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***Rapid paper***

# Identification of a WRKY Protein as a Transcriptional Regulator of Benzylisoquinoline Alkaloid Biosynthesis in *Coptis japonica*

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**Selected cultured *Coptis japonica* cells produce a large amount of the benzylisoquinoline alkaloid berberine. Previous studies have suggested that berberine productivity is controlled at the transcript level of biosynthetic genes. We have identified a regulator of transcription in berberine biosynthesis using functional genomics with a transient RNA interference (RNAi) and overexpression of the candidate gene. The 24 primary candidate clones were selected from 1,014 expressed sequence tags (ESTs) that were obtained from a *C. japonica* cell line producing high levels of berberine. Further characterization of the expression profiles of these ESTs suggested that five ESTs would be good candidates as regulators of berberine production. A newly developed transient RNAi system with *C. japonica* protoplasts indicated that double-stranded RNA of an EST clone significantly reduced the level of transcripts of 3′-hydroxy *N*-methylcoclaurine 4′-*O*-methyltransferase. Sequence analysis showed that this EST encoded a group-II WRKY, and we named it *CjWRKY1*. When the effects of double-stranded RNA of the *CjWRKY1* gene were examined in detail, a marked reduction in the transcripts of all genes involved in berberine biosynthesis was detected, whereas little effect was found in the transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and chorismate mutase (CM) that are associated with primary metabolism. Ectopic expression of *CjWRKY1* cDNA in *C. japonica* protoplasts clearly increased the level of transcripts of all berberine biosynthetic genes examined compared with control treatment, whereas the levels of GAPDH and CM were not affected. The functional role of *CjWRKY1* as a specific and comprehensive regulator of berberine biosynthesis is discussed.**

**Keywords:** *Coptis japonica* — Functional genomics — Isoquinoline alkaloid biosynthesis — Transcriptional regulation — Transient RNAi — *WRKY*.

Abbreviations: BBE, berberine bridge enzyme; bHLH, basic helix–loop–helix; CaMV, cauliflower mosaic virus; CM, chorismate mutase; CNMT, *(S)*-coclaurine-*N*-methyltransferase; CrGBF, *Catharanthus roseus* G-box-binding factor; CYP80B2, *(S)*-*N*-methylcoclaurine 3′-hydroxylase; CYP719A1, canadine synthase; DQSDH, dehydroquinate shikimate dehydrogenase; dsRNA, double-stranded RNA; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LUC, luciferase; NCS, *(S)*-norcoclaurine synthase; 4′OMT, 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase; 6OMT, *(S)*-norcoclaurine 6-*O*- methyltransferase; PEG, polyethylene glycol; RNAi, RNA interference; SMT, *(S)*-scoulerine-9-*O*-methyltransferase; STR, strictosidine synthase; TAIL PCR, thermal asymmetric interlaced PCR; TYDC, tyrosine decarboxylase.

The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank/EMBL database under accession numbers AB267401–AB267405, CI999921–CI999931, CI999934–CI999936 and CI999939–CI999948.

## Introduction

Benzylisoquinoline alkaloids are a large group of natural products consisting of >2,500 compounds (Hashimoto and Yamada 1994, Croteau et al. 2000, De Luca and Laflamme 2001, Facchini 2001). They are pharmaceutically important alkaloids and include the analgesics morphine and codeine, the muscle relaxant papaverine (*Papaver somniferum*), the anti-microbial agent sanguinarine (*Eschscholtzia californica*) and the anti-microbial agent berberine (*Coptis japonica*), which has recently been reported to lower cholesterol levels (Kong et al. 2004). While synthetic chemistry as well as fermentation technology can produce many medicinal compounds, plant-derived chemicals, including many benzylisoquinoline alkaloids, are extracted from natural harvests. Thus, the production of these chemicals in cell culture systems may be the most likely alternative for obtaining these compounds. However, a few selected plant cells can produce sufficient quantities of metabolites for industrial applications. For example, cells of *C. japonica* can

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produce large quantities of the anti-microbial benzylisoquinoline alkaloid berberine (Sato and Yamada 1984).

Berberine biosynthesis begins with the conversion of L-tyrosine to dopamine and 4-hydroxyphenylacetaldehyde. These compounds are condensed to (*S*)-norcoclaurine by norcoclaurine synthase (NCS), and three methyltransferases [(*S*)-norcoclaurine 6-*O* methyltransferase (6OMT), (*S*)-coclaurine-*N*-methyltransferase (CNMT) and 3′-hydroxy *N*-methyl coclaurine 4′-*O*-methyltransferase (4′OMT)] and a cytochrome P450 [*N*-methyl coclaurine 3′-hydroxylase (CYP80B2)] are then involved in the conversion of (*S*)-norcoclaurine to (*S*)-reticuline, which is a common intermediate for morphine, sanguinarine, papaverine and berberine. In *C. japonica*, almost all of the enzymatic genes involved in the biosynthetic pathway have been cloned and characterized (Kutchan 1995, Takeshita et al. 1995, Morishige et al. 2000, Choi et al. 2002, Morishige et al. 2002, Ikezawa et al. 2003, Weid et al. 2004) (Fig. 1).

Using isolated genes, the molecular characterization of gene expression in benzylisoquinoline alkaloid biosynthesis has been attempted (Morishige et al. 2002, Ikezawa et al. 2003). Preliminary analysis showed that the transcriptional activation of biosynthetic genes has occurred in selected cultured cells of *C. japonica*, whereas the actual mechanism of transcriptional regulation is still not clear. We thought that our selected cultured *C. japonica* cells would provide a good system for studying transcriptional regulators in alkaloid biosynthesis. Whereas some cultured cells produce alkaloids in response to certain fungal elicitors or the wounding hormone methyl jasmonate (Pasquali et al. 1992, Shoji et al. 2000, Sakai et al. 2002, Facchini and Park 2003), the regulatory mechanism for alkaloid biosynthesis is still not clear. Only a few transcriptional factors in alkaloid biosynthesis have been isolated by promoter analysis and the cDNA-amplified fragment length polymorphism (AFLP) method, e.g. the ORCA transcriptional factor for the strictosidine synthase gene in the terpenoid indole alkaloid biosynthetic pathway (Pasquali et al. 1999, Rischer et al. 2006) and a promoter analysis of putrescine *N*-methyltransferase in tropane alkaloids (Xu and Timko 2004).

