed, it is likely to be stimulated in response to *Pseudomonas* sp. Therefore, such a clonal line is equally likely to be tolerant to *Pseudomonas* sp. Further, such a clonal line should also exhibit high proline oxidation and RA content in response to A2C and *Pseudomonas* sp. In addition, in the presence of A2C or *Pseudomonas* sp., increased activity of key enzymes G6PDH (pentose-phosphate pathway), P5C 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 stimulated. 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,[103] differentiating animal tissues,[115] and plant tissues.[85] The hypothesis of this model is that the same metabolic flux from over expression of proline-linked pentose-phosphate pathway regulates the inter-conversion of ribose-5-phosphate to E4P driving shikimate pathway. Shikimate pathway flux is critical for both auxin and phenylpropanoid biosynthesis, including RA. This hypothesis has been strengthened by preliminary results in which RA biosynthesis in several oregano clonal lines were significantly stimulated by exogenous addition of proline analogue (e.g A2C) and ornithine.[109,110] The same clonal lines are also tolerant to *Pseudomonas* sp. and respond to the bacterium by increasing RA and proline biosynthesis.[112,119] High RA-producing clonal lines selected by approaches based on this model[116-121] are being targeted for preliminary characterization of the key enzymes mentioned above. The success of this strategy will lead to access of critical interlinking metabolic pathways associated with RA biosynthesis and will allow more detailed analyses, which could lead to metabolic engineering for efficient RA biosynthesis. This strategy for investigation and stimulation of RA biosynthesis can be the foundation for metabolic engineering of other dietary phenolic phytochemicals from cross-pollinating, heterogeneous species.[3]

Proline-linked phenolic synthesis in seed sprouts

Preliminary results[3,6,109,110,122] have provided empirical evidence for a link between proline biosynthesis and oxidation, as well as stimulation of G6PDH. In light-mediated sprout studies in pea (*Pisum sativum)*, acetylsalicylic acid in combination with fish protein hydrolysates (a potential source of proline precursors) stimulated phenolic content and guaiacol peroxidase (GPX) activity during early germination with corresponding higher levels of proline and G6PDH activity.[123] In parallel light-mediated studies in pea, low pH and salicylic acid treatments stimulated increased phenolic content and tissue rigidity. Similarly, there was concomitant stimulation of G6PDH and proline.[122] This work supported the hypothesis that pentose-phosphate pathway stimulation may be linked to proline biosynthesis and that modulation of a proton-linked redox cycle may also be operating through proline-linked pentose-phosphate pathway.[122] In dark-germinated studies in pea, high cytokinin-containing anise root extracts stimulated phenolic content and antioxidant activity, which correlated with increased proline content, but inversely with G6PDH activity.[124] In further dark-germination studies in mung bean (*Vigna radiata*), dietary-grade microbial polysaccharide treatments stimulated phenolic content and enzyme activity, G6PDH and GPX compared to controls,[125] with concomitant stimulation of proline content. In addition, specific elicitors xanthan gum, yeast extract and yeast glucan stimulated antioxidant activity. In additional studies, oregano phenolic extracts were used as elicitors to stimulate phenolic content during dark germination of mung bean. Again, increased phenolic content corresponded to an increase in activity of G6PDH and GPX and phenolic-related antioxidant activity were also stimulated.[126] In studies with dark-germinated fava bean, support for the hypothesis that stimulation of proline-linked pentose-phosphate pathway would stimulate phenolic metabolism under elicitor and stress response was probed. In polysaccharide elicitor studies, gellan gum stimulated fava bean total phenolic content by 9-fold in late stages of germination with a corresponding increase in proline content and GPX activity, although the effect on antioxidant and G6PDH activity was incon-clusive.[34] In the same fava bean system, UV-mediated stimulation of phenolic content in dark-germinated fava bean sprouts indicated a positive correlation to G6PDH and GPX activities with a concomitant increase in proline content.[127] It was further confirmed that proline analogue, A2C also stimulated phenolic content in fava bean with positive correlation to G6PDH and GPX activities as well as proline content.[128] Similar to studies in clonal shoot cultures of thyme[109] and oregano,[110] the proline analogue-mediated studies in fava bean confirmed that proline over-expression was not only possible, but involved stimulating G6PDH and therefore

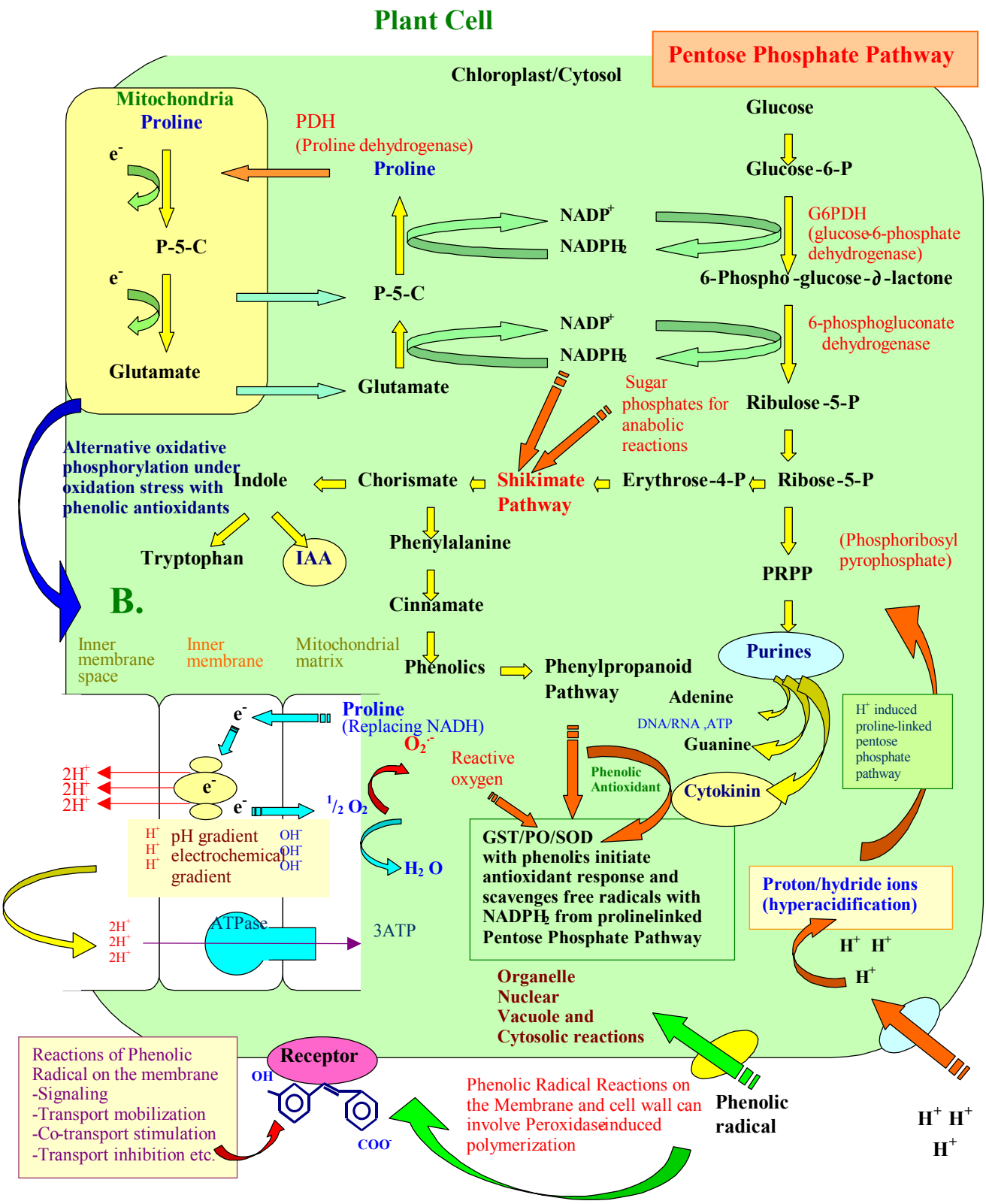

Figure 2. Improved model for the role of proline-linked pentose phosphate pathway in regulating phenolic biosynthesis, which also accommodates the mechanism of action of external phenolic phytochemicals. (Abbreviations: P5C;pyrroline-5-carboxylate, IAA; indole acetic acid, GST; Glutathione-s- transferase, PO; peroxidase, SOD; superoxide dismutase)

likely diverted the pentose-phosphate pathway towards phenylpropanoid biosynthesis. The late stage stimulation of phenolic content and GPX activity in response to microwave-mediated thermal stress in dark-germinated fava bean strongly correlated with stimulation of free radical scavenging activity of free phenolics as measured by the quenching of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay and stimulation of superoxide dismutase (SOD) activity.[129]

