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2-Acetyl-1-Pyrroline Biosynthesis: from Fragrance to a Rare Metabolic Disease

Review Article

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Abstract

2-acetyl-1-pyrroline has been reported as a major compound responsible for pleasant aroma in basmati and other scented rice varieties. The biosynthesis of this molecule is due to deletion in the betaine aldehyde dehydrogenase2 gene. This deletion leads in the accumulation of Δ^1-pyrroline which reacts non-enzymatically with methylglyoxal to form 2-acetyl-1-pyrroline. Due to non-functionality of this gene that regulates the synthesis of Gamma-amino butyric acid, the plant species synthesizing 2-acetyl-1-pyrroline suffers for the yield losses, sterility and susceptibility to biotic and abiotic stresses. Thus the non-functionality of betaine aldehyde dehydrogenase2 gene coupled with 2-acetyl-1-pyrroline synthesis serves as a metabolic disease. In this review these aspects are discussed in detail.

Keywords: 2 acetyl-1-pyrroline; Fragrance molecule; Betaine aldehyde dehydrogenase 2 (BADH2); Gamma-amino butyric acid GABA; Biosynthesis; Aromatic rice; *Pandanus amaryllifolius*

Introduction

The world is filled with flavors and fragrance, which are the result of volatile compounds produced and emitted by plants [1]. Flavors and fragrance are directly tied to food preference and palatability and to mate choice. Fragrance of aromatic rice (*Oryza sativa* L.), is a special trait with huge economic importance. Aromatic rice like Basmati of India, Khao Dawk Mali of Thailand and Kaorimai of Japan has gained wide popularity in Asia and the Middle East and are sold at higher prices. Now days these aromatic rice are in high demand from Europe and America [2]. Aroma characteristics have been determined in following three of the distinguished genetic subpopulations of rice: *Group V* (Sadri and Basmati), *indica* (Jasmine), and tropical *japonica* [3]. A comparative study of the volatile components of aromatic and non-aromatic rice varieties showed that 2-acetyl-1-pyrroline (2AP), which contributed to specific flavour in aromatic rice [4]. For decades together researchers were engaged in identification of the compound responsible for the unique fragrance and flavor of the aromatic rice. Several researchers reported hundreds of volatile compounds but failed to report the principal molecule. For the first time Ron Buttery [5] with his co workers successfully identified 2-acetyl-1-pyrroline (2AP) as the compound responsible for the unique and pleasant fragrance of the aromatic rice. The universality of this molecule was confirmed by subsequent rice aroma analyses in several aromatic varieties worldwide [6-10]. 2AP was detected beyond aromatic rice varieties from unicellular bacteria to urine of tiger [11]. Among the different natural source of 2AP, prominent natural sources of 2AP other than aromatic rice are *Pandanus amaryllifolius* Roxb. [4], *Vallaris glabra* Ktze. [12] and *Bassia latifolia* Roxb. [13].

After discovering the principal aroma compound, several researchers are engaged in revealing the mechanism for biosynthesis of this miracle molecule '2AP' in these plant systems. A major breakthrough was obtained by Bradbury et al. [14] by identifying the gene responsible for fragrance in rice. The loss of function of betaine aldehyde dehydrogenase (*BADH2*) gene on chromosome 8 of rice was found to be responsible for the accumulation of 2AP [14]. Since then focus area for several researchers has been to introduce the fragrance trait in non-scented rice and other important crops and subsequently several report has been published on biosynthetic pathways in rice and other plants. However, the fragrance trait in aromatic rice comes at the cost of lower yield and susceptible nature to biotic and abiotic

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stresses [15,16]. The levels of *BADH2* expression have a major impact on growth and performance of rice plant. It is now for breeders, whether to choose economically important fragrance trait or yield and tolerance to stress? The synthesis of 2AP, its implications on yield and tolerance to biotic and abiotic stresses and future directions are discussed in the present article.