The activation tagging method is a powerful tool for isolating the master gene in the regulation of gene expression (Kakimoto 1996), but it is not easy to isolate the true target gene, especially when multiple transcription factors are involved. For example, ORCA3 in terpenoid indole alkaloid biosynthesis controls only some of the biosynthetic genes (Van der Fits and Memelink 2000). Considerable improvements in methodology would be needed to elucidate the complicated transcriptional network. Whereas T-DNA tagging is generally used in *Arabidopsis*, most medicinal plants are not suitable for

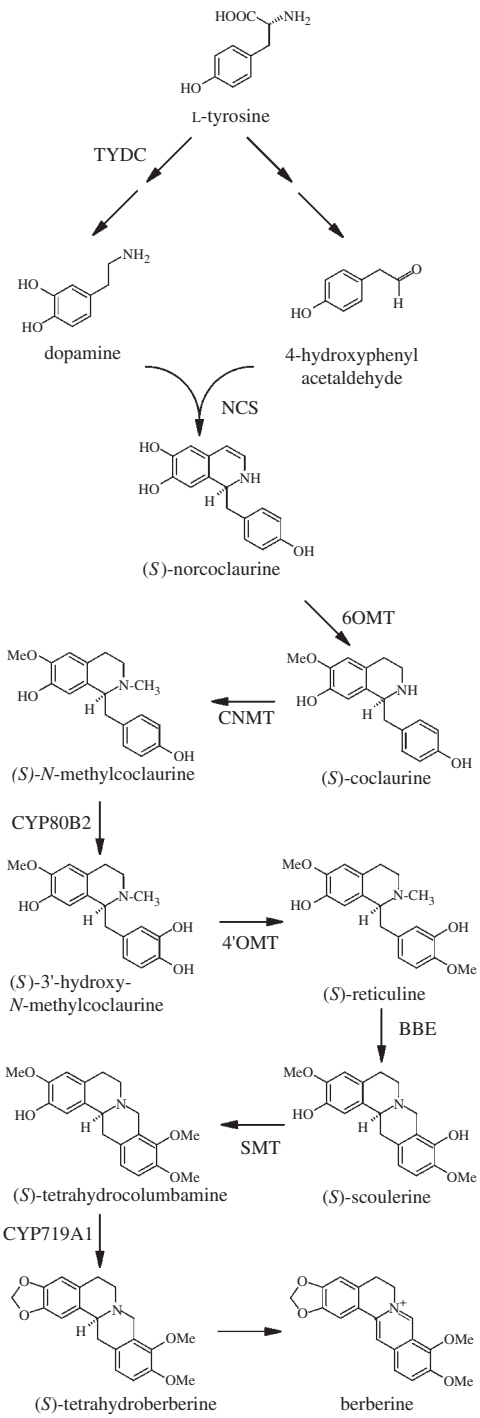


**Fig. 1** Biosynthetic pathway leading to berberine from tyrosine in cultured *Coptis japonica* cells. Enzymes for which the corresponding genes have been cloned are indicated. Enzyme abbreviations: TYDC, tyrosine decarboxylase; NCS, (*S*)-norcoclaurine synthase; 6OMT: (*S*)-norcoclaurine 6-*O*-methyltransferase; CNMT, (*S*)-coclaurine-*N*-methyltransferase; CYP80B2, (*S*)-*N*-methylcoclaurine 3′-hydroxylase; 4′OMT, 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase; BBE, berberine bridge enzyme; SMT, (*S*)-scoulerine-9-*O*-methyltransferase; CYP719A1, canadine synthase.

this approach due to the low efficiency of stable transformation, low regeneration and the large genome size. Thus, another approach using the overexpression of a candidate transcription factor in host cells has been examined with partial success (Oksman-Caldentey and Inze 2004). Again, this simple approach using the overexpression of transcription factors would not be sufficient for identifying the function of transcription factors, since biosynthetic genes in secondary metabolism are controlled by multiple transcriptional regulators, as described for terpenoid indole alkaloid biosynthesis (Pauw et al. 2004).

Thus, we examined another approach to isolate transcription regulator(s) using high-berberine-producing *C. japonica* cells as a model material with a novel transient RNA interference (RNAi) that was recently developed for *C. japonica* cells (Dubouzet et al. 2005). This newly developed RNAi system using artificially prepared double-stranded RNAs (dsRNAs) degrades target mRNA very effectively in a sequence-specific manner.

In this work, we first searched for possible transcriptional regulators from an expressed sequence tag (EST) library of high-berberine-producing *C. japonica* cells on the basis of the expression profile. We then characterized a possible candidate, a WRKY protein gene (*CjWRKY1*), as a transcriptional regulator of benzylisoquinoline alkaloid biosynthesis using the transient RNAi system. We also examined the regulatory activity of *CjWRKY1* using the overexpression of WRKY protein for both endogeneous genes and the reporter construct with the CYP80B2 promoter sequence. Our results suggest that *CjWRKY1* may be the first specific and comprehensive regulator of benzylisoquinoline alkaloid biosynthesis.

## Results

*Correlation of berberine production and the expression of biosynthetic genes in cultured C. japonica cells with different alkaloid productivities*

Berberine is synthesized from two molecules of L-tyrosine through 13 steps in *C. japonica*. Almost all of the biosynthetic genes have been isolated from cultured *C. japonica* cells (Fig. 1). To understand the molecular basis of high alkaloid productivity in 156-1 cells established by small cell aggregate cloning (Sato and Yamada 1984), a preliminary analysis of expression profiles of biosynthetic genes was performed by macroarray analysis of an EST library (E. Dubouzet et al. in preparation). These experiments suggested that high alkaloid productivity should be correlated with a high expression of biosynthetic genes.

To confirm the correlation between high berberine productivity and a high expression of biosynthetic genes, we examined the expression profiles of biosynthetic genes using three cell lines of *C. japonica* with different alkaloid productivities (Fig. 2). First, we examined the alkaloid

