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Aroma in rice: genetic analysis of a quantitative trait

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Abstract A new approach was developed which succeeded in tagging for the first time a major gene and two QTLs controlling grain aroma in rice. It involved a combination of two techniques, quantification of volatile compounds in the cooking water by gas chromatography, and molecular marker mapping. Four types of molecular marker were used (RFLPs, RAPDs, STSs, isozymes). Evaluation and mapping were performed on a doubled haploid line population which (1) conferred a precise character evaluation by enabling the analysis of large quantities of grains per genotype and (2) made possible the comparison of gas chromatography results and sensitive tests. The population size (135 lines) provided a good mapping precision. Several markers on chromosome 8 were found to be closely linked to a major gene controlling the presence of 2-acetyl-1-pyrroline (AcPy), the main compound of rice aroma. Moreover, our results showed that AcPy concentration in plants is regulated by at least two chromosomal regions. Estimations of recombination fractions on chromosome 8 were corrected for strong segregation distortion. This study confirms that AcPy is the major component of aroma. Use of the markers linked to AcPy major gene and QTLs for marker-assisted selection by successive backcrosses may be envisaged.

Key words Rice (*Oryza sativa* L.) · Aroma/fragrance/scent · Grain quality · Molecular mapping · Segregation distortion

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Introduction

Aroma in cultivated rice (*Oryza sativa* L.) is being appreciated more and more by many people and represents a high-value-added trait. Also, high milling returns and good cooking quality are often associated with aromatic or scented rice (Nagaraju et al. 1975; Tripathi and Rao 1979). Unfortunately, aromatic or scented cultivars have often undesirable agronomic characters, such as low yield, susceptibility to pests and diseases, and strong shedding (Berner and Hoff 1986). Thus, breeders wish to develop aromatic varieties with high yield and good resistance to pests.

Many studies on the genetic control of the aroma trait in rice have been reported (Ahn et al. 1992; Ali et al. 1993; Berner and Hoff 1986; Lin 1990; Nagaraju et al. 1975; Pinson 1994; Raghuram Reddy and Sathyanarayanaiah 1980; Sood and Siddiq 1978; Tripathi and Rao 1979; Yano et al. 1992). Some of the authors concluded that the trait undergoes monogenic inheritance, while others favoured the idea that two or three recessive or dominant genes participate in the construction of the trait. Thus, the problem seemed to be an interesting one to investigate with tools permitting an intensive study of the genetics of aroma, and notably, to locate the gene(s) involved in the expression of this character.

Molecular markers are a powerful tool for the study of genetic models underlying the different agronomical traits. Ahn et al. (1992) were the first investigators to combine the utilisation of molecular markers with that of near-isogenic lines (NILs), in order to locate a gene controlling aroma in rice. They found that the gene is located at the end of a linkage group belonging to chromosome 8, at 4.5 cM (centimorgans) from the restriction fragment length polymorphism (RFLP) marker RG 28.

Buttery et al. (1983) identified 2-acetyl-1-pyrroline (AcPy) as the major component of rice aroma. This compound is present in all parts of the plant (stems, leaves, grains) except roots. No linkage analysis between markers and AcPy as a quantitative trait has yet been carried out so that the monogenic or polygenic nature of the trait has yet

to be determined. In the study presented here, the genetic mapping of aroma was improved by combining for the first time the two following approaches: (1) a precise and objective aromatic analysis by gas chromatography, permitted by the utilisation of doubled haploid lines, which gave sufficient quantities of grains (Petrov et al. 1995), and (2) by saturation of the map of the chromosome 8 with several molecular markers, allowing the placement of flanking markers around the AcPy gene.

Materials and methods

Strategy

A core genetic map consists of markers covering all the genome at middle density, i.e. with no gap larger than 20–25 cM. Therefore, there will be at least 1 marker sufficiently linked at 10 cM or less to the genes(s) underlying a trait. A core rice map was developed at IRRI, Philippines (Huang et al. 1994) on the basis of 145 RFLP markers, for the most part ones that had already been placed on the interspecific saturated map (Causse et al. 1994). For this purpose, a population of 135 doubled haploid (DH) lines was used. After evaluation for aromatic compounds of the DH lines by gas chromatography at ENSIAA, France, chromosome segments of interest were identified by segregation/QTL (quantitative trait locus) analysis. Saturation of the major segment with different types of molecular markers was then done at ORSTOM-LRGAPT, France and at IRRI.

