Short Communication

The possible role of *atp6* gene in cytoplasmic male sterility in WA (Wild Abortive) type of rice (*Oryza sativa* L.)

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Abstract
The possibility of occurrence of some rearrangements inside and or around *coxII* and *atp6* genes and their relationship with male sterility in rice lines having wild abortive (WA) cytoplasm was studied. Two sterile (IR58025A and IR62829A) and two maintainer lines (IR58025B and IR62829B) were used. Radioactive Southern blotting was employed to carry out the experiments. The hybridization of coxII gene to the HindIII digests of total DNA from the maintainers and sterile lines was the same, indicating that this gene was not possibly related to male sterility. However, when the *atp6* gene was used as a probe and the same lines and procedures were employed, the probe hybridised to different fragments in maintainer lines compared to the sterile lines and their hybridization pattern was totally different. These findings could be interpreted as the occurrence of some rearrangements inside and or around *atp6* gene and its probable involvement in the male sterility of WA rice.
Keywords: *Rice; Wild abortive (WA); atp6; Cytoplasmic male sterility*

Rice (*Oryza sativa* L.) is one of the most important crops feeding over half of the world population. Rice production must increase by 40%, to keep pace with the increasing demand, as the world population is likely to pass 8 billion by the year 2030. One of the technological options to increase rice yield, is the production of hybrid rice through available male sterility systems (Rajendran *et al*., 2007). Plants that fail to produce fertile pollen grains are said to be male sterile. If male sterility does not show Mendelian inheritance, but is instead maternally inherited, it is referred to as cytoplasmic male sterility (CMS) (Laughnan and Gabay-Laughanan, 1983), which is concomitant with female fertility (Budar *et al*., 2003). Female fertility brings two advantages to the plants carrying this characteristic: first, better usage of resources in female function and an increase in female viability and second, avoiding inbreeding depression and the production of more viable seeds (Budar *et al*., 2003). In plants, CMS/fertility restoration is considered as a conflicting phenomenon between cytoplasmic and nuclear genes. In the majority of cases, mitochondrial genes act to cause male sterility; and on the opposing side, there are nuclear genes whose products restore male fertility (Budar *et al*., 2003). However, it should be emphasized that generally, the CMS feature is due to the dysfunction of tapetal cells (Kurek *et al*., 1997). Several types of CMS have been demonstrated in rice; the main types are: S1 (Chinsurah Borro II [Bo]), S2 (Wild Abortive [WA]), S3 (Gambiaca) and S4 (Young *et al*., 1983). WA type identified in the wild species *Oryza*. *sativa* f. *spontanea* is the most widely used CMS source as it is more stable and can be easily restored (Rajendran *et al*., 2007). Cytochrome oxidase subunit II (*coxII*) is a part of the cytochrome oxidase complex enzyme in the inner membrane of all aerobic organisms which binds to one heme and one copper atom to be in close contact with cytochrome c (Kao *et al*., 1984). The *atp6* gene codes for a subunit in the ATPase complex (Iwabuchi *et al*., 1993) and is encoded in the mitochondrial genome (Schuster and Brennike, 1994). In this study, an investigation on the possibility of correlation of a rearrangement around and or inside *atp6* gene and its possible involvement with male sterility induction in the WA type of CMS is

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reported.

For this purpose the seeds of all rice lines were obtained from the International Rice Research Institute (IRRI), Manila, Philippines. Sterile (A) lines included IR58025A and IR62829A and maintainer (B) lines consisted of IR58025B and IR62829B.

Total DNA was extracted from fresh leaves using the method of Doyle and Doyle (1987). Two mitochondrial genes were used as probes, coxII and atp6. The coxII gene was provided by Professor C. Leaver (Oxford, UK) and *atp6* was a wheat gene cloned into XL1-blue (Mohr *et al*., 1993). Plasmid DNA isolation and purification was carried out according to the protocol for alkaline lysis (Sambrook *et al*., 1995). Five µg of total plant DNA was digested by *Hin*dIII enzyme. The *coxII* and *atp6* probes were radiolablled with ^{32}p. For southern hybridization, a Ready-to-Go reaction mixture (Bio-Rad, UK) containing dNTPs and the FPLC-pure Klenow fragment of DNA polymerase I (4-8 units) was used. The membrane was washed by incubating at 65ºC for 30 min in each of the following 1 X saline-sodium citrate (SSC), 0.1% (w/v) sodium dodecyl sulphate (SDS); 0.5 X SSC, 0.5% (w/v) SDS and 0.1 X SSC 0.1% (w/v) SDS (Sambrook *et al*., 1995). The Southern blots were repeated twice and two separate membranes were used for each of the probes.

