Chapter 11
Breeding New Aromatic Rice with High Iron Using Gamma Radiation and Hybridization

Phuong Tan Tran and Cua Quang Ho

Abstract The goal of many breeding programmes is the combination of several improved traits to produce a cultivar that meets demands of farmer and consumer. For example, breeding aromatic rice varieties having both high quality and yield is an objective in Vietnam to satisfy domestic consumers and increase value in the export market. Genetic variation is the starting point for any breeding programme. In some cases sufficient variation exists and traditional hybridizations and introgressions are suitable for cultivar development. In other cases, new variation, such as that created through mutagenesis, is required for the development of new traits. Thus, a combinatorial approach using both hybridization and induced mutations can be considered when the goal is a new cultivar expressing several improved traits. We have taken this approach in rice breeding to generate lines with improved aroma and high iron content. Here we provide a protocol for mutation induction, hybridization and phenotypic analysis for the improvement of aroma and iron content in rice using a combined mutation and hybridization approach. Example data from this work is shown. This approach can be easily adapted for other traits of interest.

Keywords Aroma • Badh2.1 • Bioavailable iron • Iron content • High quality • Hybridization • Pedigree selection

11.1 Introduction

There is an increasing demand for the production of high-grain-quality aromatic rice to meet the domestic demand and also for export. Though there are many aspects which impact quality, key features that influence the market value of rice are aroma, kernel shape, cooking quality and taste. Aromatic rice is unique and prized in many countries. Local aromatic rice varieties in Vietnam have high value in the domestic market. There are famous varieties such as Tao Huong, Nang Thom in the south and the Tam group (Tam Xoan, Tam Thom) in the north. These

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varieties have disadvantageous traits such as photoperiod sensitivity; long growth duration (160–180 days); high stature (150–185 cm); thin, long, drooping leaves; and sparseness between grains. The consequence of these negative traits is low productivity calculated in the range of 2–3 tons/ha/crop. The advantage of these varieties is their better adaptation to the poor land and changing climatic conditions. Both genetics and environment affect traits such as aroma. For example, basmati rice loses the aroma when grown outside the Punjab in Pakistan and India. It is thought that Punjab climate and/or soil is important for producing a strong aroma (Efferson 1985). Khao Dawk Mali 105, the most important aromatic rice cultivar in Thailand, is reported to have the strongest aroma and best quality when grown in the Tung Kula Rong Hai region in northwest Thailand (Yoshihashi et al. 2004). Ideally, high-yielding, aromatic and photoperiod-insensitive varieties can be bred to serve increasing demands of customers in domestic and the world markets and to increase incomes for farmers.

The success of any breeding programme relies on genetic variation in the form of altered alleles that control or contribute to the traits of interest. There are vast genetic resources available for rice (e.g. http://irri.org/about-us/our-organization/genetic-resources-center). Traditional breeding approaches seek to generate new combinations of alleles that result in an improved variety. Yet, some traits (alleles) may not be available in existing germplasm or available only in genotypes that are recalcitrant due to linkage drag with negative traits. New alleles can be created at a high frequency using mutagenesis (*see* Chap. 1). Thus mutagenesis can be considered to support breeding objectives. In some cases mutagenesis of elite cultivars can be performed and improved varieties can be directly released (*see* Chap. 9). This is quite common and about 62 % of all officially registered mutant varieties are produced in this fashion (*see* Chap. 1 and MVD 2016). Hybridization of mutant alleles with "natural" alleles already present in germplasm is another approach. We provide an example of hybridization of mutant and natural alleles to produce high aroma and high bioavailable iron accessions in this chapter. The general approach for directed hybridizations is shown in Fig. 11.1.

In addition to requisite genetic variation, appropriate screening techniques are needed to produce improved varieties. For rice aroma, many researchers have examined the trait by a sensory test. Buttery et al. (1983) identified the characteristic aroma compound in steam volatile oils of cooked aromatic rice as 2-acetyl-1-pyrroline (2AP). Yoshihashi (2002), by a method using isotope-labelled analogues of 2AP, reported that the concentrations of 2AP are presented in milled and brown rice, rice bran, husk and seedlings. 2AP was not detected in root and it does not form during cooking or postharvest processing. Solid phase microextraction (SPME) has emerged as a rapid and efficient tool for the extraction and quantification of the aroma compounds (Stashenko and Martínez 2007). It is a rapid, simple, versatile and solvent-free technique and has integrated sampling, extraction, concentration and sample introduction of volatile compounds into gas chromatography (GC) in a single step resulting in high sample throughput (Soria et al. 2009; Picó et al. 2007).

