Differential levels of metabolites and enzymes related to aroma formation in aromatic indica rice varieties: comparison with non-aromatic varieties

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Abstract

Accounting for aroma production in different aromatic indica rice varieties based on variations in the levels of concerned metabolites and enzymes is poorly explored. The present investigation was, therefore, focused on unraveling the differential levels of metabolites and activities of enzymes related to aroma formation in eleven indigenous aromatic rice varieties, as compared with four non-aromatic varieties. The levels of metabolites such as proline (Pro) and Δ^1 -pyrroline-5-carboxylate (P5C), and the activity of related enzymes such as proline dehydrogenase (PDH), Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), and ornithine aminotransferase (OAT) were comparatively higher in the aromatic varieties, with Kalonunia and Tulaipanji registering the highest Pro, Kalonunia the highest P5C content, Gobindobhog with the highest PDH activity, Gobindobhog and Tulaipanji with the highest P5CS, and Pusa Basmati-1 with the highest OAT activity. The levels of putrescine (Put) and γ -aminobutyric acid (GABA) were comparatively lower in aromatic varieties, with concomitant higher diamine oxidase (DAO) activity, especially in the varieties Gobindobhog and Tulaipanji. The betaine-aldehyde dehydrogenase 2 (BADH2) enzyme activity was remarkably lesser in aromatic varieties, especially Radhunipagal and Gobindobhog. Though the metabolites such as glycine–betaine and higher polyamines such as spermidine and spermine showed no specific trend with respect to their quantitative level in either aromatic or non-aromatic varieties, they were notably lower in the aromatic varieties such as Gobindobhog, Kalonunia, and Tulaipanji, indicating a possibility of their involvement in aroma formation. Therefore, the levels of metabolites such as Pro, P5C and methylglyoxal (MG), and the activity of enzymes such as PDH, P5CS, OAT, and DAO were comparatively higher in the aromatic rice varieties than the non-aromatic ones, whereas the levels of Put, GABA, and BADH2 were lower. Overall, the present study showed that there exist variations in the accumulations of such metabolites as well as differential activity of enzymes controlling their production, which altogether regulate generation of aroma in aromatic varieties.

Keywords Aromatic rice · Betaine-aldehyde dehydrogenase 2 · Enzyme activity · Metabolite level

Introduction

Aromatic or fragrant rice has been in great demand in the international market and considered as an asset in many countries due to its contributing features such as pleasing aroma kernel elongation after cooking, and taste. Though the increase in demand of the indigenous aromatic rice varieties

is seen throughout the World, it is more prominent mainly in the countries of East and South East Asia, United States, and Middle East. The Indian economy also appreciates the importance of aromatic rice due to their increasing demand in the global market for desirable features such as volatile flavor and palatability. West Bengal is one of the leading Indian states in rice production, thereby earning the title of ‘rice bowl’ of India. West Bengal is especially famous for its vast diversity of indigenous aromatic rice varieties such as Kalonunia, Tulaipanji, Kanakchur, Chini atop, Gobindobhog, Badshabhog, Dhanaguri, Rupsal, Kalamkati, etc. In addition, Pusa Basmati-1 is the well-known aromatic rice, grown mainly in Punjab, Uttar Pradesh, Haryana, and Uttarakhand. India is the leading exporter of Basmati and

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Non-Basmati aromatic rice varieties in the international market.

Aromatic rice is well known for its characteristic ‘nutty or pop-corn’ flavor which is emitted while cooking (Huang et al. 2008). More than 200 volatile compounds are considered to be the contributors of rice aroma, including (E, E)-2, 4-decadienal, hexanal, octanal, nonanal, 4-vinylphenol, and 4-vinyl guaiacol, with 2-acetyl-1-pyrroline (2-AP) as the primary and principal volatile aromatic compound (Li et al. 2016). The biosynthetic pathway of aroma formation has been deciphered in recent times, where a number of metabolites such as proline (Pro), glutamate, and ornithine are assumed to be the probable precursors, since they produce the common metabolite Δ^1 -pyrroline-5-carboxylate (P5C), mediated by three respective enzymes, viz., proline dehydrogenase (PDH), Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), and ornithine aminotransferase (OAT) (Huang et al. 2008). Vanavichit and Yoshihashi (2010) proposed that polyamine (PA) pathway also contributes towards aroma production. The involvement of putrescine (Put) was also suggested, since Δ^1 -pyrroline, a product of Pro catabolism, via Put oxidation, is the immediate precursor of pyrroline ring of 2-AP (Costello et al. 2001). The diamine oxidase (DAO) is the sole enzyme responsible for the conversion of Put to γ -aminobutyraldehyde (GABald), which cyclizes to either Δ^1 -pyrroline (Gill and Tuteja 2010) or produces γ -aminobutyric acid (GABA), depending, respectively, on whether the enzyme betaine-aldehyde dehydrogenase2 (BADH2) is non-functional (mutated) or active (Nadaf et al. 2014). The same GABald, which normally is converted to GABA by functional BADH2 in non-aromatic rice, is cyclized to Δ^1 -pyrroline by truncated version of BADH2 enzyme in aromatic rice (Chen et al. 2008). The involvement of methylglyoxal (MG) as determinant of non-enzymatic conversion of all the metabolites to generate fragrance is also established (Huang et al. 2012). The other enzyme BADH1, an isoform of BADH2 with similar molecular function, is not considered as a factor responsible in the aroma pathway (Bradbury et al. 2005). BADH1 is responsible for the biosynthesis of glycine–betaine (gly–bet) from betaine aldehyde (Srivong et al. 2008). Rice plants are normally recognized as non-accumulator of gly–bet (Nakamura et al. 1997). Moreover, rice BADH2 shows more affinity towards GABald than betaine aldehyde (Bradbury et al. 2008). In addition, Put also helps in the synthesis of higher PAs, spermidine (Spd), and spermine (Spm) by the enzymes spermidine synthase (SPDS) and spermine synthase (SPMS), respectively (Gill and Tuteja 2010), the connection of which with aroma production is not reported till date.