Implications of proline-linked pentose-phosphate pathway in plants for functional food development and environmental applications

Investigations in clonal shoot culture studies using several herb species and legume sprout systems has allowed not only the development of strategies for generating consistent phytochemical profiles for functional food and nutritional studies, but also has provided some insights as to how these beneficial phenolic compounds are likely

synthesized in plants. Investigations to date have supported the hypothesis that during development and stress response when phenolic biosynthesis is stimulated, an alternative mode of oxidative phosphorylation linked to proline metabolism may be more efficient and suitable (Fig. 2). In this alternative model, the synthesis of proline is coupled to activity of the pentose-phosphate pathway, whereby diversion of carbon flux from TCA cycle at the level of α-keto glutarate to glutamic acid drives the flux towards proline biosynthesis, which requires $NADPH_2$ and is supplied by the activity of the pentose-phosphate pathway. Any proton-linked redox cycling that may occur as acidification of the cytosol from the introduction of exogenous phytochemicals, microbial elicitors or true acids could also be coupled to proline-linked pentose-phosphate pathway activity and therefore stimulate activity to support co-substrate needs ($NADPH_2$ and sugar phosphates) of anabolic pathways, including antioxidant and defence pathways (Fig 2). In this scheme, proline serves as strong RE (alternative to NADH from Krebs/ TCA cycle) for mitochondrial oxidative phosphorylation. An active ATP-producing metabolic role of proline (beyond its normally suggested role as an osmolyte) has been previously suggested.[3,65,103] Though evidence to support the proline-linked pentose-phosphate pathway hypothesis is still emerging, the general concept from the model has already been exploited for developing phytochemicals for functional (health) food applications, especially in generating dietary phenolic antioxidants and antimicrobials. In the Mint (Lamiaceae) family, which is a source of many valuable phenolic phytochemicals,[3] proline analogue and bacterial perturbation have been used to isolate high phenolic and rosmarinic clonal lines[116-121] that can subsequently be grown as single seed origin clonal phenotypes on a large scale using vegetative propagation. In the legume family, phytochemical elicitors, acid treatment, proline precursors, proline analogues and microbial elicitors have been utilized to stimulate phenolic antioxidant-type phytochemicals during the sprouting stages.[34,35,122-131] In the area of environmental applications microbial interaction, proline precursors and phytochemical elicitation have been utilized to enhance vigour response of germinating seeds[122,123,130-133] and reduce problems associated with vitrification-linked adaptation of plant tissue cultures.[134-137] In the area of phytoremediation, the clonal screening concept in *Lamiaceae* has been used to isolate high phenolic clonal lines that are tolerant to aromatic pollutant analogues[137-142] and to develop a model for pollutant (poly aromatic hydrocarbons) tolerance based on correlation between phenolic and proline content and GPX activity.[142]

Model for mechanism of action of phenolic antioxidants and role of proline-linked pentose-phosphate pathway

Research efforts on phenolic phytochemical biosynthesis have focused mainly on antioxidant-type metabolites in several food plants due to relevance of major oxidation-linked chronic human diseases such as CVD, arthritis, cognition diseases (Alzheimer and Parkinson's), cancer and diabetes.[1] Phenolic antioxidants from food plants have also been targeted as a source of antimicrobial phytochemical profiles against bacterial pathogens as an alternative and complement to antibiotics due to the increasing emergence of antibiotic resistance in such pathogens. Design of functional and health foods will take into consideration the above major health targets (oxidation-linked chronic diseases and bacterial infections). Therefore, understanding the mechanism of action of phenolic antioxidants is very important and critical for health and wellness applications.

A model has been developed for mechanism of action of phenolic metabolites based on correlation between stress-stimulated phenolic biosynthesis and stimulation of antioxidant enzyme response pathways in plant systems. In this model (Fig. 2), acid, exogenous phenolic, proline analogues and precursor combinations and microbial elicitors were used to stimulate phenolic biosynthesis and key antioxidant enzyme responses. During the phenolic response, proline content and its association to the first committed step of the pentose-phosphate pathway (G6PDH activity) was followed. Positive proline and G6PDH correlations were associated with increased phenolic content, potential polymerization of phenolics represented by GPX activity, antioxidant free radical scavenging antioxidant activity of phenolic extracts and superoxide dismutase activity.[122,125,126,129,143] In this model (Fig. 2), elicitors through various independent and/or common pathways directly or indirectly mediate a proton-linked redox cycle in the cytosol, which is coupled to the stimulation of proline-linked pentose-phosphate pathway activity. This stimulation results in proton recycling through $NADPH_2$-requiring proline biosynthesis which is replenished by the forward reactions of the pentose-phosphate pathway which generate more $NADPH_2$ for proline biosynthesis and other biosynthetic pathways, including phenylpropanoid and antioxidant response pathways involving SOD and peroxidases. Again, proline that is produced is then used as an alternative RE in place of NADH for driving mitochondrial oxidative phosphorylation for ATP synthesis similar to the previously described model.

Other plant and plant-fungal fermentation systems have been developed to understand the role of phenolic antioxidants and how the regulation of their synthesis and function may critically involve the proline-linked pentose-phosphate pathway. We have developed an *Agrobacterium rhizogenes*-induced root culture system wherein the natural auxin and cytokinin genes were transferred to anise (*Pimpinella anisum*) shoot explants and then naturally transformed and morphologically distinct roots were isolated.[111,144] The proposed advantage of this system is based on the rationale that additional flux towards auxin and cytokinin pathways would over-express the proline-linked pentose-phosphate pathway (Fig. 1). In root cultures, total phenolics and a novel phenolic metabolite, epoxypseudo-isoeugenol-2-methyl-butyrate (EPB), were highest in transformed root cultures and correlated with increased proline content.[144] Antioxidant activity was higher in late stages of root culture, but there were no significant differences between untransformed and transformed root cultures.[144]

