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Molecules and Cells

Characterization of Putative Capsaicin Synthase Promoter Activity

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Capsaicin is a very important secondary metabolite that is unique to *Capsicum*. Capsaicin biosynthesis is regulated developmentally and environmentally in the placenta of hot pepper. To investigate regulation of capsaicin biosynthesis, the promoter (1,537 bp) of pepper *capsaicin synthase* (*CS*) was fused to GUS and introduced into *Arabidopsis thaliana* (Col-0) via *Agrobacterium tumefaciens* to produce CSPRO::GUS transgenic plants. The *CS* was specifically expressed in the placenta tissue of immature green fruit. However, the transgenic Arabidopsis showed ectopic GUS expressions in the leaves, flowers and roots, but not in the stems. The CSPRO activity was relatively high under light conditions and was induced by both heat shock and wounding, as *CS* transcripts were increased by wounding. Exogenous capsaicin caused strong suppression of the CSPRO activity in transgenic Arabidopsis, as demonstrated by suppression of *CS* expression in the placenta after capsaicin treatment. Furthermore, the differential expression levels of *Kas, Pal* and *pAmt*, which are associated with the capsaicinoid biosynthetic pathway, were also suppressed in the placenta by capsaicin treatment. These results support that capsaicin, a feedback inhibitor, plays a pivotal role in regulating gene expression which is involved in the biosynthesis of capsaicinoids.

INTRODUCTION

Pungency, which is the most recognized characteristic of the pepper (*Capsicum* spp.) fruit, results from the amassment of the natural secondary metabolite capsaicin and its analogues, capsaicinoids (Thresh, 1876).

Recently, capsaicinoids came into the spotlight because of their broad range of medical benefits including alleviation of pain (Knotkova et al., 2008), blood pressure regulation (Vaishnava and Wang, 2003), fat distribution (Leung, 2008) and cancer prevention (Surh, 2003). Additionally, there is a report that capsaicin has anti-bacterial activity that is sufficient for providing an anti-fouling coating for foods (Xu et al., 2005).

Pungency is a very common characteristic in the genus *Capsicum*, but is rare in *Plantae*, even in the *Solanaceae* family. From this scarcity, very little is known about capsaicinoid biosynthesis. According to the current model of capsaicinoid biosynthetic pathway, vanillylamine from the phenolylpropanoid pathway and 8-methyl-6-nonenoyl-CoA from the branched-fatty acid pathway are condensed by an amide bond to produce capsaicin (Kopp and Jurenitsch, 1981; Leete and Louden, 1968; Sukrasno and Yeoman, 1993). Especially, putative *Capsaicin synthase* (*CS*), the key point of capsaicinoid biosynthesis, is only found in *Capsicum* species. Kim et al. (2001) first identified a cDNA clone highly related to capsaicin synthase. The clone, named SB2-66, codes for a putative acyltransferase and was one of the differentially expressed transcripts identified by suppression subtractive hybridization from a cDNA library of *C. chinenese* cv. Habanero placenta (Kim et al., 2001). In more recent studies, researches investigated the putative capsaicin synthase gene, *AT3,* an acyltransferase that co-localizes with the *Pun1* locus. In these studies, a 2.5 kb deletion of *AT3* was found to be related to non-pungency in several pepper species. The pattern of *AT3* expression might be under the same regulation as *Kas* and *Pal*, which were reported to be associated with pepper pungency (Aluru et al., 2003; Stewart et al., 2005). Stewart et al. (2007) presented further evidence supporting *AT3* as a *CS* by showing that a 4 bp-deletion in the reading frame of the *Pun1* locus was responsible for the non-pungent pepper in *C. chinense*.

In the capsaicinoid biosynthetic pathway, several genes have been reported that may be co-regulated with *AT3*. Aluru et al. (2003) reported that the expression levels of *Acl1*, *FatA* and *Kas* genes were highly related to pepper pungency. Expression levels of these genes on pepper placenta tissue through fruit development were also investigated; *Kas* and *Pal* (Stewart et al., 2005), or just *Kas* (Stewart et al., 2007) were shown to have similar expression patterns with *AT3*. Additionally, there was an attempt to confirm the role of capsaicin biosynthesis genes on the pathway through loss-of-function studies. Del Rosario Abraham-Juarez et al. (2008) researched the effect of *Comt, pAmt* and *Kas* gene expression level on pepper pungency using virus-induced gene silencing (VIGS) techniques on pepper fruits. Each *Comt*, *pAmt*, and *Kas* knocked-down plants showed a large decrease in the capsaicin contents in pepper fruits.

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Whereas pungent peppers all have the same *AT3*, the pungency levels are extremely variable within species. Even in the same variety, the pungency varies according to years, cultivation techniques and environmental states (Lindsay et al., 1995). These phenomena may be the result of regulation of *AT3* expression by external factors such as the environment, herbivores and diseases. The light condition is the most studied component influencing capsaicin accumulation. During the after-ripening period, the peppers under a fluorescent light condition had twice the capsaicin component of peppers under dark condition (Park and Kim, 1975). In the greenhouse, red color polyethylene film induces an increase in the capsaicin content per pepper fruit as compared to normal white color polyethylene film (Kim et al., 1978). The effect of light continuance was also studied. Murakami et al. (2006) reported that the capsaicin content under continuous fluorescent light is higher than that under outdoor light condition. This report indicates that light continuance is more important to capsaicin accumulation than light strength (Murakami et al., 2006). The temperature is also important to capsaicin content alteration. It was reported that keeping the suspension culture at 35°C rather than at room temperature resulted in increased capsaicin in pepper tissue cells (Lee et al., 2001). More recently, high temperature during night was reported to have more of an effect on capsaicin contents than temperatures during the daylight period (Murakami et al., 2006). Drought stress and seed formation were also reported to have an effect on capsaicin accumulation (Curry et al., 1999; Murakami et al., 2006; Wierenga and Withrow, 1985).

