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Running title: Freezing tolerance of tobacco with introduced wheat CBF

Increased freezing tolerance through up-regulation of downstream genes via the wheat *CBF* gene in transgenic tobacco

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Abbreviations: CaMV, cauliflower mosaic virus; COR, cold-responsive; CBF, CRT-binding factor; CRT, C-repeat; DRE, dehydration-responsive element; GUS, ß-glucuronidase; LEA, late-embryogenesis-abundant; MS, Murashige-Skoog; QTL, quantitative trait locus; RAPD, randomly amplified polymorphic DNA.

Abstract

The wheat (*Triticum aestivum* L.) *CBF* gene family is assumed to play important roles in development of low-temperature and freezing tolerance through activation of the downstream *Cor*/*Lea* genes. However, no direct evidence shows association of the wheat *CBF* genes with stress tolerance or any interaction between wheat CBF transcription factors and *Cor*/*Lea* gene activation. Here, we introduced *Wcbf2,* one of the wheat *CBF* genes, into the tobacco (*Nicotiana tabacum* L.) genome. Expression of *Wcbf2* significantly increased the level of freezing tolerance in the transgenic tobacco plants without phenotypic retardation, and altered the expression patterns of tobacco genes, including cold-responsive genes. A transgenic tobacco plant expressing *Wcbf2* was crossed to other transgenic plants expressing a *GUS* reporter gene under control of the wheat *Cor*/*Lea* gene promoter. Analysis of the F_1 plants showed that the WCBF2 protein positively regulated at least the expression of *Wdhn13* and *Wrab17*. These results strongly indicate that WCBF2 functions as a transcription factor in the development of freezing tolerance in common wheat.

Keywords: CBF transcription factor, *Cor*/*Lea* genes, differential display, freezing tolerance, transgenic plant, *Triticum aestivum* L.

1. Introduction

Cold acclimation is an adaptive process for acquiring freezing tolerance in higher plants. In the cold-acclimation process, a large number of low-temperature responsive genes are transcriptionally activated, and the accumulated proteins and metabolites lead to the protection of cell structures and functions from freezing damage [33]. The low-temperature responsive genes are called *Cor* (cold-responsive)/*Lea* (late-embryogenesis-abundant) genes. A functional cis-acting element, i.e., the CCGAC core motif known as a CRT (C-repeat)/DRE (dehydration-responsive element) sequence, was proven to play a pivotal role in the promoter function of *COR15A*/*RD29A* genes in *Arabidopsis* [3, 35]. A family of transcription factors called CRT-binding factors (CBFs) or DRE-binding proteins (DREBs) regulates *Cor*/*Lea* gene expression through binding to the CRT/DRE cis elements. These transcription factors contain a DNA binding domain found in the ethylene-responsive element binding protein/APETALA2 (EREBP/AP2) family [16, 28]. The CBF/DREB1 transcription factors are key regulators of cold signal transduction in various plant species [6, 17, 26, 33]. Overexpression of *Arabidopsis CBF1* not only leads to strong expression of *Cor*/*Lea* genes, but also improves freezing tolerance [8, 10, 16].

In common wheat, many low temperature-responsive *Cor*/*Lea* genes have been characterized [5, 11, 21, 31, 32]. Wheat *CBF* homologs such as *TaCBF*, *TaDREB1* and *Wcbf2* have also been isolated and characterized [7, 14, 23]. Recently, a cluster of 11 *CBF* genes in einkorn wheat was located to the *Fr-A^{m}2* locus, which maps as a quantitative trait locus (QTL) for freezing tolerance on chromosome 5A^{m} [18, 34]. The *Fr-A^{m}2* locus controls *Cor*/*Lea* gene expression and freezing tolerance [34]. Another QTL for freezing tolerance, *Fr-1*, was also assigned to the homoeologous group 5 chromosomes [4, 29], and it was strongly suggested that the *Fr-1* locus controls development of freezing tolerance and *Cor*/*Lea* gene expression through *CBF* transcriptional activation [12]. Therefore, *Fr-1* and *Fr-2* loci regulate wheat freezing tolerance through *Cor*/*Lea* expression. However, there is no direct evidence for trans-activation of *Cor*/*Lea* expression via the *CBF* genes in wheat. Our previous study showed that a reporter gene under control of the promoter sequences of two wheat *Cor*/*Lea* genes, *Wcor15* and *Wdhn13*, seemed to be slightly activated by co-transformed *Wcbf2* in wheat cultured cells, although the results were not statistically significant [14].