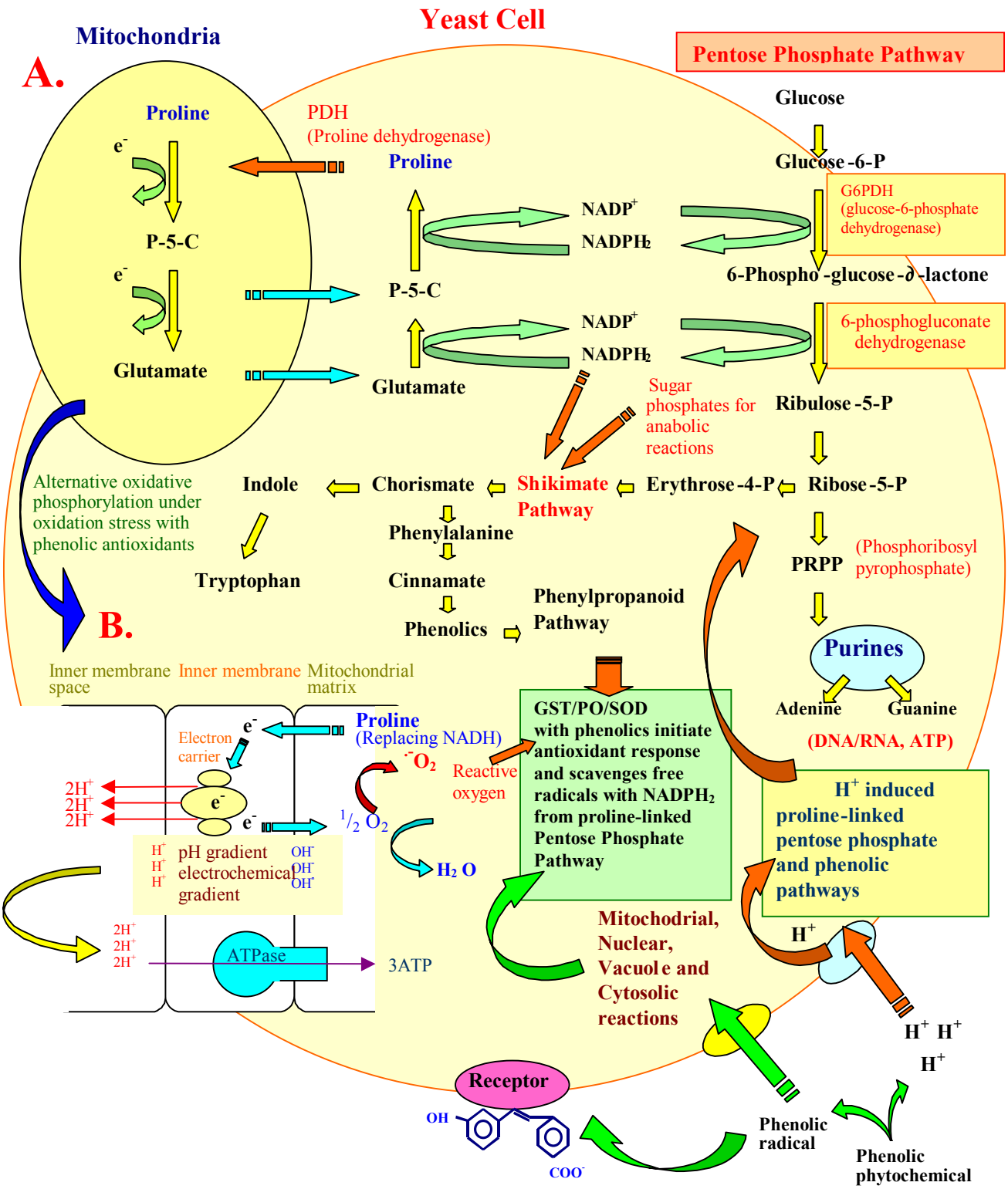

Figure 3. Extension of plant proline-linked pentose phosphate pathway model for the effect of external phenolic phytochemicals in yeast and fungal systems. (Abbreviations: P5C; pyrroline-5-carboxylate, GST;Glutathione-s-transferase, PO; peroxidase, SOD; superoxide dismutase)

Additional studies showed that elicitor treatment in the form of acetylsalicylic acid and proline precursors from likely stimulated lignification as indicated by an increase in GPX activity in transformed root cultures.[145] EPB synthesis in response to elicitors in transformed root cultures increased 3-6-fold. However, in the stimulated state, antioxidant activity and G6PDH activity did not vary between treated and control cultures.[145] Proline and proline analogues were used to stimulate somatic embryogenesis in *A. rhizogenes*-transformed root cultures. Highest embryos were produced in response to proline and proline analogue treatments and this correlated to highest total phenolic content.[111] The proline precursors ornithine and arginine in combination with proline analogue also significantly stimulated somatic embryogenesis.[111]

In another study, phenolic synthesis and antioxidant activity was investigated in snow alga, *Chlamydomonas nivalis* in response to UV light. Exposure to UV light resulted in a 5-12% increase in total phenolic content.[146]

Free proline was not affected by UV-A, but increased markedly after UV-C exposure. Antioxidant protection increased in response to UV-A, but remained constant after UV-C exposure.[146] Initial summary of this research is that UV light exposure, especially in the UV-C range, can stimulate phenolic antioxidant production in aplanospores of *C. nivalis* by affecting biochemical pathways related to proline metabolism.[146]

Solid-state bioprocessing of plant substrates using dietary fungal systems has been developed for the production of functional phenolic ingredients and enzymes for food-processing.[147-150] Additionally, we have developed solid-state bioprocessing for environmental applications.[147-150] In the context of solid-state production of phenolic antioxidants, we have found that dietary fungi *Rhizopus oligosporus* and *Lentinus edodes* can mobilize the phenolic metabolite ellagic acid from cranberry pomace.[40,41] Further, we have found that water extracts containing high phenolic content have antimicrobial effects against the food-borne pathogens, *Listeria monocytogenes, Vibrio parahaemolyticus* and *Escherichia coli* (unpublished results). The phenolics were also antimicrobial against *Helicobacter pylori,* a pathogen linked to gastric diseases. We have now extended this fungal-mediated phenolic mobilization concept to soy-bean phenolics.[151] We have proposed a model that under the conditions of high phenolic antioxidant mobilization, fungi, including dietary yeasts, may activate a proline-linked pentose-phosphate pathway for energy and reducing equivalent needs (Fig. 3). This would also support antioxidant protection of the fungi using the mobilized phenolics under higher oxidative stress. A proline-linked, phenolic antioxidant-protective response may also be enhanced by addition of proline and proline precursors to the growth medium. This model, if confirmed, has exciting potential applications for improving the bioprocessing abilities and stability of all fungal species used for diverse food, industrial and environmental applications.

Additional series of investigations using plant tissue cultures have been undertaken to determine whether proline precursors from hydrolyzed fish proteins can be used to stimulate plant tissue differentiation and phenolic antioxidant response. Initial results have practical applications for adaptation of plant tissues cultures and seeds in outdoor environments. Based on the observations that proline and proline analogues stimulated cytokinin-induced shoot organogenesis,[101] fish protein hydrolysates and proline analogues were used to similarly stimulate shoot organogenesis.[102] In both studies, proline content was stimulated when shoot organogenesis was stimulated. Studies in anise root cultures indicated that auxin-induced somatic embryogenesis was stimulated by proline and proline precursors (ornithine and arginine) in combination with proline analogues.[111] Based on this study, fish protein hydrolysates with high proline and glutamic acid in combination with proline analogue were used to stimulate auxin-induced somatic embryogenesis.[152] In other studies, fish protein hydrolysates were used to reduce vitrification or hyperhydricity-related malformation of tissue cultures that normally reduce outdoor adaptations.[153,154] In these studies, reduction of tissue malformation was suggested to relate to the stimulation of phenolic response and possibly improved liginification as indicated by enhanced GPX activity.[154] The practical success of using fish protein hydrolysates was then extended to the improvement of seedling vigour in germinating seeds.[123,130] The extent of improvement of seedling vigour and early seed performance was stimulated in response to fish protein hydrolysates. The improved response was correlated to phenolic response and stimulation of proline content, G6PDH and GPX, therefore suggesting a role for proline-linked pentose-phosphate and phenolic antioxidant response pathways.

Role of antioxidant response pathway in plants

Plants synthesize diverse phenolic metabolites, which are then compartmentalized and sequestered into specific organs and tissues and within organelles of specific cells within tissues depending on the nature and functional use of the compound. Many phenolic metabolites serve as antioxidants under various biological and physical stresses by minimizing oxidation-induced damage within tissues where they are produced.[125,126,129] Plant phenolics have the potential to behave as antioxidants by trapping free radicals from various oxidative processes and, in particular, mitochondrial-linked oxidative phosphorylation. The phenolic antioxidants can function either to trap free radicals in direct interactions mediated by direct enzymatic/non-enzymatic steps, or quench free radicals through a series of coupled antioxidant enzyme defence systems[155,156] (Fig. 4). The coupled enzymatic defence systems could involve low-molecular weight antioxidants such as ascorbate, gluathione, α-tocopherol, carotenoids and phenylpropanoids, in conjunction with several enzymes such as SOD, catalase, peroxidases, glutathione reductase and ascorbate peroxidase.[155,157-159] In a schematic representation (Fig. 4) based on UV and ozone-stress induced chemical changes,[155] SOD coverts the superoxide radical into H_2O_2 and O_2. The antioxidants like ascorbate and glutathione participate in both enzymatic and non-enzymatic H_2O_2 degradation.[155,160] Catalase converts H_2O_2 into water and O_2, whereas peroxidase degrades H_2O_2 by oxidation of co-substrates (i.e. phenolic antioxidants).[155,161] Other peroxidases, such as those specific to ascorbate but not to glutathione, are also observed in plants.[155,158,162] Ascorbate peroxidase catalyzes the first step of H_2O_2 scavenging pathway by oxidizing reduced ascorbate. Monodehydroascorbate reductase and dehydroascorbate reductase catalyzes the conversion of monodehydroascorbate or dehydroascorbate to reduced ascorbate by oxidizing glutathione.[155] Glutathione (GSH) is regenerated by glutathione reductase in a $NADPH_2$ dependent reaction. Additionally peroxidases metabolize H_2O_2 by using phenolic metabolites as co-substrates through ascorbate-dependent pathway.[155,163] Phenolic compounds such as flavonoids, in addition to their inherent antioxidant properties are believed to protect plants from UV stress.[164] These protective mechanisms are suggested to work through antioxidant response pathways involving peroxidases (as mentioned above) as well as biosynthesis of polymeric phenolics that lead to protective lignification or smaller polymers that act as antioxidants.[127,163]