In this study we attempted to show the effect of light, heat, wounding and capsaicin treatment on the putative *CS* promoter activity. The promoter region of *AT3* was fused with β-glucuronidase (GUS) gene and transformed into *Arabidopsis thaliana* since pepper transformation has not been well established. By observing the changes in GUS expression, we analyzed the regulatory activity of the *CS* promoter.

Since capsaicin is an end product of the pathway, we investigated its possible feedback inhibitory regulation by performing a transcription analysis of *Pal*, *pAmt, Kas* and *CS* from pepper placental tissue.

MATERIALS AND METHODS

Isolation of the putative *CS* promoter

We isolated promoter sequence from the full-sequenced BAC clone containing the *Pun1* locus from the 15X BAC library of *C. annuum* var. CM334 (Yoo et al., 2001; 2003). Specific primers (forward: 5′-GTGGTTTTACAACGTGACTGGG-3′, reverse: 5′-CGGGATCCCGAAATAAATATATGCTGCTG-3′) were made to isolate and amplify the *CS* promoter via PCR. The template DNA was extracted from the BAC clone using a modified alkaline lysis procedure. The PCR product was transferred into the pGEM-T easy vector (Clontech©, USA), and then transformed into *Escherichia coli* strain DH10B using electroporation. The construct was verified with *Eco*RI digestion and sequencing. This construct was used for the construction of the *CS* promoter expression vector.

Computational analysis

PlantCARE (Lescot et al., 2002) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used as a database for signal scanning of *cis*-acting elements in the *CS* promoter sequence. To determine the significance of these elements, PLACE/Signal Scan (Higo et al., 1999) (http://www.dna.affrc.go.jp/PLACE/) was used to identify and annotate previously predicted signals.

Construction of the *CS* promoter::GUS expression vector

To construct the 1,537 base pairs of the CSPRO::GUS expression vector (Jefferson et al., 1987), we amplified the *CS* promoter region using the forward primer (5′-AAGCTTGTGGTTTTACAACGTGACTGGG-3′) and the reverse primer (5′-CGGGATCCCGAAATAAATA-TATGCTGCTG-3′). The PCR product was cloned into the pCAMBIA1391Z vector (CAMBIA, Australia) using the restriction sites of *Bam*HI and *Hin*dIII (underlined). The constructed structure was then verified by sequencing. We transferred the construct into the *Agrobacterium tumefaciens* strain GV2260 using the freeze-thaw method (Holsters et al., 1978). This construct was used for the Arabidopsis transformation.

Plant material preparation and Arabidopsis transformation

Arabidopsis thaliana ecotype Col-0 seeds were sown in autoclaved soil and grown in a growth chamber under 16 h of fluorescent light at 120 μmol min^{-1} s^{-1}, 23°C of temperature and 70% of relative humidity. Arabidopsis transformation was achieved by the floral-dip method (Clough, 2005). Harvested T_1 candidate seeds were selected on Murashige and Skoog (MS) agar media with 15 mg L^{-1} of hygromycin (Duchefa, the Netherlands). Candidate T_1 plants were confirmed by PCR amplification with primers for the HPT gene (primers: 5′-GGCGAGTACTTCTACACAGCCATC-3′ and 5′-GTGCTTTCAGCTTCGATGTAGGAG-3′), GUS gene (primers: 5′-CGTGAAATCAAAAAACTCGACG-3′ and 5′-ACCTGTAATTCACACGTGGTGG-3′) and the *CS* promoter (universal primers: M13 forward and M13 reverse). Additionally, to select homozygous lines, the same antibiotic selection was performed for the T_2 and T_3 generations. The verified T_3 plants were used for the stress treatment and GUS activity assay.

Environmental stress treatment

To assay the light responsiveness of the *CS* promoter in transgenic Arabidopsis, 16 sterilized seeds were germinated on MS agar media in 90 mm Petri dishes under standard fluorescent light conditions (120 μmol min^{-1} s^{-1}) or dark conditions wrapped with aluminum foil. After a 5-day-germination, 3 seedlings were randomly selected to measure GUS activity. For additional light-sensitivity assays, 3 mature plants (4-week-old) grown on sterilized soil were treated under normal light (120 μmol min^{-1} s^{-1}) or dim light (approximately 30 μmol min^{-1} s^{-1}) for 3 d and 3 rosette leaves from every plant were harvested and GUS activity was measured. For the heat treatment experiment, three 5-day-old seedlings were submerged in 1% sucrose and 50 mM potassium phosphate buffer (pH 6.0) and incubated at 37°C or 23°C in the dark for 3 h prior to performing a quantitative GUS assay as previously described (Prändl et al., 1995). For the wounding experiment, the siliques and the inflorescence stems from fully flowered transgenic Arabidopsis were pressed several times with a surgical blade. Then the samples were immerged into 50 mM sodium phosphate buffer, pH 7.0. After incubation at 23°C for 3 h, histochemical GUS assay was performed (Lu et al., 2007). To directly assess the wound responsiveness of the pepper, nine immature green pepper fruits (~20 dpa, day post-anthesis) were pressed several times with surgical blades and were wrapped by plastic film and aluminum foil. After 3 h of incubation at 23°C, the placenta were collected from each fruit, and used for RNA extraction and reverse transcriptase PCR (RT-PCR). For a control group in the experiment using Arabidopsis, we used transgenic lines containing an empty vector and the CaMV35S promoter (35S)::GUS structure.

