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An Efficient Protocol for Genomic DNA Extraction from Santol (*Sandoricum Koetjape*) for SRAP Marker Analysis

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Santol (*Sandoricum koetjape*) belonging to the family of Meliaceae which is a common fruit crop and fruit processing in Thailand. The large numbers of santol varieties have been under cultivation and a few of molecular markers have been used to evaluate genetic diversity. PCR-based techniques require high quality and sufficient quantity of DNA. However, Satol contains high amounts of polyphenol and other secondary metabolites. Therefore, the present study was aimed to establish efficient protocol for DNA extraction and evaluate genetic diversity using Sequence-related amplified polymorphism (SRAP) markers. Three stages of leaves, include young, immature and mature leaves were tested and optimized for high quality DNA. Standard CTAB method was modified by adding step to crush sample with 2x CTAB with an aim to remove phenolic compounds. DNA purification was carried on only for young leaves before experiments. Preliminary study of SRAP marker, two varities were selected to analyse with thirty sets of primer combinations. The number of SRAP bands were observed with an average of 9.7 bands per primer combinations, sizes ranged from 100 to 2500 base pairs. Nine primer combinations showed reproducible fragments with easily recordable bands and gave polymorphisms. Therefore, those primer combinations were selected for further genetic diversity analysis.

Keywords: DNA extraction, *Sandoricum koetjape*, Santol, SRAP

Introduction

Santol or cottonfruit (*Sandoricum koetjape* (Burm.F.) Merr.) belongs to the family Meliaceae which is a tropical fruit grown in Southeast Asia. In Thai name, this fruit is called Kraton (กระท้อน) that is one of fruit were making much more income for farmers. There are over 10 different named varieties of Kratol

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such as Puifai, Tubtim, Khunthong, Khiaowan, Nimnuan, Thongbaiyai, Thongkammayi and Ela. The sweet varieties such as Puifai and Ela have gained popularity for eating fresh fruit. On the other hand, the fruit from native varieties such as Tubtim and Nimnuan tends to be smaller size and sour tasting fruit. The higher value of fruit can be added by processed into delicious product like a dried santol peel candy and chilled santol in syrup (Kathon Loy Kaew). Furthermore, morphological characters alone are not sufficient to distinguish those varieties as well as varieties names. Including, a few of molecular markers have been used to evaluate genetic diversity in this plant. DNA-based molecular techniques are require high quality and sufficient quantity of DNA. However, Kratol contains high amounts of polyphenol and other secondary metabolites that interfere with DNA extraction as well as PCR reaction **(**Rajakani *et al*., 2013). Therefore, the present study was aimed to evaluate efficient protocol for DNA extraction and to screen primers for sequence-related amplified polymorphism (SRAP) marker.

Materials and methods

Collections and DNA extraction

The young, immature and mature fresh leaves of 4 samples (KT22: Puifai, KT24: Tubtim, KT27: Khunthong, KT28: Khiaowan) of *S. koetjape* were collected from Nonthaburi, Thailand. The leaves transported to the laboratory in an icebox and stored in -80 ℃ until used. The CTAB method as described by Doyle and Doyle (1990) and its modifications have been used to obtain good quality DNA. First, grind plant tissue into a fine powder in liquid nitrogen. The samples were homogenized in CTAB buffer using a mortar and pestle before incubate the samples at 65 ℃ in water bath. The chloroform: isoamyl alcohol (24:1) step was repeated at least four times. The precipitated DNA was dissolved in 100 µL of TE buffer. Finally, the DNA concentration and quality was observed on 1% agarose gels stained with ethidium bromide.

PCR amplification and DNA sequencing

The ribulose-bisphosphate carboxylase (*rbc*L) gene that in the chloroplast genome was amplified using the rbcLN and 840R primers (Yamashita and Tamura 2000). The PCR reaction contained a final concentration of 1X PCR buffer, 0.8 µM each primer, 0.2 mM dNTPs, 1U Taq DNA polymerase and 100 ng DNA template and add ddH_2O to a final

volume of 25 µl. The PCR reaction was performed in Mastercycler ep Gradient S (Eppendorf) using one denaturation step at 94 ℃ for 3 min followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 50 ℃ for 30 s and extension at 72 ℃ for 1 min 20 s and a final extension at 72 ℃ for 10 min. The PCR products were determined by electrophoresis in 1% agarose gels in 1X TBE buffer. The quality of PCR product was purified with a GF-1 gel DNA recovery kit (Vivantis) prior to sequencing at First Base Laboratories (Malaysia).