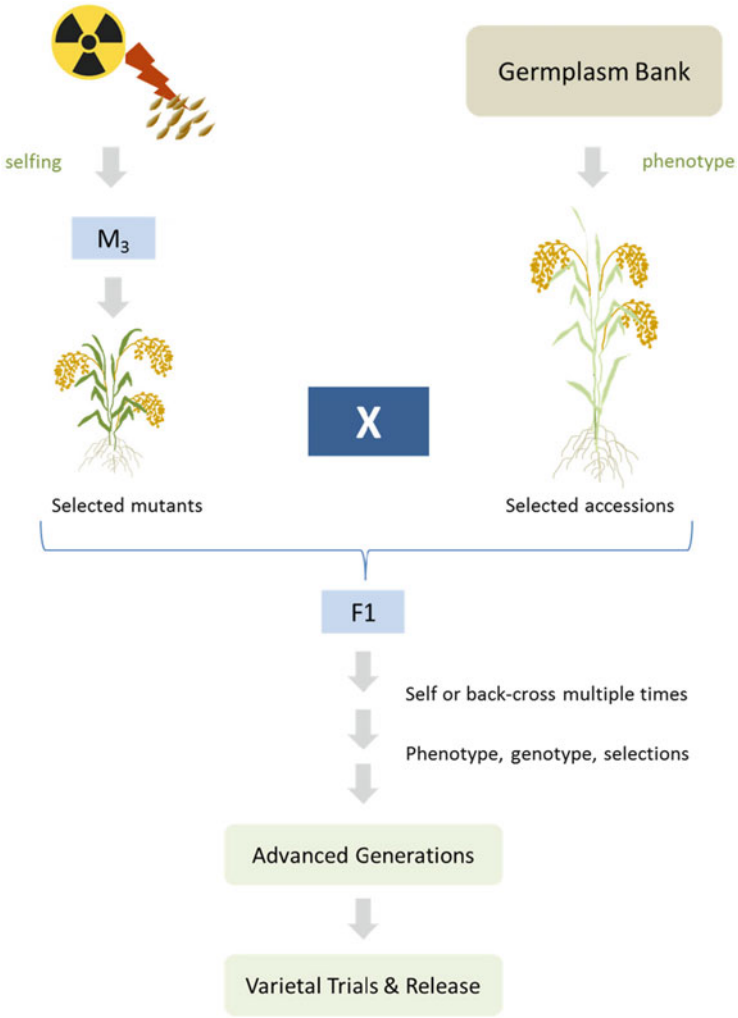

Fig. 11.1 Development of new rice cultivars through hybridization of mutant and natural alleles. Mutant populations are developed by treating seed with gamma irradiation (*top left*). Plants are self-fertilized and screened for improved traits. Phenotypic analysis is also carried out on accessions from a germplasm bank. Selected accessions are then hybridized with selected mutants. Since most traits will be recessive, the resulting F1 is further crossed. Different crossing strategies can be used such as selfing or backcrossing to the selected accession to introgress mutant alleles. Crossing and phenotypic analysis continues until traits are stabilized. At this time plants can be subjected to requisite varietal trials followed by eventual release. Figure courtesy of Dr Joanna Jankowicz-Cieslak and Dr Bradley J. Till of the FAO/IAEA Joint Programme

Micronutrient malnutrition, the result of diets poor in vitamins and minerals, affects more than half of the world's population. Women and children are especially susceptible to deficiencies in micronutrients, particularly vitamin A, iron and

zinc. As a result they are at risk of disease, premature death, lower cognitive capacity and poor quality of life. Nutritionally improved staple food provides an inexpensive, cost-effective, sustainable, long-term means of delivering micronutrients to the poor. Brown rice is a more nutritious food in comparison with white rice owing to the fact that the bran layer contains high amounts of vitamin B1, plus other nutrients and micronutrients. Other important components, γ-tocotrienol and γ-oryzanol in the bran covering of brown rice, have the effect of reducing cholesterol in the blood, an important factor causing cardiovascular disease (Chen and Cheng 2006). Brown rice is also suitable for people who follow the "macrobiotic diet" which not only brings much nutrients but also has a sweet taste created by the contribution of many enzymes on sugar and proteins inside grains. Researches on iron content in brown rice samples showed that iron content changes depending on varieties, IR64 (12.58–12.88 mg/kg), Jasmine 85 (12.84–18.50 mg/kg) and OMCS2000 (11.77–14.78 mg/kg), and about 2/3 of iron content is lost through milling (Tran et al. 2004). However, it is difficult to stabilize the iron content and aromatic level because iron and zinc contents vary in different regions and growing seasons (Liang et al. 2006). Based on research and evaluation of iron-enriched rice domestically and internationally, and responding to the national strategy on nutrition, we have conducted cross-breeding for iron-enriched aromatic rice varieties by using mutant materials, local aromatic varieties and improved aromatic varieties to contribute to nutrition security for households.

11.2 Materials

1. Rice seed from accessions with desired traits (*see* **Note 1** and Table 11.1 for examples).
2. Rice mutant lines (*see* **Note 2**).
3. KOH (potassium hydroxide).
4. A cobalt-60 facility (*see* **Note 3**).
5. Scale for measuring seed weight.
6. Ruler.
7. Hygrometer.
8. Test tubes (1.3 × 10 cm).
9. Dehuller (e.g. Satake).
10. Atomic absorbance spectrophotometer.
11. Taq polymerase.
12. $MgCl_2$.
13. dNTPs.
14. Gene-specific primers.

Table 11.1 Rice accessions with variation in aroma

SDK[b]	Name of Vietnamese local rice variety	Aroma[a]	SDK[b]	Name of Vietnamese local rice variety	Aroma[a]
Vietnamese local aromatic rice varieties					
	Nang Thom Cho Dao	+	5121	Tam Con	+
233	Tam Tuc Tay Bac	+	5122	Tam Nghia Lac	+
268	Tam Den Ha Dong	+	5124	Tam Hai Giang	+
274	Tam Ap Be Ninh Binh	+	5126	Tam Ap Be	+
314	Tam Xoan Bac Ninh	+	6212	Tam Co Rut	+
316	Tam Nghe Hat Do	+	6216	Tam Thom	+
5117	Tam Xuan Dai	+	6240	Tam Cao Cay	+
5119	Tam Xuan Hong	+	6250	Tam Tieu	+
5120	Tam Nghia Hong	+	2376	Du Nghen	+
219	Tam Tron Hai Duong	−		Tam Thom Hai Hau TT1	+
Vietnamese improved aromatic varieties					
	Soc Trang 3 (ST3)	+		Soc Trang 13 (ST13)	+
	Soc Trang 5 (ST5)	+		Soc Trang 14 (ST14)	+
	Soc Trang 6 (ST6)	+		Soc Trang 15 (ST15)	+
	Soc Trang 10 (ST10)	+		Soc Trang 17 (ST17)	+
	Soc Trang 12 (ST12) Huong Com Hoa Sua	+ + +		Soc Trang 18 (ST18)	+
Rice control					
	Jasmine 85	+			
	Khao Dawk Mali 105	+		VND95-20	-