Capsaicin treatment

Sterilized transgenic Arabidopsis seeds were sown on MS agar

media in 90 mm plastic Petri dishes and grown under the same conditions as the plant material preparation. The 3-day-old seedlings were transplanted to MS agar media each containing 0, 0.1, 1, 5, and 10 μg ml^{-1} of capsaicin (Fluka, Switzerland). Every dish held 16 seedlings in regular spacing and was kept under the same growth conditions. After 5 days, 3 of 16 seedlings from every dish were collected and quantitatively measured for their GUS activities. Arabidopsis containing void vector and the 35S::GUS structure were used as control groups. For capsaicin treatment on pepper placenta tissue, immature green pepper fruits were harvested and their fresh placental tissues were immediately collected. Every 3 tissues were immersed into the 1/2 MS solution containing 0, 0.15, 0.3 or 0.6 mg ml^{-1} capsaicin. Then the containers were wrapped with aluminum foil and incubated at 23°C with shaking at 20 rpm for 3 h. After incubation, total RNA extraction and RT-PCR were performed with every individual placenta tissue to determine the changes in expression of several genes including the putative *CS*. As a control, placenta tissues with no immersion treatment were used.

Plant total RNA extraction and semi-quantitative RT-PCR

Total RNA extraction from plant materials was achieved with the RNeasy® mini kit (QIAGEN, USA) according to the user's handbook. The extracted total RNA (2 μg) was used as a template for reverse transcription with the *AccuPower*® RT-PCR PreMix (Bioneer, Korea) according to the protocol included. The pepper *actin* gene (GenBank accession no. AY572427) was amplified as an internal equal-loading control using the primers (5′-GGTATTGTGCTTGATTCTGGTGA-3′, 5′-CATCACGAACAATTCCCCGTTC-3′) for semiquantitative RT-PCR. To detect the expression activity of several genes in the pepper fruit, amplifying with PCR was accomplished with gene-specific primers [*CS*: 5′-CTCCCATTTTCAGTTCATAGACCA-3′, 5′-GGATCCCTCTCTCTCCTCTTCAAT-3′, *Kas* (GenBank accession no. AF085148): 5′-ATCAGGGTCATGGCTTCTCC-3′, 5′-CTTGTTGGCCAAGGTTAGCA-3′, *Pal* (GenBank accession no. AF081215): 5′-GAACAACGGTGAAAGCGAGA-3′, 5′-AGGAGCACCATTCCAGCTCT-3′, and *pAmt* (GenBank accession no. AF085149): 5′-TAGCGGATCAGAAGCCAATG-3′, 5′-GCTACTGTTTCAGGCCCCTC-3′]. For quantifying and comparing the intensities of the PCR results, Quantity One© program version 4.0.3 (Bio-Rad, USA) was used. Every experiment was performed at least three times and the data are displayed with the average and standard error.

Histochemical and quantitative assay of GUS activity

For the histochemical GUS assay, plant samples, in total or by organs, were collected and submerged into 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-$_D$-glucuronide), 100 mM phosphate buffer pH 7.2, 0.1% Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 10 mM EDTA for 12 h in the dark at 37°C (Jefferson et al., 1987). Plant samples were photographed after removing chlorophylls with 70% ethanol. For the quantitative GUS assay, fresh plant samples were ground with a plastic pestle and the protein was extracted with lysis buffer (50 mM phosphate buffer pH 7.2, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sodium laurylsarcosine, and 10 mM β-mercaptoethanol). The homogenate was centrifuged twice at 18,000 × *g* for 15 min and the clear supernatant was used for quantification of crude protein (Bradford, 1976) and GUS activity. The GUS activity assay was carried out by incubating 200 μl of protein extract with the substrate 4-methylumbelliferyl-β-$_D$-glucuronide (4-MUG) at 37°C. The released 4-methylumbellifrone (4-MU) was quantified with a Hoefer® DyNA Quant® 200 fluorometer (Amersham Pharmacia Biotech, Sweden), according to the manufacturer's instructions. The GUS specific activity was expressed as 4-MU pmol min^{-1} μg^{-1} of protein. Every experiment was read at least 3 times and the values were presented with the average and standard error.

RESULTS AND DISCUSSION

Sequence characterization of the *CS* promoter

The putative *CS* promoter region (1,534 bp) was isolated from a fully sequenced BAC clone of the *Capsicum annuum* 'CM334' BAC library (Yoo et al., 2001; 2003) and named "CSPRO". This sequence was analyzed for possible *cis*-elements using PlantCARE (Lescot et al., 2002) and PLACE/ Signal Scan (Higo et al., 1999). Several motifs had clear similarities to previously identified *cis*-acting elements and their locations were annotated (Fig. 1). Half of the predicted elements, such as the 3-AF1 binding site, ACE, Box 4, Box I, CATT motif, G Box and GT1 motif have light responsiveness. Moreover, two heat stress consensus elements (HSE) and a single element that responds to mechanical stress (WUN-BOX) were also detected.