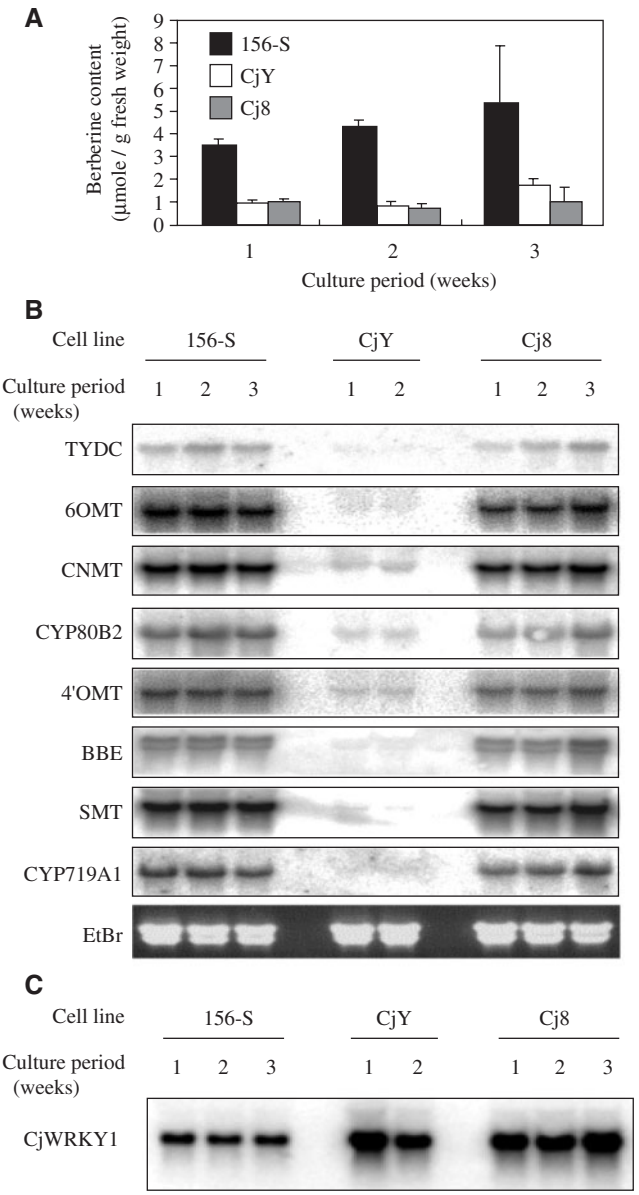


**Fig. 2** Berberine alkaloid productivity and transcript levels of biosynthetic genes in cultured *C. japonica* cells with different levels of alkaloid productivity. (A) Quantification of berberine content in cultured *Coptis japonica* cells: 156-S, a high-producing line (black bar), CjY, a non-selected line (white bar), Cj8, a selected line which lost high berberine productivity (gray bar). Each value is the average of three measurements. (B) RNA gel blot analysis of biosynthetic genes in cultured *C. japonica* cells. Total RNA was isolated from 1- to 3-week-old 156-S and Cj8, and 1- to 2-week-old CjY. RNA gel blots were hybridized with $^{32}$P-labeled cDNA probes for TYDC, 6OMT, CNMT, CYP80B2, 4′OMT, SMT and CYP719A1. Equal loading of RNA was assessed by ethidium bromide staining and by hybridization with an actin probe. (C) RNA gel blot analysis of *CjWRKY1* in cultured *C. japonica* cells. Total RNA was isolated from 1- to 3-week-old 156-S and Cj8, and 1- to 2-week-old CjY, and equal loading of RNA was assessed as in (B).

productivities of 156-S (a high alkaloid producer derived from 156-1), Cj8 (a previously high-producing line which had lost such productivity) and CjY (a non-selected low alkaloid producer). HPLC of cell extracts clearly indicated that the high-producing line 156-S maintained high berberine productivity whereas Cj8 and CjY produced only low amounts of berberine (Fig. 2A). Berberine content in 156-S reached >5 μmol (g FW)$^{-1}$, whereas other lines showed <1 μmol (g FW)$^{-1}$ throughout the culture period.

RNA blot analysis showed that all of the biosynthetic genes in the berberine pathway were highly expressed in 156-S cells, whereas very low expression was detected in non-selected CjY cells. Interestingly, selected Cj8 cells showed substantial expression of all of the biosynthetic genes examined (Fig. 2B). These results suggest that a high expression of biosynthetic genes is required, but not sufficient, for high alkaloid productivity (see Discussion).

*Screening of regulatory factors in berberine biosynthesis by RNA blot analysis*

Since 156-S cells showed a high expression of biosynthetic genes compared with low-producing cells and a similar coordinated change in gene expression was also found in different plant organs with different alkaloid contents (Fujiwara et al. 1993, Ikezawa et al. 2003), we speculated that the gene expression of biosynthetic enzymes in the berberine pathway was regulated in a coordinated manner by a single or a few transcriptional regulator(s). Thus, we tried to isolate such transcriptional regulator genes from an EST library constructed from high-berberine-producing 156-1 cells.

A sequence analysis of 1,014 EST clones and a homology search showed that the EST library was highly enriched with berberine biosynthetic genes (data not shown; E. Dubouzet in preparation) and contained about 24 putative regulatory genes that encode putative receptors, signal transduction molecules, transcription factors, and so on (Supplementary Table 1). We further analyzed the expression of these putative regulatory genes to screen possible regulatory factors in berberine biosynthesis. A preliminary macroarray analysis showed that nine genes were up-regulated and six were down-regulated in the high-berberine-producing cell line, whereas four did not change significantly and five genes were not detected in the cells examined. Further RNA blot analysis of 18 ESTs showed marked changes in the expression level in 156-S cells in comparison with CjY, and five of these ESTs (ESTs 3, 6, 14, 28 and 32) would be good candidates as regulators of berberine biosynthesis, since their expression was correlated with the expression of biosynthetic genes under different productivities (Fig. 2C and Supplementary Fig. S1). Three ESTs, *CjCalmodulin* (EST 14;CI999930), *CjRING-H2 finger* (EST 28;CI999943) and *CjZinc finger* (EST 32;CI999946), showed positive correlations, whereas two ESTs, *CjWRKY1* (EST 6;CI999923) and putative *AT-hook DNA-binding protein* (EST 3;CI999922), showed negative correlations with berberine productivity.

*Identification of regulatory factors using transient RNAi*

To identify the function of the putative regulatory factor genes, a newly developed transient RNAi technique (Dubouzet et al. 2005) was applied. Preliminary experiments using real-time PCR as a tool to measure transcripts indicated that 50 μg of dsRNA for $10^6$ protoplasts was sufficient to suppress endogenous gene expression, while the expression of biosynthetic and other regulatory candidate genes increased during protoplast incubation after treatment with polyethylene glycol (PEG; data not shown). Since the RNA silencing effects of dsRNA were observed after 24 h of incubation, 72 h of incubation was used here. Under optimized conditions, we analyzed the effects of five candidate genes on the expression of a biosynthetic gene, 4′OMT, as a reporter (Supplementary Fig. S2). The introduction of dsRNA of a candidate gene clearly reduced each corresponding target transcript level (Supplementary Fig. S2A), e.g. the dsRNA of *CjWRKY1*, *CjCalmodulin*, *CjRING-H2 finger* and *CjZinc finger* reduced transcript levels by >90%, whereas the dsRNA of *CjAT-hook DNA-binding protein* was less effective.

When we measured the RNA silencing effect on the level of the biosynthetic gene 4′OMT, most dsRNAs of candidate transcriptional regulator genes did not affect the level of the 4′OMT transcript. Only the dsRNA of *CjWRKY1* significantly reduced the 4′OMT level (42 ± 8.2%) (Supplementary Fig. S2B). Repeated experiments confirmed the silencing effect of dsRNA of *CjWRKY1* on the expression of 4′OMT, although this silencing effect showed some fluctuation. Interestingly, when the expression level of *CjWRKY1* was measured during incubation for up to 72 h, the *CjWRKY1* gene showed a steady increase in transcript in control protoplast culture, while dsRNA of *CjWRKY1* showed a marked silencing effect at 24 h after dsRNA treatment (data not shown).

*Characterization of CjWRKY1 based on the nucleotide sequence*

Since 72 WRKY genes are found in the *Arabidopsis* genome and this gene family is the ninth largest group of transcriptional factors (Eulgem et al. 2000, Ülker and Somssich 2004), we determined the full-length sequence of *CjWRKY1* to clarify its sequence homology (see Supplementary Fig. S3). As shown in Fig. 3, *CjWRKY1* (accession No. AB267401) has a typical WRKY domain, a C2H2 zinc finger motif and 52.9% homology with AtWRKY75 (AAL50784). Further sequence analysis (~4,000 ESTs; Y. Kokabu et al. in preparation)