2-acetyl-1-pyrroline; the fragrance molecule

2 acetyl-1-pyrroline [2AP; IUPAC name 5-acetyl-3,4-dihydro-2*H*-pyrrole and 1-(3,4-dihydro-2*H*-pyrrol-5-yl)-ethanone] was identified for the first time as the major flavor compound of cooked rice [5]. The odour threshold of 2AP is 0.1 part (ml) of compound per 10^9 parts (ml) of water [5]. The compound 2AP, usually described as a "pop-corn" or "roasted" flavour compound, was also identified in the leaves of *Pandanus amaryllifolius* Roxb. [4], *Bassia latifolia* Roxb. [17], spinach [18], taro corms [19], bread flowers (*Vallaris glabra* Ktze.) [12], soybean [20], mung bean [21], sorghum [22] and cucumber [23]. 2AP was identified in bacterial strains of *Bacillus cereus* isolated from cocoa fermentation boxes [24], *Lactobacillus hilgardii* [25], and several fungal strains [26,27]. Interestingly, it was also identified in urine of tigers [28]. The 2AP also reported in large variety of cereal products, vegetable-derived and animal-derived products. e.g. wheat bread crust, rye bread [4,5] bread crust [29], toast [30], wet milled millet [31], popcorn and Sweet corn based products [33], moderately roasted sesame [34], baguette crusts [35], cooked tail meat of freshwater crayfish (*Procamarus clarkia*) [36], boiled potatoes [37], roasted seeds of wild mango (*Irvingia gabonensis*) [38], cooked blue crab [39], maize flour [40], taro volatiles (*Colocasia esculanta* (L.) Schott) [19], boiled Crap fillet (*Cyprinus carpia* L.) [41], oyster cooker effluent [42], cooked tail meat of American lobster (*Homarus americanus*) [43], heat-treated nonfat dry milk [44], Iberian dry-cured ham [45], cooked acha (*Digitaria exilis* Stapf) [46].

Besides living systems, 2AP has been reported as an important Maillard flavour compound in large number of heated and processed food products [47,48]. 2AP is highly unstable compound which makes it unsuitable for long term storage and commercial utilization. Hence it is used in natural form wherever present e.g. the leaves of *P. amaryllifolius* are known to harbor highest 2AP contents in the plant kingdom and are widely used in South East Asia for flavouring various foods such as bakery products, sweets, drinks, ice-creams etc. [49,50]. The leaves are also added to ordinary rice while cooking to impart basmati rice aroma. The 2AP contents vary considerably in premium aromatic rice viz. Basmati rice (0.34 ppm), Jasmine rice (0.81 ppm) and Texmati (0.53 ppm) [51]. In *P. amaryllifolius* leaves, relative 2AP contents recorded were 10 - 12 ppm [12,52].