Genetic material and DNA extraction

The mapping population of (DH) lines was derived from the F_1 hybrid 'IR 64'×'Azucena' through anther culture at IRRI (Guiderdoni et al. 1992). Azucena is a scented *japonica* landrace from Philippines, and IR 64 is a non-scented *indica* variety obtained from IRRI. DNA was isolated from lyophilised leaves using the CTAB method (Murray and Thompson 1980).

Restriction fragment length polymorphism markers

In this study, we used probes from the saturated RFLP rice map developed by Causse et al. (1994). Probes were kindly provided by Dr. S. McCouch (Cornell University, USA). Southern transfers, hybridisations and non-radioactive DNA labelling used for revelation of hybridisations were done according to IRRI or CIMMYT protocols (Hoisington et al. 1994). Six restriction enzymes were used for the core map: *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I, *Xba*I. Fourteen additional enzymes were used to test for polymorphism with probes showing a monomorphic pattern.

Bulked segregant analysis

A complementary tool is the bulked segregant analysis (Michelmore et al. 1991) coupled with randomly amplified polymorphic DNA (RAPD) markers. This method permits a rapid analysis of the progeny for several markers and therefore the saturation of the map in the regions of interest. Two bulks (aromatic/non-aromatic) of eight individuals were constituted. Positive primers were verified by amplifying all 16 lines individually. Polymerase chain reaction (PCR) amplifications were carried out in 25 µl, with the following reagents: 0.4 µ*M* primer, 0.02 U/µl *Taq* polymerase (Appligene), 1×buffer mix (Appligene), 150 µ*M* dNTP. Conditions were 95 °C–5 min; 45 (95 °C–1 min; 35 °C–1 min; 72 °C–2 min); 72 °C–6 min.

Sequence-tagged sites (STSs)

STSs are PCR markers obtained by the amplification of DNA between two primers corresponding to the two bounds of a genomic probe. STSs have the advantages of RAPD markers (they are based on PCR) and those of RFLP markers (they are locus-specific and codominant). Such 20-mer primers were published by Inoue et al. (1994). These primers correspond to probes of the saturated map of the Rice Genome Research Program – Japan (Kurata et al. 1994). PCR conditions were the same as in Inoue et al. (1994). As polymorphism between parents was not detected directly, digestion of amplification products was done with 4 bp restriction enzymes.

Isozymes

Isozyme analyses were performed following the protocol of Guiderdoni et al. (1989).

Evaluation of aroma

The evaluation of rice aroma is not easy, and classical smelling or chewing methods are not supposed to be totally reliable because of their subjective nature. The method used here, detailed in Petrov et al. (1995), is based on a gas chromatography quantification of volatile compounds contained by the cooking water of 100 g. of grains. By this technique, the presence of 2-acetyl-1-pyrroline at the ppb (1 ppb equals to 1 ng/g) level can be detected with good repeatability (Petrov et al. 1995). This compound is known to be the major agent of aroma in rice (Buttery et al. 1983, 1988). As several DH lines were partially sterile, we could not obtain sufficient amounts of grains for the analysis of these lines. Consequently, the analysis was performed on only 84 lines of the mapping population. Measurements on parents were replicated 12 times.

The method of scent revelation with KOH (Sood and Siddiq 1978) using leaves and seeds was also applied on a replication of the population grown in the glasshouse. For the test using leaves, one or two leaves per line were cut into small pieces and put into petri boxes with 10 ml of 1.7% KOH. After 30 min, the boxes were opened and immediately smelled. DH lines were then scored as aromatic or non aromatic. For the test using seeds, ten seeds per line were ground and aroma revelation and scoring were identical to that used for leaves. Each line was evaluated by a minimum of four persons chosen for their capacity to easily distinguish between the two parents. Since these tests need only ten seeds or one or two leaves from each line, the entire population (135 lines) was evaluated for both leaves and seeds. These two tests permitted us to compare the results of sensitive tests using leaves or seeds to those of gas chromatography.