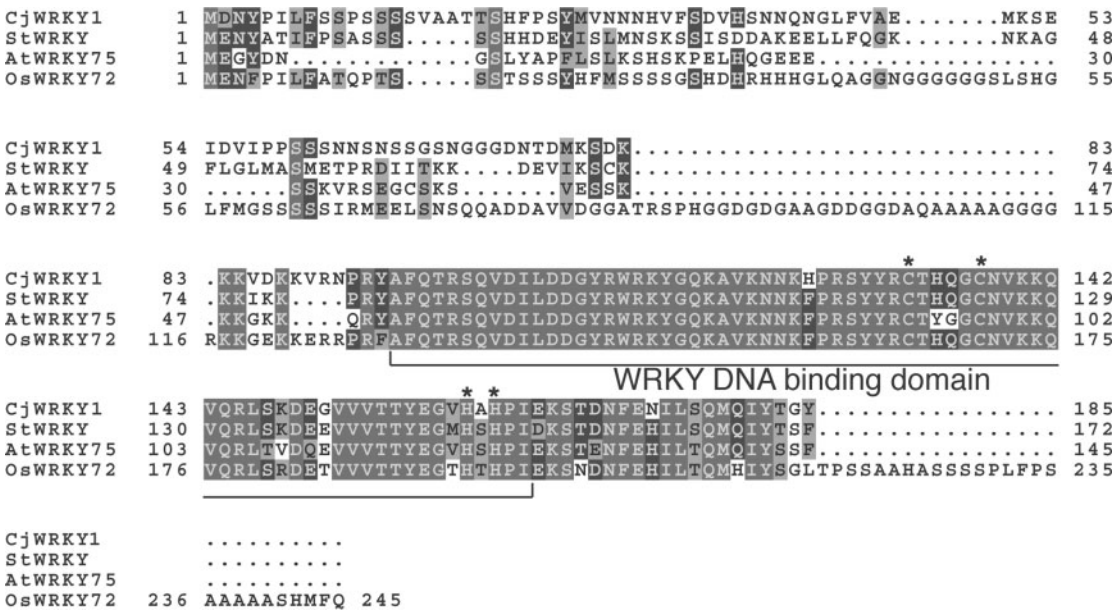


**Fig. 3** Predicted amino acid sequence of *CjWRKY1* and sequence comparison with WRKY proteins. Sequences of WRKY proteins from *Coptis japonica, Solanum tuberosum, Arabidopsis thaliana* and *Oryza sativa*. Identical residues are shown as white letters shaded in gray, and similar residues as white letters shaded in black. Highly conserved WRKY DNA-binding domains are shown. Full-length similarities were 54.5% for *S. tuberosum* WRKY (accession No. BAC23031), 52.9% for *A. thaliana* WRKY75 (accession No. AAL50784) and 46.1% for *O. sativa* WRKY72 (accession No. DAA50784). Asterisks indicate conserved cysteine and histidine residues (C-$X_{4-5}$-C-$X_{22-23}$-H-X-H) in a potential zinc finger-like motif of group II members of the WRKY family.

indicated the presence of an additional three genes with WRKY domains, *CjWRKY2– CjWRKY4* (accession Nos. AB267402–AB267404) in *C. japonica*, although their sequence homology was rather low (~50% identity on a nucleotide basis). When the effects of dsRNAs of the other *CjWRKY* genes (2–4) on 4′OMT and *CjWRKY1* expression were examined, those dsRNAs did not affect the expression of 4′OMT or *CjWRKY1* (Fig. 4). Thus, we concluded that the RNA silencing effect of the dsRNA of *CjWRKY1* was unique to berberine alkaloid biosynthesis in *C. japonica* protoplasts.

### *Effects of RNA silencing of CjWRKY1 on transcript levels of berberine biosynthetic genes*

To investigate the functional role of *CjWRKY1* in benzylisoquinoline alkaloid biosynthesis, the expression levels of all of the biosynthetic genes isolated were examined by transient RNAi with dsRNA of *CjWRKY1* (Fig. 5). Quantitative real-time PCR analysis of the eight biosynthetic genes investigated clearly showed the simultaneous and marked reduction of transcripts of all of the biosynthetic genes; TYDC ($60 \pm 28.7\%$ of control), NCS ($59 \pm 9.0\%$), 6OMT ($47 \pm 7.0\%$), CNMT ($34 \pm 4.7\%$), CYP80B2 ($64 \pm 3.5\%$), 4′OMT ($42 \pm 8.2\%$), berberine bridge enzyme (BBE; $64 \pm 4.9\%$) and CYP719A1 ($34 \pm 4.9\%$). RNA silencing effects were repeatedly confirmed, and the reduction of transcript was statistically significant at $P < 0.05$. On the other hand, the expression levels of genes involved in primary metabolism including tyrosine biosynthesis were not affected by dsRNA of *CjWRKY1* and their transcript levels actually increased during culture as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; $96 \pm 6.1\%$ of control), 3-deoxy-D-arabino heputulosonate 7-phosphate synthase (DAH7PS; $114 \pm 22\%$), dehydroquinate shikimate dehydrogenase (DQSDH; $90 \pm 14\%$) and chorismate mutase (CM; $107 \pm 11\%$) (Fig. 5).

### *Effects of the overexpression of CjWRKY1 on transcript levels of berberine biosynthetic genes*

Since transient RNAi analysis suggested that *CjWRKY1* may be a general regulator of berberine biosynthesis, we tried to investigate whether *CjWRKY1* regulates the expression of berberine biosynthetic genes using a transient overexpression system (Fig. 6). For this purpose, we constructed the overexpression vector *CjWRKY1* under the control of the cauliflower mosaic virus (CaMV) 35S promoter in pBI221 for a transient assay. When this construct was introduced into protoplasts of 156-S cells, transcripts of *CjWRKY1* increased about seven times more than the control after 72 h of incubation. Analysis of the expression levels of biosynthetic genes by quantitative real-time PCR showed that the expression level of all of the genes involved in berberine biosynthesis

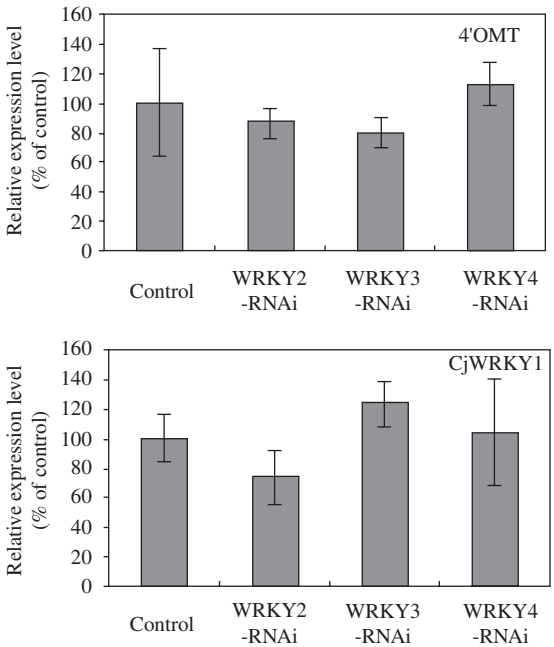


**Fig. 4** Transient RNAi assay of *CjWRKY2–CjWRKY4* genes on berberine biosynthesis. Effects of transient RNAi of *CjWRKY2–CjWRKY4* genes on the expression of the 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase (4′OMT) gene in berberine biosynthesis and *CjWRKY1* were analyzed by real-time PCR analysis. The results shown are mean values ± SD of four measurements. The relative transcript level was calculated using the arabinogalactan protein gene as an internal control. Similar results were obtained in at least two independent experiments.

significantly increased with the ectopic expression of *CjWRKY1* (Fig. 6) as follows: NCS (4.5 ± 0.5-fold of control), 6OMT (22 ± 1.3-fold), CNMT (8.7 ± 1.7-fold), CYP80B2 (4.1 ± 1.3-fold), 4′OMT (16 ± 1.5-fold), BBE (26 ± 3.7-fold), scoulerine 9-*O*-methyltransferase (SMT; 15 ± 1.5-fold) and CYP719A1 (21 ± 4.7-fold). The effects of ectopic expression of *CjWRKY1* were statistically significant at $P < 0.05$. However, genes that are not involved in berberine biosynthesis, such as GAPDH (1.5 ± 0.4-fold), DQSDH (0.4 ± 0.07-fold), DAH7PS (1.9 ± 1.1-fold) and CM (1.1 ± 0.2-fold), showed modest changes in transcript compared with the control (Fig. 6). These data confirmed that the expression of *CjWRKY1* regulated the expression of berberine biosynthetic genes comprehensively and specifically.