Genetics and Biochemistry of 2AP synthesis

Several studies were conducted by the researchers worldwide to map fragrance trait in rice. Genetic analyses have repeatedly revealed that the primary fragrance trait is controlled by recessive monogenic inheritance, independent of cytoplasmic genes. Among the rice varieties studied, some showed monogenic control [53,54] whereas, in some others it was found to be a quantitative trait and many genes were involved in its expression [55], thus, indicating complex genetic control of the aroma trait [56]. Initially, a single recessive gene located on chromosome 8 was identified by different researchers using different techniques viz. RFLP [57], translocation and trisomics lines from non aromatic rice cultivar IR36 [58] and by using SSR markers RM210 and RM515 [59]. Now the rice genome was sequenced and finally physical fine mapping study revealed that a candidate gene homologous to betaine aldehyde dehydrogenase (*badh2*) on chromosome 8 is responsible for 2AP formation in aromatic rice [3, 4,16,60,61]. They found that an eight base pair deletion in *badh2* leads to the generation of a premature stop codon that would, if translated, produce a truncated non-functional protein. The *badh2* locus of rice constituting the *fgr* gene has been recognized as a major genetic determinant of fragrance [3,14,16,60,61]. On the long arm of chromosome 8 the *fgr* gene is located [61–65] and codes for the enzyme BADH, that also renders the *badh2* gene product non-functional and leads to synthesis of 2AP [62,66]. It was also found that two null fragrant of recessive alleles exist in rice are responsible for non-functional *badh2* [67], *badh2-E7* [3,68] with an 8 bp deletion and three single nucleotide polymorphisms (SNPs) in the 7th exon [69,70]. *badh2-E2* with a sequence identical to the *badh2* allele but containing a 7 bp deletion in the 2nd exon [67]. These both *badh2* alleles contribute to rice flavor [67,71]. *BADH1* is homologous with *BADH2* [71] and located on chromosome 4 in rice, both having similar molecular function [62] but its role in aroma is not yet confirmed [14,72,73]. The several conflicting reports suggested the possibilities of controlling rice aroma by several dominant or recessive genes, viz. one major QTL on chromosome 8 and two minor QTLs on chromosomes 3 and 4 [62], two to three recessive or dominant genes [74], two recessive genes [55,75,76], one major QTL located on chromosome 8 and two minor QTLs on chromosomes 4 and 12 [77], a single dominant gene [78], a dominant suppression epistasis interaction between two genes and an interaction between two genes [79]. For the first time, the confirmation if the reduced expression of *badh2* for 2AP accumulation was studied by Vanavichit et al. [80] using, RNA interference (RNAi) technology in Jasmine rice. They observed that, the strongest RNAi expression gave the strongest suppression and the highest accumulation of 2AP. In another study, transgenic rice containing RNAi by an inverted repeat of cDNA encoding Os2AP accumulated 2AP in considerable amounts [81]. Chen et al. [82] confirmed the reduced expression of the *badh2* using RNAi in rice, resulted in 2AP accumulation. Therefore, it is confirmed that *badh2* determines the accumulation of 2AP in rice. The genes responsible for fragrance in sorghum has also been characterized, as like rice, sorghum contain a premature stop codon in *BADH2* which impairs the synthesis of full length functional BADH2 protein leading to the accumulation of 2AP [73,83,84]. Yundaeng et al. [84] reported an association between the *BADH2* gene and fragrance in sorghum. Through QTL analyses they confirmed the association between BADH2 and fragrance. For the first time Juwattanasomran et al. [73] reported amino acid substitution in soybean, which is essential for functional activity of BADH2 protein, suggesting that the SNP in BADH2 is responsible for the fragrance.

BADH2, is basically involved in synthesis of an osmolyte glycinebetaine from betaine aldehyde [85]. But rice is a non accumulator of glycine betaine [86,87]. Bradbury et al. [88] found that rice BADH2 has higher activity towards gamma aminobutyraldehyde

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(GABald) and moderate activity on betaine aldehyde. Rice BADH2 was found to regulate metabolism of gamma-amino butyric acid (GABA) from GABald in rice. GABAld spontaneously cyclises to Δ^1-pyrroline, the key precursor of 2AP. This was recently confirmed by the absence of 2AP in an aromatic variety after transformation with functional *badh2* [82] and suppression of *badh2* transcript in a non-aromatic *japonica* rice callus (*O. sativa* japonica cv. Nipponbare) increases 2AP level [80].

Romanczyk et al. [24] showed increase in 2AP contents in *B. cereus* cultures when supplemented with high levels of proline, ornithine and glutamate. They also found high amounts of 2AP when carbon sources of amylose and glucose were used in cultures. Later proline, ornithine and glutamate were identified as the basic precursors for 2AP biosynthesis in rice [89,90] and *P. amaryllifolius* [91]. Costello and Henschke [25] suggested the role of ornithine as nitrogen source of 2AP via γ-amino butyraldehyde (GABAld), a product of putrescine degradation pathway in *Lactobacillus hilgardi*. GABAld spontaneously cyclises to Δ^1-pyrroline followed by to acylation at C-2 position of acyl-CoA derivatives, leading to the synthesis of 2AP. Precursor studies by Cheetangdee and Chaiseri [92] also showed glutamic acid, proline, glucose and fructose as possible precursors of 2AP in *P. amaryllifolius*. Methylglyoxal, a degradation product of sugars was identified as a carbon source for 2AP [93]. The accumulation of 2AP in rice is due to the absence of BADH2 activity, leading to an increased level of its substrate, GABald/Δ^1-pyrroline [82,88]. Availability of free GABald/Δ^1-pyrroline has been observed to be the rate controlling factor in synthesis of 2AP. The availability of GABald/Δ^1-pyrroline, is dependent on degradation of polyamines and proline and the activity of BADH2 enzyme. Utilization of GABald/Δ^1-pyrroline by BADH2 for conversion to GABA inhibits 2AP synthesis, whereas, accumulation of GABald/Δ^1-pyrroline results in increased 2AP synthesis [82].