Map construction

The map was constructed with MAPMAKER 3.0 (Lander et al. 1987). The two-point LOD *score* threshold was equal to 5, and r_{max} to 0.3. Ordering of markers was done using the 'order' and 'try' commands. Conversion of recombination fractions into centimorgans (cM) was obtained with Kosambi's mapping function (Kosambi 1944). In order to verify the impact of segregation distortion, we calculated two-point estimates and likelihoods for three-point orders in a similar manner as in Lorieux et al. (1995). An appropriate model of selection based on the observation of the frequencies of genotypic classes generated by the segregation analysis of two markers was defined to derive the estimates and likelihoods. For chromosome 8, which showed strong deficit on the *ab* (both markers carrying Azucena bands) class for all pairs of markers, we used the following consistent estimate:

$$\hat{r}_c = \frac{b+c}{2a+b+c},$$

where a, b and c are the frequencies of AB, Ab and aB classes, respectively. The variance of this estimate is

$$V_{\hat{r}_c} = \frac{r(1+r)(r-1)(ru-r-u-1)}{2n},$$

where u is the selection coefficient of class ab and r is the corrected estimate. This variance is slightly smaller than that of the classical estimate under the conditions of selection observed on chromosome 8 ($u<1$).

The standard deviation of the Kosambi map distance $\hat{d}_c$ associated to the estimate $\hat{r}_c$ is:

$$s_{\hat{d}_c} = \frac{1}{(1-4r^2)}\sqrt{\frac{r(1+r)(r-1)(ru-r-u-1)}{2n}}.$$

Cosegregation analysis between markers and characters

Two approaches were used:

– A QTL detection approach based on quantitative evaluation of AcPy by gas chromatography. The interval mapping method (MAPMAKER/QTL for Unix v. 1.1; Lander and Botstein 1989) was used. As AcPy was not normally distributed, ANOVA 1 (SAS-IML®) and the Kruskall & Wallis test (MapQTL for Unix v. 2.4 ; van Ooijen 1992) were used to confirm results of interval mapping. In order to detect putative minor QTLs, we repeated the interval mapping after putting the loci detected by the first analysis as cofactors.

– A Mendelian approach based on coding AcPy in the presence/absence and on sensitive tests. The data were included in the marker data matrix and analysed using MAPMAKER v. 2.0 for a Macintosh computer.

Results

Sensitive tests using leaves and seeds gave the same results: 40 lines and the 'Azucena' parent were found to be aromatic, and 90 lines plus the 'IR 64' parent were found to be non-aromatic; 5 lines were difficult to evaluate and thus were considered as missing data.

AcPy was distributed in the progeny as follows: all lines unambiguously found to be aromatic contained 3–40 ppb AcPy, while the non-scented lines never contained AcPy (see Fig. 1 for AcPy distribution). 'IR 64' and 'Azucena' contained 0.0±0.0 and 24.2±3.9 ppb AcPy, respectively (mean of 12 evaluations). AcPy evaluated by gas chromatography was therefore perfectly correlated with scent evaluation and moreover, it was more accurate since unambiguous data for all of the lines tested were obtained (see Petrov et al. 1995 for detailed information on volatile compounds evaluation).

The first QTL analysis performed on the core map of the whole genome revealed only one "QTL" located on chromosome 8, with a peak between the markers RG 28 (RFLP)/Y5 (RAPD) and RG 1 (RFLP) (Fig. 2A). This fragment was detected with a maximum LOD score of 14.5 at 10 cM from RG 28 (map distances calculated with MAPMAKER) and explained about 69% of the variance of the character. Thus, it may be considered as a major gene. Two other QTLs for AcPy were found after putting the QTL on chromosome 8 as a cofactor. The first QTL was on chromosome 12 (Fig. 2B), with an associated LOD=1.8. The second QTL was on chromosome 4, with an associated LOD=1.6. The associated probabilities to the LODs of these two QTLs were 0.004 and 0.008, respectively.