In our previous study, overexpression of *Wcor15*, a member of the wheat *Cor*/*Lea* gene family, improved freezing tolerance in transgenic tobacco plants, but the increase in freezing tolerance was observed only under limited conditions [24]. Restricted but significantly improved levels of freezing tolerance were also reported in transgenic *Arabidopsis* plants expressing the *Cor15a* and *Wcs19* genes [2, 20]. Therefore, overexpression of the CBF transcription factor might lead to higher levels of freezing tolerance than that of individual COR/LEA proteins. In fact, expression of an *Arabidopsis CBF* gene greatly increased transcript accumulation levels of many *Cor*/*Lea* genes and the freezing tolerance level in transgenic plants [8, 10, 16, 22].

The aim of the present study was to clarify the in vivo interaction between *Wcbf2* and *Cor*/*Lea* genes. Here, we report production of transgenic tobacco plants expressing *Wcbf2* and/or *Cor*/*Lea* promoter-containing reporter genes, trans-activation of the reporter gene and alteration of freezing tolerance.

2. Results and discussion

2.1. Alteration of freezing tolerance in Wcbf2*-expressing transgenic tobacco*

A plasmid construct containing a wheat *CBF* gene, *Wcbf2,* under the control of a cauliflower mosaic virus (CaMV) 35S promoter was introduced into the tobacco genome by an *Agrobacterium*-infection method; 8 transgenic plants named *35S::Wcbf2* were recovered on selection medium. Integration and expression of the introduced *Wcbf2* gene were confirmed in the recovered transformants by Southern blot analysis (data not shown) and reverse transcription (RT)-PCR (Fig. 1A), respectively. No phenotypic alteration was observed in the transgenic tobacco plants expressing *Wcbf2*. Freezing tolerance was greatly improved in *Wcbf2*-expressing transgenic tobacco plants (Fig. 1B, 1C). We then compared the freezing tolerance level of the most freezing-tolerant plant (line #4) with that of a previously produced transgenic plant which was the most freezing-tolerant line (line #6) of a set of *35S::Wcor15* transgenic plants [24]. The two types of transgenic lines were treated at -15˚C for 1 or 2 h without cold acclimation, after which some transgenic plants recovered (Fig. 1B, 1C). Therefore, the level of freezing tolerance in the transgenic line #4 expressing *Wcbf2* was similar to that in the transgenic line #6 expressing *Wcor15* under these stress conditions. After a 3-day cold acclimation, the two types of transgenic lines were treated at -15˚C for 2 h, and the number of transgenic plants that recovered was slightly more for *35S::Wcbf2* than that for *35S::Wcor15* (Fig. 1D), whereas the recovery rates were very low in both types of transgenic tobacco plants and the difference was not significant.