Indian-scented rice constitutes a diverse group, with short-, medium-, and long-sized grains, and varying aroma content, with some varieties showing more pronounced aroma than the others. However, whether such differences in

aroma intensity among the varieties could be accounted for by the variation in the levels of aforementioned metabolites or enzymes has not been explored earlier, despite the characterization of aroma pathway. There is dearth of adequate experimental reports on the different indigenous aromatic rice varieties, especially with regard to the associated biochemical pathways related to aroma production, as compared with non-aromatic rice varieties. Therefore, the main purpose of the current study was to investigate the different biochemical parameters, viz., metabolites and enzymes, connected with aroma production in both the seedlings and seeds of eleven aromatic rice varieties, as compared with four non-aromatic rice varieties. The metabolites such as Pro, P5C, PA (Put, Spd and Spm), GABA, MG and gly–bet, and activity of enzymes, viz., PDH, P5CS, OAT, DAO, and BADH2, related to aroma pathway were examined and compared among the different aromatic rice varieties as against the non-aromatic varieties. The differences in metabolite or enzyme levels hypothesized in the present study, both in the seedlings and seeds of the aromatic cultivars varying in their aroma level, will definitely lead to aroma characterization based on the levels of several regulatory components of aroma biosynthetic pathway.

Materials and methods

Plant materials and growth conditions

Seeds of *Oryza sativa* L. aromatic varieties, Gobindobhog (GB), Kalonunia (KN), Tulaipanji (TP), Radhunipagal (RP), Seetabhog (SB), Kataribhog (KB), Pusa Basmati-1 (PB-1), Gandheshwari (GS), Radhatilak (RT), IET-21261, and Paramanya (PM) were collected from Bidhan Chandra Krishi Viswavidyalaya, Nadia, West Bengal or Chinsurah Rice Research Station, Hooghly, West Bengal. The non-aromatic rice varieties, IR-64, Satabdi (SA), Khitish (KT), and Swarna (SW), were procured from Chinsurah Rice Research Station, Hooghly, West Bengal. The seeds were surface sterilized with 0.1% (w/v) HgCl_2 for 20 min, washed extensively, imbibed in deionized water for 18 h, and allowed to germinate over water-soaked sterile gauge in Petridishes at 37 °C in dark for three days. The uniformly germinated seedlings were further grown at 32 °C under 16 h light and 8 h dark photoperiodic cycle with 50% relative humidity and 700 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a plant growth chamber as standardized in our earlier report (Roychoudhury et al. 2008). The 12-day-old seedlings were harvested and samples of equal fresh weight were homogenized in liquid nitrogen for different biochemical assays. Equal weight of dehusked rice seeds of the above-mentioned varieties was also used for the same assays. Since our main intention in this work was to make a relative comparison of concerned

metabolites and enzymes for aroma production among different varieties, it could be performed irrespective of early or late stage of seedling development. Srivong et al. (2008) have also used in their study 14-day-old seedlings of four cultivars of Thai rice for characterization of fragrant gene and BADH activity assay.

Estimation of endogenous proline (Pro)

Free Pro content was determined according to the procedure of Bates et al. (1973). About 0.5 g of seedlings or 3 g of seeds were homogenized in 3% (w/v) aqueous sulfosalicylic acid and the absorbance of the red chromophore was measured spectrophotometrically at 520 nm. Pro content was estimated by comparing against a standard curve. The contents were expressed as $\mu\text{g g}^{-1}$ fresh weight (FW).

Proline dehydrogenase (PDH, EC 1.5.99.8) assay

The activity of PDH enzyme was determined according to the method of Costilow and Cooper (1978). 0.5 g of seedlings or seeds were extracted with 0.1 M potassium phosphate buffer, pH 7.5, containing 0.5% (w/v) polyvinylpyrrolidone (PVPP) and 3 mM ethylene diamine tetraacetic acid (EDTA). The reaction mixture contained 0.1 M sodium carbonate, pH 10.3, 20 mM L-Pro and 10 mM nicotinamide adenine dinucleotide (NAD). The enzyme extract was added and the increase in absorbance was recorded at 340 nm. The PDH activity was expressed as units mg^{-1} protein (one unit is defined as an increase in absorbance of 0.01 per min at 340 nm).

Estimation of endogenous Δ^1 -pyrroline-5-carboxylate (P5C) levels

About 0.5 g of seedlings or 3 g of seeds were homogenized in 3% (w/v) aqueous sulfosalicylic acid (Miller et al. 2009). The reaction mixture comprised of enzyme extract, 10% (v/v) trichloroacetic acid (TCA) and 0.1 M *ortho*-aminobenzaldehyde. The absorbance was noted at 440 nm. The concentration of P5C was calculated using the molar extinction coefficient of P5C (molar extinction coefficient 2580 mol cm^{-1}) (Mezl and Knox 1976).

Estimation of Δ^1 -pyrroline-5-carboxylate synthetase (P5CS, EC 1.5.1.12) activity

The P5CS activity was done following the method of Stines et al. (1999). 0.5 g of seedlings or seeds were extracted with 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 10 mM β -mercaptoethanol, 1% (w/v) PVPP, 5 mM MgCl_2 , and 0.6 M KCl. The total reaction mixture contained 75 mM glutamic acid, 0.1 M Tris Cl, pH 7.2, 20 mM MgCl_2 ,

5 mM ATP, 0.4 mM NADPH, and enzyme extract. The absorbance was measured at 340 nm. The enzyme activity was estimated in terms of the consumption of NADPH. The P5CS activity was expressed as units μg^{-1} protein (one unit is defined as an increase in $0.001 A_{340}$ per minute).

Estimation of ornithine aminotransferase (OAT, EC 4.1.1.17) activity

The activity assay of OAT enzyme was performed according to the modified method of Chen et al. (2001). 0.5 g each of seedling and seed tissues were extracted with a buffer constituting 50 mM Tris-Cl, pH 7.5, together with 7 mM MgCl_2 , 1 mM KCl, 3 mM EDTA, 1 mM DL-DTT, and 5% (w/v) PVP. The reaction mixture contained 100 mM potassium phosphate buffer, pH 8.0, 50 mM ornithine, 20 mM α -ketoglutarate, 1 mM pyridoxal-5-phosphate, and the enzyme extract (100 μg of total protein). The reaction was terminated by adding 10% (w/v) TCA and 0.25% *o*-aminobenzaldehyde and incubating for 60 min. The absorbance was noted at 440 nm. The activity was calculated on the basis of the molar extinction coefficient of $2.68 \text{ mM}^{-1} \text{ cm}^{-1}$. OAT activity was expressed as $\text{mmol g}^{-1} \text{ FW}$.

Estimation of endogenous polyamine (PA) levels

The PA level was estimated according to the earlier method (Roychoudhury et al. 2012) with minor modifications. About 2.5 g of seedlings or 5 g of seeds were homogenized with 10% (v/v) perchloric acid. The dansylated amines were separated on an activated silica gel TLC plate and the individual PAs were quantified spectrofluorometrically at the excitation and emission wavelengths of 360 and 506 nm, respectively.