SRAP marker

Thirty combinations of five forward primers (ME1-ME5) and six reverse primers (EM1-EM6) (Li and Quiros, 2001) were initially screened in KT22 and KT28. The PCR reaction was carried out in a final volume of 20 µl with 100 ng template DNA, 0.8 µM each primer, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 1U Taq DNA polymerase and 1X PCR buffer. The PCR amplification program was as follows: 3 min of denaturation at 94 ℃; five cycles of 1 min of denaturation at 94 ℃, 1 min of annealing at 35 ℃, and 1 min of elongation at 72 ℃, then 35 cycles of 1 min of denaturation at 94 ℃, 1 min of annealing at 50 ℃, and 1 min of elongation at 72 ℃ followed by one final extension of 10 min at 72 ℃. The amplified SRAP fragments were separated on a 2% agarose gel stained with ethidium bromide and made SRAP profile by PowerPoint program.

Results and Discussions

DNA extraction

Generally, the mature leaves were collected for DNA extraction due to their continued availability whole year round. On the other hand, the young leaves are not always available from limit the time of collection. However, mature leaves are not preferred for DNA extraction due mainly to the presence of high concentrations of polysaccharides, polyphenols, and other secondary metabolites (Souza *et al*., 2012; Moreira and Oliveira, 2011). These compounds bind tightly to nucleic acids during the isolation of DNA and interfere with subsequent reactions. Like, the isolation of genomic DNA from mature leaves in Meliaceae was complicated due to high contamination of polyphenolics, polysaccharides and proteins (Rawat *et al*., 2016). Therefore, most of the reported DNA extraction protocols use young leaves for DNA extraction.

Initiation for genomic DNA extraction, the fresh young (Fig. 1A), immature (Fig. 1B) and mature (Fig. 1C) leaves of *S. koetjape* were collected and isolated from 2 varities, KT24 (Tubtim) and KT27 (Khunthong). When leaves crushed using liquid nitrogen, the young leaves powder turned dark brown color (Fig. 1D). While, the immature and mature leaves powder were are dark green (Fig. 1E-F). After the homogenization step, the color of solution were deep brown and deep green (Fig. 1G-I). Finally, the genomic DNA from immature and mature leaves were white color pellet. The light brown color were observed only in the DNA pellet of young leaves showing very high contamination of polyphenolics (Fig. 1J-L). This is according to previous reported of Kit and Chandran (2010) which found in Chokanan mango (*Mangifera indica* L.) extraction. The quality and quantity of extracted DNA were checked by agarose gel electrophoresis. The higher amount of genomic DNA can be obsearved from young leaves follow by immature and mature leaves, respectively as in Fig. 2.

PCR amplification and DNA sequencing

First step, modified CTAB extraction protocol by crush sample with 2x CTAB before incubated in water bath and 4 times repeat of chloroform: isoamyl alcohol (24:1). DNA samples were used as template for PCR with rbcLN (5'-ATGTCACCACAAACAGAAACT-3') and 840R (5'-TTGTCGCGGCAATAATGAGCC-3') primers. The result shown that DNA from immature and mature leaves were showed good amplification. The averages of PCR products size was 900 bp. While, no PCR product was obtained from young leaves DNA (Fig. 3A). Next, we attempted to modify the protocol for young leaves DNA with purified DNA by GF-1 Gel DNA Recovery Kit (Vivantis, Malaysia). PCR amplification was successful after DNA pass purification (Fig. 3B). No inhibition of Taq DNA polymerase activity was observed. So, the polysaccharides and polyphenols are problematic contaminants associated with DNA isolated from young leaves. In contrast, other reports that accumulation of polyphenolics increases with leaf development and it reduces DNA quality in mature leaves than in young leaves (Kit and Chandran, 2010; Moreira and Oliveira, 2011) From this study, indicated that DNA extraction from Santol young leaves that have secondary metabolites reduce the quality of the DNA. By *rbc*L region sequencing, the size of the DNA fragments are 840 bp and the results obtained using BLASTn program to identify the species. Sequence similarity searching, the entire query has coverage only in the *S. koetjape* (DQ238068) (Muellner *et al*., 2006) with 100% identity.