[a](+) aroma, (-) non-aroma
[b]SDK: seed bank number

11.3 Methods

11.3.1 Preparing a Mutant Population

1. Irradiate seed (*see* **Note 4**).
2. Sow seed in a field nursery.
3. Transplant mutant plants to an experimental field after 25 days nursery growth.
4. Propagate material until the M_3 generation (*see* **Note 5**).
5. Begin phenotypic evaluation in the M_3 generation (*see* **Note 6**).
6. Select interesting plants for further characterization and hybridization (*see* **Note 7**).
7. Continue phenotypic evaluations (*see* **Note 7**).

11.3.2 Phenotypic Analysis of Aroma

1. Harvest seed when 90 % in panicles are ripe. Note that M_1 plants from seed mutagenized material is not suitable for phenotypic analysis as it is chimeric.
2. Air-dry until humidity reaches 14 %.
3. Dehull seed from individual plants.
4. Combine 40 seeds and 5 ml 1.7 % KOH in a test tube.
5. Cover tube and let stand for 15 min at room temperature.
6. Evaluate aroma by smell (*see* **Note 8**).

11.3.3 Genotypic Analysis of Aroma

1. Extract genomic DNA from selected material (*see* **Note 9**).
2. Design primers for a PCR-based marker assay (*see* **Note 10**).
3. Combine 0.25 µl Taq DNA polymerase, 1 µl of genomic DNA, 2.5 µl of 10× buffer, 3 mM $MgCl_2$, 4 µl of dNTPs and 2.0 µl of each primer in a total volume of 25 µl.
4. Incubate samples: 94 °C for 2 min followed by 35 cycles of 5 s at 95 °C, 5 s at 58 °C and 5 s at 72 °C, concluding with a final extension of 72 °C for 5 min.
5. Analyse PCR products using a 2 % agarose gel containing 0.5× TBE (*see* **Note11**).

11.3.4 Chromatographic Analysis of Aroma Compounds

1. Place 3.5 g of milled rice with 500 µl of water in a 10 ml vial (*see* **Note 12**).
2. Equilibrate samples at 80 °C for 5 min.
3. Introduce a Supelco® VB/Carboxen/PDMS (divinylbenzene/Carboxen/polydimethylsiloxane) fibre in the headspace surrounding the rice at the same temperature for 15 min for solid phase microextraction (SPME) of aroma compounds.
4. Analyse extracts using a Hewlet Packard 5890 Série II gas chromatograph using a non-polar DB-5 (J&W Scientific) capillary column (length 60m, 0.32 mm, film thickness 0.25 µm).
5. Use helium as a carrier gas at a flow rate of 1.9 ml min^{-1} at 25 °C.
6. Perform injection in splitless mode first (5 min for SPME), then in split mode to the end of the cycle (38.5 min for SPME).
7. Warm the column at 40 °C for 5 min, then apply the following temperature programmes for SPME: from 40 to 115 °C at a rate of 3 °C/min, then from115 to 220 °C at 30 °C/min, and finally maintain at 220 °C for 5 min.
8. Maintain the detector port at 250 °C.

9. Calculate 2AP from the area ratio between 2AP's peak and the internal standard (*see* Sect. 11.5 for example data).

11.3.5 Combination of Traits Through Hybridization

1. Select materials for crossing (*see* **Note 13**).
2. Perform crosses to generate F_1 material.
3. Perform phenotypic evaluation of F_1 material (*see* **Note 14**).
4. Self-fertilize material to produce a segregating F_2 population.
5. Begin phenotypic and genotypic evaluations and selections to choose material for further propagation.
6. Continue propagation for several cycles (e.g. F_{11}) to ensure that traits are fixed and pure bred (*see* Fig. 11.1 and Sect. 11.5.1 crossing schemes to combine and fix traits in rice).

11.4 Notes

1. It is good practice to choose, when available, multiple accessions having similar traits. This material will be used in hybridizations with mutant lines. Having a diverse set of starting material diversifies the alleles for introgression and should increase the chances of success. In the example, 20 Vietnamese local aromatic rice varieties that were tall, bold grain shape and short-day length photoperiod sensitive were chosen. They have had hard texture when cooked and the average yield is approximately 3–4 tons/ha. Nine aromatic ST rice varieties and Hoa Sua, Huong Com have growth duration of 105–115 days, improved phenotype with 5–6 ton/ha yield and high-quality grain length 7.5–7.8 mm, slender grain, non-chalkiness of endosperm and amylose content of 17–19 %.
2. Seed for irradiation should be homogeneous and crossable with the other accessions chosen. In the example provided in this protocol, Zazu and Huyet Rong, a wild-type, tall and photoperiod-insensitive variety, was selected. The average yield is approximately 3–4 tons/ha. This is a local aromatic variety specific to Vietnam, having hard texture when cooked, aroma, long grains, dark-red bran layer and purple-yellow husk.
3. Other sources of gamma irradiation such as caesium and other types of mutagenesis such as X-ray irradiation and also chemical mutagenesis can be used. It is important that the mutagenic treatment is optimized.
4. The procedure to obtain the mutants may be summarized as follows: Seeds of Zazu and TT1 were incubated in a water bath at 33 °C for 48 h to induce germination to obtain high-frequency gene mutation. They were then irradiated by gamma-rays from a ^{60}Co facility at two doses: 12 krad (120 Gy) and 15 krad

(150 Gy). After 24 h, the seeds were sown in a field nursery to obtain the first generation (M_1). Phenotypic selections began at the M_3 generation selected by staff Le Xuan Tham and Nguyen Thi Thu Hien.

