Dissecting the biosynthetic pathway and regulatory switches of 2-acetyl-1-pyrroline, the potent aromatic compound in rice

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Abstract

Since the discovery of Os2AP as the gene regulating the accumulation of 2-acetyl-1-pyrroline (2AP), the major aromatic compound in rice, cereals, pandan, bread flowers, bacteria and fungi, the enzymatic and biosynthetic pathway have not been revealed so far. The enzymatic analysis of Os2AP isogenic lines and Nipponbare *Os2AP* RNAi confirmed *Os2AP* is a member of amino aldehyde dehydrogenase (AMADH). Using RNA interference of the *Os2AP* Nipponbare was converted into aromatic japonica rice that synthesizes 2-acetly-1-pyrroline both in leaves and grains in T_1, T_2 and T_3. To understand the biosynthetic pathway of *Os2AP*, seedlings of Khoa Dawk Mali in the presence of enzyme inhibitors revealed that 2AP is synthesized via polyamine pathway. In addition, the N^{15} lebelling tracer experiments using isogenic and transgenic lines confirmed that ornithine was the prime precursor of N in 2AP via 1-pyrroline (1P), the immediate precursor of 2AP. The inhibitory effects were more pronounced in the light than in the dark. The function of Os2AP was revealed when 4-aminobutyric acid (GABA) was detected in small amount in seedling of the aromatic isogenic line than the non-aromatic counterpart. As 4-aminobutyraldehyde was the immediate precursor of both 1-pyrroline and GABA, it is likely that *Os2AP* controls 1P pool, the immediate precursor of 2AP, by converting 4-aminobutyraldehyde, the immediate precursor of 1P, to GABA. The comparative experiment in transgenic Nipponbare is on-going.

Media Summary Map-based cloning of aromatic genes revealed functions of aromatic gene leading to discovery of biosynthesis of aromatic compound, 2-acetyl-1-pyrroline.
Key words aromatic rice, proline, grain aroma, 2-acetyl-1-pyrroline, RNA inference, Map-based cloning,

Introduction

Grain aroma is the most attractive characteristic of high quality rice. Cooked rice fragrance composed of more than 100 volatile compounds including hydrocarbons, alcohols, aldehydes, ketones, acids, esters, phenols, pyridines, pyrazines, and other minor constituents. Of all these volatile compounds, the "popcorn-like" aroma determined as 2-acetyl-1-pyrroline (2AP) was the major potent flavor component of all aromatic rice, crust of bread wheat and rye bread (Buttery et al, 1982). This rice fragrance were identified as major aromatic compound in pandan leaves (Buttery *et al*., 1983), bread flowers (*Vallaris Glabra* Ktze.) (Wongpornchai *et al*., 2003), wet millet (Seitz *et al*., 1993), popcorn (Schieberle, 1991), fungi (Nagsuk et al., 2003) and *Bacillus ceres* (Romanczyk *et al*., 1995).

Positional cloning of rice grain aroma is one of the most challenging project in rice because the volatility of the aroma made it difficult to quantify accurately. The first successful report on the map-based cloning of aromatic gene in rice was narrowed down into the 8 bp deletion in the exon 7 of an aldehyde dehydrogenase located with 27.5 kb with a BAC clone developed from KDML105 (Vanavichit et al., 2004). A set of isogenic lines (ISL) carrying such deletion accumulated 2AP. In support of this finding, the aldehyde dehydrogenase (BAD2) was later reported using in silico mapping (Wanchana et al, 2005) and positional candidate gene analysis (Bradbury et al., 2005). However, functional analysis was not completely revealed.

Hypotheses of how a single recessive gene can bring out the most stimulating characteristic in rice were proposed. The aromatic compound has a pyrroline ring similar to

amino acid proline. Two evidences linking proline as the precursor of 2AP was reported in callus feeding experiments (Suprasanna et al, 1998; Suprasanna et al., 2002) and *in planta* isotopic labeling (Yoshihashi et al., 2002). However, the exact biosynthetic pathway of 2AP *in planta* is yet to be elucidated. Here we report the functional analysis of the *Os2AP* in modulating the accumulation of 2AP. By disrupting the expression of *Os2AP*, Nipponbare, a non-aromatic rice, has become an aromatic rice by enhancing the synthesis of 2AP.