It was suggested that freezing tolerance is developed through the collective action and the cumulative effect of individual COR/LEA proteins [2, 17, 20, 24]. If the increased level of freezing tolerance in the *35S::Wcbf2* transgenic line #4 was due to a collective, cumulative effect, the introduced *Wcbf2* cDNA should activate or enhance some tobacco genes. Therefore, differential display with randomly amplified polymorphic DNA (RAPD) primer sets was conducted to compare gene expression profiles between *Wcbf2*-expressing transgenic and non-transformed tobacco plants. RNA samples were isolated from 5-day cold-acclimated and non-acclimated seedling leaves. In total, 50 RAPD primer combinations were examined, and 78 cDNA fragments were amplified. Two sets of the primer combinations, OPR15/OPU15 and OPU5/OPV5, showed a difference in gene expression patterns between the transgenic and non-transgenic plants (Fig. 2A). The OPR15/OPU15 primer set produced a 550-bp fragment named R15U15 only in the cold-acclimated leaves of the two *35S::Wcbf2* transgenic lines. Two fragments with the OPU5/OPV5 primer set, named U5V5-1 and U5V5-2, were amplified in both cold-acclimated and non-acclimated seedlings of transgenic plants and in cold-acclimated non-transgenic plants. These fragments were cloned and sequenced. The cDNA sequences of R15U15 and U5V5-1 showed high homology respectively to that of rice *LIP19* (GenBank accession no. **X57325**) and rice cDNA clone J023025N02 (GenBank accession no. **AK069543**). Specific primer sets for the R15U15 and U5V5-1 sequences were designed and the expression patterns of R15U15 and U5V5-1 were analyzed by RT-PCR analysis. Transcripts of both cDNA sequences accumulated after low-temperature treatment in non-transgenic tobacco, and constitutive accumulation of the transcripts was observed in the two *35S::Wcbf2* transgenic lines (Fig. 2B). The U5V5-1 transcript was abundantly expressed in cold-accumulated leaves of the most freezing-tolerant lines of the *35S::Wcbf2* transgenic plants. Rice *Lip19* encoding a bZIP-type transcription factor was strongly responsive to low temperature in seedlings [1] and OsOBF1, another type of bZIP protein, forms a heterodimer with LIP19, which binds to a C/G hybrid containing the ACGT core sequence of the ABA responsive element [25]. The wheat *LIP19* homolog, *Wlip19*, is also transcriptionally activated by low temperature [13] and some wheat *Cor*/*Lea* genes are clearly controlled by trans-activation of WLIP19 in wheat seedlings (our unpublished results). In this study, it was found that a tobacco *LIP19* homolog was also responsive to low temperature. Transcript accumulation of the *LIP19* homolog was observed in both cold-acclimated and non-acclimated seedlings of *35S::Wcbf2* transgenic tobacco, suggesting that the cold-responsiveness of *LIP19* gene expression might be due to a CBF transcription factor, at least in tobacco. The interaction of *CBF* and *LIP19* should be clarified by further study.

Wcbf2 expression altered both freezing tolerance and gene expression patterns in transgenic tobacco plants. These results indicated that the introduced *Wcbf2* gene precisely translates the WCBF2 protein, functioning as a transcription factor and activating expression of the endogenous genes in tobacco cells. The high levels of freezing tolerance might be due to ectopic expression of endogenous tobacco genes via WCBF2. *Wcbf2* could alter endogenous gene expression patterns in heterologous plant species, resulting in improved tolerance to freezing stress as previously reported for other *CBF* genes [8, 10, 16, 36]. It was previously reported that *CBF* overexpression under control of the CaMV35S promoter causes severe growth retardation under normal growth conditions [16], but *Wcbf2* expression could avoid such phenotypic retardation, at least in tobacco.

2.2. Trans-activation of a Cor/Lea *promoter-containing reporter gene via WCBF2*

There is no direct evidence for activation of *Cor*/*Lea* gene expression through binding of CBF transcription factors to *Cor*/*Lea* promoters in wheat. In our previous studies, four types of ß-glucuronidase (GUS) chimeric gene constructs were produced, with *GUS* expression under the control of 5' upstream sequences of four wheat *Cor*/*Lea* genes, *Wcor15*, *Wdhn13*, *Wrab17* and *Wrab19* [31, 32]. These four 5' upstream sequences contained core motifs of CRT/DRE *cis*-elements. The promoter regions of *Wcor15*, *Wdhn13* and *Wrab17* enhance GUS activity in leaves of transgenic tobacco plants in response to low temperature treatment [31, 32]. The *35S::Wcbf2* transgenic tobacco produced in this study was crossed with the four types of *Cor/Lea pro::GUS* transgenic plants, and the F_1 plants were assessed to clarify the interaction between the wheat CBF transcription factor and *Cor*/*Lea* promoter regions.

A histochemical GUS staining assay showed that *Wcbf2* expression enhanced *GUS* expression under the control of the 5' upstream sequences of *Wdhn13* and *Wrab17* at normal growth temperature in the F_1 plants (Fig. 3A, 3B). Under normal temperature conditions, the four types of *GUS* transgenic plants represented low levels of *GUS* expression. The *Wcor15* and *Wrab19* promoter regions led to no visible enhancement of GUS staining levels with the *35S::Wcbf2* construct in the F_1 plants (data not shown). Next, the GUS activities were quantified and estimated relative to GUS activity in a *Wrab19* promoter*::GUS* chimeric construct (Fig. 3C). The 5' upstream sequences of *Wdhn13* and *Wrab17* significantly increased GUS activity in F_1 seedlings compared with those in the parental transgenic plants. This heterologous tobacco system clearly indicated that the WCBF2 protein directly and positively regulates *Wdhn13* and *Wrab17* gene expression.