Role of BADH2 in GABA synthesis

The synthesis of functional *badh2* in rice is associated with synthesis of GABA and if non-functional leads to synthesis of 2AP. Srivong et al. [94] also demonstrated that *BADH2* shows higher activity with GABAld. Bradbury et al. [88] expressed rice *BADH2* in *E. coli* expression vector and analyzed the purified enzymes for a range of substrates including BADH and GABAld. They reported that the optimum activity of *BADH2* with BADH was at pH 10 and that *BADH2* showed greatest activity with GABAld. Chen et al. [82] also determined the *BADH2* activity of *E. coli* expressed protein to be higher with GABAld. A functional BADH reacts with GABald to form GABA, where as a non functional form leads to accumulation of GABald which exists in equilibrium with Δ^1-pyrroline, which reacts with methylglyoxal to form 2AP [88]. GABA is a four carbon non-protein amino acid and has been shown to play a role in pollen tube growth and guidance and flower development [95]. GABA synthesis is also responsible in pH regulation, nitrogen storage, plant development and defense, as well as a compatible osmolyte and an alternative pathway for glutamate utilization [96]. GABA also plays a role in detoxification of free radicals [97], intracellular signal transduction [98] and as a natural pesticide [96]. The synthesis of GABA is regulated by two independent pathways- 1) via GABA shunt and 2) via GABald by degradation of polyamines [99]. Due to several physiological functions, GABA was thought to be involved in maintaining crop yield and tolerance to stress and diseases. BADHs assist in osmotic stress resistance by increasing the accumulation of in response to salt stress in rice [100]. But, a recent study by Fitzgerald et al. [16], no significant difference in the concentration of GABA in leaf tissue from aromatic and non-aromatic plants was detected and they concluded that inferior salt tolerance of aromatic rice is unlikely to be related to steady-state GABA levels in the whole plant. However, they came out with the possibility that this phenomenon is somewhere related to GABA metabolism and the metabolic pathway surrounding GABA production in aromatic rice may cause decreased salt tolerance without affecting steady-state GABA levels.