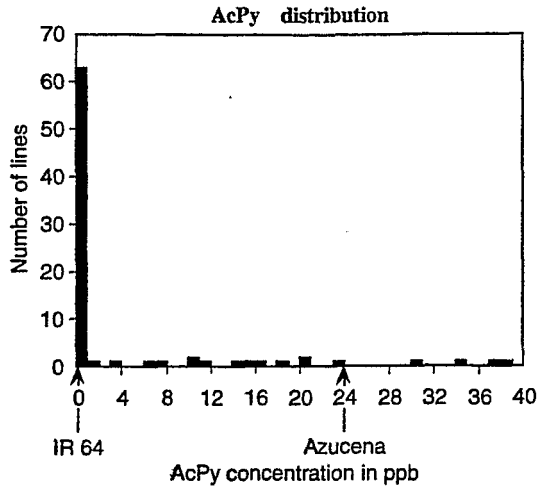

Fig. 1 Distribution of 2-acetyl-1-pyrroline concentration in the DH lines coming from the F_1 hybrid 'IR 64'×Azucena. The deviation in favor of non scented lines (AcPy concentration=0 ppb) is due to strong segregation distortion on chromosome 8 (see text for details). Among the scented lines (AcPy concentration > 0 ppb), some lines are intermediate between 'IR 64' and Azucena, some contain as much AcPy as Azucena and others contain more AcPy than Azucena

As the major gene was located on chromosome 8, the mapping effort was concentrated on this linkage group. Thus, sixteen markers were mapped. The minimum two-point map LOD was always found to be greater than 10, except between RG 20 and A5J560 (LOD=3.45). The probe RG 28, which was found to be close to aroma (4.5 cM) by Ahn et al. (1992), was not polymorphic with the six enzymes used for the map construction. Nevertheless, polymorphism was found for this probe with enzyme *Hae*III. The RAPD marker Y5 (Operon) showed complete linkage with RG 28. Moreover, this marker had a codominant behaviour as two bands (840 bp and 800 bp) were clearly allelic. As this marker provides repeatability, it may therefore be used advantageously in rice F_2 populations, for which dominant markers give very poor information on recombination. Considering that (1) AcPy was perfectly correlated with aroma, (2) AcPy exhibited a roughly bimodal distribution (Fig. 1), and (3) the QTL on chromosome 8 was a major gene regarding the percentage of explained variance, we encoded it as a monogenic trait, i.e. in presence/absence. Segregation analysis allowed us to locate AcPy on chromosome 8 unambiguously between RG 28/Y5 and RG 1 (at 6.4±2.6 and 5.3±2.7 cM, respectively; recombination fractions corrected for segregation distortion and map distances obtained with Kosambi mapping function). Logically, the aroma trait obtained by sensitive tests was located in the same interval with very similar but more precise distances due to the larger population size (at 5.8±1.9 and 6.2±2.1 cM, respectively; Fig. 3).

All loci except RZ 143 and RG 20 showed segregation distortion (Table 1). The total length of the linkage group was 161.3 cM (estimated with MAPMAKER). The use of