The predicted responsiveness of the *CS* promoter included actual environmental responsive characteristics of capsaicin accumulation such as response to light (Kim et al., 1978; Lee et al., 2001; Murakami et al., 2006; Park and Kim, 1975) and heat (Lee et al., 2001; Murakami et al., 2006). On the other hand, although the *CS* is expressed only in placental tissues of immature green fruit in pepper, we could not identify any specific element that restricts gene expression according to tissue, organ or developmental stage. This can be explained in three ways: (1) from the uniqueness of the *CS*, there might be some unidentified *cis*-elements that limit gene expression in the pepper, (2) the *CS* may not be limited by the promoter, but rather, by other factors, such as an enhancer, substrates or terminator, or (3) the regulation of gene expression may differ between the pepper and Arabidopsis.

Spatial and temporal expression patterns of CSPRO::GUS in *Arabidopsis thaliana*

The developmental stages of pepper fruits were divided into 5 steps according to the days-after anthesis (dpa), coloration and seed ripening (Fig. 2A). *CS* expression, which is regulated by the *CS* promoter, was intensively restricted spatially and temporally. The *CS* was expressed only in the placental tissue and its expression was measurable from young green to mature green stages (Figs. 2B and 2C) (Lee, 2006; Stewart et al., 2005). In contrast, transgenic Arabidopsis carrying the CSPRO::GUS construct displayed different expression patterns in comparison to the pepper (Fig. 3). As shown in Figs. 3A and 3B, GUS expression was detected in 1-day-old seedlings and 3-day-old seedlings, with strong blue staining in whole cotyledons and hypocotyls and relatively weak expression in the roots. Similar GUS activities were maintained in whole parts of 5-day-old (Fig. 3C) and 10-day-old seedlings (Fig. 3D). Strong GUS expression was observed in the 4-week-old transgenic plants, on the rosette and cauline leaves (Figs. 3E and 3F), two taper points (abscission zone and stigmatic papillae) of immature and mature siliques (Figs. 3J and 3K) and the emerging apical shoot (Fig. 3H). There was less strong expression in the roots (Fig. 3G) and flowers (Fig. 3I) and no expression in the seeds, pericarps and septum (Fig. 3L).

Although *CS* expression is very limited spatially and temporally in pepper (Fig. 2), the *CS* promoter showed a more ectopic expression pattern in transgenic Arabidopsis. In particular, we

Fig. 1. The CS promoter sequence and construct used for *A. thaliana* transformation. The '**A**' in the bold '**ATG**' indicates the translational initiation site, and the 'a' in the gray box indicates the putative transcription initiation site. The *cis*-acting elements that were computationally analyzed are underlined.

examined the septum and the replum (Fig. 3L), as the homologous organs to the pepper placenta, and no chromogenic expression was detected. There are 3 explanations for this discrepancy. First, the promoter fragment used in this study might be too short to totally limit gene expression. More than 2.5 kb of intergenic sequence exists between the *CS* gene and the upstream hypothetical gene (data not shown), thus there is an additional 1 kb region that was not analyzed. We cannot rule out the possibility that a strong regulatory element may be located in this unanalyzed region, causing the uncontrolled expression pattern observed in transgenic Arabidopsis. Second, the regulatory element may not be in the promoter region of the *CS*. It is possible that the regulatory element is a *trans* factor, in an intron or in the terminator region. Another possibility is that *CS* expression is regulated by substrate or other signals. Third, the genetic diversity between pepper and Arabidopsis may explain this phenomenon. Even if there were some *cis*-acting elements working upon the CSPRO in pepper, these elements may not be utilized in Arabidopsis. These interpretations could be verified with more certainty if transgenic pepper plants could be used. Since the pepper transformation system is not well established and consumes too many seeds, and too much time and effort, we have to employ other systems like the tomato and tobacco in the same *Solanaceae* family.

Expression regulation in response to light

Computational analysis of the CSPRO sequence revealed that several motifs are identical or similar to previously recognized light-response motifs (Fig. 1), which suggests that the *CS* promoter may be activated by light. To test this possibility, we compared the GUS activities of the seedlings germinated under normal light and dark conditions (Fig. 4). In 5-day-old seedlings, the activities of both the 35S promoter and the *CS* promoter

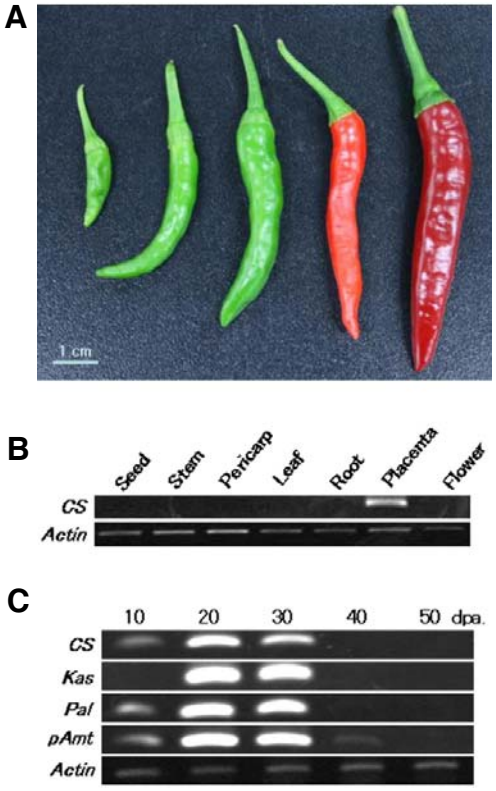

Fig. 2. The developmental stages of pepper fruit, and spatial and temporal expression of capsaicinoid biosynthetic genes in pepper. (A) The developmental stages of pepper fruit. Each fruit from left to right represents a developmental stage: young green (~10 dpa; days post-anthesis), immature green (~20 dpa), mature green (~30 dpa), immature red (~40 dpa) and mature red (~50 dpa). (B) *CS* transcripts were expressed only in placenta. (C) The spatial and temporal expression patterns of *Kas*, *Pal* and *pAmt* were very similar to that of *CS*. The bar indicates 1 cm.

were suppressed under dark conditions by 46.4% and 78.7%, respectively (Figs. 4A and 4B). If the 35S promoter activity is used as a positive control, the additional 32% suppression of the *CS* promoter activity could be interpreted as being due to the effect of the light.

