

CHAPTER II

GENERAL MATERIALS AND METHODS

The experimental program was based on four varieties: Thasano 1 (TSN 1), Thadokkham 1 (TDK 1), Hom Nang Houane (HNN) and Kai Noy Leuang (KNL). HNN is a traditional Lao variety that is aromatic and carries the fragrance gene commonly found in most aromatic types. KNL is also a traditional aromatic Lao variety which does not carry the common mutation in the fragrance gene but is highly aromatic, TSN 1 and TDK 1 are improved varieties, high yielding, but not aromatic. Populations were made and progressed through generations at the Rice and Cash Crop Research Centre, National Agriculture and Forestry Research Institute (NAFRI), Vientiane, Lao PDR, The study was undertaken in two parts:

Part 1: Analysis of the parent varieties: study on the effects of N on yield, and development of populations and grain quality (aroma (2AP), protein, gelatinisation temperature, texture and viscosity) to determine the best improved variety to be the agronomically adapted background for mapping populations, and determining the compound in the grain of these parents by using metabolomic profiling

Part 2: Determining compounds in the pathways of aroma of KNL and HNN using introgression lines (ILs) derived from crosses with TSN1 until BC₄F₂, and the genetic basis of fragrance in KNL.

2.1 Comparing the effect of nutritional conditions on yield and grain quality (aroma (2AP), protein, gelatinisation temperature, texture and viscosity), and determining the compound of in the grain of these parents by using metabolomic profiling.

2.1.1 Plant growth

Seeds of TDK1, TSN1, HNN and KNL were sown in 2006 rainy season in Rice and Cash Crop Research Centre (RCCRC), Vientiane, Lao PDR. One month later, seedlings were transplanted in a split plot randomised design in RCBD of sub-plots within four main plots. Each main plot contained three sub-plots of each variety.

Each sub-plot was 2 m x 5 m, and 10 rows of 25 plants were transplanted at a spacing of 20 cm between plants and between rows. The main plots received 30 kg ha⁻¹ of P and K at transplanting. Different levels of N fertilisation were applied to each main plot: 0, 30, 60 or 90 kg N ha⁻¹. For the two high N treatments, 30 kg of N was applied at transplanting and the remainder was applied in two equal top-dressings at 25 and 45 days after transplanting, and for the low N treatment, N was applied in two equal top-dressings at 25 days and 45 days after transplanting.

The harvested grain was then sent to the International Rice Research Institute in the Philippines for analysis of quality traits. Paddy from each sample was dehulled (Satake Rice Machine, Tokyo, Japan), milled (Grainman 60-230-60-2AT, Grain Machinery Mfg. Corp., Miami, FL), and a sub-sample ground to flour (Udy Cyclone Sample Mill 3010-030, Fort Collins, CO) to pass through a 0.5 mm sieve. Reagent-grade chemicals were used. Reverse osmosis water, filtered through a 0.22 µm Millipore (Billerica, MA) filter, was used throughout the study.

2.1.2 Data collection

During plant growth, the following parameters were recorded:

Tillering number per hill, day to flowering, plant height, % filled grain, number of panicles per plant, and yield.

2.1.3 Data analysis

Mature grain was analysed for grain quality - aroma (2AP), protein content, gelatinisation temperature, texture and viscosity. For each set, 200 g of each sample of mature grain was milled, vacuum packed and shipped to the EU consortium for metabolomic profiling to analyse for compounds (described in Chapter IV).

2.2 Determining compound in the pathways of aroma using ILs derived from crosses with TSN 1 until BC₄F₄

Three varieties of rice (*Oryza sativa* L.) from Laos, TSN 1, a high yielding but non-aromatic variety, and HNN and KNL, traditional aromatic varieties, were used as parental lines to construct two populations. Backcrossing was performed to transfer the aroma alleles from HNN to TSN 1, and from KNL to TSN 1. The first crosses were made in 2006 rainy season at RCCRC in Vientiane, Lao PDR. At each subsequent generation, progeny with the TSN 1 plant type that were fragrant were

selected for the next generation of backcrossing. For the set where HNN was the donor parent, the presence of the fragrance gene was detected with markers at each generation, exactly as previously described (Fitzgerald et al., 2008). The F₁ progeny heterozygous for the deletion were selected and crossed back to TSN 1. This procedure was repeated from BC₁F₁ up to BC₄F₁. For the set with KNL as the donor parent, fragrant progeny were selected based on a sensory test for fragrance. Those found to be fragrant were back-crossed to TSN 1 in the same way as for the HNN set. For both sets the BC₄F₁ progeny were allowed to self, to give BC₄F₂, which were grown in the Lao PDR in the 2009 rainy season. At early tillering, leaf material were harvested from each line for DNA extraction for SNP genotyping to estimate the similarity of the ILs to the recurrent parent for selecting ILs, with the fragrance mutation and minimal introgression from HNN. At BC₄F₄, seed were planted and at the three leaf stage, leaves were collected for DNA extraction and SNP profiling at the 384 loci to determine the segregation pattern, and grain of these ILs were sent to METAPHOR for determining compounds involved in the pathway of fragrance by metabolomic profiling (described in Chapter IV).

In order to determine the genetic basis for aroma in KNL, the BC₄F₂ lines from that population were genotyped at 384 SNP loci, and then 2AP was detected in the grains by gas chromatography. Associations were then made between the presence of 2AP and the genotype.

In order to assess the quality of the 2AP from KNL, and population was made between HNN and KNL and grown to F₅ at IRRI. Once the gene for aroma was discovered in KNL, markers were selected for the mutation and 100 progeny carrying the fragrance gene from HNN and 100 that carried the fragrance gene from KNL were selected and grown at IRRI in the 2010 dry season. 2AP in the grains of each line was determined with gas chromatography.