5. A bulking procedure can be used until interesting phenotypes are identified. At this point a pedigree approach should be taken whereby mutations are fixed. Individuals are propagated through self-fertilization, and phenotypes are evaluated and selected at each generation to develop pure-bred lines. In our work we carried mutant lines to M_{10} and hybridized material to F_{11}. Further description of creating pure-bred material from initially bulked material is described in Chap. 9.
6. In addition to specific traits of interest, it is useful to collect data on general traits. Data on plant height (cm), number of effective tillers/plant, panicle length (cm), number of filled grains/panicle, 1000 seed weight (gr), days to maturity and grain yield (ton/ha) were recorded in our example. After harvesting, the seeds of each genotype were dehulled for evaluation of the grain quality, viz. grain size (grain length), grain shape (grain length-breadth ratio) and also aroma. In addition, in our work analysis for bioavailable iron was conducted by atomic absorbance spectrophotometer (AAS) method and the related methods at Da Lat Nuclear Research Institute. See Sect. 11.5 for example data.
7. It is useful to phenotypically characterize accessions prior to using in hybridizations to ensure plants actually show the desired traits. For example, in our work we chose to perform analysis for bioavailable iron conducted by atomic absorbance spectrophotometer (AAS) method and the related methods at Da Lat Nuclear Research Institute. See Sect. 11.5 for example data. Analysis of mutant plants can continue until desired mutant traits are found. During the early stages of propagation (e.g. M_3), there is a chance that other mutations (alleles) are co-segregating and epistatic interactions may limit the ability to identify plants with interesting traits. As propagation proceeds, alleles should segregate away from each other and phenotypes stabilize. At some point the chance of finding new traits is low. We typically do not carry observations past the 11th generation.
8. The detection of fragrance can be carried out *via* sensory or chemical methods, although each has their disadvantages. Chemical methods involving smelling leaf tissue or grains after heating in water or reacting with solutions of KOH (Sood and Sidiq 1978) can cause damage to the nasal passages. Sensory methods therefore have their limitations when processing large numbers of samples, but it gives results in a shorter time and at lower costs for the rice breeder. Aroma evaluation by smell is subjective. It is ideal to select a group of people for the evaluation and average the scores. For example, there were five people in our testing team, they smelled at well-aired places and classified the aroma by four groups: strongly aromatic (score 7), moderately aromatic (score 5), lightly aromatic (score 3) and non-aromatic (score 1). The score of one sample is the average score of three repeated times of smelling, each time is 10 min apart. It is useful to include control material known to have good aroma

and control material known to have poor aroma when performing subjective sensory methods.

9. Methods for low-cost extraction of genomic DNA are described in Chap. 14.
10. Molecular markers may have been developed for traits arising from natural alleles that have spread in populations through evolution and breeding. In the case of rice aroma, the accumulation of 2AP in aromatic rice is explained by the loss of function mutations in the badh2 gene (Bradbury et al. 2005; Chen et al. 2008). At least ten non-functional alleles of the badh2 gene have now been identified (Shi et al. 2008; Sakthivel et al. 2009; Kovach et al. 2009). We used this data to develop four primer sets to evaluate aroma markers in varieties from Vietnam using a simple PCR assay to evaluate differences in amplicon mobility (*see* Sect. 11.5). It is important to note that this approach is inefficient and difficult when evaluating new alleles created by mutation because newly induced mutations did not previously exist in the population and could be in any gene or regulatory region. To create markers for mutant alleles, the best approach is traditional mapping/cloning or through the aid of genome sequencing (*see* Chap. 1).
11. Suitable primer combinations show clearly different band mobilities. Higher-resolution gels such as polyacrylamide can be used when agarose is insufficient.
12. For samples analysed by SPME-GC, collidine was added as an internal standard.
13. It is best to choose well-characterized materials for hybridization.
14. This is important when using mutant material to confirm if alleles are dominant or recessive. If desired combinations of traits are observed in the F_1, one can consider doubled haploid approaches to instantly fix alleles. Doubled haploidy (DH) can also be applied in the F_2 or later generations. Chapter 16 of this book provides a protocol on validation of putatively DH plants.

11.5 Example Data

11.5.1 Breeding New Aromatic Rice in High Bioavailable Iron by Using Gamma Radiation and Crossing

11.5.1.1 Results of Aroma Testing by Sensory from M_3 to M_{10} Generations

We selected dark-red bran layer rice grains and tested aroma in rice. Results of line choice and aroma testing by sensory from M_3 to M_{10} generations (Table 11.2) showed that the rate of strong aroma increased from 8.22 to 12 % and aromatic lines of total aroma tested lines increased correlatively from 30.14 to 60 %, and the mean aroma score of populations increased from 3.55 to 4.68. From M_3 we got 5311 M_3 plants (Table 11.2). From this, 111 M_3 plants were chosen for testing aroma.