The effect of light was further examined under dim light conditions (Fig. 4C). In 4-week-old transgenic plants with the CSPRO::GUS construct, reduction of GUS activity by 54.76% was observed under dim light condition, whereas the reduction of GUS activity in the transgenic plant with the 35S::GUS construct was only 2.32%.

These responses of the *CS* promoter to light are consistent with those reported in previous reports on the relationship between light and capsaicin accumulation (Iwai et al., 1977; Kim et al., 1978; Lee et al., 2001; Murakami et al., 2006; Park and Kim, 1975). According to these reports, the length of the light period, as well as the quality of the light are critical for determining capsaicin content. Therefore, the CS promoter plays an important role in the light-dependent transcriptional regulation of the capsaicin synthase gene.

Expression regulation in response to heat stress

Two Heat Stress Consensus Elements (HSE) of the *CS* promoter were analyzed in the next validation study (Fig. 1). Measurement of GUS activity under the CSPRO construct in 5-day-old seedlings after a 3-hour incubation at high temperature (37°C) showed an impressive 98.61% increase over normal growth temperature (23°C) (Fig. 4D). However, there was little change in activity (3.12%) with the 35S promoter. This observation is consistent with the high capsaicin accumulation in outdoor pepper cultivation during hot summer days when 37°C is near to daylight period temperature.

Expression regulation in response to wounding

Strong GUS staining has often been observed at the cutting edges of transgenic plants. Moreover, a single wound-response element (WUN-Box, -1062~-1053, TCACTACGAA) was predicted from the CSPRO sequence (Fig. 1). Thus, we characterized the wounding-inducible activity of the *CS* promoter. Strong GUS expression was present at the cutting edges of stems and siliques of transgenic plants containing the CSPRO (Figs. 5B, 5C, 5E, and 5F). The control plants transformed with empty vector, however, were not stained anywhere (Figs. 5A and 5D). It is plausible that the *CS* promoter activity is up-regulated under mechanical stress. Since wound-stimulation on capsaicin accumulation has not been reported, we need to directly confirm this possibility with the pepper fruit where CSPRO originated. Wounding stress can be created by pressing surgical blades against the young green fruits of *C. annuum*. The transcript levels of the *CS* gene increased by 48.66% in wounded placenta over control placenta (Fig. 6).

Furthermore, there is other supporting evidence that *CS* expression might be regulated by mechanical wound or pathogen invasion. During virus-induced gene silencing in pepper fruits, up-regulation of capsaicinoid concentration was detected by virus invasion containing only empty vector (Del Rosario Abraham-Juarez et al., 2008). This suggests that capsaicin accumulation can be stimulated by external mechanical damage inducing *CS* promoter activity. Additionally, these data support a role for capsaicin in plant defense.

Expression regulation in response to capsaicin

The peak of *CS* transcript expression and that of capsaicin concentration do not coincide during fruit development (Kim et al., 2001). The *CS* expression starts at 10 dpa, reaches a maximum at 20 dpa, then suddenly decreases and almost stops at 40 dpa. On the contrary, capsaicin concentration begins to increase at 20 dpa and reaches the maximum at 40-50 dpa (Kim et al., 2001). This phase difference suggests that capsaicin may have a role in suppressing *CS* expression. Thus, a negative feedback relationship between *CS* expression and capsaicin concentration may exist. It would be very interesting to elucidate whether the negative feedback of capsaicin affects expression of *CS*, *Pal*, *pAmt* and *Kas* genes. Transgenic Arabidopsis seedlings (3-day-old) were transplanted on agar media containing various concentrations of capsaicin (0.1-10 μg ml^{-1}) (Fig. 7). A change in GUS activity was observed 5 days after incubation. The negative control containing empty vector did not show any GUS activity at any capsaicin concentration. GUS activity under the 35S promoter (547.21 pmol min^{-1} μg^{-1} of protein) showed little change at low levels of capsaicin, and only a 13.23% increase at 5 μg ml^{-1} and a 19.73% decrease at 10 μg ml^{-1} of capsaicin. However, GUS activity (178.87 pmol min^{-1} μg^{-1} of protein) under the control of CSPRO was decreased by 53.98% at 0.1 μg ml^{-1} of capsaicin. This decrease was consistent for all concentrations of capsaicin (0.1-10 μg ml^{-1}). These observations suggest that (1) the activity of the *CS* promoter can be reduced by capsaicin treatment, (2) a concentration of only 0.1 μg ml^{-1} of capsaicin can suppress the *CS* promoter in

