Original Papers

Plant Biotechnology, 14(3), 157-161(1997)

Antisense and Sense *CHS* cDNA Expressed by a Tapetum-specific Promoter Causes Partial Male Sterility in Transgenic Tobacco

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Received 20 February 1997; accepted 23 May 1997

Abstract

Chalcone synthase (CHS) is a key enzyme in the biosynthesis of all classes of flavonoids in plants. An antisense or sense *CHS* cDNA of tea under the regulation of a tapetum-specific promoter of rice was introduced into tobacco by *Agrobacterium*-mediated transformation. A reduction in the number of fertile pollen grains was observed in four out of 27 antisense CHS transformants and in five out of 26 sense CHS transformants. The distorted pollen grains of transformants lacked starch and flavonols. Fluorescein diacetate staining indicated abortion started at the late uninucleate microspore stage. The antisense or sense transgene would affect the pollen fertility.

1. Introduction

Flavonoids which are initiated by the enzyme chalcone synthase (CHS, EC 2. 3. 1. 74.) as a side branch of the phenylpropanoid pathway are a principal component of pollen pigments. It has been suggested that one role of these flavonoids is to function during pollen germination and pollen tube growth in many plant species [1, 2]. It has also been suggested that the transition of phenylpropanoids to flavonoids is essential for the development of viable pollen grains [3, 4]. Kehrel and Wiermann [5] demonstrated that the CHS in tulip anthers is predominantly located in the tapetum cells, indicating that the CHS activity plays an essential role in the development of microspores into mature pollen grains. If this hypothesis is supposed to be correct, the artificial reduction of CHS activity in tapetum cells of anthers should also result in reduction of the number of fertile pollen grains in transgenic plants. An approach of genetic transformation with an antisense or sense *CHS* cDNA under the regulation of a tapetum-specific promoter will be useful and powerful to perturb the expression of endogenous *CHS* genes only in the tapetum.

CHS cDNA has been isolated from many species of plants, such as parsley [6], petunia [7] and pea [8]. We used a cDNA clone for CHS (*CHS1*) isolated from young leaves of tea, since cDNAs from tobacco anthers were not available. The *CHS* cDNA of tea showed 73 % homology with *CHS* cDNA from parsley, 76 % homology with one of petunia and 75 % homology with one from pea [9]. On the other hand, some tapetum-specific promoters have also been isolated, such as *TA29* promoter of tobacco [10], *A9* promoter of *Arabidopsis* [11] and *Osg6B* promoter of rice [12]. We demonstrated previously that the *Osg6B* promoter was active in tapetum of transgenic tobacco from the tetrad stage to the uninucleate microspore stage just before microspore mitosis [12].

In this report, we fused the *Osg6B* promoter to *CHS* cDNA of tea in antisense or sense orientation, and the resulting chimeric genes were introduced into tobacco. The transformants reduced the number of fertile pollen grains. It is possible that *CHS* cDNA under the regulation of tapetum-specific promoter affects the development of microspores.

2. Materials and Methods

2. 1 Construction of chimeric genes

An expression cassette pAS6-101E containing *Osg6B* promoter sequence was constructed by Tsuchiya *et al.* [13]. A 1.5-kb *Eco*R I fragment containing the full-length cDNA of *CHS* gene (*CHS1*) from tea (*Camellia sinensis*) [9] was ligated at the same site of pAS6-101E, which exists downstream of the *Osg6B* promoter, in an antisense (**Fig. 1-A**) or sense orientation (**Fig. 1-B**). *Osg6B* promoter-*GUS* gene fusion plasmid [12] was used for the control.

A hygromycin resistant cassette containing gene for hygromycin phosphotransferase [14] was inserted at the *Hin*dIII site of the constructed vectors (**Fig. 1-A** to **C**).