Expression of Os2AP

The expression Os2AP was detected in leaves, roots, stems and florets in non-aromatic ISLs. The 8 bp deletions in *Os2AP* created a premature stop codon that could lead to non-sense mediated degradation (NMR) against its own mRNA and may lead to the loss-of-function of this gene among aromatic varieties as a consequence. As expected, the expressions of the Os2AP were less in all plant parts for the aromatic ISLs. In florets of the non-aromatic ISLs, the expression of the Os2AP was constitutive in 10, 15 and 20 days after pollination (DAF) whereas that in the aromatic ISLs, the expression was much less, in particular in 20 DAF. The expression patterns reported here agreed well with the recessive nature the aroma in rice. It is interesting to observe that the expression of the Os2AP in roots of aromatic ISLs was highly suppressed at the same level as in the leaves but several reports confirmed that no 2AP was detected in roots (Yoshihashi et al., 2002). This is one of several examples of post-transcriptional control play crucial roles in regulating gene expression in plants.

Using RNA interference (RNAi) against *Os2AP* transcript converted Nipponbare into aromatic japonica rice. RNAi directly suppressed the transcript accumulation of Os2AP in all transgenic events. The accumulation of 2-acetyl-1-pyrroline was determined by the expression level of Os2AP transcripts in transgenic Nipponbare. of both in leaves and grains in T_1, T_2 and T_3.

Precursor of 2AP: Proline hypothesis

Isotopic labeling in aromatic rice revealed that proline is a possible precursor of the nitrogen in the pyrroline ring of 2AP (Yoshihashi et al., 2001). In this experiment, they used 15N-proline and carboxyl carbon 13 labeling . These results indicated that the N source of 2AP was inherited from L-proline but not the acetyl group in Thai Hom Mali Rice. In higher plants, proline is synthesized via both the glutamic acid and ornithine pathways. The former is considered to be a major pathway, especially under osmotic stress. In the glutamine pathway (Figure 5), proline is synthesized from glutamine via two intermediates, glutamic-gamma-semialdehyde (GSA) and delta1-pyrroline-5-carboxylate (P5C). Two enzymes catalyze this pathway, P5C synthetase (P5CS) in the first step and *P5C* reductase (*P5CR*) in the final step. Genes encoding P5CS and P5CR were isolated from various plants, and their expression and functions were characterized. It has been shown in several plants that P5CS is the rate-limiting enzyme in proline biosynthesis. On the other hand, proline is metabolized to glutamine via *P5C* and *GSA* by Proline dehydrogenase (*ProDH)* in the first step and P5C dehydrogenase (*P5CDH*) in the final step. There are strong evidences linking *Os2AP* to the synthesis of 2AP in rice. First, we identified strong homology between aldehyde dehydrogenase domains between *P5CDH* and *Os2AP*. Second, the 8 bp deletion in the exon 7 in the *Os2AP* have strong association with the ability to synthesize 2AP in aromatic ISLs and all rice varieties compared today. Third, the pattern of gene expression of *Os2AP* in all parts of the non-aromatic ISLs plants correspond well with the ability to synthesize 2AP, except in roots.

RNAi for functional analysis

RNA interference was set to minic the in vivo NMD in aromatic rice. The *Os2AP*-ihpRNAi (*Os2AP*i) was constructed from the genomic DNA spanning exons 6 to 9 joined in the opposite direction to its cDNA. The *Os2AP*i was inserted behind the 35S promoter in the pCAMBIA1302. The whole vector was bombarded to scutella-derived calli prepared from Nipponbare. Monitoring of the GFP, endogenous *Os2AP* and *Os2APi* revealed that the expression of GFP and *Os2APi* were negatively correlated to the expression of endogenous *Os2AP*. The expression levels of endogenous Os2AP were negatively correlated to the accumulation of 2AP in leaves of these transgenic plants. The transgenic Nipponbare enhanced the levels of 2AP by 5 to almost 20 folds. Segregation analysis of grain aroma from T_0RNAi2(10) confirmed this result. Therefore, the suppressive expression caused by NMD and RNAi in *Os2AP* had similar effects on the accumulation of 2AP in rice plants.

Precursor of 2AP: Polyamine hypothesis

To understand the biosynthetic pathway of *Os2AP*, seedlings of Khoa Dawk Mali in the presence of enzyme inhibitors revealed that 2AP is synthesized via polyamine pathway. In addition, the N^{15} lebelling tracer experiments using isogenic and transgenic lines confirmed that ornithine was the prime precursor of N in 2AP via 1-pyrroline (1P), the immediate precursor of 2AP. The function of Os2AP was revealed when 4-aminobutyric acid (GABA) was detected in small amount in seedling of the aromatic isogenic line than the non-aromatic counterpart. As 4-aminobutyraldehyde was the immediate precursor of both 1-pyrroline and GABA, it is likely that *Os2AP* controls 1P pool, the immediate precursor of 2AP, by converting 4-aminobutyraldehyde, the immediate precursor of 1P, to GABA. The enzymatic analysis of Os2AP isogenic lines and Nipponbare *Os2AP* RNAi confirmed *Os2AP* is a member of amino aldehyde dehydrogenase (AMADH). Enzyme activities of AMADH and amino acid sequence confirmed.

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