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**Review**

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# Genetic Modification of Coffee Plants

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## Abstract

**An efficient molecular breeding technique for coffee plants was developed. In order to produce transgenic coffee plants, we established a model transformation procedure via *Agrobacterium* method. We isolated a gene encoding a protein possessing 7-methylxanthine methyltransferase (theobromine synthase) activity, and it was designated as Coffea arabica *7-methylxanthine methyltransferase; CaMXMT*. Using this clone, we produced transgenic coffee plants, in which the expression of *CaMXMT* is suppressed by double-stranded RNA interference (RNAi) and/or anti-sense methods. The expression pattern of *CaMXMT* was analyzed by reverse transcription-PCR method and we found that, in the transformed cell lines, the level of transcripts were obviously suppressed by RNAi. The endogenous level of caffeine in the transformed cells was dramatically reduced in comparison with non-transformed cells.**

## Introduction

The coffee plant is one of the most important crops on the world market, and is the most widely cultivated tropical tree species. The most economically important varieties are *Coffea arabica* and *C. canephora*. *C. arabica* is the only tetraploid (2n=44) and autogamous coffee species. It produces high quality coffee with low caffeine content, but it is susceptible to diseases. *C. canephora* is a diploid (2n=22) allogamous species that produces coffee with higher caffeine content, making poorer in quality than *C. arabica*. Its advantage, however, is resistance to diseases. These two species account for 75% and 25% of the market, respectively (Berthouly and Etienne, 1999). Since coffee is economically valuable crop, a traditional breeding, mainly by crossing, has been carried out and some superior varieties have been selected. However, due to the life cycle of these woody species, it takes long periods for the selection, usually more than 25 years (Spiral et al., 1999). Therefore, a new breeding method for a rapid selection of disease tolerant varieties and/or an improvement of coffee in quality is highly demanded. Over-intake of caffeine is thought to induce functional diseases such as insomnia and dizziness. Hence, caffeine-free coffee is demanded on the world market, and is normally produced by the chemical extraction method. However, such product is poor in quality, and other methods to produce caffeine-free coffee are intensively investigated. For this purpose, we have been trying to construct transgenic coffee plants, in which caffeine synthesis is repressed. Caffeine is synthesized through sequential three-step methylation of xanthine derivatives: first, xanthosine into 7-methyl xanthine, then into 3,7-dimethyl xanthine (theobromine), and finally into 1,3,7-trimethyl xanthine (caffeine). We identified and characterized a gene encoding a key enzyme for the caffeine biosynthetic pathway (Ogawa et al., 2001). Mean-while, we established a model system for coffee transformation, using the GUS reporter gene by adopting a transformation procedure via *Agrobacterium* method (Hatanaka et al., 1999). We also developed an efficient somatic embryogenesis system for the transformation (Ogita et al., 2001; 2002). We report

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here our current progress for genetic modification of coffee plants, i.e. production of caffeine-free coffee by double-stranded RNA interference (RNAi) and/or anti-sense methods.

## Materials and Methods

### Plant materials

Coffee plants, *C. arabica* L. and *C. canephora*, were cultivated in a greenhouse (Figure 1A). Young leaves of approximately 5-9 cm long were collected from the mother trees and used for plant materials.