IIn the CMS-[Bo] type, a chimeric copy, *urf-rmc* (unidentified reading frame of rice mitochondria associated with CMS) of *atp6* as well as the normal atp6 gene were reported to be present (Kadawaki *et al*., 1990). The appearance of CMS character has been correlated to reorganizations around the *atp6*, suggesting that recombination downstream from the *atp6* gene is involved in CMS [(Iwabuchi *et al*., 1993, Akagi *et al*., 1994)]. Recently, Wang *et al*., (2006) showed that in rice with BoroII cytoplasm, an abnormal mitochondrial reading frame, *orf79*, is cotranscribed with a duplicated *atp6* gene which eventually produce a cytotoxic peptide. They further found that the expression of *orf79* in CMS lines and those rice plants that had been transformed with *orf79*, caused gametophytic male sterility.

In comparison to the findings on the [Bo] type of CMS, few studies have focused on WA CMS. The presence of plasmid like DNAs, in mitochondria suggested to be associated with male sterility (Mignouna *et al*., 1987), however, this idea was soon disproved by Saleh *et al*., (1989). Narayanan *et al*., (1996) found a major rearrangement in the *coxI* locus, by comparing the sterile and fertile lines. Rajandrakumar *et al*., (2007) found a mitochondrial DNA sequence containing a repeat motif that was polymorphic between WA -CMS lines and their cognate isonuclear maintainer lines. As two of the important genes responsible for the mitochondrial function, we investigated *coxII* and *atp6* in order to discover any possible differences between sterile and maintainer lines of WA rice

In this study, two sterile lines (A) and two corresponding maintainer (B) lines were used. The two mitochondrial genes were hybridized to *HindIII* and *BamHI* digests of total DNA from all lines. Three Southern blotting experiments were carried out. In the first experiment, the *atp6* gene was used as a probe to hybridize with *Hin*dIII digest of total DNA from the sterile and maintainer lines. In IR62829B one strong signal of 7.5, and two weak signals of 4.8 and 2.4 kb were detected (Fig. 1A, lane 1) and in IR62829A one strong signal of 8.4, and two weak signals of 4.8 and 2.4 kb (Fig. 1A, lane 2) were observed. In IR58025B, one strong signal of 7.0, and two weak signals of 4.6 and 2.2 kb were detected (Fig. 1A, lane 3). In IR58025A, one strong signal of 8.0, and three weak signals of 6.0, 4.6 and 2.2 kb were observed (Fig. 1A, lane 4). In the second hybridization experiment, *Bam*HI digests of all lines were hybridized with the *atp6* probe. In IR62829B, 2 signals of 12.0 and 7.7 kb (Fig. 1B, lane1), in IR62829A, 3 signals of 11.2, 10.0 and 7.7 kb were detected (Fig 1B, lane 2). In IR58025 B (Fig. 1B lane 3), 3 signals of 11.2, 10.0 and 7.7 kb and IR58025A (Fig 1B, lane 4), 2 signals of 9.2 and 7.5 kb were observed, respectively. The hybridized signals include signals with high intensities (strong signals) and low intensities (weak signals). It can be suggested that the strong signals are the fragments containing the *atp6* gene and the weak signals contain sequences with some homology with the *atp6* gene. In this regard, in the sterile lines (A) larger fragments were hybridized to the atp6 probe, when their DNA was restricted with HindIII (Fig 1a, lanes 2 and 4) compared to the maintainer lines (B). However, the main signals were not necessarily larger in the sterile lines compared to the maintainer lines, when their DNA was restricted with BamHI (Fig 1b). In the third experiment, digested DNA was hybridized to coxII gene. In IR62829B and A lines, a strong fragment of 3.5 and a weak fragment of 3.3 kb were hybridized to the probe (Fig. 1c, lanes 1 and 2). This extra 3.3 kb signal in the IR62829B and A lines may be due to duplication or the presence of another copy of this gene (or a part of this gene) in the nucleus or chloroplast of these lines (Covello and Gray, 1992; Nugent and Palmer 1991). In IR58025B and A lines, only one strong fragment of 3.5 kb was hybridized to the probe

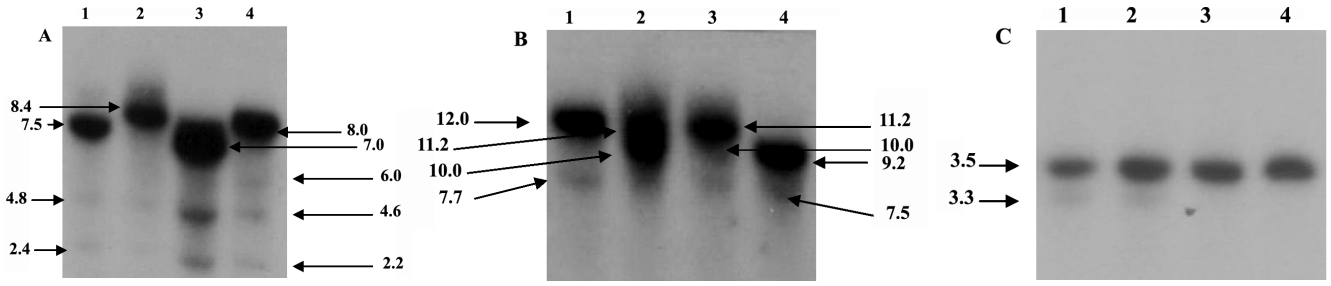

Figure 1. A: Southern blot hybridization of *Hin*dIII digests of total DNA from IR62829B (lane 1), IR62829A (lane 2), IR58025B (lane 3) and IR58025A (lane 4) lines with *atp*6. B: Southern blot hybridization of *Bam*HI digests of total DNA from IR62829B (lane 1), IR62829A (lane 2), IR58025B (lane 3) and IR58025A (lane 4) lines with *atp*6. C: Southern blot hybridization of *Hin*dIII digests of total DNA from IR62829B (lane 1), IR62829A (lane 2), IR58025B (lane 3) and IR58025A (lane 4) lines with *coxII*. All sizes are given in kb.