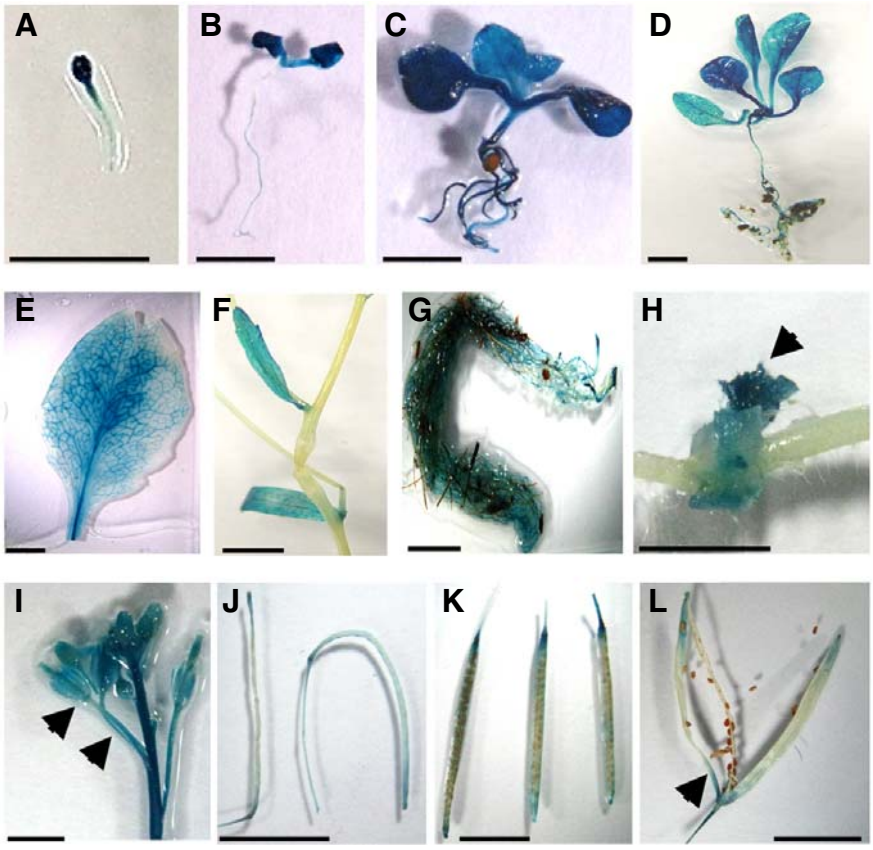

Fig. 3. GUS expression regulated under the *CS* promoter in transgenic Arabidopsis. GUS histochemical stainings were observed in 1-day (A), 3-day (B), 5-day (C), and 10-day-old (D) seedlings. Additional stainings in a 4-week-old plant are shown in rosette leaves (E), inflorescent stems and cauline leaves (F), roots (G), apical shoot (H), reproductive organs containing flowers (I), immature siliques (J), mature siliques (K), and dehiscensed silique (L). The arrow points to the apex (H), the receptacle and the stalk (I) and the septum (L), respectively. The bar is 0.5 cm.

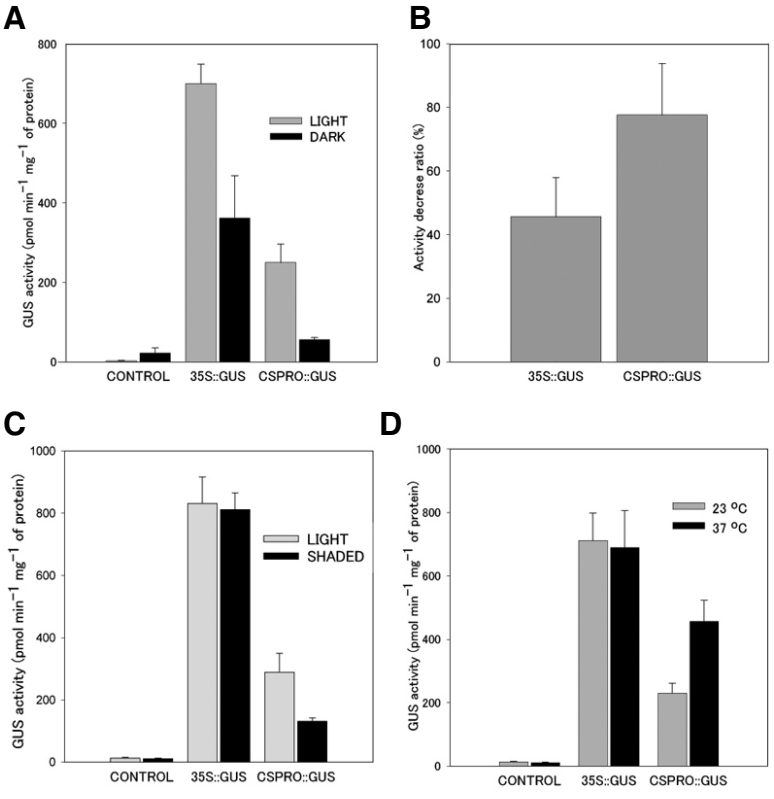

Fig. 4. Effect of light and temperature on the GUS activity in CSPRO::GUS transgenic Arabidopsis. (A) The GUS activities under the control of the CSPRO and CaMV35S promoters decreased in darkness, compared to that under standard light conditions. (B) Decreased ratio of CSPRO activity displayed as a percentage. (C) Effect of shading on CSPRO activity. GUS activity was measured 3 days after the transfer of 4-week-old plants from light to shade. (D) Heat responsiveness to CSPRO activity. GUS activity was measured 3 h after incubation of the 5-day-old seedlings at 23°C or 37°C.

