短　報（Short Communication）

Application of STH-PAS, a novel chromatographic visualization system of PCR products for rapid screening of male-sterile lines in *Cryptomeria japonica*

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Abstract

To construct a simple method for the genotyping *MALE STERILITY 1* (*MS1*), a major causative gene of male sterility in Japanese cedar (*Cryptomeria japonica*), this study attempted to reduce the labor required for the post-PCR procedure using a single-stranded tag hybridization chromatographic printed array strip (STH-PAS), which allows for visual confirmation of PCR products. PCR was performed using tagged DNA- or biotin-modified primers to develop amplification products on stick membranes with complementary tags. After ~15 min the detection line was colored and the *MS1* genotype was determined. The diagnostic results were consistent with the electrophoresis results. STH-PAS can significantly reduce the time and labor required for *MS1* determination.

Key words : *Cryptomeria japonica*, DNA chromatography, male sterility, simple genotyping method, STH-PAS

Introduction

Since almost 40% of the population suffers from cedar pollinosis in Japan (Matsubara et al. 2020), the use of pollen-free (male-sterile) sugi (Japanese cedar, *Cryptomeria japonica* (L.f.) D.Don) cultivars that do not release pollen is expected as a countermeasure from the forestry field. However, the number of cultivars available for breeding is limited (Saito 2010). DNA marker-assisted selection is an effective way to search for breeding materials, namely, male-sterile trees or heterozygotes of the causative gene for male sterility (Mishima et al. 2018, Moriguchi et al. 2020). In addition to searching for breeding materials, a large supply of male-sterile seedlings is essential for its widespread use. Maruyama et al. (2022) developed a method for mass propagation of pollen-free sugi seedlings by somatic embryogenesis. Post-zygotic immature seeds are cultured as explants to regenerate the plantlets. During the process of somatic embryogenesis, it is necessary to determine whether the cultured cell line is male-sterile or fertile (Maruyama et al. 2020, Tsuruta et al. 2021). The development of a simple and rapid marker diagnostic method is necessary for the large-scale screening of breeding materials and the detection of male-sterility in large numbers of cultured cell lines.

Recently, *MALE STERILITY 1* (*MS1*), a major causative gene of male sterility in sugi was identified as a mutation in the CJt020762 gene (Hasegawa et al. 2021, Wei et al. 2021). While comparing alleles (*ms1*) in male-sterile trees to those in normal wild types (*Ms1*), deletions of 4 bp in the first exon or 30 bp in the third exon were observed and were designated *ms1-1* and *ms1-2*, respectively. Based on these mutations, DNA markers have been developed that can determine the presence of male-sterile alleles with 100% accuracy (Hasegawa et al. 2020). Currently, large-scale screening of sugi carrying male-sterile alleles has been performed using these markers (Moriguchi et al. 2020, Hasegawa et al. 2021, Watanabe et al. 2022). However, this method requires an expensive sequencer, and is not suitable for laboratories with insufficient genetic experiment equipment. Although Hasegawa et al. (2020) further developed allele-specific PCR markers that can be determined by agarose gel electrophoresis, discrimination is still labor-intensive. Furthermore, the determination of *MS1* genotype requires PCR amplification of the wild-type and mutant alleles in separate tubes. Tsuruta et al. (2021) simplified the extraction of DNA and developed a new marker that can identify the *MS1* genotype using a single PCR reaction and electrophoresis with agarose gel (one-step indel genotyping marker: ING marker).

This study attempted to simplify the post-PCR manipulation by using a single-stranded tag hybridization chromatographic printed array strip (STH-PAS, TBA Co., Ltd., Sendai, Japan) to further simplify *MS1* genotyping. This STH-PAS method is an alternative to electrophoresis where the presence or absence

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of PCR amplified products is visually confirmed by developing the PCR product on a stick membrane (C-PAS). PCR is performed using primers with a tag DNA. The complementary tag DNA to the primer tag is fixed in a line (i.e., detection line) on C-PAS, and when the PCR product is captured by the tags the detection line turns blue. This method has been used for various diagnoses such as detection of microorganisms and identification of crop cultivars (e.g., Hayashi et al. 2013, Monden et al. 2014, Tian et al. 2014, Sasai et al. 2017). Thus, we applied STH-PAS to *MS1* genotyping of sugi and compared the results with conventional agarose gel electrophoresis to confirm the utility of this new method.