Estimation of diamine oxidase (DAO, EC 1.4.3.6) activity

The DAO activity was measured according to the method of Holmstedt et al. (1961). 0.5 g of seedlings or seeds was extracted with 50 mM potassium phosphate buffer, pH 7. The total reaction mixture contained 0.1 M phosphate buffer, pH 7, 10 mM Put and the enzyme extract (100 μg of total protein). The reaction was terminated by adding 10% (w/v) TCA followed by 10 mg ml^{-1} of *o*-aminobenzaldehyde. The absorbance was measured at 430 nm and DAO activity calculated using the extinction coefficient of $1.86 \times 10^3 \text{ mol}^{-1} \text{ cm}^{-1}$.

Estimation of endogenous γ -aminobutyric acid (GABA) content

The endogenous GABA content was estimated according to the method of Kitaoka and Nakano (1969). The absorbance

was recorded at 690 nm, with ethanol as blank. The endogenous GABA levels were quantified by comparing the absorbance against a standard curve.

Determination of methylglyoxal (MG) content

Methylglyoxal was measured following the method of Wild et al. (2012) by extracting plant samples in 5% (v/v) perchloric acid. The MG estimation was done by adding sodium dihydrogen phosphate and *N*-acetyl-L-cysteine to a final volume of 1 ml. The absorbance was recorded after 10 min at wavelength 288 nm and MG content was calculated using a standard curve of known concentration of MG.

Estimation of betaine-aldehyde dehydrogenase2 (BADH2, EC 1.2.1.8) activity

The BADH2 enzyme activity was determined according to Bradbury et al. (2005). About 0.5 g of seedlings or seeds were extracted in a buffer containing 50 mM HEPES–KOH, pH 8, 1 mM EDTA, 20 mM sodium metabisulfite, 10 mM sodium borate, 5 mM sodium ascorbate, and 5 mM dithiothreitol (DTT). The total reaction mixture of 1 ml contained 50 mM HEPES–KOH, pH 8, 10 mM DTT, and 37 mM 4-aminobutyraldehyde (AB-aldehyde substrate) (Srivong et al. 2008). The reaction was initiated by adding 1.5 mM NAD⁺. The freshly prepared enzyme extracts (10 µg of total protein) were examined against the AB-aldehyde substrate. The BADH2 activity was measured by recording the increase in NADH consumption at 340 nm for 10 min. The enzyme activity was calculated using the molar extinction coefficient of NADH (6220 M⁻¹ cm⁻¹) and the activity expressed in nmol min⁻¹ µg⁻¹ protein.

Estimation of endogenous glycine–betaine (gly–bet) content

The gly–bet content was estimated according to the method of Grieve and Grattan (1983) with minor modifications. The seedlings or seeds (0.5 g) were homogenized, and the supernatant was diluted 1:1 with 2 N H₂SO₄, cooled in ice water for 1 h, followed by the addition of 0.2 ml of cold KI–I₂ (potassium tri-iodide) reagent. The periodide crystals obtained were dissolved in 1, 2-dichloroethane. After 2 h, the absorbance was measured at 365 nm with gly–bet as standard. The gly–bet content was expressed as µg g⁻¹.

Statistical analyses

All the experimental data values were means from three independent series, each done with three replicates, and the results presented as mean ± standard error. The significance of data was statistically evaluated by Student's *t* test

at $p \leq 0.05$, using the Windows 98/Microsoft Excel 97 computer package for significance.

Results

Pro content and PDH activity

The Pro content was found to be 1.2–2.3 times higher in the seedlings of aromatic rice varieties as compared to non-aromatic ones. In case of seedlings, the aromatic varieties KN and TP showed the highest level of Pro, while PM the lowest level (2.3 times lower than KN and TP). The overall trend of Pro content in the seedlings can be represented as: KN = TP > GB > SB > GS > RP > PB-1 > RT > KB > IET-21261 > PM > SA > IR-64 > KT > SW (Fig. 1a). In case of seeds, the aromatic variety KN showed the maximum endogenous Pro content, while GS the least (2.3 times lower than KN). The overall trend of Pro content in the seeds is represented as: KN > PB-1 > KB > RP > GB > SB > TP > PM > RT > IET-21261 > GS > KT = SW > SA > IR-64 (Fig. 1b).

The aromatic rice varieties showed higher PDH enzyme activity in both the seedlings and seeds, as compared to the non-aromatic varieties. GB showed the highest PDH activity, while PM the lowest amongst all the aromatic varieties. The overall trend in case of seedlings was: GB > KN > KB > TP > RP > PB-1 > SB > GS > RT > IET-21261 > PM > IR-64 > KT > SW > SA (Fig. 1c), while that in seed was: GB > KN > RP > TP > KB > SB > IET-21261 > PB-1 > RT > GS > PM > KT > IR-64 > SA > SW (Fig. 1d).

Endogenous P5C level, P5CS, and OAT activity

The seedlings and seeds of the aromatic variety KN showed the maximum amount of endogenous P5C content, while PM the least (2.0 times lower than KN). The P5C content in seedlings followed the trend: KN > GB > RP > TP > IET-21261 > SB > GS > KB > PB-1 > RT > PM > IR-64 > SA > KT > SW (Fig. 2a). The overall trend of the P5C content in the seeds can be represented as: KN > KB > GB > RP > TP > PB-1 > RT > SB > GS > IET-21261 > PM > SW > IR-64 > KT > SA (Fig. 2b).

The results of P5CS assay showed aromatic rice varieties to have 1.3–2.6 times higher enzyme activity in the seedlings and up to 1.3 times higher enzyme activity in the seeds, as compared to non-aromatic varieties. In case of seedlings, GB showed the highest P5CS activity, while RT the least (2.6 times lower than GB) among the aromatic varieties. The examined varieties showed the following trend: GB > KN > KB > SB > TP > PB-1 > GS > RP > IET-21261 > PM > RT > IR-64 > SA > KT > SW (Fig. 2c). In case of seeds, TP showed the highest P5CS activity, while IET-21261 the least (1.3 times lower than TP) among the aromatic varieties. The

Fig. 1 Accumulation of Pro in the seedlings (a) and seeds (b) and estimation of PDH activity in the seedlings (c) and seeds (d) of aromatic rice varieties, viz., GB, KN, TP, RP, SB, KB, PB-1, GS, RT, IET-21261, and PM, compared with non-aromatic varieties, IR-64, SA, KT, and SW. Data are the mean value ($n = 3$) $\pm$ SE. The SE in each case is represented by the vertical bar in each graph. The different letters (a–k) indicate statistically significant differences at $p \leq 0.05$

trend of P5CS activity in seeds can be represented as: TP > GB > KN > GS > RT > RP > KB > SB > PB-1 = PM > IET-21261 > SW > SA > KT > IR-64 (Fig. 2d).