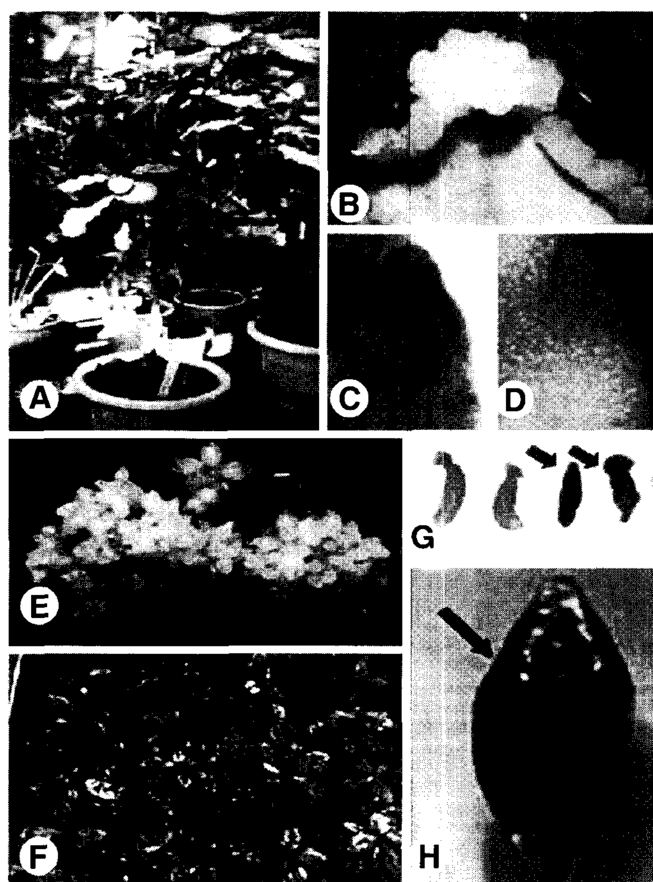


**Figure 1.** Somatic embryogenesis and *Agrobacterium*-mediated transformation of coffee plants. A; Coffee plants grown in a greenhouse. B; Embryogenic tissue of *Coffea arabica* derived from leaf explant. C & D; Morphological characteristics of embryogenic tissue of *C. arabica* observed under white light (C) and fluorescent light (D). DAPI staining results in blue fluorescence from the tissue indicating that the cells are cytoplasmic with large nuclei. E; Somatic embryos of *C. canephora* directly induced from leaf explant. F; Somatic seedlings of *C. canephora* grown in soil. G & H; X-Gluc staining of somatic embryos (G) and leaf (H) of transgenic coffee line. GUS-positive reactions (arrows in G and H) are observed as deep blue in color. Non-transgenic somatic embryos in G show GUS-negative reactions.

### Cloning and characterization of a key enzyme for the biosynthetic pathway of caffeine

Using degenerate primers designed on the basis of conserved amino acid regions of tea caffeine synthase (TCS1; Kato et al., 2000, accession number AB031280) and *Arabidopsis* hypothetical proteins (Z99708 and AC008153), a particular DNA fragment was amplified from a mRNA population of coffee plants. After probing with this fragment, four independent clones were isolated from a cDNA library derived from young coffee leaves. Glutathione S-Transferase (GST) fusion proteins of these four clones were produced in *Escerichia coli*, N-methyltransferase activities were then determined. One of them was found to encode a protein possessing 7-methylxanthine methyltransferase activity, and designated as *CaMXMT* (AB048794). Reverse transcription (RT)-PCR, HPLC, and a microscopic observation methods were detailed somewhere else (Ogawa et al., 2001).

### Embryogenic tissue induction and maintenance

Leaf explants of coffee (*C. arabica* and *C. canephora*) were prepared from the mother trees grown in a greenhouse. Collected leaves were surface sterilized first with 70% ethyl alcohol for 1 min followed by a 2% NaClO solution for 10 min. The leaves were then washed 3 times with sterile water. The modified half strength Murashige and Skoog (modified1/2MS) containing 3% sucrose was applied for embryogenic cultures of both species. As plant growth regulators, various concentrations of 2-isopentenyl-adenine (2ip), N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU), and 2,4-dichlorophenoxyacetic acid (2,4-D), were supplemented in the modified1/2MS medium. The pH of the media was adjusted to 5.7 with several drops of 0.1M KOH and/or 1 N HCl. The complete media containing 0.3% gellan gum were autoclaved for 20 min at 121°C, then each of 20 mL volume of media was poured into a petri dish of 90 mm diameter. The sterilized explants were cut into piece of approximately 5-7 $mm^2$ and placed, the upper side of a leaf explant down, on the media. The cultures were incubated at 25°C, in dark condition.

### Maturation of somatic embryos and plant recovery

In order to induce further somatic embryo development, embryogenic tissues and/or immature somatic embryos were transferred to modified 1/2MS medium of hormone-free. The cultures were incubated at 25°C, in dark

condition or with a 16-h/8-h light/dark photoperiod under fluorescent illumination at 30-35 mol $m^{-2} \cdot s^{-1}$.

### Constructs

Gene-specific sequence in a sense (S) and an anti-sense (A) fragments of *CaMXMT* were first amplified and then anti-sense and RNAi (S-GUS-A) constructs for suppressing the expression of *CaMXMT* were constructed. A partial ®-glucuronidase (GUS) fragment was used as a linker of the RNAi sequence. Intron-GUS fragment of pIG121Hm (Ohota et al., 1990) was digested at the *Xba*I and *Sac*I sites, then the anti-sense and the RNAi constructs were inserted to the sites, respectively.

### *Agrobacterium* transformation

*Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986) carrying DNA constructs in pIG121Hm was used to transform coffee plants as described (Hatanaka et al., 1999). Embryogenic tissue of *C. arabica* and somatic embryos of *C. canephora* were co-cultured in bacterial suspension (preferably absorbance of 0.5 at 600 nm) for 30 min in liquid modified 1/2MS medium with plant growth regulator(s) and acetosyringon, respectively. Transformed coffee tissues and somatic embryos were selected on modified 1/2MS medium containing hygromycin. Histochemical GUS assay and genomic DNA amplification were carried out as described (Hatanaka et al., 1999). RT-PCR for analyzing the expression pattern of *CaMXMT* was performed by referring Ogawa et al. (2001).