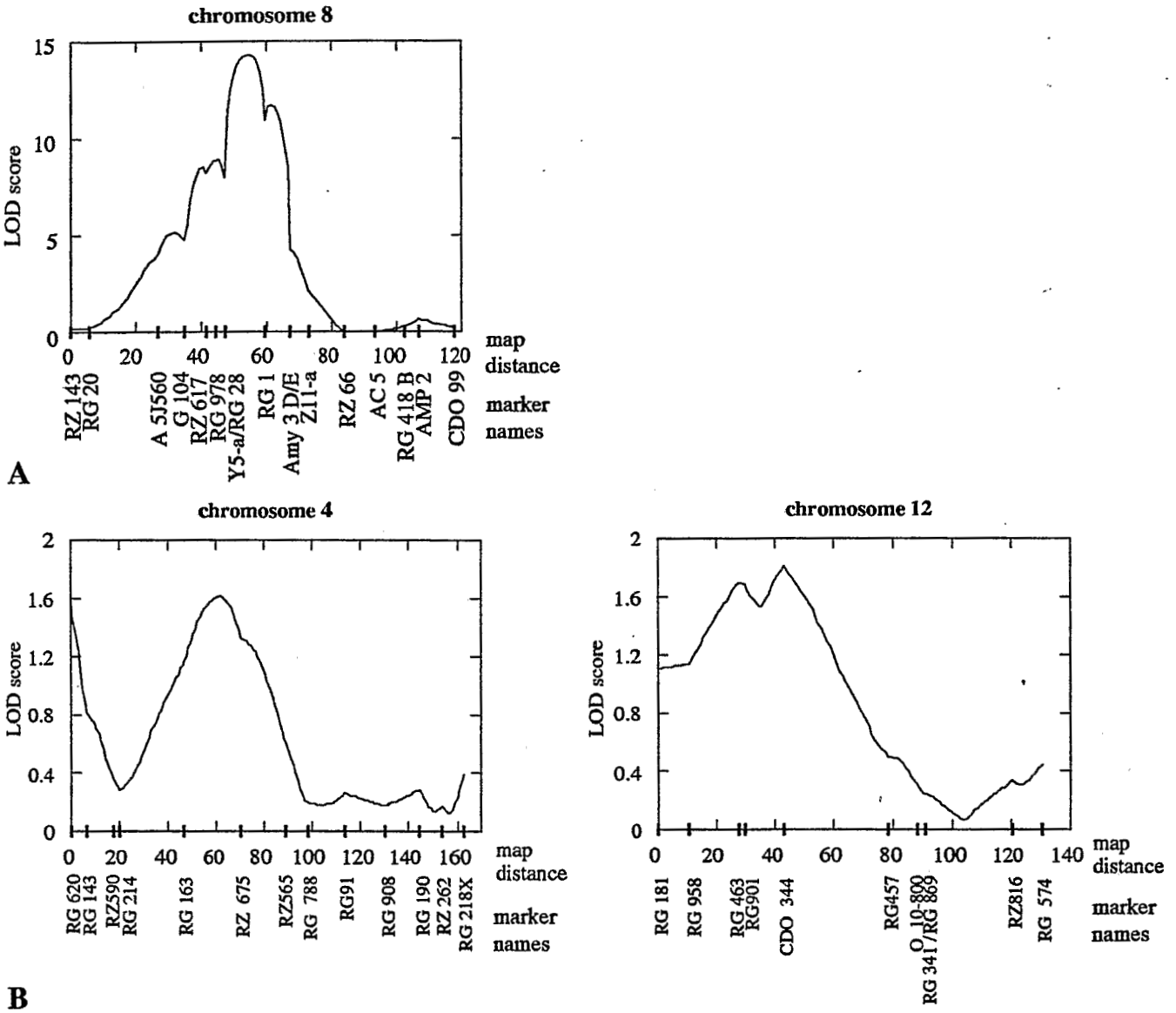

Fig. 2A, B A QTL found for AcPy on chromosome 8, corresponding to a major gene controlling aroma in rice. **B** QTLs on chromosomes 4 and 12 obtained after putting QTL on chromosome 8 as a cofactor in the analysis

the two-point map distance estimate corrected for segregation distortion, $\hat{d}_c$, gave the total length of the group as 117.5 cM, leading to a reduction of map distances of about 27%, compared with those obtained by MAPMAKER. Three-point models corrected for distortion revealed that the order was not modified by segregation distortion.

Discussion

This study permitted us to tag a major gene for aroma between close flanking markers. Moreover, two QTLs were identified that may affect the strength of aroma in rice varieties. Although these QTLs were not highly significant, it is very probable that we would have obtained more significant tests if the numbers of scented and non-scented DHs had been roughly the same (it can be shown that segregation distortion affects the power of QTL detection but does not generate false positive detection). It was also confirmed that 2-acetyl-1-pyrroline (AcPy) is the major component of aroma in rice, since *AcPy* and *Aroma* were perfectly correlated and mapped at the same locus.

The map length of chromosome 8 corrected for segregation distortion (117.5 cM) is in good accordance with that found in the intraspecific saturated map (124.8 cM; Kurata et al. 1994), indicating that the proposed estimate of recombination fractions was appropriate for this linkage group. The corrected length is logically larger than chromosome 8 of the interspecific map (Causse et al. 1994), which shows a reduction of recombination due to the genetic distance of the parentals involved in the cross.