The aromatic rice varieties showed comparatively higher OAT activity, up to 1.4 times higher in case of seedlings and 1.3 times higher in case of seeds, as compared to the non-aromatic varieties. The highest OAT activity was observed in PB-1 seedlings and seeds. The overall trend of the OAT activity in seedlings was as follows: PB-1 > TP > RP > GB > KN > GS > IET-21261 > KB > RT > SB > PM > KT > SW > SA > IR-64 (Fig. 2e), while seeds showed the following trend: PB-1 > GS > RP > GB > TP > KN > KB > IET-21261 > RT > PM > SB > KT > SW > IR-64 > SA (Fig. 2f).

Endogenous Put, DAO activity, and GABA

The endogenous Put content was estimated to be higher in the non-aromatic rice varieties, viz., 2.1–7.3 higher in seedlings and 1.4–5.0 times higher in seeds, as compared to the aromatic rice varieties. The highest Put content in seedlings and seeds was registered in IR-64. The Put content in seedlings followed the trend: GB < GS < TP < KN < RP < SB < RT < IET-21261 < PB-1 < KB < PM < SW < SA < KT < IR-64 (Fig. 3a), while in seeds, the general trend was: TP < GB < RP < KN < GS < RT < IET-21261 < PM < SB < PB-1 < KB < SW < SA < KT < IR-64 (Fig. 3b).

The aromatic rice varieties showed comparatively higher DAO activity, 1.2–1.4 times higher in seedlings and almost up to 1.2 times higher in seeds, than the non-aromatic varieties. The highest DAO activity among aromatic varieties was recorded in TP seedlings, while the lowest in PB-1 (1.4 times lower than TP). The overall trend of the DAO activity in the seedlings was as follows: TP > GB > RP > KN > KB > SB > PM > GS > IET-21261 > RT > PB-1 > KT > IR-64 > SW > SA (Fig. 3c). In case of seeds, GB showed the highest DAO activity and KB the lowest (1.2 times lower than GB) among aromatic varieties. The DAO activity in seeds followed the trend: GB > RP > TP > PB-1 > KN > SB > RT = IET-21261 > PM > GS > KB > KT > IR-64 > SW > SA (Fig. 3d).

The GABA content in seedlings appeared to be higher in the non-aromatic varieties, viz., 1.7–2.0 times higher in seedlings and 2.3–3.6 times higher in seeds, as compared to the aromatic varieties. The overall trend can be depicted as: GB < IET-21261 < PB-1 < PM < TP < KN < RT < RP < KB < SB < GS < SW < SA < IR-64 < KT (Fig. 3e) in seedlings, while in case of seeds, GB < SB = GS < KB < KN < RP < TP = PB-1 < RT < IET-21261 < PM < SW < IR-64 < KT < SA (Fig. 3f).

MG content

The aromatic rice varieties showed comparatively higher MG content, viz., 1.4–2.8 times higher in seedlings and

Fig. 2 Accumulation of P5C in the seedlings (a) and seeds (b) and estimation of P5CS and OAT activity in the seedlings (c and e, respectively) and seeds (d and f, respectively) of aromatic rice varieties, viz., GB, KN, TP, RP, SB, KB, PB-1, GS, RT, IET-21261, and PM, compared with non-aromatic varieties, IR-64, SA, KT, and SW. Data are the mean value ($n = 3$) $\pm$ SE. The SE in each case is represented by the vertical bar in each graph. The different letters (a–o) indicate statistically significant differences at $p \leq 0.05$

1.1–2.9 times higher in seeds as compared to non-aromatic varieties. In case of seedlings, the highest MG content was recorded in RP, while the lowest in PM (2.8 times lower than RP) among aromatic varieties. The overall trend of the endogenous MG content in the seedlings was as follows: RP > PB-1 > GB > SB > TP > KB > GS > IET-21261 > KN > RT > PM > SA > KT > SW > IR-64 (Fig. 4a). In case of seeds, IET-21261 showed the highest MG content and PM accounted for the lowest (2.9 times lower than IET-21261) among aromatic varieties. The overall trend of the MG content in the seeds was as follows: IET-21261 > GB > KB > TP = GS > RP > SB > RT > KN = PB-1 > PM > KT > SA > SW > IR-64 (Fig. 4b).

BADH2 enzyme activity

The BADH2 enzyme activity in the seedlings and seeds was 3.7–7.4 times and 4.0–14.7 times higher, respectively, in the non-aromatic varieties with respect to the aromatic ones. The highest BADH2 activity in both seedlings and seeds was documented in KT. The overall pattern in seedlings was as follows: RP < GB < TP < KN < GS < SB < RT < IET-21261 < KB = PB-1 < PM < IR-64 < SW < SA < KT (Fig. 4c). The order of the varieties in terms of BADH2 activity in seeds was: RP < GB < RT < KN = GS < TP < KB < SB < IET-21261 < PM = PB-1 < IR-64 < SA < SW < KT (Fig. 4d).

Fig. 3 Accumulation of Put and GABA in the seedlings (**a** and **e**, respectively) and seeds (**b** and **f**, respectively) and estimation of DAO activity in the seedlings (**c**) and seeds (**d**) of aromatic rice varieties, viz., GB, KN, TP, RP, SB, KB, PB-1, GS, RT, IET-21261, and PM, compared with non-aromatic varieties, IR-64, SA, KT, and SW. Data are the mean value ($n = 3$) $\pm$ SE. The SE in each case is represented by the vertical bar in each graph. The different letters (a–o) indicate statistically significant differences at $p \leq 0.05$

Endogenous gly–bet, Spd, and Spm content

The level of gly–bet was comparable and quite similar in both aromatic and non-aromatic varieties. The highest gly–bet content was recorded in the seedlings of SA, while the lowest in GB. The non-aromatic varieties in general showed slightly higher levels of gly–bet in the seedlings following the trend: GB < GS < PM < RP < IET-21261 < KN < RT < SB < KB < PB-1 < TP < SW < IR-64 < KT < SA (Fig. 5a). In case of seeds, the non-aromatic variety SA again showed the highest amount of endogenous gly–bet; however, most of the varieties, whether aromatic or non-aromatic (except GB, GS, RT, IET-21261, and PM) showed negligible differences in the level of gly–bet in seeds. GB constituted the variety with the lowest gly–bet level and the

overall trend can be depicted as: GB < GS < PM < IET-21261 < RT < SB < RP < KN < TP < KB < PB-1 < KT < SW < IR-64 < SA (Fig. 5b).