It was recently reported that some members of the barley *CBF* gene family, the *HvCBF*s, can induce *Cor*/*Lea* gene expression in transgenic *Arabidopsis* plants but other *CBF* genes caused no alteration in gene expression patterns [27]. *HvCBF3* effectively induced expression of all four examined *Cor*/*Lea* genes in *Arabidopsis*, but *HvCBF6* did not increase the transcript accumulation level of *Arabidopsis COR47*. *Wcbf2* also showed such a target specificity in its enhancement of the expression levels of wheat *Cor*/*Lea* genes. *Wcor15* contains three putative CRT/DRE elements in its 5’ upstream region, and its expression is specifically induced by low temperature [31]. In both this and previous studies, no significant increase in GUS expression could be observed when the *Wcor15 pro::GUS* gene co-existed with the *35S::Wcbf2* gene [14]. These observations indicate that *Wcbf2* did not bind to the putative CRT/DRE elements in the *Wcor15* 5’ upstream region, and suggest that different *CBF* copies, for example *TaCBF2* and *TaCBF4* [18, 27], might be associated with low temperature-specific regulation of *Wcor15* gene expression.

In our previous study, a transient expression assay using wheat callus was conducted to assess trans-activation of *Cor/Lea pro::GUS* chimeric genes via *Wcbf2* [14]. No significant interaction was observed, even between the *Wdhn13* promoter region and WCBF2, in a transient expression assay using wheat callus. The heterologous transgenic approach using transgenic tobacco was more effective in demonstrating a significant effect of the wheat CBF transcription factor on downstream gene expression than the transient expression assay. The transient expression assay using a particle delivery apparatus gave mean values with a wide standard deviation, and the target homologous cells showed high background GUS activity. The transgenic tobacco system used in this study decreased both the standard deviation and the background GUS activity. These results demonstrated effectiveness of the heterologous tobacco system to clarify the positive relationship between wheat transcription factors and their target downstream genes.

In this study, overexpression of *Wcbf2* significantly improved freezing tolerance through alteration of endogenous gene expression patterns in transgenic tobacco. Moreover, WCBF2 enhanced expression of downstream genes through interaction with *Cor*/*Lea* promoter regions. It was concluded that WCBF2 functions as a transcription factor for the *Cor*/*Lea* genes to develop freezing tolerance in common wheat.

3. Methods

3.1. Vector construction and tobacco transformation

Wcbf2 cDNA clones were introduced into the *Xba*I/*Sac*I site of pBI121 (Clontech) to produce a *CaMV35S::Wcbf2* construct. The construct was introduced into leaf discs of tobacco (*Nicotiana tabacum* L.'Petite Havana') using *Agrobacterium tumefaciens* LBA4404. Transformants were selected on Murashige-Skoog (MS) medium [19] containing 0.1 mg L^{-1} alpha-naphthalene acetic acid, 1.0 mg L^{-1} 6-benzyl aminopurine and 250 mg L^{-1} kanamycin, and regenerated on hormone-free MS medium containing 50 mg L^{-1} kanamycin.

For Southern blot analysis, total DNA extracted from tobacco leaves was digested with a restriction enzyme *Hin*dIII. The digested DNA was fractionated by electrophoresis through an 0.8% agarose gel, transferred to Hybond N^+ nylon membrane (GE Healthcare, Piscataway, NJ, USA) and hybridized with ^{32}P-labeled *Wcbf2* cDNA as a probe. Probe labeling, hybridization, washing and autoradiography were performed according to Kume *et al.* [14].

3.2. Bioassay for freezing tolerance

Two types of transgenic tobacco plants were used for determination of freezing tolerance. The *CaMV35S::Wcbf2* transgenic plants were produced in this study. Another transgenic tobacco, *35S::Wcor15*, was previously reported [24]. Two-week-old seedlings of transgenic tobacco plants were grown on the MS medium in a controlled-climate cabinet at 25 ˚C with a 16 h photoperiod at a light intensity of 110-120 μm photons $m^{-2} s^{-1}$ provided by cool white fluorescence lamps. The seedlings were treated with or without cold acclimation at 4˚C for 3 days and then frozen at -15˚C for 1 or 2 h in the dark. The frozen seedlings were thawed overnight at 4˚C and transferred back to normal temperature conditions (25˚C). On the 14th day after transfer, survival of seedlings was recorded. The whole experiment was repeated three times and the data were statistically analyzed by Student's *t*-test between the wild-type plant and transgenic lines.