Table 1 Segregation patterns of markers on chromosome 8. Strong segregation distortion is observed on all markers except for RZ143 and RG20. Markers are listed in map order

Marker name	IR 64	Azucena	Total	χ^2	Probability
RZ 143	73	55	128	2.53	0.11161
RG 20	62	58	120	0.13	0.71500
A 5J560	39	18	57	7.74	0.00541
G 104	84	49	133	9.21	0.00241
RZ 617	84	34	118	21.19	$< 1 \cdot 10^{-5}$
RG 978	85	40	125	16.20	0.00006
Y 5-a	89	44	133	15.23	0.00010
RG 28	87	43	130	14.89	0.00011
RG 1	73	36	109	12.56	0.00039
Amy 3 D/E	94	30	124	33.03	$< 1 \cdot 10^{-5}$
Z 11-a	75	27	102	22.59	$< 1 \cdot 10^{-5}$
RZ 66	84	43	127	13.24	0.00027
AC 5	73	41	114	8.98	0.00273
RG 418 B	93	40	133	21.12	$< 1 \cdot 10^{-5}$
AMP 2	81	31	112	22.32	$< 1 \cdot 10^{-5}$
CDO 99	76	47	123	6.84	0.00893

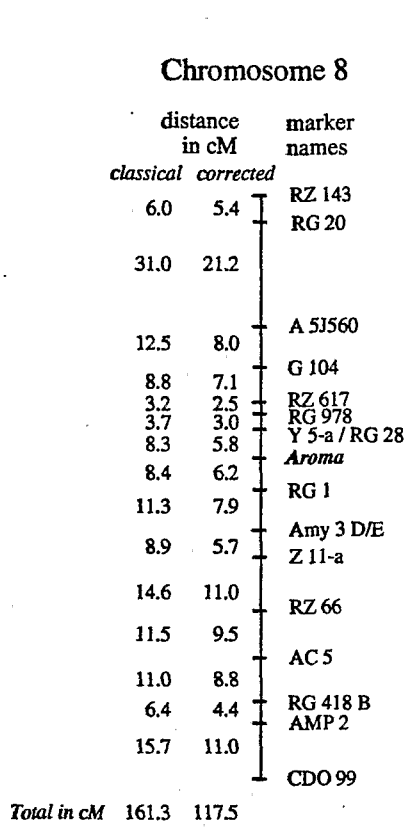

Fig. 3 Genetic map of rice chromosome 8. Classical distances and distances corrected for segregation distortion between markers were calculated using the Kosambi mapping function. Correction leads to an overall reduction in chromosome length of about 27%

The relative orders of common markers were found to be identical in the two maps, indicating that segregation distortion did not disturb the ordering calculations.

The exact function of the '*AcPy gene*' is not known at this time. Very little is known about the biosynthesis chain of AcPy. This compound may be experimentally obtained by different reactions. For instance, it can be derived from pyruvaldehyde and proline, from pyruvaldehyde and ornithin or from fructose and ornithin (Schieberle 1990).

Some lines were difficult to evaluate for aroma by the sensitive tests. This may be due to different parameters influencing the quantity of AcPy in leaves or seeds. An important factor is the temperature in the glasshouse, due to the volatile nature of AcPy (Basmati or Thaï rice are harvested at the beginning of winter in order to obtain more aroma). Another factor is the age of the plants: for instance, young leaves are more aromatic than old ones. Moreover, some known volatile compounds may develop other aromas, which may interfere with AcPy and make the sensitive analysis more difficult (Petrov et al. 1995). The difficulties in evaluating aroma were probably due to an unfavourable combination of these factors.