The Spd content in seedlings was the highest in IET-21261 followed by a slightly lowered content in PB-1 and SW. The high aroma-containing varieties such as GB, KN, TP, RP, and GS were in general amongst the lower Spd-containing varieties, with the lowest content recorded in GB. The Spd accumulation in seedlings followed the trend: GB < KN < TP < GS < RP < KB < PM < SB < IR-64 < SA < RT < KT < SW < PB-1 < IET-21261 (Fig. 5c). In case of seeds, the highest Spd level was detected in the non-aromatic variety KT. The varieties such as GB, KN, TP, and RP with high aroma were amongst the low Spd-containing varieties with KN

Fig. 4 Estimation of MG content and BADH2 enzyme activity in the seedlings (**a** and **c**, respectively) and seeds (**b** and **d**, respectively) of aromatic rice varieties, viz., GB, KN, TP, RP, SB, KB, PB-1, GS, RT, IET-21261, and PM, compared with non-aromatic varieties, IR-64, SA, KT and SW. Data are the mean value ($n = 3$) $\pm$ SE. The SE in each case is represented by the vertical bar in each graph. The different letters (a–o) indicate statistically significant differences at $p \leq 0.05$

registering the lowest Spd content. However, the non-aromatic variety IR-64 also showed less Spd accumulation in seeds, even lesser than RP. The trend in seed Spd level was: KN < TP < GB < IR-64 < RP < GS < PB-1 < PM < RT < IET-21261 < SB < SW < KB < SA < KT (Fig. 5d).

The highest level of Spm in seedlings was recorded in the non-aromatic variety SW, followed by the aromatic variety PB-1. The aromatic varieties such as GB, KN, and KB were amongst the lower Spm-containing varieties with the lowest level in KN. The overall trend was as follows: KN < KB < GB < TP < RP < GS < PM < KT < IR-64 < IET-21261 < SB < SA < RT < PB-1 < SW (Fig. 5e). In case of seeds, the non-aromatic variety KT showed the highest Spm content. The high aroma-containing varieties such as GB, KN, TP, and RP were amongst the low Spm-containing varieties; the lowest level documented in TP seeds. The seeds of IR-64 also accumulated less Spm, showing a similar pattern as Spd accumulation in seeds. The observed trend can be represented as: TP < KN < RP < GB < IR-64 < GS < IET-21261 < PB-1 < RT < PM < SB < SW < KB < SA < KT (Fig. 5f).

In a nutshell, the levels of metabolites such as Pro, P5C, and MG, and the activity of enzymes such as PDH, P5CS, OAT, and DAO were comparatively higher in the aromatic rice varieties than the non-aromatic ones, whereas the levels of Put, GABA, and BADH2 were considerably lower.

Discussion

Aromatic rice with their characteristic volatile flavor and pleasant aroma is of considerable demand in the international market in recent times. The aroma trait is dependent on the type of the aromatic cultivar, with some cultivars registering considerably higher aroma level than the others. Given the demand for fragrant rice, a comprehensive study of the different biochemical pathways and the level/activity of candidate metabolites/enzymes that are determinants of fragrance in several aromatic rice cultivars would enable us to screen such cultivars in terms of superior aromatic trait, against the non-aromatic cultivar. Aroma accumulation is attributed to inactive version of the BADH2 enzyme, which in turn is connected with the formation of the compound Δ^1 -pyrroline, which further undergoes a non-enzymatic reaction with MG to generate aroma in aromatic rice (Nadaf et al. 2014). To derive an integrated picture of the overall biochemical pathways related to aroma formation in indigenous aromatic indica rice varieties, the level of several metabolites and enzymes connected with aroma has been investigated in eleven aromatic rice cultivars and compared with four non-aromatic cultivars as control.

Experiments with seedlings and callus of a fragrant rice variety (Khao Dawk Mali 105) showed that Pro is

Fig. 5 Accumulation of gly-bet, Spd and Spm in the seedlings (a, c, and e, respectively) and seeds (b, d, and f, respectively) of aromatic rice varieties, viz., GB, KN, TP, RP, SB, KB, PB-1, GS, RT, IET-21261, and PM, compared with non-aromatic varieties, IR-64, SA, KT, and SW. Data are the mean value ($n = 3$) $\pm$ SE. The SE in each case is represented by the vertical bar in each graph. The different letters (a–o) indicate statistically significant differences at $p \leq 0.05$

the main amino acid precursor and nitrogen source of the major aroma compound, 2-AP (Yoshihashi et al. 2002). A threefold increase in aroma content was reported with Pro supplementation. Romanczyk et al. (1995) also reported an increase in aroma generation in *Bacillus cereus* cultures when agar medium was supplemented with Pro. L-Pro was also found to improve aroma in callus of rice cells grown in vitro (Nadaf et al. 2014). Our data is in uniformity with earlier observations, since all the eleven aromatic rice varieties (KN and TP in particular) exhibited higher Pro content in both seedlings and seeds, as compared to non-aromatic varieties. Such higher level of Pro is largely channelized towards aroma production in all the aromatic varieties, since Pro is the main nitrogen source for aroma compound (Yoshihashi et al. 2002). P5C, the key precursor

of Δ^1 -pyrroline, can be synthesized from the oxidation of L-Pro by the enzyme PDH (Huang et al. 2007). Mo et al. (2016) have reported that supplementation of Zn and La in culture medium of aromatic rice increased the Pro, PDH, and aroma level of the detached aromatic panicles, thereby showing a positive correlation among the three parameters. Our data also showed comparatively much higher PDH activity in the seedlings and seeds of aromatic rice varieties, with GB recording the highest.