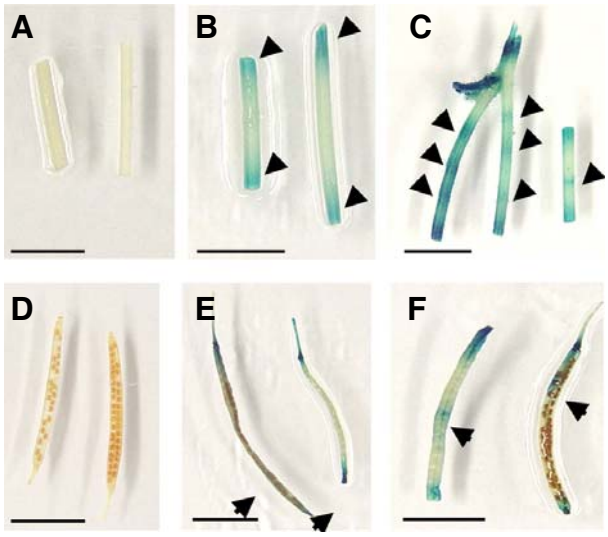

Fig. 5. Effect of mechanical wounding on CSPRO activity in CSPRO::GUS transgenic Arabidopsis. GUS staining reveals a strong response of the *CS* promoter to blunt-end cutting (B and E) and half-way cutting (C and F), compared with empty vector control (A and D). The arrow indicates strong GUS staining at wounding sites in stems and siliques. Bars are 0.5 cm.

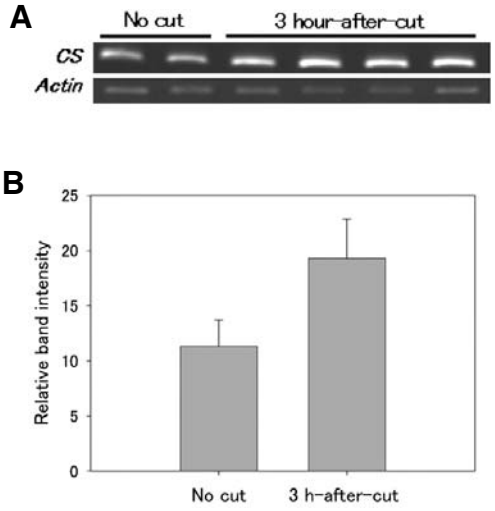

Fig. 6. Effect of wounding on the expression of *CS* transcripts in immature green placenta. (A) Gel electropherogram shows induction of *CS* transcripts 3 h after wounding. (B) Relative intensity of *CS* expression was measured quantitatively by the ratio of [intensity of *CS* transcript's band] / [intensity of pepper *actin* transcript's band]. The experiment was repeated twice with similar results.

transgenic Arabidopsis, and (3) the signal transduction between capsaicin and the *CS* promoter should be conserved in Arabidopsis.

The suppression effect of capsaicin on CSPRO activity was further tested with pepper placenta. The concentration of capsaicin was adjusted to the normal capsaicin concentration (0.6 mg ml^{-1}) found in mature pepper fruit (Kim et al., 1997).

The *CS* transcript level was dramatically suppressed after incubation of placenta tissue with three levels of capsaicin concentration (0.15, 0.30, 0.60 mg ml^{-1}) for 3 h. In contrast, any significant difference was hardly observed in the no-treatment-control and mock-treated placenta samples (Fig. 8). The *CS* transcript level was diminished by 85.30% with 0.15 mg ml^{-1} capsaicin treatment. It is plausible that the activity of the *CS* promoter is directly or indirectly affected by capsaicin presence in the pepper, even in the phylogenetically distant plant species, Arabidopsis. This also explains why there is a phase difference between *CS* expression peak and capsaicin concentration peak; it is due to negative feedback of the *CS* expression by capsaicin.

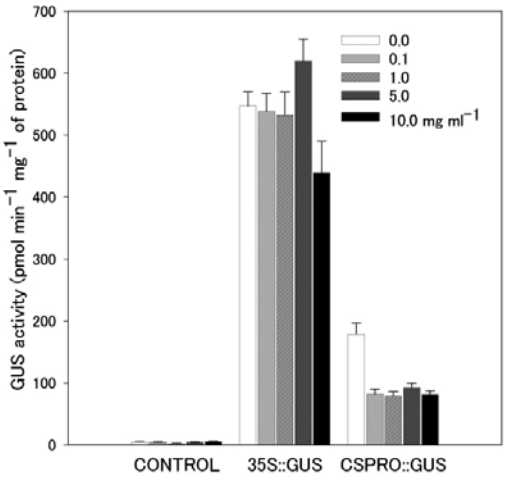

Fig. 7. Effect of capsaicin concentration on CSPRO activity. There was a dramatic reduction in GUS activity with the *CS* promoter, but strong GUS activity with the 35S promoter, regardless of capsaicin concentration tested. Each bar represents 3 independent measurements from 3 individual samples. The error bar indicates the standard error.

Possible co-regulation of *Kas*, *Pal*, and *pAmt* expression by capsaicin was further tested, since these genes are known to show temporally similar expression patterns to *CS* (Lee, 2006; Stewart et al., 2005; 2007). The transcript levels of the *Kas*, *Pal* and *pAmt* gene were down-regulated drastically by more than half at all levels of capsaicin treatment, except for *Kas* at 0.30 mg ml^{-1} of capsaicin treatment where no suppression was observed for an unexplainable reason (Fig. 8). These overall results demonstrate that expressions of *CS*, *Kas*, *Pal* and *pAmt* are all under the same temporal regulation and feedback inhibition by capsaicin.