*Trans-activation of the CYP80B2 promoter:LUC reporter gene by the overexpression of CjWRKY1*

To examine whether the *CjWRKY1* protein can cause trans-activation of a biosynthetic gene in vivo, *C. japonica* protoplasts were co-transformed with a CYP80B2$^{pro}$:LUC (luciferase) reporter gene and an overexpression construct of *CjWRKY1* (35S:*CjWRKY1*), as shown in Fig. 7. The 5′ upstream region of the CYP80B2 promoter was obtained by TAIL (thermal assymetric interlaced) PCR and the precise sequence (accession No. AB267405) was confirmed by direct PCR of genomic DNA. This sequence contained multiple *cis*-elements including a W-box [TTTGACC(C/T); Yoshida et al. in preparation]. Relative LUC activity of CYP80B2$^{pro}$:LUC in *C. japonica* protoplasts was significantly induced to 3.5 ± 0.6-fold of that in the control by co-transformation with 35S:*CjWRKY1*, indicating that *CjWRKY1* trans-activated the CYP80B2 gene through its *cis* regulatory sequence in vivo (Fig. 7B).

## Discussion

In this study, we examined whether the expression of genes involved in berberine biosynthesis was regulated in a coordinated manner. Our RNA blot data (Fig. 2B, Fujiwara et al. 1993, Ikezawa et al. 2003) clearly indicated that the expression levels of biosynthetic genes coincided with alkaloid productivity except in the selected line Cj8, which had lost high alkaloid productivity during non-selective culture after selection. The result in Cj8 is very suggestive, since a high level of expression of biosynthetic genes has been established by cellular selection, but productivity may be lost during non-selective subculture, especially when careful handling is not strictly followed. Our preliminary investigation of the metabolic profile suggested that the supply of the substrate would be rate limiting in Cj8 cells (data not shown).

Since the coordinated gene expression of biosynthetic enzymes involved in berberine synthesis in *C. japonica* cells was observed, we tried to isolate the transcriptional regulator(s) that control alkaloid productivity in berberine biosynthesis. First, we screened the transcriptional regulators expressed in high-berberine-producing 156-1 cells. Next, we characterized their expression profiles to select the most likely regulator(s) in berberine production. After RNA blot analysis, we obtained five regulatory gene candidates that showed either a positive or negative correlation with alkaloid productivity (Supplementary Table S1 and Fig. S1). Among these five candidates, two zinc finger protein genes showed a very good correlation with alkaloid productivity. However, an RNAi assay did not support our speculation that these zinc finger genes were positive regulators in berberine biosynthesis (Supplementary Fig. S2). Thus, downstream biosynthetic gene expression was not affected even when dsRNA of these zinc finger protein genes clearly reduced the target transcripts. Unexpectedly, the *CjWRKY1* protein gene, the expression profile of which showed a rather complex relationship with alkaloid productivity in cultured *C. japonica* cells, strongly affected the expression of all of the biosynthetic genes examined (Fig. 5). Thus, RNA

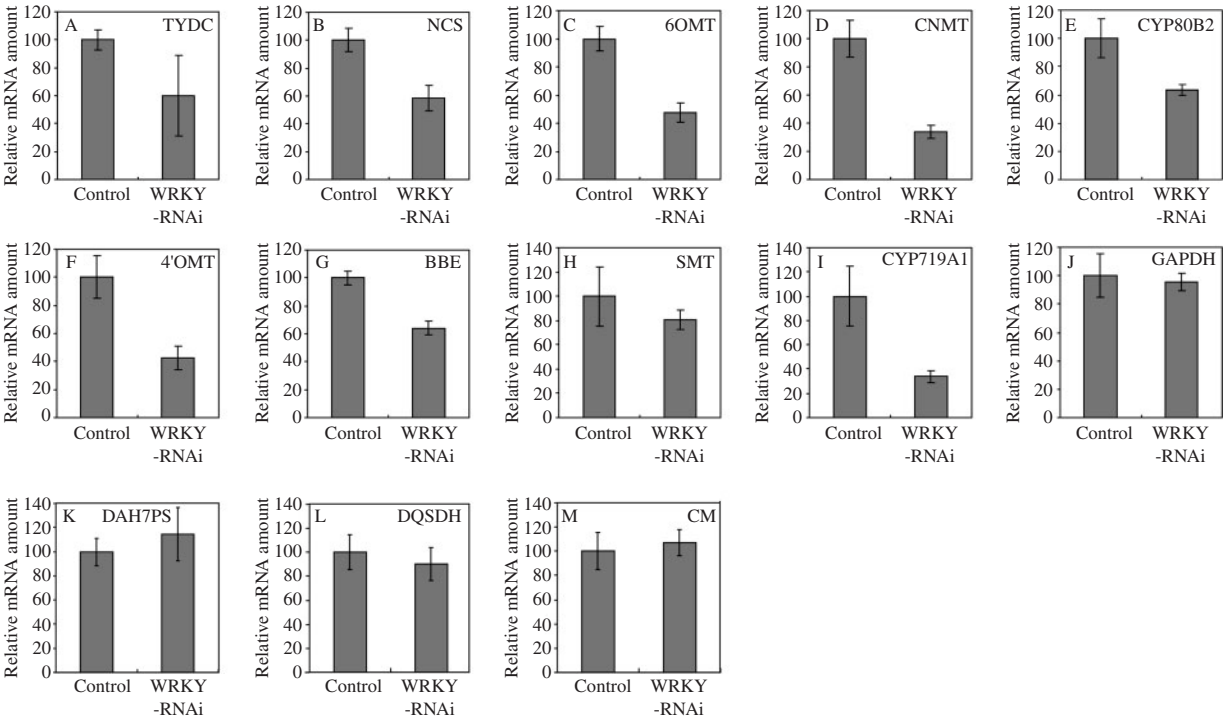


**Fig. 5** Transient RNAi of *CjWRKY1* significantly reduced the expression level of biosynthetic genes in berberine biosynthesis, but not of those in primary metabolism. Transcript accumulation of biosynthetic genes was analyzed by real-time PCR. The genes analyzed were (A) tyrosine decarboxylase (TYDC), (B) norcoclaurine synthase (NCS), (C) norcoclaurine 6-*O*-methyltransferase (6OMT), (D) coclaurine *N*-methyltransferase (CNMT), (E) *N*-methylcoclaurine 3′-hydroxylase (CYP80B2), (F) 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT), (G) berberine bridge enzyme (BBE), (H) scoulerine 9-O-methyltransferase (SMT), (I) canadine synthase (CYP719A1), (J) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (K) 3-deoxy-D-arabino heputulosonate 7-phosphate synthase (DAH7PS), (L) dehydroquinate shikimate dehydrogenase (DQSDH) and (M) chorismate mutase (CM). The relative transcript level was calculated by using β-actin as an internal control as reported elsewhere (Dubouzet et al. 2005). Double-stranded RNA of the green fluorescent protein gene was used as a control. The results shown are mean values ± SD of four measurements.