Materials and methods

The applicability of STH-PAS for *MS1* genotyping was verified using ING markers. The ING marker amplifies the *Ms1* and/or *ms1-1* allele-specific bands and a control band (Tsuruta et al. 2021). STH-PAS was designed to detect each allele-specific amplified product. A tag sequence was added to the allele-specific primers of the ING marker (F1-tag: Mt+rightPrimer_2_F, F4-tag: WT+leftPrimer_2_R), and the common primers (Mt+rightPrimer_2_R and WT+leftPrimer_2_F) were modified with biotin that binds to the colored beads in the latex solution (Table 1). C-PAS4 (C-PAS with four detection lines, TBA Co., Ltd.) was used to detect the tagged PCR amplified products.

DNA extracted using the modified CTAB method from the young leaves of sugi cultivars (‘Ōi 7’: *Ms1*/*ms1-2*, ‘Suzu 2’: *Ms1*/*ms1-1*) and somatic embryogenesis-derived cell lines (SSD-18: *ms1-1*/*ms1-1*, SSD-100: *ms1-1*/*ms1-1*, FO7-141: *ms1-1*/*ms1-2*, and FO7-144: *ms1-1*/*ms1-2*) whose genotype of *MS1* has already been determined (Hasegawa et al. 2021, Tsuruta et al. 2021) were used in the experiments. Note that the allele *ms1-2* does not have a 4 bp deletion and is therefore typed as *Ms1* by the ING marker. A total of 20 µL of reaction solution containing 10 µL of 2x QIAGEN Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.2 µM of each primer with a tag sequence or biotin modification, and template DNA (1-10 ng) was reacted under the following conditions: initial denaturation at 95°C for 15 min followed by 35 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 5 min.

Ten µL of PAS Dilution Buffer (Mod.) (TBA Co., Ltd.) prepared to a NaCl concentration of 150 mM and 1 µL of pre-vortexed latex solution (TBA Co., Ltd.) were added to 10 µL of the PCR product. The C-PAS4 membrane stick was dipped into the mixture. The developments were performed under three conditions: 1) 26°C with 60% humidity, 2) 22°C with 38% humidity of laboratory (Fig. 1A), and 3) humidified simple developer bath (a 500 mL beaker filled with water about 5 mm, Fig. 1B). The remaining 10 µL of the PCR reaction products were loaded into a 1.5% agarose gel for electrophoresis.

Results and discussion

Approximately 15 min after the PCR product was developed in C-PAS, the color of the detection line was observed and the *MS1* genotype was successfully determined (Fig. 2A, B). The *MS1* genotypes determined by STH-PAS were consistent with agarose gel electrophoresis (Fig. 2C) and previous marker analysis (Tsuruta et al. 2021). Although in electrophoresis, insufficient separation of close bands sometimes happens due to similar length of PCR products, in STH-PAS, the intervals between the detection lines were sufficient to precisely determine genotypes. Therefore, STH-PAS may be superior to discriminate multiple alleles with similar or even the same amplified fragment lengths. There were no differences in the development time (data not shown) and genotyping results depending on the experimental environment (Fig. 2A, B). However, C-PAS were slightly bluish at the 38% humidity condition (Fig. 2A). In low humidity (below 40%), non-specific signals are reported to be occur (TBA Co., Ltd.). In

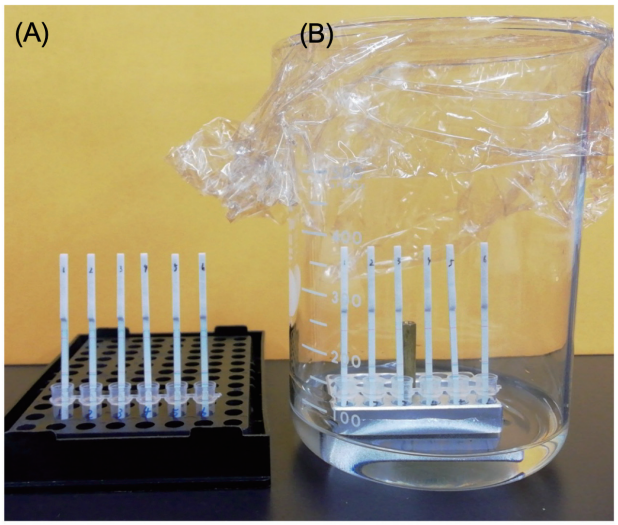

Fig. 1. An image of development of PCR products on a stick membrane under laboratory condition (A) and the humidified simple developer bath (B).

Table 1. Primer sequences and modifications for detecting *Ms1* and *ms1-1* alleles.