3.3. cDNA differential display with RAPD primers

Low temperature treatment for 3 days was given by transferring 14-day-old seedlings from normal temperature conditions to cold acclimation conditions (4˚C) for 5 days. Total RNA was extracted by guanidine thiocyanate from cold-acclimated and non-acclimated tobacco leaves. First-strand cDNA was synthesized from DNase I-treated RNA samples with oligo-dT primers using ReverTra Ace® (Toyobo, Osaka, Japan). A differential display method [15] was performed using the first-strand cDNA as template. A total of 50 random 10-mer primer combinations (Operon Technologies, Inc., CA, USA) were used for identification of transcripts abundant in transgenic tobacco plants. PCR amplification was initiated at 95˚C for

1 min, followed by 35 cycles of 94˚C for 1 min, 40˚C for 1 min, and 72˚C for 2 min, and terminated at 72˚C for 1 min. After amplification, the resulting fragments were separated on a 1.5% agarose gel. These fragments were cloned into pGEM-T Easy vector (Promega, WI, USA) and sequenced. Expression of these clones was studied by RT-PCR using the following two gene-specific primer sets: 5'-ATGTCGTCGCCGTCGCGCCG-3' and 5'-CTCCGGGGATGTCCACGGGG-3' for the R15Y15 fragment and 5'-GAGATCTGAGTAGGTGA-3' and 5'-CTAGCAATCCATCCATC-3' for the U5V5-1 fragment. The annealing temperatures for the RT-PCR amplification were 55˚C and 45˚C for the R15Y15 and U5V5-1 fragments, respectively. Thirty-five cycles of PCR were performed and the amplified products were separated by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide.

3.4. Crossing of transgenic tobacco plants and GUS assay

The CaMV35S promoter of pBI121 was exchanged for the 5' upstream sequence of wheat *Cor*/*Lea*, i.e., *Wcor15*, *Wdhn13*, *Wrab17* or *Wrab19*, and four chimeric *GUS* genes were constructed using these sequences, then introduced into the tobacco genome by the *Agrobacterium*-infection method [31]; these were named, respectively, *Wcor15 pro::GUS*, *Wdhn13 pro::GUS*, *Wrab17 pro::GUS* and *Wrab19 pro::GUS*. *CaMV35S::Wcbf2* transformants were used as pollen parent in crosses with transgenic tobacco plants expressing a chimeric GUS gene under the control of the *Cor*/*Lea* promoter. The F_1 transgenic tobacco plants were selected on hormone-free MS medium containing 50 mg L^{-1} kanamycin under normal temperature conditions.

First, GUS activity was assessed histochemically as described previously [30]. The chlorophyll of histochemically stained leaves was removed with ethanol. Next, GUS activity was quantified according to Jefferson [9]. Means with standard error were calculated based on 3 independent experiments, and the data were statistically analyzed by Student's *t*-test between the F_1 and parental plants.

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Figure Legends

Figure 1. Freezing tolerance of transgenic tobacco plants expressing *Wcbf2*. (A) RT-PCR analysis of the introduced *Wcbf2* gene in 8 *35S::Wcbf2* transgenic lines. WT; wild-type tobacco plants. (B) Increased freezing tolerance in transgenic tobacco plants expressing *Wcbf2*. The introduced *Wcbf2* and *Wcor15-GFP* genes were controlled by a CaMV35S promoter. Non-acclimated transgenic and wild-type plants were treated at freezing temperature (-15˚C) for 1 h. Pictures were taken on the 14th day of recovery after freezing treatment. (C) Comparison of survival rates after freezing treatment for *35S::Wcbf2* and *35S::Wcor15* transgenic plants. The non-acclimated transgenic and wild-type plants were treated at freezing temperature (-15˚C) for 1 and 2 h. The means ± standard deviations were calculated from data in 3 experiments. Student's *t*-test was used to test for statistical significance (*$P < 0.05$) between the wild-type plant and transgenic lines. (D) Comparison of survival rates after freezing treatment between *35S::Wcbf2* and *35S::Wcor15* transgenic plants acclimated for 3 days. Pictures were taken on the 4th and 14th day of recovery after freezing treatment.