SRAP marker profile

To develop markers for reliable and rapid detection and to investigate the genetic diversity using Sequence-Related Amplified Polymorphism (SRAP) marker. In a preliminary study, two varities, KT22 (Puifai) and KT28 (Khiaowan) which have different fruit size were selected to study. Thirty sets of primer combinations that were combined by 5 forward primers and 6 reverse primers were screened, out of which only 9 primers combinations showed reproducible fragments with easily recordable bands and gave polymorphisms. These distinguish banding patterns can be successfully, especially in 5 primer combinations (Fig. 4). The number of SRAP bands were observed with an average of 9.7 bands per primer combination, size of the amplification products ranged from 100 to 2500 base pairs. Therefore, nine primer combinations were selected for further genetic diversity analysis of Santol varities.

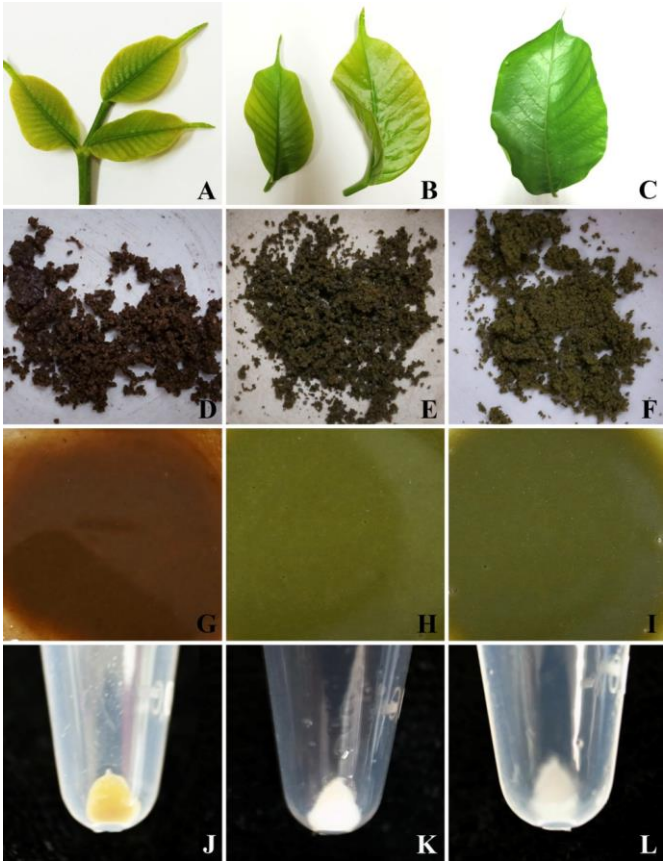

Fig. 1 *Sandoricum koetjape* (KT 24: Tubtim) DNA extraction protocol: young leaves (A), immature leaves (B), mature leaves (C), young leaves powder (D), immature leaves powder (E), mature leaves powder (F), the color of homogenize solution from young

(G), immature (H) and mature (I) leaves, light brown DNA pellet of young leaves (J), white DNA pellet of immature (K) and mature (L) leaves.

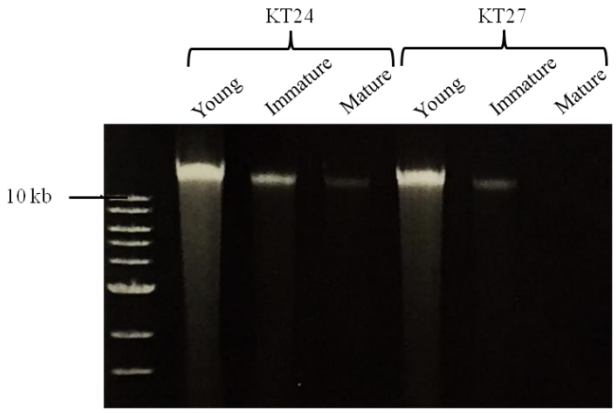

Fig. 2 Genomic DNA isolated from young, immature and mature *S. koetjape* leaves: 1 kb marker (lane 1), KT24 sample (lane 2-4) and KT27 sample (lane 5-7) resolved under 1% agarose gel.