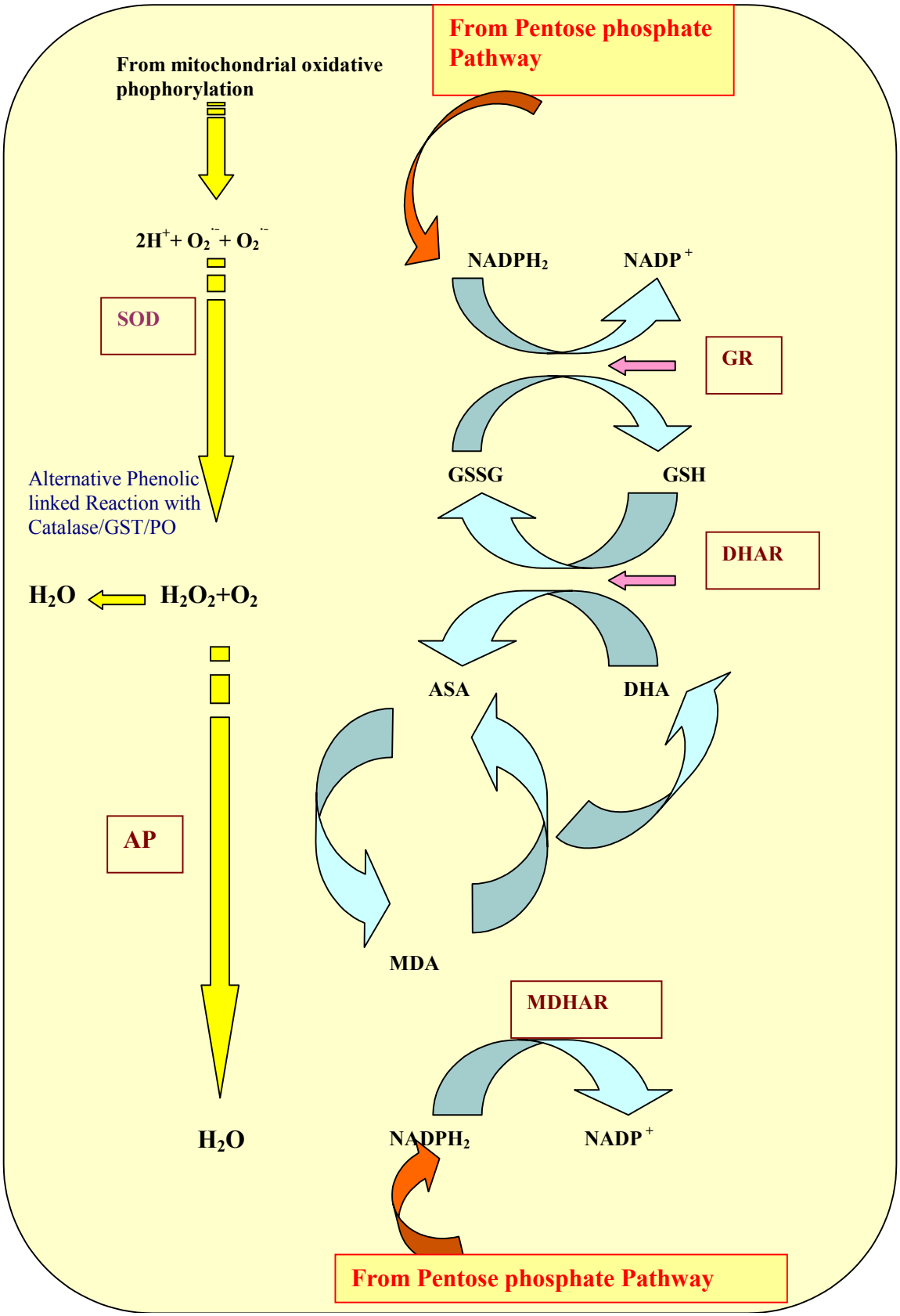

Figure 4. Model for specific steps in antioxidant response pathway in plants. (Abbreviations: SOD; superoxide dismutase, AP; ascorbate peroxidase, GR; glutathione reductase, GSSG; oxidized glutathione, GSH; reduced glutathione, DHAR; dehydroascorbate reductase, ASA; reduced ascorbate, DHA; dehydroascorbate, MDA; monodehydroascorbate, MDHA; monodehydroascorbate reductase).

It is clear from current investigations in plant systems that the antioxidant response pathway is dependent on important $NADPH_2$-requiring enzymes, similar to proline biosynthesis. From our initial investigations in food plant systems,[34,122,125-129] we have proposed that proline-linked pentose-phosphate pathway activity could provide the $NADPH_2$ and sugar phosphate needs of phenolic synthesis and antioxidant pathway activity. This proposal is supported by enhanced activity of G6PDH in response to various elicitor stresses and concomitant stimulation of

phenolics, proline and GPX in several food sprout systems. In this model, proline could replace NADH from Krebs/TCA cycle as the RE for oxidative phosphorylation for ATP synthesis. The proline-coupled synthesis of phenolics and antioxidant response pathways could also serve to scavenge and protect from any free radicals that may be generated during proline-linked oxidative phosphorylation or other free radical-generating processes active in the cell during a stress-induced state. It is also conceivable, based on pH and salicylic-induced stimulation of proline-linked phenolics, G6PDH and GPX activity,[122] that cytosolic acidification could be involved in the activation and coupling of $NADPH_2$-generating steps that are part of the pentose-phosphate pathway.

Role of proline in animal systems

The role of proline in human and mammalian physiology is being investigated by Phang and co-workers at the National Institutes of Health.[103] Early studies showed that activities of enzymes degrading and synthesizing proline were related to development in rat liver and kidney.[165] It was reported that proline oxidase/P5C dehydrogenase ratios were 25 to 50-fold higher in adult central tissue than fetal tissue indicating that proline degradation was favored in adult stages and during synthesis in fetal stages.[165] Another important study showed the transfer of P5C as oxidizing potential from hepatocytes to erythrocytes by inter-conversion from proline forming a redox cycle.[166] The resulting couple to the pentose-phosphate pathway in erythrocytes was suggested to enhance production of 5-phosphoribosyl pyrophosphate (PRPP), a key substrate for purine synthesis.[166] Later studies showed that P5C could stimulate glucose oxidation through pentose-phosphate pathway in cultured human fibroblasts, Chinese hamster ovary cells and rabbit kidney cells.[166] Further, it was shown that reducing equivalents could be transferred into mitochondria by inter-conversions of proline to P5C.[103,104,108] From these studies, it was proposed that the shuttling of proline via P5C could function to increase ribose-5-phosphate by the oxidative limb of the pentose-phosphate pathway for PRPP and purine synthesis during growth.[108] In another interesting study, P5C markedly increased the activation of purine anti-metabolites, 6-thiohypoxanthine, 6-thioguanine and azathiopurine, to their respective nucleotides in intact human erythrocytes,[167] and later confirmed that P5C can stimulate PRPP to support purine biosynthesis in erythrocytes.[168] From these studies it is clear that as a redox couple, proline and P5C provide a mechanism for the inter-compartmental and intercellular transfer of redox potential.[103] The transfer of the redox potential alters the ratio of $NADP^+/NADPH_2$, which can then meet the needs of anabolic pathways by producing more $NADPH_2$. It was also confirmed that even though reduction of P5C is important for the transfer of redox potential, the metabolic inter-conversions of proline, ornithine and glutamate might also play an important role.[103] The end point of the animal studies showed that this redox cycle favoured the formation of purine ribonucleotides.[103] From these mammalian system studies, it was hypothesized that synthesis of plant phenolic metabolites may be linked to the proline-linked pentose phosphate pathway[3] (Fig. 1). In the animal model, proline is synthesized by a series of reduction reactions from glutamate. In this sequence, P5C and proline function as a redox couple and are known as metabolic regulators.[103,104,108] During respiration, oxidation reactions produce hydride ions, which aid reduction of P5C to proline in the cytosol. Through activity of proline dehydrogenase /proline oxidase,[103,105] proline could enter mitochondria and support oxidative phosphorylation. The reduction of P5C to proline in the cytosol increases NADP+, which activates G6PDH, an enzyme that catalyzes the rate-limiting step of the pentose-phosphate pathway. Proline synthesis is therefore hypothesized and partly shown to regulate pentose-phosphate and purine pathways in erythrocytes[106] and cultured fibroblasts.[107] Proline synthesis was shown to stimulate purine metabolism via ribose-5-phosphate, which affects cellular physiology and therefore function.[103,108] A role of proline metabolism in modulation of cellular physiology has been further confirmed in p53-dependent initiation of apoptosis in a colorectral cancer cell line.[169] In this study, p53-dependent initiation of apoptosis was accompanied by the induction of proline oxidase, a mitochondrial enzyme catalyzing the conversion of proline to P5C with concomitant transfer of electrons to cytochrome c.[169] Based on other studies, the up-regulation of proline oxidase during p53-dependent apoptosis has been suggested, indicating a role in redox regulation.[169-171] In another interesting study, proline oxidase was up-regulated in a p53-sensitive bladder carcinoma cell line but not in a p53-resistant cell line.[172] Further, it was shown that P5C generated by proline oxidase, inhibited the proliferation and survival of these bladder carcinoma cells and induced apoptosis in both cell lines.[172] This study directly implicated proline oxidase and proline/P5C interconversions in p53-induced growth suppression and apoptosis.[172] A recent study has further indicated that proline oxidase induces apoptosis in tumour cells and that its expression is frequently absent or reduced in renal carcinomas.[173] In the context of other diseases, molecular genetics studies based on patients with adult or childhood onset of schizophrenia strongly suggest that genetic variations in proline oxidase gene may increase the risk of susceptibility to schizophrenia.[174]