(Fig. 1c, lanes 3 and 4).

The results of this study are in agreement with the previous studies, such as the occurrence of mutations in atpa, atp6 and coxII genes (Levings and Dewey 1988), a chimeric open reading frame in maize (Gallagher *et al*., 2002), a 5′ leader sequence in the *atp6* gene in sugar beet (Yamamoto *et al*., 2005), a pseudo *atp6* gene in pepper (Kim and Kim, 2005) and *coxI* gene and *orf* 256 in wheat (Rathburn and Hedgcoth, 1992). In rice, the [BO] type of CMS has been shown to be associated with an additional chimeric gene *urfrmc* which consists of the 5' flanking noncoding region of *atp6* (Kadowaki *et al*., 1990). Kowaki *et al*., (1990) and Akagi *et al*., (1994), reported that recombination events around some mitochondrial genes specially *atp6*, are involved in causing male sterility in Chinsurah BoroII type of CMS in rice. Wei *et al*., (2006) also found results similar to those of this study, using several rice lines with gametophytic and sporophytic types of CMS. They used 12 sterile (A) and maintainer (B) rice lines and 5 mitochondrial genes including *atp6*, *atpA*, *cob*, *coxI* and *coxII* in Southern blot experiments. Significant differences were observed between A and B lines in the *atp6* and *cob* genes. The CMS phenotype has been related to a homologous recombination hotspot domain in the *atp6* gene, with a conserved sequence of 7 base pairs 5′-TTCCCTC-3′ which can induce the formation of chimeric genes in mitochondrial DNA (Hanson and Folkers, 1992). Processing of the abnormal copies of mitochondrial gene products could restore male fertility in some CMS lines of rice. In a study, Wang *et al.,* (2006) reported that an abnormal mitochondrial open reading frame, *orf79* is cotranscribed with an abnormal copy of *atp6* (B-*atp6*) gene and encode a cytotoxic peptide which accumulates in CMS lines. The rf1 gene product could restore fertility by endonucleolytic cleavage or degradation activity on dicistronic B-*atp6*/*orf79* mRNA. Kazama and Toriyama (2003) reported that a 4.7 kb fragment of a rice restorer line promoted the processing of aberrant *atp6* RNA when introduced into a CMS line. Editing of the *atp9* mRNA gene has also been shown to restore fertility in CMS lines in purple rice (Wei *et al*., 2008) and in tobacco (Zabaleta *et al*., 1996). Yashitola *et al*., (2004), using PCR (polymerase chain reaction), identified a mitochondrial DNA sequence in the WA type of CMS in rice that differed in A and B lines and their hybrids. They used IR58025A, IR58025B, IR62829A, IR62829B, and DRRH1 hybrid lines. This DNA fragment showed 97% homology to a region of the rice mitochondrial DNA, located at the 5′ end of the *rps3-rp116-nad3-rps12* gene cluster. The results of this investigation are supported further by the findings of Kazama and Toriyama (2003) and Wang *et al*., (2006) who showed that in the BoroII cytoplasm a pseudo copy of the *atp6* gene is causing male sterility. The clear differences in the A and their corresponding B lines in and/or around *atp6* gene could show a new insight in the cause of CMS into the WA type of rice. When the *coxII* gene was used as a probe, identical signals were detected in the A and their cognate isonuclear B lines. In the IR62829B and A lines, one strong fragment of 3.5 and a weak fragment of 3.3 kb were hybridized to the probe (Fig. 1C, lanes 1 and 2). This extra 3.3 kb signal in the IR62829B and A lines may be due to duplication or the presence of another copy of this gene (or a part of this gene) in the nucleus or chloroplast of these lines (Covello and Gray, 1992; Nugent and Palmer 1991). In IR58025B and A lines, only one strong fragment of 3.5 kb was hybridized to the probe (Fig. 1C, lanes 3 and 4).

Even though it has been shown in other types of CMS in rice that mitochondrial genes can cause male

sterility, but the findings of this study draw more attention to the possible relation between the *atp6* gene and CMS in the WA rice. This line of evidence needs to be verified by other techniques such as Northern and Western blot analyses. Furthermore, the sequence of atp6 gene could be determined in both A and B lines, in order to find sequence differences. RNA editing in A and B lines of WA rice is another option for future investigations.

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