The quantitative differences in AcPy content observed between the DH lines may result from different sources: (1) the lines were not grown under the same conditions, (2) the lines were not stored under the same conditions and (3) the lines bear different genetic factors controlling aroma. The two first sources may be eliminated, since all lines were grown in the same field, at the same time, and the seeds were harvested and stored under the same conditions. Moreover, the measurement for each line is a mean of the large number of plants needed to obtain 100 g of grains. For the first time, QTLs have been identified for this trait. These QTLs explain why some DH lines are less scented than 'Azucena', the aromatic parent. Indeed, it can be seen from the raw marker data that the most scented DH lines are those which bear alleles coming from Azucena for both QTLs and for the major gene. On the other hand, lines with intermediate fragrance are those which have the 'IR 64' allele for one or both QTLs. We therefore believe that our results could help to gain an understanding of why a gradient of strength of aroma is observed among scented cultivars. For example, the variety 'Jasmine 85' is derived from the Thaï rice, 'Khao Dawk Mali 105', but is less scented. It is possible that QTLs analogous to those we identified were lost by recombination during the creation of 'Jasmine 85'.

The question may be asked if there is a general genetic model underlying aroma as a quantitative trait. Pinson (1994) observed a difference in AcPy concentrations between 'Jasmine 85' and 'Dragon Eyeball 100'. He supposed that differences between varieties are due to mutations of the same gene on chromosome 8. Moreover, he observed segregations corresponding to one or two independent recessive genes in crosses between different aromatic/non-aromatic varieties. Several authors have found contradictory results for segregation patterns of aroma (Ahn et al. 1992; Ali et al. 1993; Berner and Hoff 1986; Lin 1990; Nagaraju et al. 1975; Pinson 1994; Raghuram Reddy and Sathyanarayanaiah 1980; Sood and Siddiq 1978; Tripathi and Rao 1979; Yano et al. 1992). To our knowledge, all recent linkage studies using molecular marker data have lead to the conclusion that aroma is governed by a single recessive gene located on chromosome 8. In addition to the results of Ahn et al. (1992), aroma was

located at the same locus by Yano et al. (1992) who used an F_2 population and RFLP markers. Moreover, aroma was found to be linked at 7 cM to a RAPD marker that mapped close to aroma in our DH progeny using a cross involving 'Basmati 370' (data not shown). Pinson (1994) found that all scented varieties, including 'Basmati', 'Jasmine 85' and a mutant of 'Della' share the same gene (which is that of chromosome 8). According to several authors, the contradictory conclusions are due to problems in the handling of the endospermic nature of aroma. In F_2 progenies, this confusion may lead, for instance, to the conclusion that the gene is dominant instead of recessive. Segregation distortion can also strongly modify the segregation pattern of a gene (e.g. a 1:1 segregation may be interpreted as a 3:1 segregation in a DH or backcross population). In our study, without molecular marker information we would have to conclude the existence of two complementary aromatic genes coming from Azucena. It is not impossible that the two-gene segregations observed by Pinson (1994) were due to this phenomena, which could not be detected since no marker data were available.

An immediate application of our results is the introgression of the major gene for aroma in a high yielding variety using successive backcrosses, with the aid of the flanking markers. RG 28 and RG 1 are RFLP markers and may be converted into STSs in order to perform a rapid succession of gene introgression. Y5 is a RAPD marker and could be used as it is or after conversion into STS. The advantages of a such approach are: (1) a twofold decrease in the number of necessary generations by choosing in the BC1 progeny the individual with the higher percentage of recurrent alleles, (2) the complete elimination of linkage drag (Ragot et al. 1994) and (3) the direct following of the allele for aroma in successive generations by markers, without the need of the self-pollination steps that are necessary in classical breeding schemes involving recessive characters. Another promising application is marker-assisted selection (MAS), which can integrate major gene and QTL information in selection indexes. It can not be excluded that other QTLs for aroma may be revealed by using a larger progeny. The problem of small quantities of seeds provided by partially sterile lines could be solved by modifying the protocol conditions of aroma extraction, i.e. by using a method that needs less material per DH line. Finally, a fine mapping of the chromosome segment between RG 28 and RG 1 could be initiated using a bulked segregant analysis approach in combination with RAPD and AFLP™ markers. Candidate markers of the saturated intra-specific map (Kurata et al. 1994) which can be identified with the help of common markers with the inter-specific map (Xiao et al. 1992) could also be used. Isolation and map-based cloning of the gene would then be possible, and every high yielding rice variety with good grain quality could then be genetically transformed for this gene.

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