2. 2 Transformation of tobacco plants

The constructed vectors (**Fig. 1-A** to **C**) were transferred to *Agrobacterium tumefaciens* strain A136

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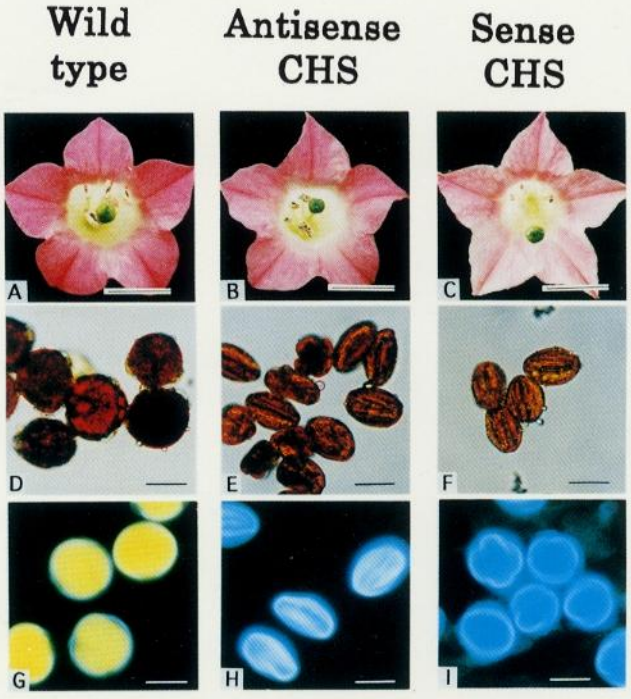

Fig. 2 The pollen grains of the untransformed control tobacco (A, D, G), antisense CHS transformants (B, E, H) and sense CHS transformants (C, F, I).
Bars (A to C) indicate 1 cm and bars (D to I) indicate 25 μm.
(A) Open flower of untransformed control tobacco.
(B) Open flower of antisense CHS transformant no. 12.
(C) Open flower of sense CHS transformant no. 86.
(D) The pollen grains of untransformed control tobacco which were stained with I_2-KI solution.
(E) The pollen grains of antisense CHS transformant no. 12 which were stained with I_2-KI solution.
(F) The pollen grains of sense CHS transformant no. 86 which were stained with I_2-KI solution.
(G) The pollen grains of untransformed control tobacco under UV light after flavonol staining.
(H) The pollen grains of antisense CHS transformant no. 12 under UV light after flavonol staining.
(I) The pollen grains of sense CHS transformant no. 86 under UV light after flavonol staining.