silencing of *CjWRKY1* reduced the expression of all of the biosynthetic genes, and ectopic expression of this gene induced a marked increase in biosynthetic genes (Fig. 6). Furthermore, these effects were only found in berberine biosynthetic genes, and no other genes in primary metabolism, including the biosynthetic gene for tyrosine, which is a substrate of berberine, were affected.

While our transient assays indicate that CjWRKY1 is a positive regulator, RNA blot analysis of selected cultured lines (Fig. 2C) and different plant tissues (Supplementary Fig. S4) indicates no direct correlation between CjWRKY1 and downstream biosynthetic genes. To address this discrepancy, we overesxpressed *CjWRKY1* in low-berberine-producing CjY protoplasts. Our preliminary results (Supplementary Fig. S5) indicated that CjY protoplasts showed little response to a marked increase of *CjWRKY1*. This result clearly supported our findings that high CjWRKY1 expression in CjY cells was not sufficient for the induction of berberine biosynthetic genes, and additional transcription factor(s) and/or post-transcriptional regulation would be required for the regulation of those genes as reported for indole alkaloid biosynthesis (Pauw et al. 2004).

*CjWRKY1* showed the highest homology to potato WRKY (BAC23031; Fig. 3), and the physiological function of potato WRKY is not known. The transcription of WRKY genes is strongly and rapidly up-regulated in response to wounding, pathogen infection or abiotic stresses in many plant species (Ülker and Somssich 2004). However, the exact biological functions of most WRKY genes are still not clear, since Ülker and Somssich (2004) reported that >40 WRKY knock-out lines rarely showed phenotypic alteration. Thus, our transient analysis in *C. japonica* protoplasts should provide useful information regarding the biological function of WRKY protein in secondary metabolism. Whereas the direct interaction of *CjWRKY1* and W-box element(s) in the biosynthetic gene promoter has not yet been confirmed, our system may be useful for determining the regulatory network through WRKY genes. In particular, our system may be useful for characterizing

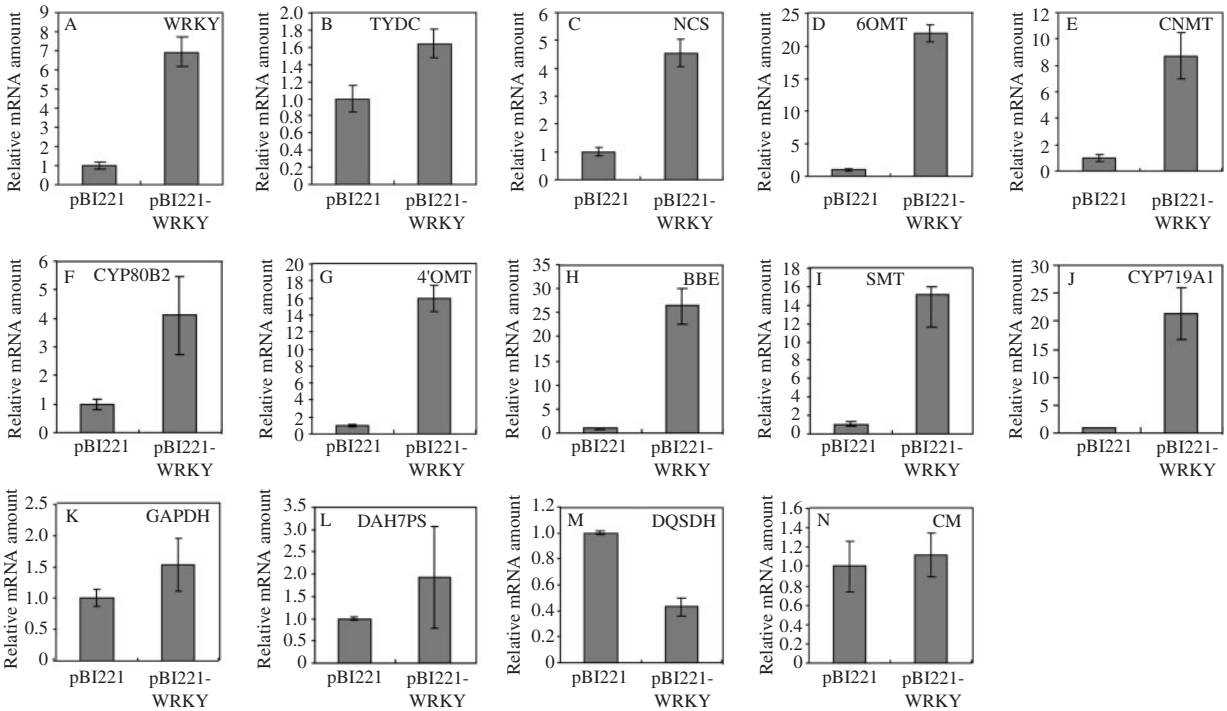


**Fig. 6** Ectopic expression of *CjWRKY1* significantly induced the gene expression of biosynthetic enzymes in the berberine pathway, but not of those in primary metabolism. Transcript accumulation of biosynthetic genes was analyzed by real-time PCR. (A) WRKY, (B) TYDC, (C) NCS, (D) 6OMT, (E) CNMT, (F) CYP80B2, (G) 4′OMT, (H) BBE, (I) SMT, (J) CYP719A1, (K) GAPDH, (L) DAH7PS, (M) DQSDH and (N) CM were analyzed. The relative transcript level was calculated by using β -actin as an internal control. The pBI221 vector was used as a control. The results shown are mean values ± SD of four measurements.

the post-translational activation of WRKY through the mitogen-activated protein kinase cascade and/or calcium-dependent protein kinase (Ludwig et al. 2004). Stable transformants of potato, tobacco or California poppy under propagation may provide additional cues to understand better the physiological function of the WRKY family in alkaloid biosynthesis, if these alkaloid-producing plants have a similar genetic regulation system.

In this study, we used a transient RNAi system to screen candidate genes for transcriptional regulators. Our successful identification of *CjWRKY1* showed that our transient RNAi system is useful for the initial verification and characterization of transcription factor activity. This rapid and simple analytical technique may be a more direct method for the identification of candidates from the EST library. We have recently screened additional transcriptional regulator genes without further RNA blot analysis, and identified a basic helix–loop–helix (bHLH) gene (*CjbHLH1*) as another general transcriptional regulator (Y. Kokabu et al. in preparation). *CjbHLH1* and *CjWRKY1* independently regulate berberine biosynthesis, and we suspect that more than two components are involved in the regulation of berberine biosynthesis in *C. japonica*.