Role of phenolic antioxidant metabolites and proline-linked pentose-phosphate pathway in human health: a hypothesis

Preliminary studies in food-grade clonal herb systems[3] and legume sprouts[125,126,129] led to the development of the model that activity of proline-linked pentose-phosphate pathway is important for stress-induced phenolic biosynthesis and that this stimulation of phenolics may be closely linked to stimulation of antioxidant response pathways.[125,126,129] Further research has indicated that the proline biosynthesis pathway coupled to stress-induced antioxidant response pathways could be also stimulated in legume sprouts using exogenous treatment of phenolic extracts from clonal oregano.[35,126,130] Phenolic extracts from these clonal oregano lines have high free-radical scavenging activity. Proline-linked stimulation of antioxidant response pathways may also be stimulated by low

pH and salicylic acid.[122] Further, exogenous seed treatment with oregano phenolic antioxidant extracts enhanced endogenous phenolic content, GPX activity, and consequently, enhanced seedling vigour during germination.[130] From these initial plant studies and plant model (Fig. 2), a human/mammalian cell model has been developed (Fig. 5) wherein a proton donation by phenolic antioxidants at the outer plasma membrane initiates a proton/hydride ion influx into the cytosol which activates an antioxidant response through the stimulation of the proline-linked pentose-phosphate pathway. Demand for $NADPH_2$ by stimulated proline biosynthesis also drives the production of precursors for phenolic (only in plants/fungi), purine and antioxidant pathways. In this model, proline can be used as a RE to support oxidative phosphorylation for ATP synthesis. Using this model, we have developed several phenolic over-expressing plant systems for functional food and agro-environmental applications. The optimized phenolic phytochemical profiles can be used as antioxidants and antimicrobials in biological systems and have implications for human health and wellness.

From the assumptions based on the animal antioxidant response model (Fig. 6) and the plant antioxidant response model (Fig. 2 & 4), a new, integrated model for the mechanism of action of phenolic antioxidants for human health involving the proline-linked pentose-phosphate pathway has been proposed (Fig. 5). It is clear that many of the major diseases afflicting humanity today in an age of increasingly sufficient and, perhaps, excess calories, are oxidation-linked chronic diseases such as cancer, CVD, arthritis, cognition diseases, and diabetes. Oxidation-linked immune dysfunction and the inability to fight pathogenic infection under a very low calorie and protein diet remains a problem in several parts of the developing world and needs continued and serious attention. Oxidation-linked and infectious diseases involve free-radical reactions. Free radicals are potential carcinogens because they can facilitate mutagenesis, tumour promotion and progression.[175-177] For example, in the case of cardiovascular diseases, free radicals are implicated in the pathogenesis of atherosclerosis, which is characterized by the hardening of the arterial wall.[175,178,179] Rheumatoid arthritis, a systemic autoimmune disease characterized by chronic joint inflammation with infiltration of macrophages and activated T cells. Production of free radicals at sites of inflammation is thought to contribute to the pathogenesis.[175, 180,181] Free radicals are implicated in the pathogenesis of Alzheimer disease.[175,182] As significant amounts of lipid peroxidation in brain tissues have been observed and may help explain the progressive decline in cognition function and excessive neuronal loss in afflicted patients. The brain tissue shows numerous amyloid plaques. Free radicals have been implicated in Diabetes Mellitus.[175,183,184] In this disease, oxidative stress is associated with a pro-oxidative shift of glutathione redox state in the blood.[175,185] Elevated glucose levels are associated with increased production of free radicals by several different mechanisms.[175,186,187]

All of the above diseases are in part diet- and lifestyle-influenced and improvement of diet is important in the prevention and management of these diseases. It is evident that a variety of plant-based phenolic antioxidants have a positive impact on the prevention and modulation of various oxidation-linked diseases[10,18,19,43,188,189] and must be considered an important part of the dietary management of these diseases. Currently the modes of action and early stage effects of these phenolic antioxidants in positively modulating and preventing various diseases are not completely clear. Extensive research is underway to ascertain how free radicals modulate physiological control of cell function at the level of cell proliferation and deterioration[175] and at the level of gene expression.[190] Phenolic antioxidants have been targeted to control the free radical-linked cellular deterioration that can lead to major oxidation-linked chronic diseases.

One way to develop diet-based interventions is through design of functional foods (conventional foods with clinically defined health promoting components) based on the understanding of phenolic antioxidant biosynthesis in food plants (Fig. 2) and the effect of such phenolic antioxidants in human and mammalian systems (Fig. 5). In order for diet-based interventions (through functional foods) to be effective, it is also important to understand the early stage modes of action of these functional compounds and how to deliver them at consistent levels and with no toxicity problems. In the antioxidant response model for human health, proposed herein a consistent and defined phytochemical profile of phenolic antioxidants can be developed using clonal shoot, sprout and fermented systems using various dietary botanicals as discussed earlier (Fig. 2). In the human model (Fig. 5), the early stage mode of action of these phenolic antioxidants in human cells has parallels to the models for plant and fungal systems (Fig. 2, 3 & 5), but within the scope of human cellular physiology, function, and diversity. By this model, plant phenolic antioxidants similarly initiate an inward proton flux at the outer human cell membrane, which increases the cytosolic proton/ hydride ion concentration and activates the proline-linked pentose-phosphate pathway. Some phenolic antioxidant radicals, depending on their size, may penetrate the plasma membrane along with the proton/hydride ion flux (co-transport) into the cytosol. The cytosolic proton/ hydride ion flux then drives proline-linked pentose-phosphate pathway generating $NADPH_2$, sugar phosphates for anabolic reactions and proline as an alternative RE to generate ATP via oxidative pentose-phosphate pathway. The products of the pentose-phosphate pathway are important for purine biosynthesis and for stimulating antioxidant enzyme response pathways in conjunction with action of the dietary phenolic antioxidants (Fig. 6). The control of free-radicals that is likely associated with proline or TCA cycle generated NADH-linked mitochondrial oxidative phosphorylation at this early stage could have a positive effect on any subsequent oxidation-linked cellular deterioration and consequent oxidation-linked chronic disease manifestations. Other roles for phenolic radicals that penetrate the membrane could involve: a) stability and protection of organelle membranes and proteins from free-radical damage; b) participation in the antioxidant response pathway to quench superoxide and peroxide radicals; c) protection of DNA and protein stability and/or d) stimulation of proline-linked pentose-phosphate pathway activity to