2AP biosynthesis and yield performance in aromatic rice

There are several aromatic cultivars, only a few of them have made it to the world market because current aromatic rice varieties produce significantly less yield than non-aromatic varieties and are also susceptible to biotic and abiotic stress and also susceptible to diseases and insects [15,16,101,102]. Basmati rice, for example, is susceptible to blast, bacterial leaf blight, stem borer and white backed plant hopper. Jasmine rice is also susceptible to brown plant hopper, blast, and bacterial leaf blight. Both traditional Basmati rice and Jasmine rice are photosensitive. They require short day length during flowering; thus, the harvest season is limited to only one crop per annum. Another important reason is because the market of aromatic rice is highly competitive; import regulations and technical trade barriers have made it difficult for newly developed aromatic rice [102]. BADH1 transcript levels exhibit a consistent increase in response to salt treatment in both aromatic and non-aromatic rice varieties [103]. However, BADH2 transcript levels did not increase. In contradiction, transgenic non-aromatic rice with inhibited expression of BADH2 by RNAi to confirm the role of BADH2 in rice fragrance [81]. These plants with inhibited BADH2 expression were shown to have decreased ability to tolerate salt stress and they concluded that BADH2 contributes to salt tolerance in rice. Recently, Fitzgerald et al. [16] also revealed that non-aromatic rice lines with specifically inhibited BADH2 are more susceptible to salt stress than wild type with normal BADH2 expression. They also found that aromatic rice lines produce very low mature seeds in comparison to non-aromatic rice lines. When aromatic rice lines were exposed to 17 mM and 22 mM NaCl stress, the mature seed production decreased by 92 % and 96.5 % respectively in comparison to non-aromatic rice lines. These results strongly suggest that BADH2 has a role in protecting rice from the effects of salt and also on producing mature seeds. Rice being non-accumulator of glycine betaine, BADH function was correlated with the synthesis of GABA from GABald [88]. Since then it was thought that the decrease in salt tolerance and less yield in rice, lacking functional BADH2 could be due to a decrease in the ability of aromatic plants to accumulate GABA. However, no significant difference in the concentration of GABA levels in aromatic and non-aromatic rice was reported by Fitzgerald et al. [16]. It is a challenge for the researchers worldwide to understand the mechanism of 2AP synthesis in aromatic rice and its correlation with reduced yield and susceptible nature to biotic and abiotic stress.

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Effect of 2AP biosynthesis on performance of *P. amaryllifolius*

P. amaryllifolius is known to harbor 10 times of 2AP more than aromatic rice [4]. However, no information on the mechanism of higher 2AP synthesis in *P. amaryllifolius* is available till the date. In our study in *P. amaryllifolius*, we first histochemically localized the major aroma compound 2AP in lower leaf epidermal papillae [104] and confirmed that lower epidermal papillae act as the storage site for 2AP [105]. The developmental pattern of papillae was also worked out in *in vitro* regenerated seedlings. It was observed stomata act as an epicenter for the development of the papillae followed by their lateral epidermal cells [106]. Further, a method was developed for identification and quantification of 2AP through headspace-solid phase microextraction (HS-SPME) coupled with Gas Chromatography- Flame Ionization Detector (GC–FID). In this analysis, along with 2AP 21 other volatiles were reported for the first time in *P. amaryllifolius* [52]. Wakte et al. [52,106] reported that *P. amaryllifolius* is susceptible to abiotic stress conditions like water deficit, elevated temperatures. The seedlings of *P. amaryllifolius* were also found susceptible to the attacks of ants and aphids. The susceptible nature to biotic and abiotic stresses might be the reason for its absence in wild as natural population. Low genetic diversity (assessed by ISSR and AFLP molecular markers) was also recorded among the clonal populations of *P. amaryllifolius* collected from peninsular India, Sri Lanka and Thailand [107]. Bradbury et al. [88] predicted that 2AP biosynthesis is the reason for loss of fertility in *P. amaryllifolius.* This view can be supported by the fact that this species is not reported to occur in the wild condition [107]. Bradbury et al. [88] reported that non-functioning of BADH2 has adverse effect on plant growth especially yield and survival. In the recent study, for the first time we reported coexistence of non-functional and functional BADH2 isoforms in *P. amaryllifolius* [108].

Future Research Directions

Fragrance character in rice is now known to affect the plant performance at larger extent. This rare metabolic disease caused due to synthesis of 2AP in rice is very complex to interpret. For better understanding, the differentially expressed genes due to mutation in BADH2 should be studied. Transformation of fragrant rice with functional BADH2 or silencing of BADH2 in non-fragrant rice can be done and differentially expressed genes can be studied. The *in-silico* analysis of these differentially expressed genes will help in better understanding the effect of 2AP biosynthesis on yield and low tolerance to stress in rice. As reported by several researchers, Δ^1-pyrroline pool is considered important for synthesis of 2AP and GABA. The over expression of ornithine decarboxylase (ODC) and diamine oxidase (DAO) genes in rice will help in increasing Δ^1-pyrroline pool and thus development of super rice variety with elevated 2AP concentration and yet with higher yield and tolerance to stress.

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