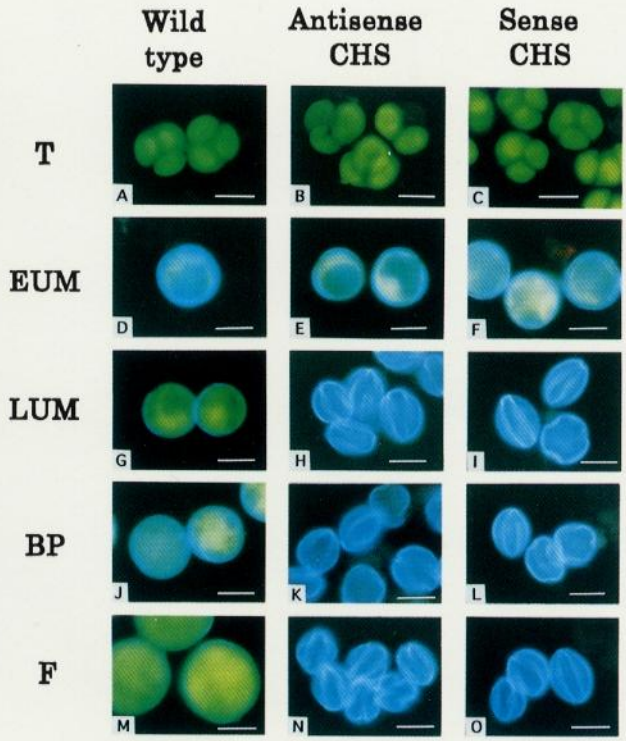

Fig. 4 The microspores and pollen grains of untransformed control tobacco and transformants after FDA staining.
(A to C) The microspores at tetrad stage.
(D to F) The microspores at early uninucleate microspore stage.
(G to I) The microspores at late uninucleate microspore stage.
(J to L) The pollen grains at binucleate pollen stage.
(M to O) The pollen grains at flowering stage.
The pollen grains of untransformed control tobacco (A, D, G, J, M), that of antisense CHS transformant no. 12 (B, E, H, K, N) and that of sense CHS transformant no. 86 were used for analysis. Abbreviations used: T, tetrad stage; EUM, early uninucleate microspore stage; LUM, late uninucleate microspore stage; BP, binucleate pollen stage; F, flowering stage.

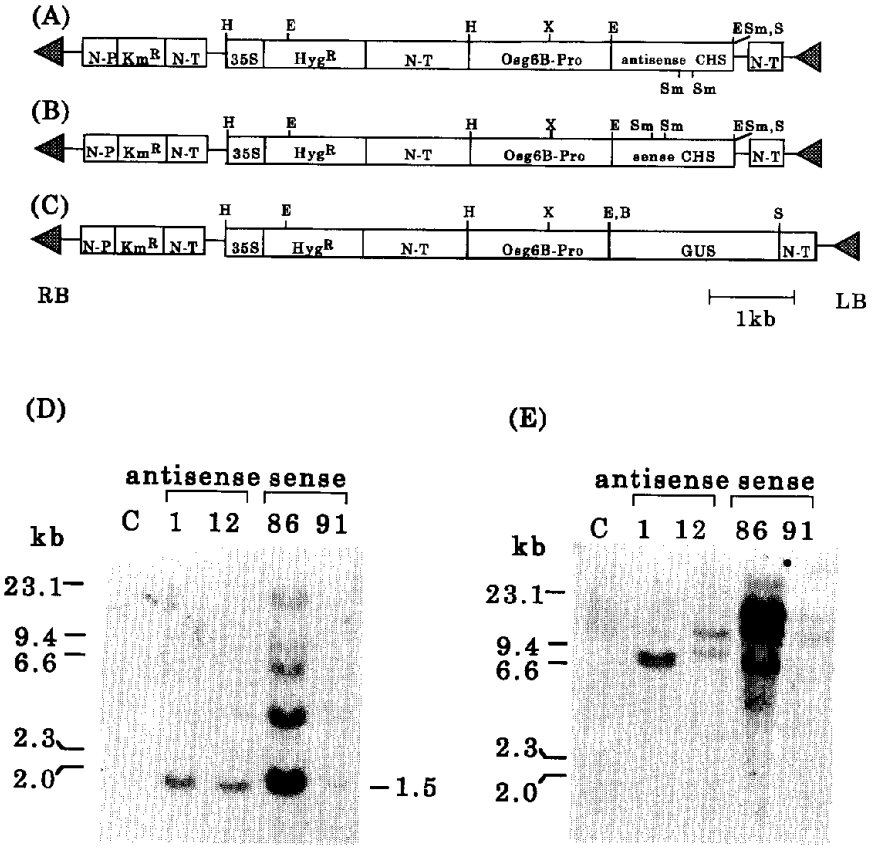

Fig. 1 Plasmid construction and genomic DNA gel-blot analysis.
(A) *Osg6B* promoter-antisense *CHS* cDNA construct.
(B) *Osg6B* promoter-sense *CHS* cDNA construct.
(C) *Osg6B* promoter-*GUS* gene construct.
(D) and (E) DNA gel-blot analysis.

Three μg of total DNA was digested with *Eco*R I (D) and *Hin*dIII (E), and then fragments were probed with *CHS* cDNA. Plants (nos. 1 and 12) were transformed with *Osg6B* promoter-antisense *CHS* cDNA construct. Plants (nos. 86 and 91) were transformed with *Osg6B* promoter-sense *CHS* cDNA construct. A capital letter C above the lane indicates untransformed control tobacco.