Del Rosario Abraham-Juarez et al. (2008) shed some light on our exceptional observation of *Kas* at 0.30 mg ml^{-1} of capsaicin treatment (Fig. 8). They studied the capsaicin biosynthetic pathway with a knocked-down pepper lines. The *Kas*-silenced lines showed extreme growth retardation, whereas the *pAmt*-suppressed lines did not show any physical defects. The authors explained this finding by (1) unexpected suppression on other analogous genes, or (2) different versatility between genes. With the second possibility, *Kas* could be involved with other important metabolic pathways, which means a more complicated regulation mechanism may exist.

Although *CS* is still regarded as a putative capsaicin synthase, all circumstantial evidence indicates that *CS* is the real capsaicin synthase (Bennet and Kirby, 1968; Iwai et al., 1979; Kim, 2004; Kim et al., 2001; Lee et al., 2005; Leete and Louden, 1968; Stewart et al., 2005; 2007). The CSPRO in transgenic Arabidopsis was sensitive to light and high temperature. Light

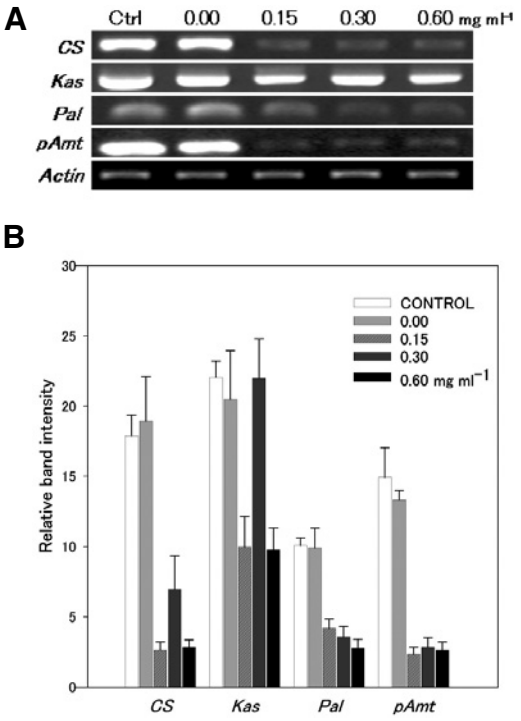

Fig. 8. Effect of capsaicin concentration on expression of *CS*, *Kas*, *Pal* and *pAmt*. (A) Gel electropherogram of *CS*, *Kas*, *Pal* and *pAmt* transcripts. There is strong correlation between suppression of *CS*, *Kas*, *Pal* and *pAmt* transcripts and capsaicin treatment. (B) Relative expression of *CS*, *Kas*, *Pal* and *pAmt* transcripts. The white column represents the non-treated control, whereas the dim gray column represents the mock-treated control. Each bar represents three experiments for each of the 3 placental samples. The bar indicates standard error.

and temperature are known to be the basic environmental factors affecting plant physiological states, photomorphogenesis and secondary metabolite accumulation. Relationships between capsaicin accumulation and these two factors have already been reported as introduced earlier (Cotter, 1980; Jeong et al., 1996; Kim et al., 1978; Murakami et al., 2006; Yun et al., 2002a; 2002b). Cotter (1980) reported that high temperatures at time of maturity increase pungency. It has been reported that in the higher light intensity, the content of capsaicin in *Capsicum annuum* cv. Taemyong, Komyong, and Tabok was higher than in lower light intensity, and that the content of capsaicin also accumulated higher in high temperature than in low temperature (Jeong et al., 1996). It makes sense now that the *CS* promoter is found in this study to be sensitive to light and high temperature stimuli. The transient and strong response of the *CS* promoter to wounding in transgenic Arabidopsis and pepper placenta tissue, however, has never been reported. It would be interesting to correlate the wound stimulation of *CS* promoter activity to seed protection and dispersal in nature.

Capsaicin, the end product of the capsaicinoid biosynthetic pathway, exerts dramatic feedback suppression on *CS*, *pAmt*, which is an immediate upstream gene, and *Pal,* which is the first determining step of the phenylpropanoid pathway (Fig. 9). In the valine pathway, *Kas* is suppressed by capsaicin treatment in a similar method. However, it is unlikely that every gene in both pathways is suppressed by capsaicin in a similar pattern. There, further testing is needed to better understand these findings.

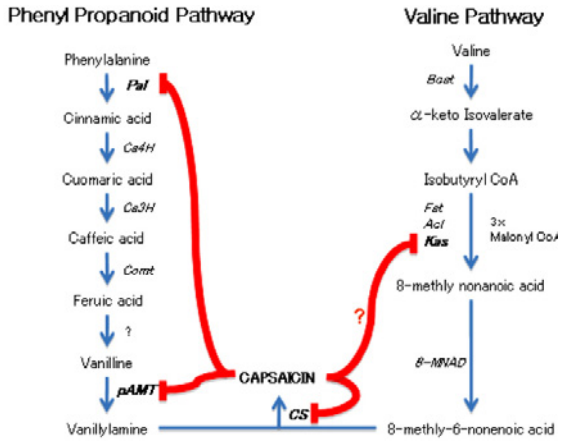

Fig. 9. Model for feedback regulation in the capsaicinoid biosynthetic pathway

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