Non-aromatic lines fluctuated from 35.14 % in the M_3 generation to 8.61 % in M_5 generation; therefore, average aroma score of the population continuously increased after each generation. From seventh to tenth generations, there were no non-aromatic lines and five strong aromatic lines were selected in M_{10}. These strong aromatic lines were planted consecutively and without replications to evaluate several agronomic criteria such as length of flag leaf, length of dynamic leaf, panicle length, number of tillers/hill and panicles/hill, hypothetical yield and actual yield. External characteristics such as grain length, grain width, grain shape and zero-score chalkiness of endosperm indicated that zero-score chalkiness of endosperm of these mutant rice lines fluctuates from 65.2 to 78.1 % and long and slender grains (ratio of length to width was 3.2–3.4), so these lines have nice grain shape and good quality and value. They have low gelatinization temperature, medium gel consistency and amylose content between 17.3 and 21.1 %; therefore, they are classified as soft rice which is still soft over 24 h after cooking. Moreover, they have long-lasting aroma and strong aroma. Comparing evaluated characteristics including the growth duration time (105 days) and bioavailable iron content, we chose two new aromatic rice varieties named as Red 06 (from 12 krad irradiation dosage) and Red 156 (from 15 krad irradiation dosage). Bioavailable iron content of Red 06 and Red 156 are 44.4 ppm and 35 ppm, while bioavailable iron content of Zazu is 19.5 ppm.

11.5.1.2 Hybridizations to Create High Aroma and Iron Rice

The availability of the rice mutant resource is already helping researchers in their quest to gain insights into the biology of this commercially important crop. These efforts are critical to understand gene function and breeding. Some promising mutant rice lines are discovered, but some characters need to be modified. Therefore we elaborated a strategy in which we use these promising mutant rice lines for crossing with other rice varieties (e.g. Fig 11.2).

Results of lines chosen and aroma testing (Table 11.3) showed that the rate of slightly aromatic and non-aromatic lines was high at early generations, and it gradually decreased until traits were fixed in selected lines.

Nine strongly aromatic lines which were selected were also taken for analysis for bioavailable zinc and bioavailable iron contents by AAS method (Table 11.4). The results showed that zinc content of the nine lines is the same as that of existing inbred varieties.

11.5.1.3 Further Hybridizations to Combine Traits

In the scope of this research, we selected four different parental lines for breeding. Parental lines selected for breeding were evaluated for their agronomic and quality traits. The results are shown in Tables 11.5 and 11.6.

Table 11.2 The result of aromatic lines selected over segregating generations

G[a]	Lines used for aroma testing	Aromatic scale								Mean aroma of populations (scale)
		7	%	5	%	3	%	1	%	
Using gamma Co^{60} at 12 krad dosage										
M2	22	1	4.55	4	18.18	11	50.00	6	27.27	3.00
M3	73	6	8.22	22	30.14	31	42.47	14	19.18	3.55
M4	479	43	8.98	165	34.45	185	38.62	87	18.16	3.69
M5	246	25	10.12	99	40.08	91	36.84	32	12.96	3.95
M6	166	19	11.45	81	48.80	57	34.34	9	5.42	4.33
M7	71	8	11.27	34	47.89	29	40.85	0		4.41
M8	34	4	11.76	19	55.88	11	32.35	0		4.59
M9	26	3	11.54	16	61.54	7	26.92	0		4.69
M10	25	3	12.00	15	60.00	7	28.00	0		4.68
Using gamma Co^{60} at 15 krad dosage										
M2	27	2	7.41	5	18.52	5	18.52	15	55.56	2.56
M3	111	2	1.80	11	9.91	59	53.15	39	35.14	2.57
M4	432	8	1.85	55	12.73	254	58.80	115	26.62	2.80
M5	267	10	3.75	54	20.22	180	67.42	23	8.61	3.38
M6	379	12	3.17	102	26.91	265	69.92	0		3.66
M7	132	14	10.61	74	56.06	44	33.33	0		4.55
M8	173	17	9.83	105	60.69	51	28.48	0		4.61
M9	121	12	9.92	76	62.81	33	28.70	0		4.65
M10	52	5	9.62	33	63.46	14	26.92	0		4.65

[a]*G* Mutant generation

According to quality characteristics shown in Table 11.5, mutant Tam Thom lines T1 and T2 have a very high rate of chalkiness of endosperm which is an obstacle in cross-breeding. However, T1 and T2 are not photosensitive to short-day light and have several other important characteristics such as maintenance of aroma of Tam Thom, closeness between grains, moderate susceptibility to leaf blast (caused by *Pyricularia oryzae*) and low amylose content. Mutant Tam Thom T1 line has brown–yellow colour of grain husk and aroma score of 4.1 which is nearly equal to the aroma score of Tam Thom Hai HauTT1. Tam Thom Hai HauTT1 is highly resistant to leaf blast, and Hoa Sua is an extra long and slender grain, very early maturing and semidwarf. These are the basic characteristics transferred into progenies to create specific characteristics of new rice varieties (Fig. 11.3).

In classical plant breeding, selection typically involves evaluating a breeding population for one or more traits at field trials. In pedigree breeding method, selection of desirable plants is made at early generations for traits of higher heritability. So, effective phenotypic screening will be less expensive for selection in large populations. These F_2 populations were developed, and stringent phenotypic selection based on phenotypic preference (like early maturity, panicle length, grain shape, aroma, etc.) was carried out on segregating populations to obtain