Abbreviations used: Km^R, gene for neomycin phosphotransferase II; Hyg^R, gene for hygromycin phosphotransferase; antisense CHS, *CHS* cDNA in reverse orientation; sense CHS, *CHS* cDNA in forward orientation; N-P and N-T, promoter and terminator sequences of the nopaline synthase gene of pBI121. N-T at the downstream of Hyg^R is derived from pLAN101MHYG [16] ; 35S, cauliflower mosaic virus 35S promoter, Osg6B -pro, *Osg6B* promoter, RB and LB, right and left border of T-DNA. The restriction sites indicated are as follows: H, *Hin*dIII; E, *Eco*R I ; X, *Xba* I ; Sm, *Sma* I ; S, *Sac* I .

containing helper plasmid pCIB542 [15]. The transformation of *Agrobacterium tumefaciens* and tobacco plants were carried out as described by Matsuda *et al.* [16].

2. 3 *DNA gel blot analysis*

Isolation of total plant DNA and DNA gel blot analysis were carried out as described previously [16].

2. 4 *Analysis of pollen fertility*

Starch accumulation of pollen grains was observed by staining with a solution of I_2-KI (0. 5 % I_2, 3 % KI) [17] and the number of pollen grains per flower was counted with a Burker-Turk type hemocytometer [13].

2. 5 *Detection of flavonols in pollen grains*

Pollen grains at the flowering stage from T_0 transformants and untransformed control tobacco were selected. We used a saturated solution (<0. 5 % w/

v) of diphenyl boric acid 2-amino-ethyl ester (Tokyo Chemical Industry, Tokyo, Japan) for detection of flavonols [2, 16, 18].

2. 6 FDA staining of pollen grains

The viability of microspores and pollen grains of T_0 transformants and untransformed control tobacco were also analyzed by FDA staining [19]. They were stained with 0. 1 % fluorescein diacetate (FDA; Sigma, MO, USA) in 10 % sucrose for 5 min. After staining, fluorescein was visualized under blue excitation. Referring to the detailed descriptions of the development of tobacco anthers [10], we selected anthers at five different stages: anthers at the tetrad stage, taken from buds of 6-mm in length, anthers at the early uninucleate microspore stage, taken from 10-mm buds, anthers at the late uninucleate microspore stage, taken from 15-mm buds, anthers at the binucleate pollen stage, taken from 22-mm buds and dehiscent anthers at the flowering stage.

3. Results

3. 1 DNA gel blot analysis

We obtained 27 independent antisense CHS transformants and 26 sense CHS transformants of tobacco. Two plants from each of the antisense CHS transformant and sense CHS transformant were analyzed by DNA gel blot analysis (**Fig. 1-D** and **E**).

The *CHS* cDNA of tea was used as a probe for DNA gel blot analysis. Only a faint band was observed in untransformed control tobacco, indicating that the *CHS* gene of tea barely detected endogenous *CHS* genes of tobacco in our experimental condition (**Fig. 1-D** and **E**). *Eco*R I digestion, which was designed to give a 1. 5-kb *CHS* cDNA fragment, showed a band of 1. 5 kb in all the tested transgenic plants (**Fig. 1-D**). An extra band of unexpected size was also observed in plant no. 86 showing genetic rearrangement. *Hin*dIII digestion was also carried out to obtain a unique fragment for each integrated copy because there is no *Hin*dIII site in *CHS* cDNA (**Fig. 1-E**). The numbers and intensities of bands are considered to represent the copy number. Plant nos. 1 and 12 from antisense transformants and plant no. 91 from sense CHS transformants were considered to contain two or three copies of *CHS* cDNA from tea, but more than 5 copies were also estimated to be integrated in plant no. 86 of sense CHS transformant.