Experimental evidences earlier have shown that in addition to L-Pro, glutamic acid and ornithine are candidates for aroma formation in rice (Nadaf et al. 2014). In general, OAT and P5CS enzymes are involved, respectively, in the conversion of ornithine and glutamate into the common metabolite, P5C. Huang et al. (2008) showed higher P5C

content in aromatic rice calli compared with non-aromatic ones. Huang et al. (2012) also correlated higher levels of P5C with aroma formation and found positive relationships of P5C content with P5CS and OAT activities. In rice, the bi-functional activity of P5CS converts glutamate to glutamate γ -semialdehyde (GSA) which cyclizes to P5C spontaneously. This P5C is later reduced to Pro by the catalytic activities of P5C reductase (Poonlaphdecha et al. 2012). Kaikavoosi et al. (2015) reported that the overexpression of *P5CS* gene led to two-fold elevation in aroma level in transgenic seeds of aromatic rice variety. Li et al. (2016) reported positive correlation between aroma content and enzyme activity of OAT and P5CS, along with P5C contents of rice varieties. Our results also showed increased amounts of P5C, along with higher P5CS and OAT activity in the seedlings and seeds of all the eleven aromatic varieties, as compared with the non-aromatic ones. The P5C level was the highest in the aromatic variety KN, P5CS activity in GB seedlings, and TP seeds, while OAT activity in PB-1. Overall, the aromatic rice varieties such as GB, KN, TP, RP, and GS showed higher Pro and P5C levels and increased activity of enzymes such as P5CS, OAT, and PDH, as compared to non-aromatic varieties. This depicts a possible interrelation between P5C level and P5CS and OAT activity with aroma production in the indigenous aromatic rice varieties chosen for our studies.

Costello and Henschke (2002) suggested the role of ornithine as nitrogen source of 2-AP via γ -amino butyraldehyde (GABald), a product of Put degradation pathway in *Lactobacillus hilgardii*. The enzyme DAO is involved in the conversion of Put to GABald (Struve and Christophersen 2003). The GABald further cyclizes spontaneously to Δ^1 -pyrroline or it produces GABA, depending on the absence or presence of functional BADH2 enzyme. Utilization of GABald by BADH2 for conversion to GABA inhibits aroma production in non-aromatic rice varieties, whereas accumulation of Δ^1 -pyrroline due to non-functional BADH2 enzyme results in enhanced aroma production (Chen et al. 2008). Deshmukh et al. (2015) have shown that rhizobacterial isolates from Basmati and Non-Basmati rice influenced the production of aroma in the culture when the media was supplemented with Put and some other precursors such as Pro, ornithine, and glutamate. Hence, Put is degraded and utilized for aroma production, which accounts for its consistent lower levels in all the eleven aromatic rice varieties (especially GB and TP) with comparatively higher levels in non-aromatic rice varieties, as observed in our experiment. Roychoudhury et al. (2008) have also shown earlier that Put level was significantly lower in the control seedlings of the aromatic rice variety GB, as compared to two other non-aromatic rice varieties, Nonabokra and M-1-48, thus supporting our present observation. Fogel et al. (1979) proposed that accumulation of Δ^1 -pyrroline is a direct indication of the enhanced DAO activity for the production of more GABald from Put. In

the current study, increased DAO activity was noted in all the aromatic rice cultivars, particularly in TP and GB, as compared to non-aromatic counterparts. Su and Bai (2008) showed increasing DAO activity with decrease in Put content. This supports our data which also shows increase in DAO activity with a decrease in Put level in aromatic cultivars, thus indicating exhaustion of Put for more production of GABald via DAO activity, while lesser Put is utilized for this purpose in non-aromatic cultivars. The decreased BADH2 enzyme activity (especially in RP), concomitant with lowered GABA level (particularly in GB), was strikingly noteworthy in our study in all the aromatic rice cultivars, whereas the BADH2 activity and GABA levels were higher in the non-aromatic cultivars. While the lowered BADH2 activity is responsible for more aroma production in aromatic rice varieties, GABA synthesis occurs at the expense of aroma production in the non-aromatic rice due to the availability of functionally more active BADH2 (Wakte et al. 2011). Low GABA content was also noted in isogenic lines of aromatic rice, as compared to non-aromatic lines (Chen et al. 2008). Wakte et al. (2011) showed that *Brassica latifolia* petals with higher aroma levels showed lower BADH2 activity. Vanavichit et al. (2005) also reported that suppression of BADH2 through RNAi technology in Jasmine rice led to reduced BADH2 activity and accounted for higher aroma production.

The BADH isozymes, viz., BADH1 and BADH2 have been shown to catalyze the conversion of betaine aldehyde to gly-bet. Betaine aldehyde is particularly acted upon by NAD⁺-dependent BADH1 to ultimately form gly-bet (Nakamura et al. 1997). Rice is normally considered as a non-accumulator of gly-bet (Nadaf et al. 2014). A very negligible difference was observed between aromatic and non-aromatic rice cultivars with regard to gly-bet accumulation, signifying that this metabolite or the main isozyme, BADH1 involved in its production, are of less importance with context to aroma production. Wakte et al. (2011) showed that the enzyme BADH2 is substrate specific and helps in the formation of GABA by the oxidation of GABald rather than utilizing betaine aldehyde to form gly-bet. Bradbury et al. (2008) reported that BADH2 showed most specificity towards GABald, moderate affinity towards betaine aldehyde and γ -guanidinobutyraldehyde (GGBald) and no affinity for NAGABald (*N*-acetyl- γ -aminobutyraldehyde).

The higher PAs, namely, tri-amine Spd and tetra-amine Spm, are synthesized from diamine Put via the enzymes SPDS and SPMS, respectively. Although the role of Spd and Spm in plant growth, development, and regulating abiotic stress tolerance in plants is well characterized, their probable involvement in aroma production in aromatic rice cultivars is not reported. In the present study, though no regular trend with respect to the level of Spd and Spm content was noted in either aromatic or non-aromatic rice cultivars, some of

the aromatic cultivars such as GB, KN, TP, and RP showed comparatively lower Spd and Spm content than the other aromatic cultivars. Duhaze et al. (2002) reported the formation of Put from Spd in their study. The lower level of Spd in the aforementioned varieties may indicate the possibility of greater cyclization of Spd to Put and hence more GABald formation. Such cyclization of Spd to Put also accounts for lesser Spm content in such varieties as noted in our study, since lesser Spd is available for conversion to Spm by the enzyme SPMS. Specific polyamine oxidase (PAO) enzymes have been found only in grasses (Smith 1983). These PAOs oxidize Spd to 1, 3-diaminopropane (DAP), along with the release of hydrogen peroxide and pyrroline, or in case of Spm oxidation, a substituted pyrroline (Smith 1983). Even, Put is directly oxidized by DAO to H_2O_2 , NH_3 , and pyrroline. In case of aromatic cultivars, probably this PA degradation pathway is highly operative, so that more Spd and Spm are broken down into pyrroline, which subsequently may contribute towards aroma production. Huang et al. (2012) suggested that the involvement of acetyl-CoA groups or MG determine the enzymatic or non-enzymatic conversion of Δ^1 -pyrroline to aroma compound 2-AP. This fact also justifies the higher MG content as observed in the seedlings and seeds of all the eleven aromatic varieties in our observation, as compared to non-aromatic varieties. Acetylation reactions are quite common in PA pathway and acetylated PAs have actually been reported in higher plants (Del Duca et al. 1995). In fact, the presence of Spd/Spm *N*-acetyltransferases (SSAT) has been reported that uses acetyl CoA to form *N*-acetylspermidine and *N*-acetylspermine (acetylated PAs). *N*-acetylspermidine is further converted to Put via the enzyme PAO (Duhaze et al. 2002). This provides a clue regarding the possible role of Spd and Spm in aroma production in at least some of the aromatic rice cultivars, if not all. However, further analysis involving the regulation of expression of genes involved in PA metabolism, together with the quantitative estimation of the aroma content in different aromatic rice cultivars in the future will further strengthen the foundation of our proposition.