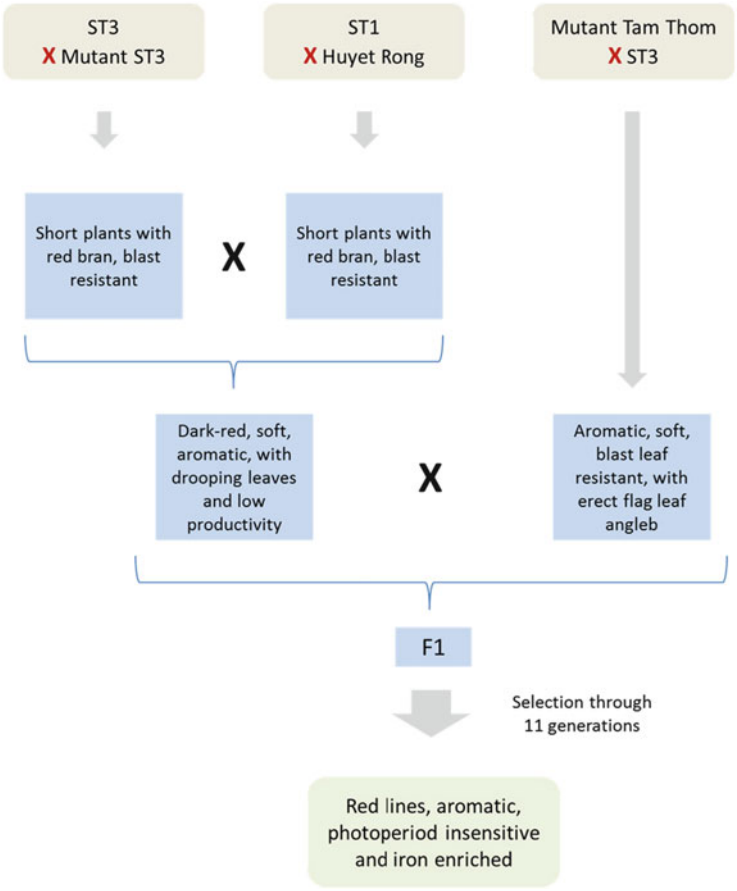

Fig. 11.2 Hybridization between five parents by cutting husks method. Crossing begins between ST3 and mutant ST3 (*top left*), ST1 and Huyet Rong (*middle*) and ST3 and Mutant Tam Thom (*right*). Resulting plants are phenotyped and plants with desired traits are selected (*blue boxes*). Hybridization of these materials is done and then is propagated through 11 generations to produce a line with the desired combination of traits (*bottom box*). Since the F_2 population contains heterozygotes, and heterozygote and dominant homozygote are not easily distinguishable, crossing is continued. Strongly aromatic lines continued to separate highly into four groups such as strongly aromatic, medium aromatic, slightly aromatic and non-aromatic at early generations, and the separation decreased through propagation until traits are pure bred. The rate of strongly aromatic and aromatic lines increased from 1.79 to 8.04 % of total aroma tested lines correlatively from F_2 generation to F_{11} generation, and the mean aroma score of populations increased from 2.35 to 4.69. To the F_{11} generation, we have selected 9 strongly aromatic and 75 aromatic lines. Figure courtesy of Dr Joanna Jankowicz-Cieslak and Dr Bradley J. Till of the FAO/IAEA Joint Programme

agronomically desirable plants and reduce the population size. Selection of desirable plants is also similar in the next segregating populations until pure breeding lines are produced.

Table 11.3 The result of aromatic lines selected over segregating generations

G[a]	Lines used for aroma testing	Aromatic scale								Mean aroma of populations (scale)
		7	%	5	%	3	%	1	%	
Separating generations of five parents (RED ST) ST3/Mutant ST3//ST1/Huyet Rong/// Mutant Tam Thom T5/ST3										
F2	3120	56	1.79	312	10.00	1320	42.31	1432	45.90	2.35
F3	4651	74	1.59	501	10.77	1812	38.96	2064	44.38	2.26
F4	4551	79	1.74	513	11.27	2013	44.23	1946	42.76	2.44
F5	3656	65	1.78	451	12.34	1751	47.89	1389	37.99	2.56
F6	3541	79	2.23	564	15.93	2015	56.90	883	24.94	2.91
F7	3621	110	3.04	662	18.28	2068	57.11	781	21.57	3.06
F8	2893	211	7.29	711	24.58	1720	59.45	251	8.68	3.61
F9	2113	189	8.94	611	28.92	1142	54.05	171	8.09	3.77
F10	1723	162	9.40	752	43.64	743	43.12	66	3.83	4.17
F11	112	9	8.04	75	66.96	29	25.89	0	0	4.69

[a]*G* mutant generation

Table 11.4 The evaluation results of bioavailable zinc and bioavailable iron contents of the nine lines plus control

No.	Name of lines	Bioavailable zinc content (ppm)	Bioavailable iron content (ppm)
1	R110-755	21.2 ± 2.1	14.6 ± 1.0
2	R34RD-840	25.6 ± 2.2	24.8 ± 1.9
3	R35RD-869	21.1 ± 1.7	19.8 ± 1.2
4	R75-797	20.8 ± 2.0	21.8 ± 1.1
5	R76-696	21.0 ± 1.8	65.1 ± 2.3
6	R75-747	21.0 ± 2.1	15.7 ± 1.2
7	R51-723	24.5 ± 1.9	25.8 ± 1.4
8	R857-821	29.8 ± 2.5	44.3 ± 2.2
9	R8-786	23.7 ± 1.6	22.2 ± 1.2
10	Jasmine 85		12.8 ± 0.9

Table 11.5 Agronomic characteristics of rice materials

Name of parents	Growth duration (days)	Plant height (cm)	Brown grain length (mm)	Reaction to brown plant hoppers (scale)	Reaction to leaf blast (scale)
Mutant T1	95	115 ± 5.7	7.0 ± 0.2	7	5
Mutant T2	99	101 ± 3.2	7.1 ± 0.2	7	5
Tam Thom Hai HauTT1	118	145 ± 6.2	5.6 ± 0.2	3	2
Hoa Sua	90	100 ± 1.0	7.8 ± 0.1	9	6