3. 2 Pollen phenotype

A reduction in the number of fertile pollen grains in dehiscent anthers was clearly visible in four (plant nos. 1, 6, 12 and 85) out of 27 independent antisense CHS transformants in comparison with anthers of untransformed control tobacco (**Fig. 2-A** and **B**). Most of the pollen grains in the four antisense CHS transformants were not stained with a solution of I_2-KI, indicating the absence of starch accumulation (**Fig. 2-E**), whereas pollen grains of untransformed control tobacco were deeply stained (**Fig. 2-D**). The pollen grains obtained lacking starch showed distorted shape (**Fig. 2-E**). The number of fertile pollen grains of these antisense CHS transformants was reduced to 1.6×10^3 to 2.3×10^5, while that of untransformed control tobacco and of transgenic plants containing the *Osg6B* promoter-*GUS* gene construct was more than 3.1×10^5 (**Fig. 3**). Such distorted pollen grains did not show yellow fluorescence after being stained with a solution of diphenyl boric acid 2-amino-ethyl ester, which indicated the absence of flavonols (**Fig. 2-H**). In contrast, the pollen grains of untransformed control tobacco (**Fig. 2-G**) showed yellow fluorescence of flavonols.

A reduction in the number of fertile pollen grains was also observed in transgenic tobacco containing sense *CHS* cDNA driven by the *Osg6B* promoter (**Fig. 2-C**). The number of fertile pollen grains judged from the accumulation of starch was reduced to 4.0×10^4 to 2.5×10^5 in five (plant nos. 4, 27, 60, 86 and 91) out of 26 sense CHS transformants (**Fig. 3**). The pollen grains from sense CHS transformants lacking starch showed distorted shape (**Fig. 2-F**) and did not show any flavonol staining (**Fig. 2-I**) in the same way as those observed in antisense CHS transformants.

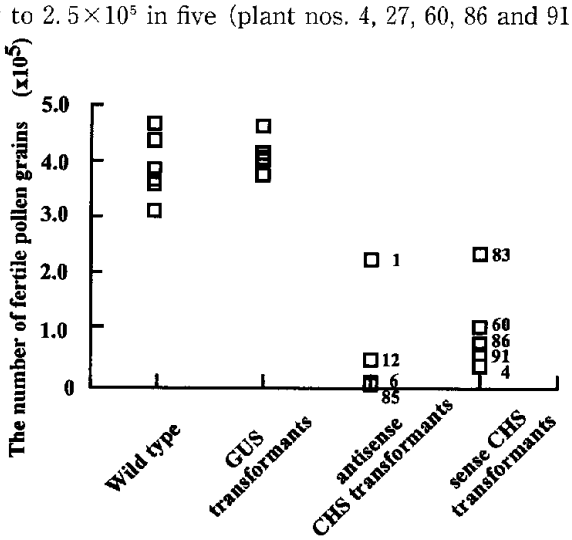

Fig. 3 The number of fertile pollen grains per flower of transformants and untransformed control tobacco. The number of fertile pollen grains per flower were counted three times per plant. Open squares are the average of the number of fertile pollen grains per flower. The numbers beside the open squares are the identification numbers of transformants.

The viability of microspore and pollen during development was investigated by FDA staining. It was

found that the microspores taken from tetrad stage of both transgenic plants and untransformed control tobacco emitted yellowish green fluorescence, indicating their viability (**Fig. 4-A** to **C**). Weak fluorescence was observed in the uninucleate microspores and there was no significant difference between untransformed control tobacco and transgenic plants (**Fig. 4-D** to **F**). However, the microspores and pollen grains after late unimicrospore stage of transgenic plants did not emit yellowish green fluorescence after staining with FDA, indicating abortion of their microspores (**Fig. 4-H, I, K, L, N** and **O**). In contrast microspores of untransformed control tobacco emitted yellowish green fluorescence during development (**Fig. 4-G, J** and **M**).

