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Isolation and characterization of an *AGAMOUS* homologue from cocoa

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Abstract

We report the cloning of a cDNA from *TcAG*, an *AG* (*Arabidopsis thaliana* MADS-box C-type transcription factor gene *AGAMOUS*) homologue from cocoa (*Theobroma cacao* L.). *TcAG* was in the cocoa flower expressed primarily in stamens and ovaries, comparable to *AG* in Arabidopsis. Additionally, we found that *TcAG* is also expressed in the fruit (pod) wall and during its entire development, as well as in the fruit pulp. Ectopic expression of *TcAG* in transgenic *A. thaliana* plants resulted in a range of weak to strong *apetala2* (*ap2*) mutant-like phenotypes as well as early flowering and curly leaves, as observed in other studies of plants overexpressing a functional *AG* homologue. The severity of the phenotypes correlated positively with the *TcAG* transcript level in the transgenic plants.

Keywords: Flower development; Cocoa; AGAMOUS; MADS-box

1. Introduction

Cocoa trees produce large numbers of flowers, at certain times of the year, from the age of 3 years and on, under good growing conditions. Flowers emerge from floral cushions (Fig. 1A), a meristematic tissue in the bark of the stem and branches at the location of old leaf axils [1], a habit called cauliflorous or truncate. The flowers are small, about 15 mm in diameter and form long pedicels (Fig. 1B). The flowers have 5 unfused sepals and petals, 10 stamens and an ovary of 5 united carpels (Fig. 1B). The petals are very narrow at the base but expand into a cup-shaped pouch and end in a broad tip or ligule. The 10 stamens are in 2 whorls, the outer whorl is consisting of 5 long non-fertile staminodes and the inner whorl of 5 fertile stamens. Cocoa trees produce flowers throughout the year, but flowering peaks in certain times of the year, when flowering progresses in waves from the lower stem flowering cushions to the branches. Flowers are open pollinated by small insects, but only 1–5% of the flowers are successfully pollinated and proceed to produce a pod [2].

In most higher eudicotyledonous flowering plants the floral organs are arranged in four different whorls, containing sepals, petals, stamens and carpels, respectively. The specification of floral organ identity is explained by the ABC model [3,4] and extensions thereof [5]. Classes A–C genes have been isolated from several eudicotyledonous model plants such as *Arabidopsis*, *Antirrhinum* and *Petunia*, and most of them belong to the family of MADS-box genes, encoding transcription factors containing a conserved 56 amino acid motif within their DNA-binding domains, named MADS after the four original members (for a review: see Ref. [5]). In *Arabidopsis thaliana* C-function, establishing identity of the reproductive organs, as well as determinacy of the floral meristem, is performed by the MADS-box gene *AGAMOUS* (*AG*) [6]. Moreover, *AG* functions in a redundant manner with the closely related genes *SHATTERPROOF* (*SHP*) *1/2* in the control of carpel development and with *SHP1/2* and *SEEDSTICK* (*STK*) in determining ovule identity [7].

In this manuscript we describe the cloning and characterization of a cDNA from flowers and fruits of the cocoa tree (*Theobroma cacao* L.), homologous to the C-type (*AG*) MADS-box gene. Results from expression analysis as well as from the phenotype caused by the ectopic expression in transgenic *Arabidopsis* plants suggest that the gene represented by this

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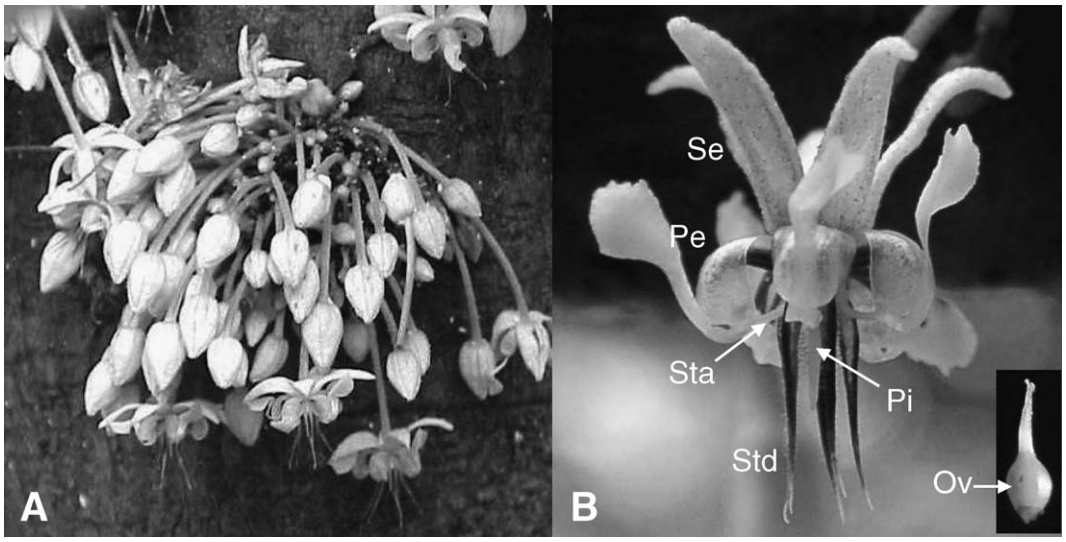