## Results and Discussion

### Isolation and characterization of cDNA encording theobro-mine synthase from *Coffea arabica*

A particular DNA fragment was amplified from a mRNA population of coffee plants by using degenerate primers designed on the basis of conserved amino acid regions of tea caffeine synthase and *Arabidopsis* hypothetical proteins. This fragment was used as a probe, and four independent clones, clones 1, 6, 35, and 45, were isolated from a cDNA library derived from young coffee leaves. The GST fusion proteins of the clones were produced *E. coli*, and only the product of clone 45 was found to encode a protein possessing 7-methylxanthine methyltransferase activity, and designated as *CaMXMT*. It consists of 378 amino acids with a relative molecular mass of 42.7 kDa and shows low similarity to tea caffeine synthase (35.8%) and salicylic acid methyltransferase (34.1%). The bacterially expressed protein exhibited an optimal pH for activity ranging between 7 and 9, and methylated almost exclusively 7-methylxanthine with low activity toward paraxanthine. *Km* values were estimated to be50 and 12 $\mu$M for 7-methylxanthine and S-adenosyl-L-methionine, respectively. Accumulation of *CaMXMT* transcripts was investigated by RT-PCR and detected in young leaves and buds. Furthermore, to identify the cellular localization of *CaMXMT*, green fluorescent protein (GFP) fusion protein assay was performed by a biolistic bombardment. The expression of GFP was observed as the strong green fluorescence in the cytoplasm of the onion epidermal cell.

### Somatic embryogenesis of coffee plants

Somatic embryogenesis has been widely used for micropropa-gation and genetic modification of higher plants. Various plant organs and tissues can be use as a source of explants for the initiation of embryogenic cultures. For breeding purpose, a somatic tissue (leaves, stems etc.) which has a certain genetic background is preferred. In the case of coffee plants, fortunately, young leaves proved to be more effective in embryogenic tissue and/or somatic embryo induction (Berthouly and Etienne, 1999). In our current experiments, yellowish cell tissues (Figure 1B) were induced from young leaves of *C. arabica* on the modified 1/2MS medium containing 1-10 $\mu$M of 4-CPPU and 1-5 $\mu$M of 2,4-D during 4-8 weeks of cultures. In order to determine morphological characteristics of the tissues, small amount of the tissue was stained with DAPI and observed under a fluorescence microscopy. Strong blue fluorescence shown in the cells (Figure 1C and D) indicates that the tissues were mainly consisted of cytoplasmic cells with large nuclei. These tissues were highly embryogenic as described by Ogita and Sasamoto (2001), and they proliferated suitably on the same medium condition as the induction.

For large-scale micropropagation, indirect somatic embryo-genesis, i.e. embryogenic callus (or tissue) induction that allows somatic embryo formation in high frequency is generally recommended. However, the irregularity of developmental stages of somatic embryos and the difficulty of regeneration still remains. Therefore, we are concentrating on direct formation of somatic embryos without producing calli or embryogenic tissues for the future transformation work. Direct somatic embryogenesis from young leaves of both *C. arabica* and *C. canephora* was established on the modified 1/2 $\mu$MS medium with 5-20 $\mu$M of 2ip. 20 M of 2ip was the most successful to induce somatic embryos directly from the leaf explants (Figure 1E). In order to induce further somatic embryo develop-

ment, somatic embryos were transferred to modified 1/2MS medium of hormone-free. They vigorously germinated and developed into small plantlets on the modified1 1/2MS medium. These plantlets could maintain healthy in soil (Figure 1F).

### Genetic modification of coffee via *Agrobacterium*

Genetic modification by molecular techniques is thought to be one of the most beneficial breeding methods for a rapid production of superior varieties. In order to obtain transgenic coffee plants, we first established a model system with the GUS reporter gene by adopting a transformation procedure via *Agrobacterium tumefaciens* (EHA101). The plasmid vector, pIG121-Hm (Ohota et al., 1990), was effective to transform coffee plants. The selected somatic embryos and plantlets exhibited a strong GUS activity (Figure 1G and H) in leaves and roots, suggesting a stable integration of the GUS gene into coffee genome (Hatanaka et al., 1999).

Anti-sense and RNAi methods were then applied to produce *CaMXMT* knock-out coffee plants. Transformed lines of both *C. arabica* and *C. canephora* were selected on modified11/2MS media containing hygromycin (final concentration of 100 mg/L). We investigated expression pattern of *CaMXMT* by RT-PCR method and found that, in the transformed lines, its transcripts were obviously suppressed by RNAi method. Antisense method also seemed to be effective to produce *CaMXMT* knock-out coffee plants. In fact, endogenous level of caffeine in the transformed cells was dramatically reduced less than a tenth in comparison with non-transformed cells.

### Concluding remarks

Our present results clearly show that somatic embryogenesis and molecular techniques are applicable for coffee breeding. In practice, the technique was used to produce caffeine-free coffee plants by suppressing the caffeine biosynthetic pathway. Our current success would open general ways to develop new valuable varieties of this species in the near future.

## Acknowledements

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