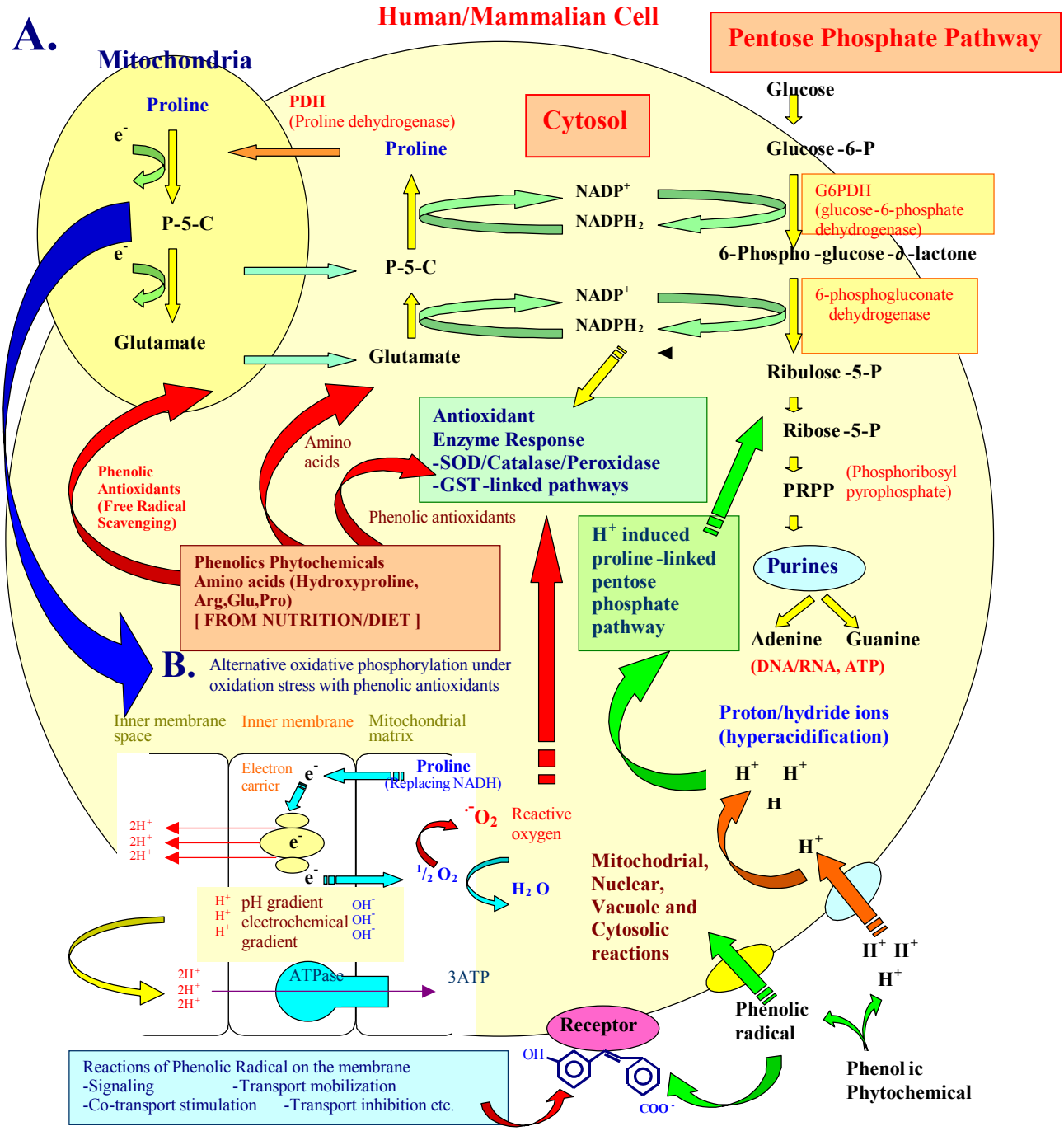

Figure 5. Extension of plant proline-linked pentose phosphate pathway model for the effect of external phenolic phytochemicals in human and mammalian systems. (Abbreviations: P5C; pyrroline-5-carboxylate, GST; Glutathione-s-transferase, SOD; superoxide dismutase)

satisfy demand for $NADPH_2$ in reactions involving penetrating phenolic radicals.

In specific cases where phenolic radicals cannot normally penetrate the outer plasma membrane, other conceivable roles could include: a) stability and protection of the outer membranes and membrane proteins from free-radical damage, b) modulation of membrane transport, c) inhibition of specific membrane proteins, including those involved in PMF and electron transport chain in Prokaryotes, d) modulation of signal transduction, e) modulation of membrane receptors, f) co-transport with H^+, sugars and/or amino acids and g) passive membrane transport through damaged membranes. An inward proton flux to the cytosol could be created even without phenolic radical penetration, which then could stimulate the proline-linked pentose-phosphate pathway and couple its action to the various reactions and roles that may be initiated and modulated through interactions of phenolic radicals at the outer plasma membrane.

Implications of phenolic antioxidants as antimicrobials

Current theory and emerging data suggests that eukaryotes evolved from prokaryotes.[191] Genetic evidence suggests that plant organelles like chloroplasts, mitochondria, and even vacuoles may have origins as free prokaryotes. The compartmentalized organization and cellular differentiation (into tissues) of plants apparently evolved in terrestrial environments about 500 million years ago. It is likely that through the millennia, plastic

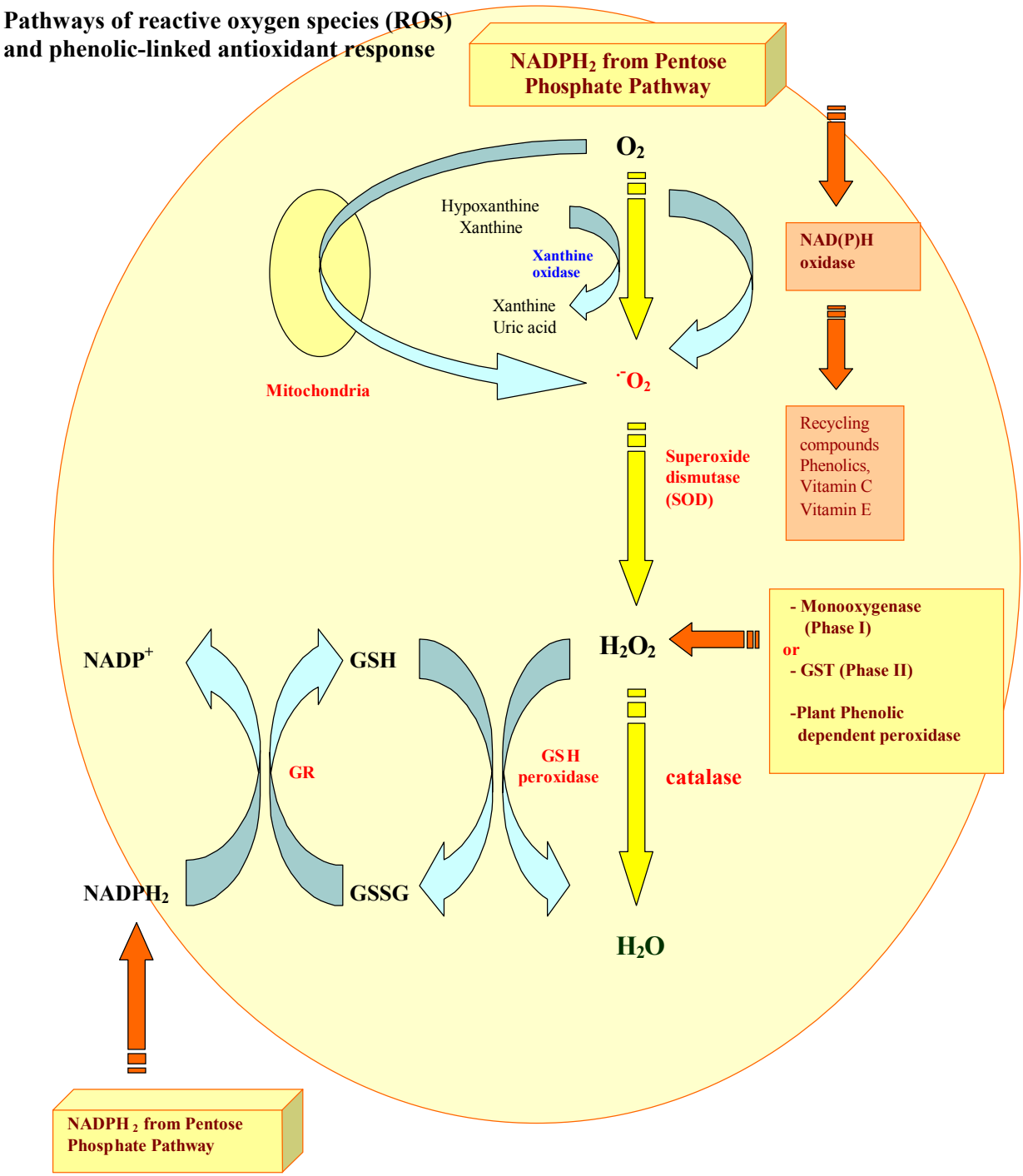

Figure 6. Model for specific steps in antioxidant response pathways in human and mammalian systems. (Abbreviations: SOD; superoxide dismutase, GST; glutathione-s-transferase, GR; glutathione reductase, GSH peroxidase; glutathione peroxidase, GSSG; oxidized glutathione, GSH; reduced glutathione)

plant species constantly interacted with many micro-organisms and environmental stresses, especially UV radiation and oxidative stress. The speculation is that constant environmental stress in many ways may have shaped the antimicrobial and phenolic antioxidant responses of plants through the evolutionary process. Natural selection of plants under various changing environmental conditions over millions of years, in many critical ways (directly or indirectly) may have shaped the current mammalian systems, including human nutrition, health, and dietary evolution.