Figure 2. Differential display of transcripts for *35S::Wcbf2* transgenic and wild-type tobacco seedlings. (A) RAPD RT-PCR pattern with the OPR15 and OPU15 (upper) and OPU5 and OPV5 (middle) primer combinations in *35S::Wcbf2* transgenic tobacco and wild-type tobacco (WT) plants. (B) RT-PCR analysis with gene-specific primers. An actin gene (lower) was used as an internal control. M; 100-bp ladder marker, NA; no acclimation, CA; cold acclimation at 4˚C for 5 days.

Figure 3. Trans-activation of *Cor/Lea* promoter-GUS chimeric genes in transgenic tobacco plants. (A)(B) Comparison of histochemical GUS staining in F_1 seedlings for *Cor/Lea pro::GUS* and *35S::Wcbf2* transgenic plants and parental transgenic plants. (C) GUS activity in F_1 seedlings and parental *Cor/Lea pro::GUS* transgenic plants. The means ± standard deviations were calculated from data in 3 experiments. Student's *t*-test was used to test for statistical significance (**P* < 0.05, ***P* < 0.01) between the F_1 and parental plants.

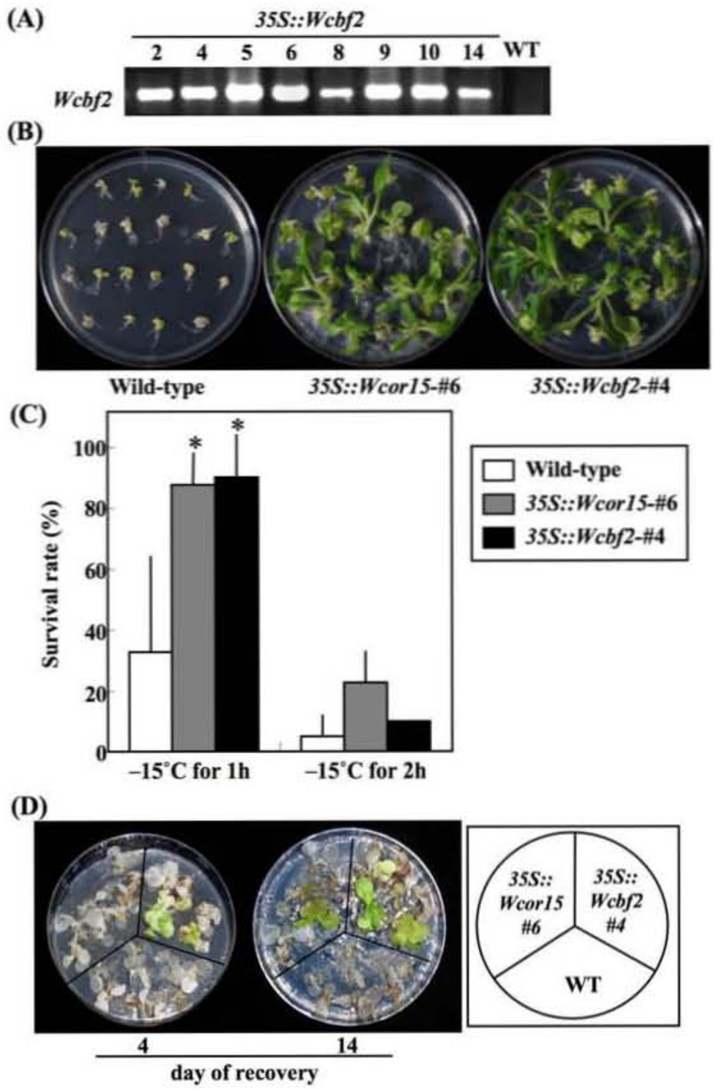

Fig. 1 (Takumi et al.)