Based on all the above observations, a model has been proposed (Fig. 6) so as to correlate and integrate the different metabolites and enzymes altogether, thereby establishing the probable metabolic pathway of aroma production in indigenous aromatic rice varieties examined in the present study. The higher Pro content, together with higher activity of PDH, OAT, and P5CS in the aromatic rice varieties, might also result in higher accumulation of P5C, which is known to be synthesized from Pro, ornithine, and glutamate utilizing the enzymes PDH, OAT, and P5CS, respectively. The low accumulation of Put in all the aromatic varieties is attributed to higher DAO activity, so that more GABald is produced than non-aromatic varieties. Because of the lower BADH2 enzyme activity in all the aromatic varieties, GABald is

Fig. 6 Model depicting the possible metabolic pathway of aroma production in indigenous fragrant rice varieties and crosstalk among the metabolites and enzymes. The different enzymes involved are as follows: I: proline dehydrogenase (PDH); II: Δ^1 -pyrroline-5-carboxylate synthetase (P5CS); III: ornithine aminotransferase (OAT); IV: ornithine decarboxylase (ODC); V: diamine oxidase (DAO); VIA: inactive (non-functional) betaine-aldehyde dehydrogenase (BADH2); VIB: active (functional) BADH2; VII: spermidine synthase (SPDS); VIII: polyamine oxidase (PAO); IX: spermine synthase (SPMS); X: Spd/Spm *N*-acetyltransferases (SSAT)

converted to Δ^1 -pyrroline (immediate precursor of aroma compound), and not to GABA, whose level has, therefore, been detected to be lower in all the aromatic varieties examined, as compared to non-aromatic varieties. The non-enzymatic mechanism of conversion of Δ^1 -pyrroline via MG to aroma compound explains the reason for higher MG levels in the aromatic varieties. The Spd and Spm observed at higher levels in at least some of the aromatic varieties might have converted to Put via enzymatic conversion of Spd/Spm first to *N*-acetylspermidine/*N*-acetylspermine with the help of the enzyme spermidine/spermine *N*-acetyltransferases, followed by degradation of *N*-acetylspermidine/*N*-acetylspermine by PAO. The Put formed through this route might have led to more and more GABald production because of higher DAO activity. Overall, the present investigation clearly establishes the crosstalk among various intermediate metabolites and enzymes of diverse metabolic pathways related to aroma production, as well as highlight their differential levels in several aromatic indica rice cultivars, compared against non-aromatic rice cultivars. A more intensive and comprehensive study at the molecular and transcriptome level, together with quantitative detection of aroma content, will be undertaken in the future in understanding the genetic determinants of fragrance and differential expression of genes encoding enzymes for diverse metabolites in different aromatic rice cultivars. This will certainly throw more light in accounting for variations in aroma level and help in implementing

strategies for efficiently optimizing aroma production in aromatic rice as well as enhancing aroma level in non-aromatic cultivars by manipulating such metabolite/enzyme levels.

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Author contributions The corresponding author Aryadeep Roychoudhury designed and analyzed all the experimental results and drafted the final manuscript. Puja Ghosh performed all the experimental works and generated data.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest in publishing the manuscript.