It is from these assumptions that we are exploring the mechanism of antimicrobial action of plant phenolics on prokaryotic pathogens and the activation and maintenance of plant and eukaryotic antioxidant responses, at a similar phenolic concentration needed for bacterial inhibition. Plant phenolics and related synthetic food-grade phenolics have been suggested to inhibit bacterial pathogens (particularly at low pH) by disrupting the proton motive force (PMF). This implies that regulation of H^+-ATPases and co-factors at the external membrane is critical, and may be a logical point to study the mechanism of inhibition by plant phenolics. Additionally, plant phenolics

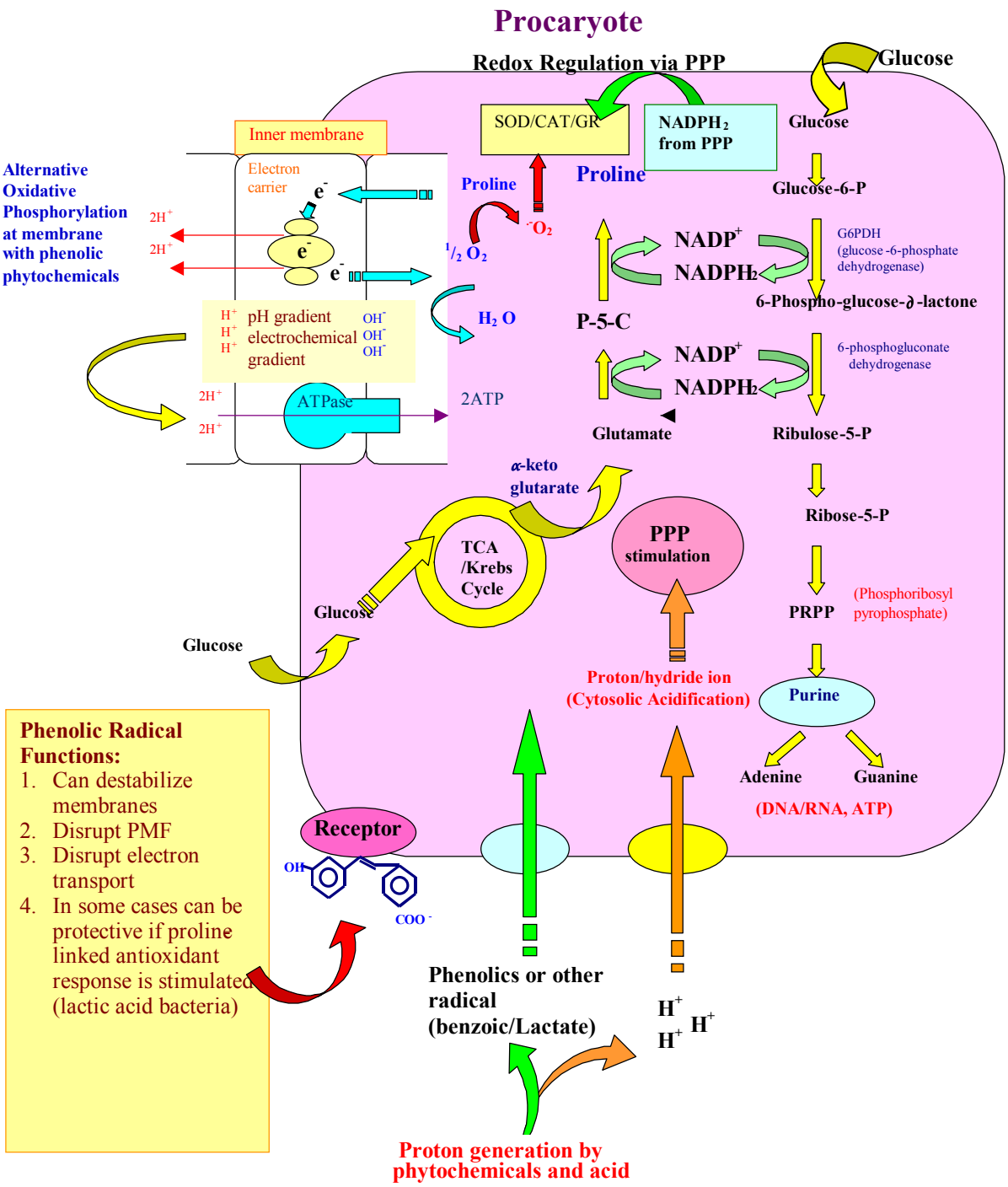

Figure 7. Extension of plant proline-linked pentose phosphate pathway model for the effect of external phenolic phytochemicals in prokaryotic bacterial systems. (Abbreviations: P5C; pyrroline-5-carboxylate, SOD; superoxide dismutase, CAT; catalase, GR; glutathione reductase, PPP; pentose phosphate pathway; Krebs {TCA-tricarboxylic acid} cycle).

may disrupt the electron transport chain or destabilize the plasma membrane.[192] At the same time it is important to investigate the role of plant H^+-ATPases at both the external membrane and organelle (chloroplast, mitochondria and vacuolar tonoplast) membrane levels to determine their potential involvement in modulation of a phenolic radical and proton-linked redox cycle. This cytosolic phenolic radical/proton-linked redox cycle (as described earlier) is hypothesized to activate the proline-linked pentose-phosphate pathway, to utilize proline as an alternative RE for mitochondrial ATP synthesis, and to generate precursors and co-substrates ($NADPH_2$ and sugar phosphates) for anabolic reactions, including a phenolic-linked antioxidant response and purine synthesis.[3,122] The genetic comparisons of H^+-ATPase alleles from bacterial and eukaryotes could provide clues towards understanding their susceptibility to plant phenolics in bacteria and the antioxidant response-linked tolerance of plants and other eukaryotes. Such knowledge could facilitate the development of better structure-function strategies for the development of better botanical phenolic profiles (i.e herb and legume clonal extracts) that may provide multiple beneficial activities. A better conceptual understanding of phenolic antimicrobial effects in bacteria and antioxidant responses in eukaryotic hosts could also facilitate the development of diet-based nutritional and

functional food strategies to control bacterial pathogens with reduced potential for antibiotic resistance due to the concerted effect of a phenolic profile, instead of a single compound, as in case of antibiotics. Such an integrated approach involving phenolic phytochemicals has excellent potential to compliment current antimicrobial strategies.

Taking into account the above rationale, a phenolic antimicrobial activity model (Fig. 7) that incorporates the role of proline-linked pentose-phosphate pathway provides a better perspective than current putative antimicrobial models for phytochemical action.[192] The phytochemical profiles that have the potential to inhibit pathogenic microorganisms[192-194] contain secondary metabolites that are defensive and inducible anti-microbials produced against both invading pathogens and stress. Therefore the methods for exploiting them must take this also into account. In certain cases, the induction is associated with action of diphenolic oxidases and resulting modified compounds can have antimicrobial activity.[195] In some cases, dihydroxy phenolics are oxidized to highly reactive quinones, which can interact with proteins of the invading pathogens and form melanoid polymers.[192] As the compounds responsible for the enzymatic browning reaction of cut fruits and vegetables and an intermediate in melanin pigment production in humans,[192] quinones are a source of stable free-radicals and complex irreversibly with nucleophillic amino acids[196] leading to inactivation of proteins and loss of function.[192] Therefore, the potential antimicrobial benefits of quinones are substantial.[192] Potential targets for inhibition in the bacterial cell are surface adhesions, cell wall polypeptides, and membrane-bound enzymes.[192] In herb species of the *Lamiaceae* family, phenolic derivatives are largely responsible for antimicrobial activity.[4,197] Thymol, present in the essential oil of thyme, oregano, savory, sage and related species, has excellent antimicrobial activity. The essential oil containing thymol can inhibit *Vibrio parahaemolyticus.*[198] The addition of 0.05% of alcoholic extracts of thyme can inhibit the growth of *Staphylococcus aureus.*[199] Sage extract was inhibitory to *Bacillus cereus* and *S.aureus.*[200] Rosemary extract of 0.1% substantially inhibited the growth of *S.aureus* and *Salmonella typhimurium.*[201] Hydroxy-cinnamic acid derivatives such as caffeic acid, ferulic acid and *p*-coumaric acid inhibited *E.coli, S.aureus* and *B.cereus.*[202] Polymeric phenolics, such as tannins, were inhibitory toward *Listeria monocytogenes, E.coli, S.aureus, Aeromonas hydrophila* and *Streptococcus faecalis.*[203] Hydroxylated phenols, such as catechol and pyrogallol, are known to be toxic to microorganisms.[192] The site and number of hydroxyl groups is linked to the antimicrobial effect and in some cases more oxidized forms are more inhibitory.[204,205]

From all the above studies it is evident that the mode of action of phenolics against bacterial pathogens has not been clearly defined or understood and many modes of action have been suggested. One major model has focused on the suspected changes in membrane permeability through membrane-localized hyperacidity, which may affect PMF across the membrane resulting in energy depletion.[206,207] Another has proposed that enzyme inhibition by oxidized compounds through reaction with enzyme sulfhydryl groups or through non-specific interactions with membrane proteins may explain the inhibition.[192,208] Even with better models on the mechanism of action, another major limitation of using many phytochemicals is that they are derived from mixed heterogeneous genetic sources and consistency cannot be guaranteed.[3,4] Now, with the emergence of antibiotic resistance from overuse of single antibiotics new strategies incorporating plant-based antimicrobials are both promising[192] and essential.[4] However, plant-based antimicrobial strategies will require a better model to understand the mechanism of antimicrobial action involving consistent profiles of phytochemicals.