4. Discussion

In this study, we used a gene transfer approach to perturb CHS activity only in tapetum. Introduction of the *CHS* cDNA from tea under the regulation of *Osg6B* promoter into tobacco produced transgenic plants with the reduced number of fertile pollen grains (**Fig. 2** and **3**). The reduction in the number of fertile pollen grains was not observed in transgenic plants containing the other construct, *Osg6B* promoter-*GUS* gene fusion (**Fig. 3**). Thus the partial male-sterile phenotype will not be ascribed to a insertional mutagenesis nor somaclonal variation, and it is possible that the phenotype was due to the properties of the inserted transgene for CHS.

It is likely that the reduction of CHS activity prevents development of fertile pollen grains because CHS is a key enzyme for flavonoid biosynthesis. It has been reported that the accumulation of the flavonols is observed in stigma, ovaries, pollen grains and pollen tubes [20-22]. Moreover, it has also been reported that flavonols stimulate development, germination, and pollen tube growth of tobacco [1]. Reduction of flavonols has been demonstrated to assign to abnormal microspore, pollen development and pollen tube growth [23-25]. On the other hand, it has been recently argued that some components of the pollen wall are made from flavonoids [3, 4]. The CHS activity in tapetum would play an important role in the normal development of microspores into fertile pollen.

When the *CHS* cDNA was inserted in antisense orientation, a reduction in the pollen fertility was observed in four out of twenty-seven transgenic plants (**Fig. 3**). Some examples were also reported for an antisense inhibition of the expression of genes for flavonoid biosynthesis [26]. An antisense *CHS* gene of petunia was fused to modified CaMV 35S promoter containing the anther box has reported to be introduced into petunia [24]. An antisense *CHS* was also fused to *CHSA* promoter and introduced back into petunia [2]. In both cases, endogenous *CHS* expression was suppressed, resulting in induction of partial male-sterility in these cases. So we consider that introduction of *CHS* cDNA into tobacco caused the suppression of endogenous *CHS* expression.

A reduction of pollen fertility was also induced in five out of 26 transgenic plants by introducing sense *CHS* gene (**Fig. 3**). The reduction of the number of fertile pollen grains might be caused by co-suppression as reported earlier in many transgenic plants [27-30]. It is also possible that the inappropriate timing, location, or level of transgene expression might lead to a collapse of biosynthesis of phenylpropanoid.

We introduced *CHS* cDNA of tea into a heterologous host, tobacco. We consider that the specific regions of homology would exist between the transgene and the target gene to achieve sense/antisense effects, although the degree of homology was not known between the *CHS* cDNA of tea and the tobacco *CHS* genes expressed in the anther tapetum. Several examples of sense/antisense inhibition by heterologous genes have been reported in some other genes [16, 31]. Our result also indicates that the cDNA from different species was able to use the sense/antisense inhibition of the target gene expression.

A tapetum-specific promoter was employed in this study, because immunohistochemical study has demonstrated that CHS is located predominantly in the tapetum [5]. It has been demonstrated that the *Osg6B* promoter used in this study directs GUS expression exclusively in the tapetum of anthers at the tetrad and the uninucleate microspore stages in transgenic tobacco [12]. So transgenic plants obtained in this study were normal except for the anther as expected from the previous study. The *Osg6B* promoter should be useful for estimating the function of genes in the anther tapetum by altering the level of gene expression, as well as for producing male-sterile plants. In addition, perturbation of flavonoid metabolism by this promoter and other transgenes will provide a powerful approach to dissect the functions of flavonoids in pollen.

Acknowledgments

We acknowledge Atsuko Takeuchi, National Institute of Vegetables, Ornamental Plants and Tea, for providing *CHS* cDNA (*CHS1*). This work was supported in part by Grants-in-Aid for Special Research on Priority Areas (nos. 07281102, 07281101 and 07281103; Genetic Dissection of Sexual Differentiation and Pollination Process in Higher Plants) from the ministry of Education, Science, Culture and Sports, Japan and by a grant pioneering

research project in biotechnology from Ministry of Agriculture, Forestry and Fisheries Japan. We thank Professor Kokichi Hinata, Tohoku University, for their comments and support.

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