References

- Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–207
- Bradbury LMT, Fitzgerald TL, Henry RJ, Jin Q, Waters DLE (2005) The gene for fragrance in rice. *Plant Biotechnol J* 3:363–370
- Bradbury LMT, Gillies SA, Brushett DJ, Waters DLE, Henry RJ (2008) Inactivation of an aminoaldehyde dehydrogenase is responsible for fragrance in rice. *Plant Mol Biol* 69:439–449
- Chen CT, Chen LM, Lin CC, Kao CH (2001) Regulation of proline accumulation in detached rice leaves exposed to excess copper. *Plant Sci* 160:283–290
- Chen S, Yang Y, Shi W, Ji Q, He F, Zhang Z, Cheng Z, Liu X, Xu M (2008) *Badh2*, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance. *Plant Cell* 20:1850–1861
- Costello PJ, Henschke PA (2002) Mousy-off flavor of wine: precursors and biosynthesis of the causative N-heterocycles 2-ethyltetrahydropyridine, 2-acetyltetrahydropyridine and 2-acetyl-1-pyrroline by *Lactobacillus hilgardii* DSM 20176. *J Agric Food Chem* 50:7079–7087
- Costello PJ, Lee TH, Henschke PA (2001) Ability of lactic acid bacteria to produce N-heterocycles causing mousy-off flavor in wine. *Aust J Grape Wine Res* 7:160–167
- Costilow RN, Cooper D (1978) Identity of proline dehydrogenase and Δ^1 -pyrroline-5-carboxylic acid reductase in *Clostridium sporogenes*. *J Bacteriol* 134:139–146
- Del Duca S, Beninati S, Serafini-Fracassini D (1995) Polyamines in chloroplast: identification of their glutamyl and acetyl derivatives. *Biochem J* 305:233–237
- Deshmukh Y, Khare P, Nadaf AB, Patra D (2015) Discrimination between 2-AP producing and non-producing rice rhizobacterial isolates using volatile profiling: a chemometric approach. *J Chemom* 29:648–658
- Duhaze C, Gouzerh G, Gagneul D, Larher F, Bouchereau A (2002) The conversion of spermidine to putrescine and 1, 3-diaminopropane in the roots of *Limonium tartaricum*. *Plant Sci* 163:639–646
- Fogel WA, Beiganski T, Maslinski CZ (1979) Effects of inhibitors of aldehyde metabolizing enzymes on putrescine metabolism in guinea pig liver homogenates. *Inflamm Res* 9:42–44
- Gill SS, Tuteja N (2010) Polyamines and abiotic stress tolerance in plants. *Plant Signal Behav* 5:26–33
- Grieve CM, Grattan SR (1983) Rapid assay for determination of water soluble quaternary ammonium compounds. *Plant Soil* 70:303–307
- Holmstedt B, Larrson L, Tham R (1961) Further studies of a spectrophotometric method for the determination of diamine oxidase activity. *Biochim Biophys Acta* 48:182–186
- Huang TC, Huang YW, Hung HJ, Ho CT, Wu ML (2007) Δ^1 -pyrroline-5-carboxylic acid formed by proline dehydrogenase from *Bacillus subtilis* ssp. *natto* expressed in *Escherichia coli* as a precursor of 2-acetyl-1-pyrroline. *J Agric Food Chem* 55:5097–5102
- Huang TC, Teng CS, Chang JL, Chuang HS, Ho CT, Wu ML (2008) Biosynthetic mechanism of 2-acetyl-1-pyrroline and its relationship with Δ^1 -pyrroline-5-carboxylic acid and methylglyoxal in aromatic rice (*Oryza sativa* L.) callus. *J Agric Food Chem* 56:7399–7404
- Huang ZL, Tang XR, Wang YL, Chen MJ, Zhao ZK, Duan MY, Pan SG (2012) Effects of increasing aroma cultivation on aroma and grain yield of aromatic rice and their mechanism. *Sci Agric Sin* 45:1054–1065
- Kaikavoosi K, Kad TD, Zanan RL, Nadaf AB (2015) 2-Acetyl-1-pyrroline augmentation in scented indica rice (*Oryza sativa* L.) varieties through Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) gene transformation. *Appl Biochem Biotechnol* 177:1466–1479
- Kitaoka S, Nakano Y (1969) Colorimetric determination of ω -amino acids. *J Biochem* 66:87–94
- Li M, Ashraf U, Tian H, Mo Z, Pan S, Anjum SA, Duan M, Tang X (2016) Manganese-induced regulations in growth, yield formation, quality characters, rice aroma and enzyme involved in 2-acetyl-1-pyrroline biosynthesis in fragrant rice. *Plant Physiol Biochem* 103:167–175
- Mezl VA, Knox WE (1976) Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. *Anal Biochem* 74:430–440
- Miller G, Honig A, Stein H, Suzuki N, Mittler R, Zilberstein A (2009) Unravelling Δ^1 -pyrroline-5-carboxylate (P5C)/proline cycle in plant by uncoupled expression of proline oxidization enzymes. *J Biol Chem* 284:26482–26492
- Mo Z, Huang J, Xiao D, Ashraf U, Duan M, Pan S, Tian H, Xiao L, Zhong K, Tang X (2016) Supplementation of 2-AP, Zn and La improves 2-acetyl-1-pyrroline concentrations in detached aromatic rice panicles in vitro. *PLoS ONE* 11:e0149523
- Nadaf AB, Wakte KV, Zanan RL (2014) 2-Acetyl-1-pyrroline biosynthesis: from fragrance to a rare metabolic disease. *J Plant Sci Res* 1:102–108
- Nakamura T, Yokota S, Muramoto Y, Tsutsui K, Oguri Y, Fukui K, Takaba T (1997) Expression of betaine aldehyde dehydrogenase gene in rice, a glycinebetaine nonaccumulator, and possible localization of its protein in peroxisomes. *Plant J* 11:1115–1120
- Poonlaphdech J, Maraval I, Roques S, Andebert A, Boulanger R, Brv X, Gunata Z (2012) Effect of timing and duration of salt treatment during growth of a fragrant rice variety on yield and 2-acetyl-1-pyrroline, proline and GABA levels. *J Agric Food Chem* 60:3824–3830
- Romanczyk LJ Jr, McClelland CA, Post LS, Aitken WM (1995) Formation of 2-acetyl-1-pyrroline by several *Bacillus cereus* strains isolated from cocoa fermentation boxes. *J Agric Food Chem* 43:469–475
- Roychoudhury A, Basu S, Sarkar SN, Sengupta DN (2008) Comparative physiological and molecular responses of a common indica rice cultivar to high salinity with non-aromatic indica rice cultivars. *Plant Cell Rep* 27:1395–1410

- Roychoudhury A, Basu S, Sengupta DN (2012) Antioxidants and stress related metabolites in the seedlings of two *indica* rice varieties exposed to cadmium chloride toxicity. *Acta Physiol Plant* 34:835–847
- Smith TA (1983) Polyamine oxidase oat seedlings. In: Tabor H, Tabor CW (eds) *Methods enzymol*, vol 94. Academic Press, New York, pp 311–314
- Srivong P, Wangsomnuk P, Pongdontri P (2008) Characterization of a fragrant gene and enzymatic activity of betaine aldehyde dehydrogenase in aromatic and non-aromatic Thai rice cultivars. *KKU Sci J* 36:290–301
- Stines AP, Naylor DJ, Hoj PB, Heeswijck RV (1999) Proline accumulation in developing grapevine fruit occurs independently of changes in the level of pyrroline-5-carboxylate synthetase mRNA or protein. *Plant Physiol* 120:923–931
- Struve C, Christophersen C (2003) Structural equilibrium and ring-chain tautomerism of aqueous solutions of 4-aminobutyraldehyde. *Heterocycles* 60:1907–1914
- Su GX, Bai X (2008) Contribution of putrescine degradation to proline accumulation in soyabean leaves under salinity. *Biol Plant* 52:796–799
- Vanavichit A, Yoshihashi T (2010) Molecular aspects of fragrance and aroma in rice. *Adv Bot Res* 56:49–73
- Vanavichit A, Yoshihashi T, Wanchana S, Areekit S, Saengsraku D, Kamolsukyonyong W, et al. (2005) Cloning of Os2AP, the aromatic gene controlling the biosynthetic switch of 2-acetyl-1-pyrroline and gamma aminobutyric acid (GABA) in rice. In: 5th International Rice Genetics Symposium, Philippines, IRRI, p 44
- Wakte KV, Tad TD, Zanan RL, Nadaf AB (2011) Mechanism of 2-acetyl-1-pyrroline biosynthesis in *Bassia latifolia* Roxb. flowers. *Physiol Mol Biol Plants* 17:231–237
- Wild R, Ooi L, Srikanth V, Munch G (2012) A quick, convenient and economical method for the reliable determination of methylglyoxal in millimolar concentrations: the *N*-acetyl-L-cysteine assay. *Anal Bioanal Chem* 403:2577–2581
- Yoshihashi T, Huong NTT, Inatomi H (2002) Precursors of 2-acetyl-1-pyrroline, a potent flavor compound of an aromatic rice variety. *J Agric Food Chem* 50:2001–2004