Table 11.6 Quality characteristic of parents

Name of parents	Weight of 1000 grains (gramme)	Zero-scale chalkiness of endosperm (%)	Gelatinization temperature (scale)	Amylose content (%)	Gel consistency (mm)	Aroma (scale)
Mutant T1	19.6 ± 0.3	14.6 ± 4.0	6.6 ± 0.5	18.4 ± 1.4	71.5 ± 1.9	4.1 ± 0.04
Mutant T2	19.7 ± 0.3	22.4 ± 2.4	6.4 ± 0.5	19.5 ± 0.9	61.2 ± 2.1	3.4 ± 0.04
Tam Thom Hai HauTT1	19.1 ± 0.3	97.6 ± 1.1	5.6 ± 0.5	16.9 ± 0.4	77.7 ± 1.7	4.4 ± 0.04
Hoa Sua	24.1 ± 0.3	99.8 ± 0.8	5.7 ± 0.2	12.1 ± 0.4	60.2 ± 3.5	2.7 ± 0.81

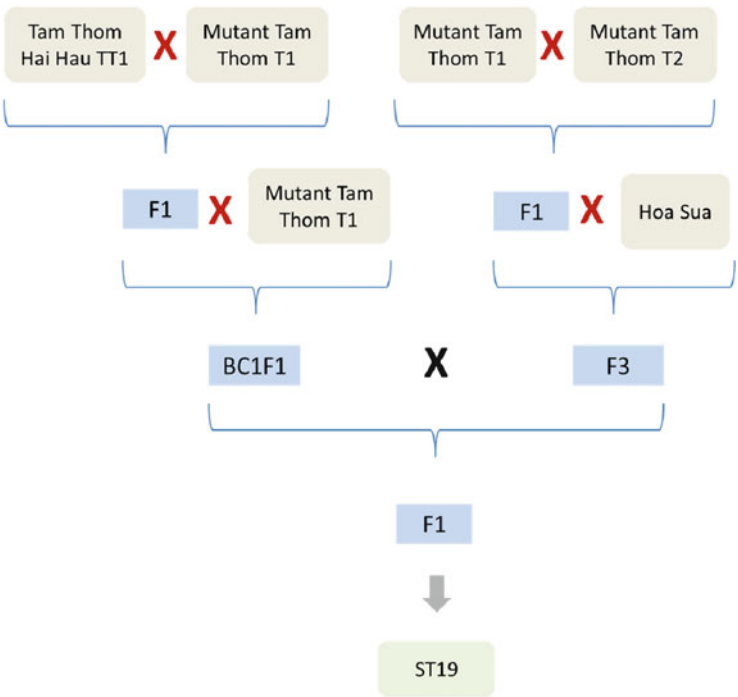

Fig. 11.3 Crosses carried out to produce the variety ST19 using varieties and advanced mutant lines. Successive rounds of back- and outcrosses were carried out to create a line with combined characteristics. Figure courtesy of Dr Joanna Jankowicz-Cieslak and Dr Bradley J. Till of the FAO/IAEA Joint Programme

Table 11.7 The result of aromatic lines selected over segregating generations

G[a]	Lines used for aroma testing	Aromatic scale								Mean aroma of populations (scale)
		7	%	5	%	3	%	1	%	
Separating generations of four parents (ST19) TT1/2*Mutant Tam Thom T1///Hoa Sua/Mutant Tam Thom T2//Mutant Tam Thom T1										
F2	2527	56	2.22	817	32.33	1326	52.47	328	12.98	3.48
F3	427	17	3.98	151	35.36	208	48.71	51	11.94	3.63
F4	268	18	6.72	122	45.52	101	37.69	27	10.07	3.98
F5	333	30	9.01	168	50.45	130	39.04	5	1.50	4.34
F6	493	53	10.75	264	53.55	176	35.70	0		4.50
F7	277	30	10.83	148	53.43	99	35.74	0		4.50

[a]*G* segregating generation

In segregating populations of three combinations, we separated aroma into four groups such as strongly aromatic (score 7), moderately aromatic (score 5), lightly aromatic (score 3) and non-aromatic (score 1) (Table 11.7). Resulting lines from the hybridizations were further characterized for traits including amylose, 1000 grain weight and iron (Table 11.8).

11.5.1.4 2-Acetyl-1-pyrroline Analysis in Aromatic Rice

The high demand for fragrant rice cultivars in markets worldwide has driven the development of methods for quantifying 2-acetyl-1-pyrroline (2AP) and distinguishing fragrant and nonfragrant cultivars. The concentration of 2AP is controlled by a recessive gene for fragrance (fgr) mapped on rice chromosome 8. Methods for the determination of the volatile compounds in rice have schemes for collection, concentration, separation and quantification. The method of preference for the pre-concentration of flavour compounds is solid phase microextraction (SPME).

We determined the concentration of 2AP in 62 samples of rice grains (brown rice). The concentration of 2AP by SPME-GC analysis varied among seasons and ecological cultivated sites (Table 11.9).

11.5.1.5 Molecular Screening for Aroma in Segregating Rice Lines

To facilitate the selection of plants with improved aroma coming from natural alleles, we used a molecular marker strategy to test for the badh2 gene. Variations in this gene are thought to have major effects on rice aroma (Bradbury et al. 2005b; Chen et al. 2008). An example gel showing size polymorphism variation between aromatic and non-aromatic rice is shown in Fig. 11.4.