An effective strategy proposed in this paper focuses on the hypothesis that high-antioxidant phenolics from single seed origin clonal lines of herbs, sprouted legumes, and fermented fruits would have excellent antimicrobial potential**.** Single seed high-antioxidant phenolic profiles have been screened, evaluated for antioxidant efficacy, and targeted to inhibit various food-borne pathogens and chronic human infections, such as those by peptic ulcer-causing *Helicobacter pylori.* The above strategy addresses both the concept of phytochemical consistency through the use of clonal lines and stress (elicitor)-based inducible phenolic antioxidants from various developmental phases of growth and also the putative impact on antimicrobial potential**.** Therefore, using our strategy for developing consistent and inducible phytochemical profiles, an alternative and more robust model (Fig. 7) for a mechanism of antimicrobial action incorporating the role of proline-linked pentose-phosphate pathway have been proposed. The mechanism of action of phenolic phytochemicals that may operate in prokaryotes involves the use hyper-acidification (protons) from acids and phenolic metabolites and transport protons inside the cell (Fig. 7), by more acid-tolerant prokaryotes (lactic acid bacteria and some moderately acid tolerant Gram negative pathogens like *H. pylori*, *Escherichia coli* and *Salmonella*) either passively or through H^+-transport membrane proteins. Such acid-tolerant prokaryotes may stimulate redox cycling through the proline-linked pentose-phosphate pathway and use proline (like the mitochondria of eukaryotes) as an alternative RE for ATP synthesis at the single outer plasma membrane with oxygen as the terminal electron acceptor. As mentioned before, the process of proline biosynthesis could also be coupled to the pentose-phosphate pathway for $NADPH_2$ recycling and to make sugar phosphates for all anabolic needs. By this model, acid-tolerant microorganisms could efficiently manage the ATP needs from stress-increased oxidative phosphorylation through coupling to pentose-phosphate pathway activity, while recycling excess proton flux from hyper-acidification. Proline-linked oxidative phosphory-lation could excrete excess protons outside the plasma membrane and augment the generation of PMF for ATP synthesis. This proline-linked metabolism model may occur in acid- and phytochemical-tolerant lactic acid bacteria (Gram-positive) and in moderately acid-and phytochemical-tolerant Gram-negative bacteria but is less likely in other acid-and phytochemical-susceptible Gram-positive bacteria.

One way to prove this model would be to determine if proline over-producing mutants are more acid-tolerant and if this tolerance is associated with increased generation of $NADPH_2$. An important difference in case of prokaryotes is that they have only one outer plasma membrane and no organelles for metabolic adjustment. Depending on the type of membrane modifications that occur between various bacterial species, phenolic radicals may negatively or positively effect membrane related functions, including transport, signalling, receptor modification and energy metabolism. Membrane-related modulation of metabolism could be closely linked to the cytosolic proton-linked modulation of proline-linked pentose-phosphate pathway. Based on this model, it is likely that Gram-positive bacteria (excluding lactic acid bacteria) would be most susceptible, followed by Gram-negative bacteria, and then by acid-tolerant lactic acid bacteria, likely being more tolerant and actually protected by phenolic phytochemicals (as antioxidants).

In eukaryotic microorganisms like yeasts, phytochemical-based inhibition is likely more challenging based on the above model. In yeasts, proton flux from acids and phytochemicals may be managed as proposed for mammals and plants, though stimulation of the proline-linked pentose-phosphate pathway (Fig. 3). Such action would support antioxidant response pathways. The implication is that, designing antimicrobial strategies for controlling yeast infections using phytochemicals becomes more challenging. For control of yeast and external fungal infections, it may be more feasible to develop phytochemicals, such as phenolics and terpenes, that disrupt external membrane transport, and to target the disruption of electron transport chain in the mitochondria only in cases where the phytochemical can penetrate the outer membrane. Alternatively, through modulation of proline-linked pentose phosphate pathway, phenolic antioxidants from food plants could be ideal for improving the stability of various yeasts and fungal systems used in food processing (i.e alcoholic beverages and dairy products) and industrial applications.

Summary and unified theory for role of proline-linked pentose-phosphate pathway in plant, animal and microbial systems

When considering the genetic, cellular and tissue divergence of various biological systems and adaptations due to environmental pressures and natural selection we must also recognize the commonalities of certain bio-chemical responses related to oxidation-linked cellular manifestations and function. During oxidation-linked stress and disease conditions, an alternative mode to regulate the pentose-phosphate pathway, as well as ATP synthesis by oxidative phosphorylation, through proline may help biological systems. Such regulation could help to efficiently manage energy needs while supporting the $NADPH_2$ and sugar phosphate requirements of biosynthetic pathways, including the antioxidant, antimicrobial, stress and adaptogenic, immune, and, consequently disease/health responses. In this unified model, all early responses under any kind of oxidation stress, including infectious diseases, are universally modulated through the proline-linked pentose-phosphate pathway. This integrated pathway would support antioxidant response pathways to manage each stressed state depending on the biological system, and human health/disease states that are dictated by specific oxidation stresses coupled to nutritional and environmental factors.

In summary, a strategy has been developed to generate consistent profiles of phenolic antioxidants from food plants to design functional health foods for improved diet as a means to potentially manage (through prevention) oxidation-linked chronic and infectious diseases. The strategy to generate consistent phenolic antioxidant profiles has driven the development of plant system models, wherein synthesis of ATP, RE, $NADPH_2$ and sugar phosphates for cellular metabolism is regulated through an alternative proline-linked pentose-phosphate pathway. The empirical insights into this alternative pathway have already been exploited for many applications in many plant species. Examples include: 1) screening high phenolic antioxidant producing food-grade clonal herbs, 2) development of biological, biochemical and stress elicitation methods to stimulate phenolic antioxidants in sprout systems, 3) development of food-grade fungal bioprocessing to generate consistent phenolic antioxidants from botanical substrates, 4) strategies for developing consistent phytochemical profiles that can be targeted against bacterial pathogens and elucidation of possible mechanisms of action of phytochemicals through the bacterial proline-linked pentose-phosphate pathway, 5) environmental applications of plants via generation of high-phenolic clonal systems for phytoremediation of aromatic pollutants, and 6) environmental applications in plants utilizing the proline-linked pentose-phosphate pathway for enhanced environmental adaptation of transplanted seedlings from tissue culture or greenhouse systems.

Advancement of this concept by further research into the alternative proline-linked pentose-phosphate pathway has numerous implications for 1) understanding the mechanism of action of phenolic antioxidants from plants in mammalian and human systems; 2) understanding whether the mechanism of action of phenolic antioxidants in mammalian and human systems as proposed in this paper is regulated through the alternative proline-linked pentose phosphate pathway; 3) understanding whether phenolic antioxidants positively modulate and prevent oxidation-linked diseases through proline-linked pentose-phosphate pathway; 4) understanding whether phenolic antioxidants from plants can control bacterial infections and whether the mechanism of action involves the proline-linked pentose-phosphate pathway; 5) understanding whether phenolic antioxidant-based functional foods can be designed for managing major oxidation-linked diseases such as diabetes, CVD, inflammatory diseases, cognition diseases and cancer; 6) understanding whether phenolic antioxidant-based functional foods can be designed for managing chronic bacterial-infection-related diseases such as ulcer, urinary tract infection and dental caries, as well as food-borne bacterial infections, and whether this can complement or reduce microbial-based single antibiotic use; 7) understanding whether phenolic antioxidant-based functional foods can be designed using probiotics and yeasts, and whether the

proline-linked pentose-phosphate pathway is important for their stability in food carrier systems and in humans; 8) understanding whether phenolic antioxidants can be used for improving bioprocessing qualities and stability of yeast and bacterial acid-fermented foods, and in such cases, what is the health benefit to humans, animals and other biological systems; 9) understanding the interactions and proper balance between plant-based phenolic antioxidants and protein foods from various sources such as legumes, fish and various meats in the human diet and whether the mechanism of action is controlled through the proline-linked pentose-phosphate pathway; 10) understanding the interactions of plant-based phenolic antioxidants with carbohydrates (particularly starch and soluble sugars) and lipids (with saturated fatty acids) and whether the mechanism of action is controlled through the proline-linked pentose-phosphate pathway; 11) understanding whether liver detoxification pathways are coupled to antioxidant response pathways; and 12) relevance to designing plant and microbial systems for Advanced Life Support and Food systems in NASA and International Space projects.

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