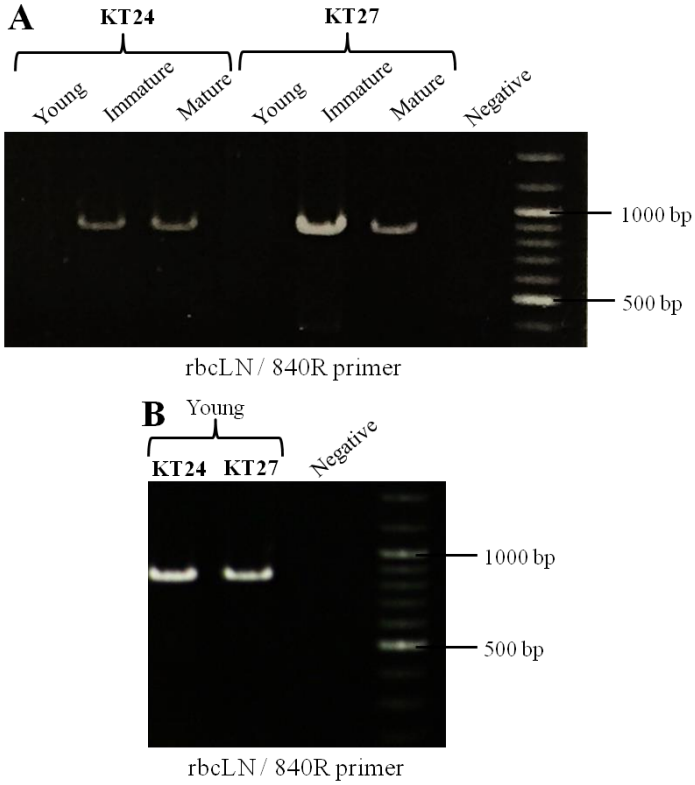

Fig. 3 Electrophoresis analysis of PCR products: from the isolated DNA by the modified CTAB method without purification (A) and with purification (B).

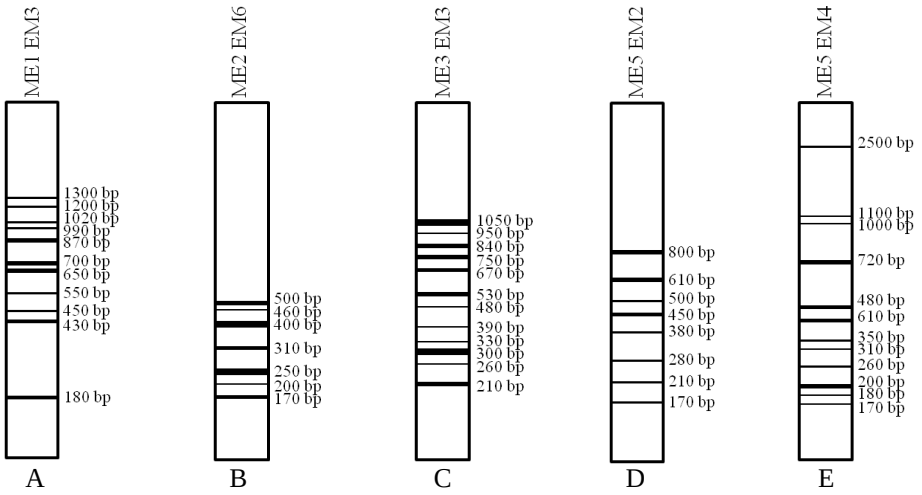

Fig. 4 SRAP profiles ME1EM3 (A), ME2EM6 (B), ME3EM3 (C), ME5EM2 (D) and ME5EM4 (E) of two different Santol varities: KT22 and KT28.

Conclusion

Generally, young leaves are the first choice to obtain good quality DNA. Because of the mature leaves contain high levels of secondary metabolite such as polyphenols, tannins and polysaccharides which makes it very difficult to isolate DNA of good quality. However, DNA extraction from Santol young leaves that have secondary metabolites reduce the quality of the DNA. So, DNA extraction of santol is necessary to consider leaf age of the santol. For SRAP marker, thirty primer combinations were screened. Those quality DNA is quite suitable for investigating the genetic profile. Nine primer combinations showed reproducible fragments with easily recordable bands and gave polymorphisms which were selected for further genetic diversity analysis.

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