Primer name	Sequence (5’ to 3’)	Modification
Mt+rightPrimer_2_F	CTCACTGGCCACAGTCACAC	F1-tag
Mt+rightPrimer_2_R	TGCAGGCAACTTATAATTAAGCAC	Biotin
WT+leftPrimer_2_F	GACGTCTTCTGCAACAACAATGG	Biotin
WT+leftPrimer_2_R	ACCCTGCGTGGGTGTTGATG	F4-tag

those cases, using the simple developer bath was effective (Fig. 2B). When nonspecific bands appear, it is necessary to further adjust the salt concentration of the PAS Dilution Buffer.

Agarose gel electrophoresis, the basic method for genetic experimentation, is simple, but requires several steps; gel preparation, loading, running, staining (in some cases), and photography. In contrast, the STH-PAS system reveals the results simply by mixing the developing solution with the PCR product and placing a stick membrane on the tube. The time required for results is also shorter than for electrophoresis (20–40 minutes). In addition, because STH-PAS does not use fluorescent dyes (toxic ethidium bromide or its substitutes) required for DNA visualization in agarose gel electrophoresis, it is advantageous and does not require waste liquid treatment.

In conclusion, STH-PAS can be applied to determine *MS1* genotypes by detecting deletions of 4 bp. In this report, a combination of STH-PAS and ING marker was used to detect two alleles of *MS1* (*Ms1* and *ms1-1*). Since C-PAS can simultaneously detect the presence of up to 12 PCR amplified products, it may be possible to construct a diagnostic system for the presence of *ms1-2*, another mutant allele of *MS1*, and other male sterility genes, namely, *MS2* to *MS4* (Saito 2010, Hasegawa et al. 2018) simultaneously. STH-PAS has also utilized for the detection of loop-mediated isothermal amplification (LAMP, Notomi et al. 2000) products (Sasai et al. 2017, Takabatake et al. 2018, Moonga et al. 2020, Takarada et al. 2020). The LAMP system could be developed for *MS1* (Hasegawa et al. 2020). To our knowledge, this is the first report on the use of STH-PAS in conifers. Consequently, the results of this study can be considered as the first step towards the ultimate goal of building a system that can identify pollen-free sugi trees anywhere in the field using only simple equipment.

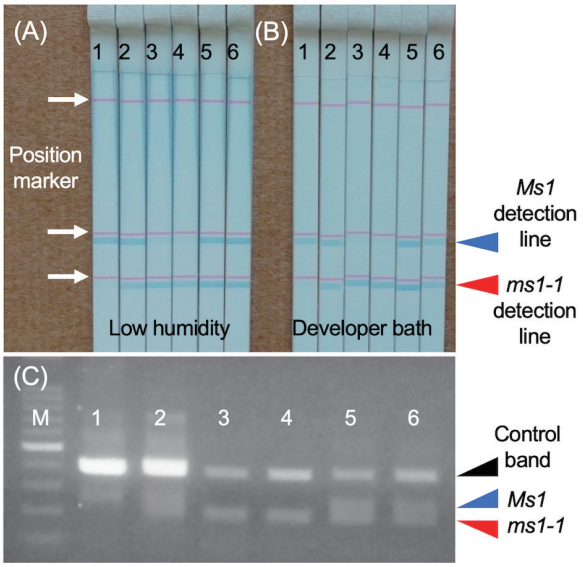

Fig. 2. An example of STH-PAS diagnosis for the presence of *Ms1* and *ms1-1* alleles under low humidity condition (A) and developer bath (B) and the result of agarose gel electrophoresis (C). Lane 1: 'Ōi 7' (*Ms1/ms1-2*), 2: 'Suzu 2' (*Ms1/ms1-1*), 3: SSD-18 (*ms1-1/ms1-1*), 4: SSD-100 (*ms1-1/ms1-1*), 5: FO7-141 (*ms1-1/ms1-2*), 6: FO7-144 (*ms1-1/ms1-2*), M: 100 bp ladder marker.

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無花粉スギ系統の迅速な判別に向けた簡易な PCR 産物可視化システム STH-PAS の適用

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要 旨

スギの雄性不稔の原因遺伝子 *MALE STERILITY 1* (*MS1*) の簡易判定法を確立するため、PCR 産物を目視で確認できる single-tag hybridization chromatographic printed array strip (STH-PAS) を用いて PCR 後の操作の省力化を試みた。タグまたはビオチンを付加したプライマーを用いて PCR を行い、増幅産物を相補タグが固相化された棒状のメンブレンに展開した。およそ 15 分で検出ラインが呈色し、*MS1* 遺伝子型が判定できた。判定は電気泳動結果と一致し、STH-PAS が判定に必要な時間と労力を大幅に削減可能なことが示された。

キーワード：スギ、核酸クロマトグラフィー、雄性不稔、簡易遺伝子型判定法、STH-PAS

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