Fig. 1. (A) Flowering stem cushion of cocoa. (B) A cocoa flower with organs indicated: Se, sepal; Pe, petal; Sta, stamen; Std, staminode; Pi, pistil; Ov, ovary. The insert shows a dissected pistil.

cDNA has similar functions in regulating cocoa flower organ specification and the ability to induce flowering when overexpressed, as its homologue in *Arabidopsis*.

2. Materials and methods

2.1. Plant materials

The plant material was obtained from the cacao tree clone DR2, which was grown in fields of Rajamandala, PTPN VIII, Bandung, Indonesia. Fruits and flowers were separated into the different tissues or organs, immediately frozen in liquid nitrogen, and stored at −80 °C until further use. Leaves were harvested from plants grown in the laboratory.

2.2. RNA extraction

The method used for RNA extraction from cocoa is a modification of a method described earlier for banana tissues [8]. After the extraction of RNA according to this method, the pellet was dissolved in MilliQ grade water and was extracted with phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), respectively, each time followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. RNA in the supernatant was precipitated with 0.1 volumes of 3 M sodium acetate pH 5.8 and 3 volumes of 100% ethanol at −70 °C for 4 h to overnight. The RNA was recovered by centrifugation at 17,000 × *g* at 4 °C for 30 min. The pellet was washed with an equal volume of 70% cold ethanol and after drying the purified RNA pellet was resuspended in water.

2.3. Isolation of pod wall-specific cDNA's by suppression subtractive hybridization (SSH)

mRNAs isolated from total RNA using oligo $(dT)_{25}$ Dynabeads (GenoVision, Philadelphia, PA, USA). Pod wall-specific cDNA-fragments were isolated using the PCR-Select cDNA subtraction kit (CLONTECH, BD Biosciences, Alphen a/d Rijn). Starting material consisted of 1 μg inner pod wall mRNA as tester and 1 μg of leaf and bean mRNA (mixed equal amounts) as a driver. All SSH products were ligated into the pGEM-T Easy vector (Promega Benelux BV, Leiden) and transformed into JM109 super competent cells.

2.4. 5′ RACE and amplification of the coding region of the cocoa homologue of AGAMOUS

For the selected SSH-fragment, corresponding 5′ cDNA-fragments were amplified using the SMART RACE-kit from CLONTECH. 5′-Ready cDNA pools were produced from cocoa flower mRNA. The cDNA-specific primer TcAG-R, complementary to the sequence beyond the stop codon of SSH contig 24 was used in the 5′ cDNA end amplification (Table 1). The PCR product was used as template in a nested PCR with the TcAG-R and NUP primers. Amplified cDNA-end fragments were purified using a QIAquick PCR purification kit (Qiagen, Benelux B.V., Venlo) and ligated into pGEM-T Easy.

To amplify a cDNA-fragment covering the full coding sequence of *TcAG*, 2 μL of the first strand cDNA from cocoa flower buds were used as a template for PCR using the kit HSRT-20 (Sigma Bioscience) with the primers TcAG-F (covering the presumed start codon and creating an *Nco*I-site) and TcAG-R (Table 1). The parameters used during the PCR reaction were as follows: 95 °C, 2 min; 36 cycles: 95 °C, 25 s;

Table 1
Oligonucleotide primers used in this study

Name	Nucleotide sequence (5′ to 3′)
TcAG-R	GGTGACCGTAGCACTTACTCCACCAGA
TcAG-F	CCATGGAGTACCAAAGTGAATCC
TcAG-F396	CGCATTGCCTATGAAGGATC
TC250F	CACCCTCGAGGTGGAAAGCTC
TC250R	CCACCACGGAGTCGCAACAAC