The molecular characterization of strictosidine synthase (STR) also indicated the involvement of multiple transcriptional regulators. The use of an enhancer domain of the STR promoter as bait in a yeast one-hybrid screen resulted in the isolation of CrBPF1, a MYB-like transcription factor (van der Fits et al. 2000). CrBPF1 expression is induced by elicitor but not jasmonate. In addition, the STR promoter contains a promoter element that is conserved in plants, called a G-box, which is located adjacent to the JERE element. A yeast one-hybrid screen using the G-box as the bait isolated G-box-binding factors (CrGBFs) of the basic leucine zipper class and MYC-type bHLH transcription factors (CrMYC1) (Chatel et al. 2003). CrGBFs have been shown to suppress STR expression (Siberil et al. 2001), whereas this factor was not sufficient to control overall gene expression in indole alkaloid biosynthesis. Regulation of the whole metabolic pathway in alkaloid biosynthesis would be more complicated (Facchini and St-Pierre 2005, Kutchan 2005).

In summary, the molecular characterization of *CjWRKY1* in a *C. japonica* protoplast expression system indicated that *CjWRKY1* is a necessary regulator to control overall gene expression in berberine biosynthesis. In addition, the promoter region of CYP80B2 was found

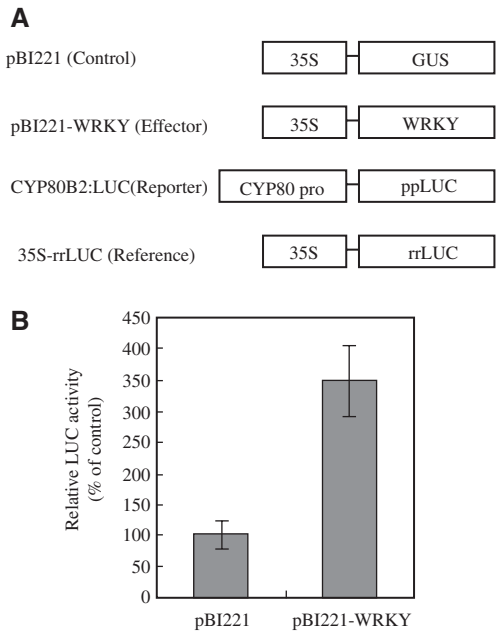


**Fig. 7** Transactivation of CYP80B2$^{pro}$:LUC by ectopic expression of *CjWRKY1*. (A) Constructs used for this experiment. *Coptis japonica* protoplasts were co-transformed with the reporter construct (CYP80B2$^{pro}$:LUC), effector construct (pBI221-WRKY) and reference plasmid (35S-rrLUC). *Photinus pyralis* (pp) and *Renilla reniformis* (rr) LUC activities were determined by a dual luciferase reporter assay. (B) The effect of overexpression of *CjWRKY1* cDNA on CYP80B2$^{pro}$:LUC expression. Relative LUC activity is shown as a percentage of the vector control. *Bars* represent means ± SD ($n = 4$).

to contain a W-box ((T)TGACC(C/T)) motif which is thought to be the target for regulation by the WRKY gene. The molecular dissection of berberine biosynthesis in *C. japonica* cells may help to reveal the secrets of the regulatory mechanisms of secondary metabolism in plants.

## Materials and Methods

*Cultured cells*

Cultured *C. japonica* cell lines 156-1 and Cj8, which were originally selected by small aggregate cloning of *C. japonica* Makino var. *dissecta* (Yatabe) Nakai, were maintained in liquid Linsmaier–Skoog medium containing 3% sucrose, 10 μM naphthalene acetic acid and 0.01 μM 6-benzyladenine (Sato and Yamada 1984). The high-berberine-producing 156-S line was developed from 156-1 cells by the introduction of extra SMT to high-berberine-producing 156-1 cells as described elsewhere (Sato et al. 2001). The high berberine productivity of Cj8 was lost during non-selective subculture. CjY was maintained without selection and produced low amounts of berberine (Yamamoto et al. 1986). For the analysis of alkaloid productivity and transcript level, 1 g (FW) of cells was inoculated in 25 ml of liquid medium and cultured for 2 weeks. *Coptis japonica* cells were cultured in the dark at 25°C with gentle shaking at 90 r.p.m.

*Alkaloid analyses*

A 750 mg aliquot of fresh *C. japonica* cells was incubated overnight in 3 ml of 0.01 N HCl-acidified methanol and centrifuged at 10,000×*g* for 15 min. The supernatant was subjected to HPLC analysis under the following conditions: mobile phase, 50 mM tartaric acid solution containing 10 mM SDS, acetonitrile and methanol (4 : 4 : 1 by vol.); column, TSK-GEL ODS-80$^{TM}$ (Toyo-Soda, Tokyo; 4.6 mm i.d. × 250 mm); temperature, 40°C; flow rate, 1.2 ml min$^{-1}$; detection, absorbance measured at 260 nm using a photodiode array detector (Shimadzu, Kyoto, Japan). Quinine sulfate was used as an internal control.

*RNA gel blot analyses*

Total RNAs were extracted from naturally grown plant tissues harvested in April at the Kyoto Herbal Garden (Takeda Chemical Industry, Japan) and from the above-mentioned cultured cells after 2 weeks of culture with an RNeasy Mini Kit (QIAGEN, Chatsworth, CA, USA) using the manufacturer's protocol. For RNA blot analysis, 10 μg of total RNA were separated on a 1% formaldehyde agarose gel, and then blotted on Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ, USA). RNA probes were radiolabeled with a Random Primed DNA Labeling Kit (Roche, London, UK) according to the manufacturer's instructions. After hybridization, the membrane was washed twice with 2× SSC (30 mM tri-sodium citrate and 0.3 M NaCl) plus 0.2% SDS for 15 min at 60°C, and then washed with 0.2× SSC (3 mM tri-sodium citrate and 30 mM NaCl) plus 0.2% SDS for 10 min at 60°C. The blots were autoradiographed on Kodak Medical X-ray film at −80°C.

*EST library and DNA sequence analysis*

For the construction of an EST library, cDNAs of high-berberine-producing *C. japonica* cells were subcloned in pDR196 vector as briefly mentioned previously (Morishige et al. 2002). Partial sequences of both ends of ESTs were sequenced by Mega Base 1000 (Amersham Biosciences) with a Dynamic ET Terminator for Megabase (Amersham Biosciences) according to the manufacturer's instructions. The primer used for sequence analysis was 5′-GAAAGAAAAAAAAATATACCCCAGC-3′. DNA sequencing analysis of the *CjWRKY1* gene was performed using a DSQ-2000L automated sequencer (Shimadzu, Kyoto, Japan) with a Thermo Sequenase$^{TM}$ Primer Cycle Sequencing Kit (Amersham Biosciences, UK)

*Transient RNAi to isolate and characterize the candidate transcriptional regulators*

dsRNA was prepared by in vitro transcription with PCR products harboring T7 RNA polymerase promoters at both ends, as reported previously (Dubouzet et al. 2005). Primers used to amplify the DNA templates are listed in Supplementary Table S2. A T7 RiboMAX Express RNA Production System (Promega, Madison, WI, USA) was used for in vitro transcription in which both the sense and antisense strands were transcribed simultaneously and self-annealed during the reaction. Residual DNA template was removed by treatment with RQ1 RNase-free DNase (Promega, Madison, WI, USA). The RNA was purified by phenol–chloroform extraction, and then precipitated with ethanol and dissolved in nuclease-free water.