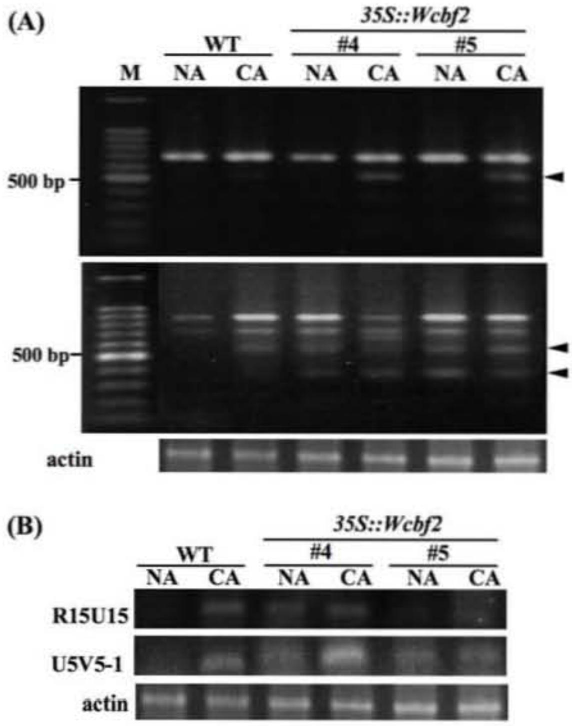

Fig. 2 (Takumi et al.)

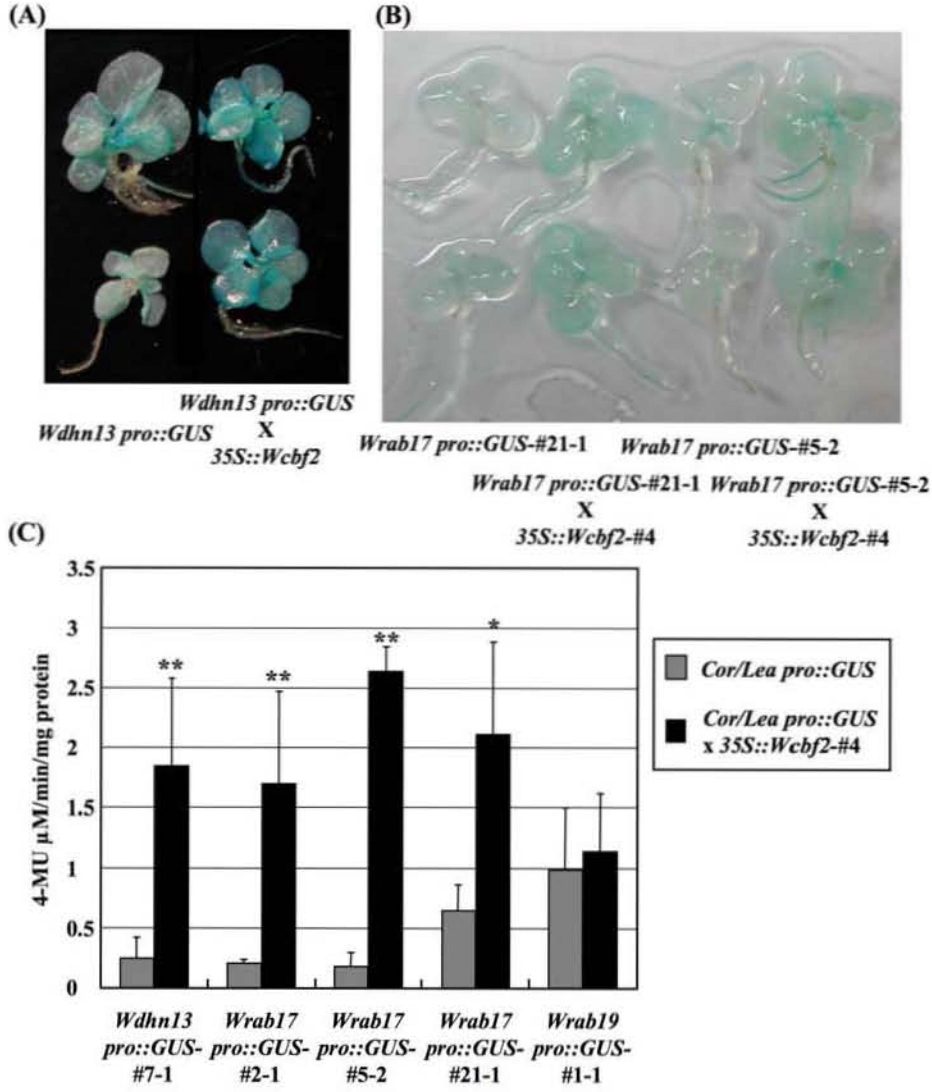

Fig. 3 (Takumi et al.)