Table 11.8 Characteristics of high bioavailable iron rice cultivars and aromatic rice cultivars

No.	Characters	Red 06	Red 11	Red 156	Aromatic rice 12	RED ST	ST16	ST19	ST20
1	Growth duration time (days)	105	105	108	110	108	112	95-105	115
2	Flag leaf length (cm)	30.4	26.0	38.1	30.9	31.0	38.1	25.48	32.1
3	Plant height (cm)	121.5	91.1	127.5	111.1	112.5	90.5	91.26	102
4	Length of panicles (cm)	28.6	26.1	28.1	29.5	27.5	26.3	26.18	25.7
5	Tillers/hill	16.5	18.5	18.4	22.2	13.7	10.3	10.79	16.2
6	Panicles/hill	11.8	14.5	15.2	14.2	9.8	8	7.76	8.5
7	Weight of 1000 grains (gramme)	24.6	23.3	25.8	27.9	27.8	25.5	23.01	25.3
8	Grains/panicle	135.6	115.0	112.2	117.5	110.0	133	151	131
9	Total filled grains/panicle	103.5	108.1	81.5	103.0	94.9	121	101.83	88.8
10	Length of kernel (mm)	6.7	6.6	6.7	7.8	7.5	8.6	7.65	8.2
11	Width of kernel (mm)	2.0	1.8	2.1	1.8	2.0	1.8	1.72	1.7
12	Zero-score chalkiness of endosperm (%)	78.1	95.0	75.2	95.2	88.0	100	100	100
13	Gelatinization temperature (scale)	6.0	6.0	6.0	7.0	7.0	7.0	7	6.8
14	Gel consistency (mm)	65.6	63.5	54.4	65.4	75.0	67	55.73	64.7
15	Amylose content (%)	18.5	15.6	17.4	19.1	13.12	14.1	10.6	12.4
16	Aroma	Strong	Strong	Light	Strong	Strong	Strong	Strong	Strong
17	Yield (ton/ha)	4.7	5.6	5.1	6.7	4.5	5.1	5.2	5.7
19	Bioavailable iron content (ppm)	44.4	55.1	35		65.1		10.8	
20	Brown rice colour	Red	Red	Red	White	Red	White	White	White

Table 11.9 The change of aroma content in aromatic rice among growing seasons

	2AP content (ppb)		Sensory test by smelling (scale)	
Cultivated name	Dry season	Wet season	Dry season	Wet season
ST3	6.03	1.77	5.3	1.9
ST10	6.80	2.40	4.5	2.5
ST12	8.73	2.86	5.1	2.5
ST16	5.97	2.84	5.8	3.4
ST17	6.83	2.63	4.2	3.3
ST18	4.47	2.07	4.9	3.7
ST19	4.90	2.57	5.0	2.6
ST20	8.83	1.58	4.3	2.9
Jasmine 85	6.10	2.52	4.7	2.9

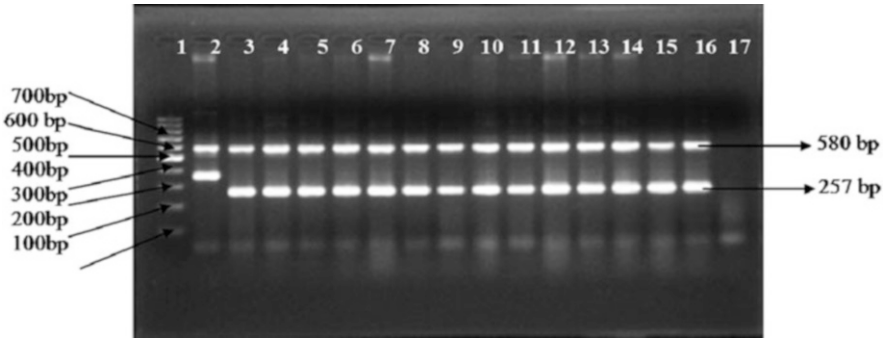

Fig. 11.4 PCR analysis of new aromatic rice varieties for the presence of aroma gene. Lanes 1, 100 bp ladder; 2, VD95-20 is non-aromatic; 3, ST10 is aromatic; 4, Red ST is aromatic; 5, 6, 7, Red 06 from 12 krad; 8, 9, 10, Red 156 from 15 krad; 11, 12, 13, Red 11 from mutant Zazu/Red ST; 14, 15, 16, aromatic rice 12 from mutant Zazu/ST10 and 17, water

11.6 Conclusion

Breeding aromatic rice varieties having high quality and yield in order to increase value and to serve increasing demand of customers domestically and for exportation is essential. Multiline crossing was carried out on mutant Tam Thom, and aroma improved rice varieties by using pedigree method. After a strict process of selection by qualitative and quantitative anticipated targets (life cycle, leaf and stem phenotypes, grain dimensions, aroma), we have selected aromatic rice varieties named ST16, ST19 and ST20. These varieties have growth duration of 95–115 days, improved phenotype (plant height of 102 cm, strongly tillering) with actual yield higher than 5 ton/ha and high-quality grain length $>$7.5 mm, slender grain, non-chalkiness of endosperm and low amylose content. Their grains are of high quality so they have high economic value.

Aroma in 20 Vietnamese local aromatic rice varieties (19 varieties of Tam group in the north of Vietnam and Nang Thom Cho Dao in the south of Vietnam) and Red

06, Red 156, Red 11, aromatic rice 12, Red ST, ST16, ST19 and ST20 are explained by the badh2.1 allele. Aroma sensory and 2AP content in the dry season were higher than in the wet season. Rice aroma was segregating in before the establishment of pure-bred lines necessitating phenotypic and genotypic screening of material. Breeding would be further aided through establishing the interrelated scale between aroma scale and 2AP content. The strategy of mutant generation and selection and hybridization with accessions harbouring natural alleles allowed us to combine novel and existing traits to create new rice varieties. The methods can be adapted for other breeding objectives in rice and other seed-propagated crops.

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