A solution of 50 μg of dsRNA was added to $1.0 \times 10^6$ protoplasts prepared from 3-week-old 156-S *C. japonica* cells as described previously (Dubouzet et al. 2005). This *C. japonica* protoplast suspension was mixed with the same volume of PEG

solution containing 40% (v/v) PEG 6000, 0.4 M mannitol and 0.1 M Ca $(NO_3)_2$, and incubated for approximately 30 min at 28°C. The protoplast suspension was diluted by the addition of W5 solution containing 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 2 mM MES (pH 5.8), and protoplasts were sedimented by centrifugation at 40×*g* for 5 min, then washed twice in W5 solution. Finally, $10^6$ protoplasts were suspended in 2 ml of W5 solution and cultured in a 6-well plate (IWAKI, Chiba, Japan) at 28°C for 72 h with gentle shaking at 30 r.p.m. The viability of protoplasts after culture was >90% by microscopic observation. After incubation for 72 h, protoplasts were harvested by centrifugation at 40×*g* for 5 min for quantitative real-time PCR.

*Quantitative measurement of target gene transcripts by real-time PCR*

Total RNAs were prepared from protoplasts according to the method described previously (Dubouzet et al. 2005). Total RNAs were treated with RNase-free DNase (Promega) to avoid contamination by genomic and/or plasmid DNA from transformation. For reverse transcription, 1 μg of total RNA was incubated at 50°C for 60 min in the presence of 1× first-strand buffer (Invitrogen, Carlsbad, CA, USA), 5 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate, 2.5 μmol of oligo(dT) primer, 40 U of RNaseOUT™ recombinant RNase inhibitor (Invitrogen) and 200 U of SuperScript™III reverse transcriptase (Invitrogen) in a total volume of 20 μl. The sequence of the oligo(dT) primer was 5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG TTTTTTTTTTTTTTTTTT-3′. Quantitative real-time PCR was performed in 0.2 ml strip tubes using a DNA Engine Opticon™ System (Bio-Rad, Hercules, CA, USA), which was equipped with a thermal cycler and real-time detector of fluorescence. A 5 ng aliquot of first-strand cDNA was specifically amplified using a DYNAmo™ HS SYBR® Green qPCR kit (Finnzymes, Espoo, Finland) in the presence of 0.3 M sense and antisense gene-specific primers (Supplementary Table S2), 2.5 mM $MgCl_2$ and 1× Master Mix (Finnzymes, Espoo, Finland) containing DYNAmo hot-start DNA polymerase, SYBR Green I, optimized PCR buffer and deoxynucleotide triphosphates in a total volume of 10 μl. After initial denaturation at 95°C for 10 min, the reaction mixture was cycled 40 times using the following parameters: 94°C for 10 s, primer annealing at 55°C for 20 s and primer extension at 72°C for 30 s. After each cycle, the fluorescence of double-stranded products was detected at 78°C. This temperature precluded the measurement of fluorescence attributed to small, non-specific DNA fragments. The final PCR products were checked for homogeneity by a melting curve analysis according to the manufacturer's instructions (Bio-Rad). Each quantitative PCR was performed at least in duplicate.

PCR primers (Supplementary Table S3) were designed for five putative regulatory molecules, 13 biosynthetic genes and an internal control using the Primer3 program (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). To facilitate real-time PCR measurement of the transcripts under standard reaction conditions, a stringent set of criteria was used for primer design. This included a predicted melting temperature of 60 ± 2°C, primer lengths of 20–24 nucleotides, guanine–cytosine (GC) contents of 45–55% and a PCR amplicon length of 60–200 bp.

The fluorescence signal from each real-time quantitative PCR is collected as a normalized value plotted vs the cycle number. Reactions are characterized by comparing threshold cycle [C (t)] values. C (t) is the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above the baseline. Quantitative values are obtained from the C (t) values at which the increase in signal associated with the exponential growth of PCR product starts to be detected according to the manufacturer's instructions (Bio-Rad). The actin, ATPase or arabinogalactan protein genes used as internal controls were isolated from an EST library of *C. japonica* cells and each transcript level was normalized with either the actin, ATPase or arabionogalactan protein gene. The final result was expressed as a relative fold by comparing the target gene transcripts with the internal control (actin/ATPase), which was determined by the equation $2^{[C\ (t)\ target\ -C\ (t)\ internal\ control]}$.

*Construction of overexpression vector and transient assay*

To examine the transcriptional regulator activity of *CjWRKY1*, we constructed an expression vector using a Dual Luciferase Reporter Assay System (Promega). *CjWRKY1* cDNA was amplified from cDNA of 156-S cells using KOD-Plus-DNA Polymerase (TOYOBO, Osaka, Japan) with the introduction of *Sal*I and *Not*I sites at the 5′ and 3′ ends, respectively. The integrity of the sequence was confirmed by nucleotide sequencing, digested with *Sal*I and *Not*I, then ligated to the pBI221-LUC$^+$ vector. The expression vectors were introduced into *C. japonica* protoplasts at a density of $10^6$ ml$^{-1}$ at a concentration of 50 μg ml$^{-1}$ with PEG solution as for the transient RNAi assay. Protoplasts that had been incubated for 72 h were harvested and the transcript levels were determined by quantitative real-time PCR.

*Luciferase assay*

CYP80B2 promoter was isolated by TAIL PCR from genomic DNA of 156-S cells as described elsewhere (Liu and Whittier 1995), and a reporter vector (CYP80B2::LUC) was constructed using a 642 bp CYP80B2 promoter sequence (accession No. AB267405) and *Photinus pyralis* LUC in pBI221-LUC+. The integrity of the promoter sequence of CYP80B2 was confirmed by nucleotide sequencing. Protoplasts of the *C. japonica* cell line 156-S were co-transformed with plasmids carrying the CYP80B2 promoter fused with *P. pyralis* LUC (reporter construct; 1 μg) and overexpression vectors carrying *CjWRKY1* cDNAs fused to the CaMV 35S promoter (pBI221-WRKY effector; 10 μg). Co-transformation with pBI221 without insert was used as a control. *Renilla reniformis* LUC cDNA under the CaMV 35S promoter (0.1 ng) was also co-introduced as an internal standard. Co-transformation was carried out in quadruplicate. Protoplasts were incubated for 72 h, and reporter activity was measured using the Dual LUC Reporter Assay System (Promega) immediately after protoplasts were harvested according to the manufacturer's instructions. Protoplasts were lysed in 1× PLB for exactly 30 s, and *P. pyralis* and *R. reniformis* LUC activities were detected using a Luminometer Lumant LB 9507 (BERTHOLD) luminometer for 10 s. The *P. pyralis* LUC activity was normalized with *R. reniformis* LUC activity.

*Supplementary material*

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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