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# Cloning and Characterization of UV-B Inducible Chalcone Synthase from Grape Cell Suspension Culture System and Its Expression Compared with Stilbene Synthase

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We performed the cloning of a chalcone synthase (CHS) gene, the key enzyme in the anthocyanin biosynthesis, from the cDNA library constructed with grape suspension cells irradiated UV-B. The PCR fragment was used to cloning the CHS gene. One CHS cDNA clone containing an open reading frame and a partial stilbene synthase (STS) cDNA, the stilbene-type phytoalexin, were isolated. The CHS cDNA clone (VCHS) showed 87% sequence homology with VvCHS (*V. vinifea*) and 72.3% identity with VSTSY (*V. vinifea*). Its amino acid sequences were longer than any other CHS genes as 454 residues. Two genes were weakly expressed in white light irradiated cells, but highly induced in UV-B irradiated condition during 32 hours. Interestingly, the STS was quickly and abundantly expressed from 2 hours when supplemented with jasmonic acid (JA) and the maximum expression was observed at 4 hours and then gradually decreased. But, the additional UV-B or white light quickly degraded the STS expression than only JA treated grape suspension cells. The CHS also was rapidly induced with JA and the synergistical effect was observed at the additional light treatment of UV-B or white light. These results are indicated that CHS and STS have different response mechanisms against the environmental stresses.

**key words:** chalcone synthase, stilbene synthase, methyl jasmonate, ultra-violet B, white light

## INTRODUCTION

In higher plants, the accumulation of secondary metabolites occurs in specific tissues or cells during a specific stage. However, the induction of defense mechanisms of plants against the stresses such as UV irradiation, pathogene attack and wound seemed to be related to rapidly accumulation of secondary metabolites.

In the grape suspension cell, anthocyanin is a major secondary metabolite. Chalcone synthase (CHS) is the key enzyme for anthocyanin biosynthesis in higher plants. External stimuli such as UV-B irradiation and pathogen attack induce CHS expression in many plants. [1,2,3]

Stilbene synthase (STS) which catalyzes stilbene biosynthesis occurs in a limited numbers of widely unrelated higher plants and closely related to CHS. CHS and STS genes catalyze the condensation of coumaroyl CoA and malonyl CoA to form the stilbene resveratrol or naringenin chalcone, respectively. STS gene have been cloned from several plants such as grape, peanut and pine. [4,5,6] The major function of STS in a pine (*Pinus sylvestris*) is the response against environmental stress. [6]

Jasmonic acid (JA) and its methyl jasmonate (Me-JA) have been identified to intracellular signal molecules that mediate the activation of plant secondary metabolism related gene expression in response to wounding, elicitor treatment and pathogene infection. [7,8,9] The application of JA and its precursors into plants is caused the accumulation of proteinase inhibitor, phytoalexin and defense response genes. [7,10]

Phytoalexins have antibiotic properties and are dominant survival factors for plants. Upon infection of pathogens in plant, stilbene type phytoalexins induced other phytoalexins such as terpenoids, flavonoids, anthraquinones and alkaloid class, [11] and increased the resistance to pathogen infection on the transgenic plants. [12]

Previously, we described effects on anthocyanin accumulation of salts concentration, [13] light irradiation, [14] salicylic acid [15] and UV-B and JA treatment [1] during the cell suspension culture of grape. The anthocyanin accumualtion in grape suspension cells was dynamically affected by culture conditions. Especially, in the case of JA and UV-B the anthocyanin accumulation was abundantly increased although cell growth was controlled. In this study, we performed a cDNA cloning of CHS, a key enzyme in the anthocyanin biosynthetic pathway, and its expression was analyzed and compared with STS which are closely related with CHS.

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†Abbreviations : CHS, chalcone synthase ; STS, stilbene synthase ; JA, jasmonic acid ; Me-JA, methyl jasmonate

## MATERIALS AND METHODS

*Plant material and treatment.* The suspension culture of grape cells was maintained as described in our previous report. [14] Subculture was performed routinely every 7 days by a 1:10 dilution, and etiolated cells was used as experiment material. Grape cells were treated with light (UV-B, white fluorescent light), JA (±jasmonic acid) and stilbene (trans-3,4, 5-Trihydroxystilbene) on the fourth day after subculture. To increase transmission of UV-B, grape cells were cultured in conical beakers sealed with vinyl wrap on shaking incubator at 28℃. Light irradiation was performed as described at previous report. [1,14]

*Library screening and cDNA analysis.* The cDNA library was constructed with Poly(A)$^+$ RNA purified from grape suspension cells irradiated with UV-B from 2 to 32 hours. The cDNA library sceerning was performed with the PCR product of CHS. The 5′ forward primer for PCR amplification of CHS was designed with 5′ -AGCAGGATCCAA(AG)GC(C/T)AT (C/T)AA(A/G)GAGTGGGG-3′ and the 3′ reverse primer was

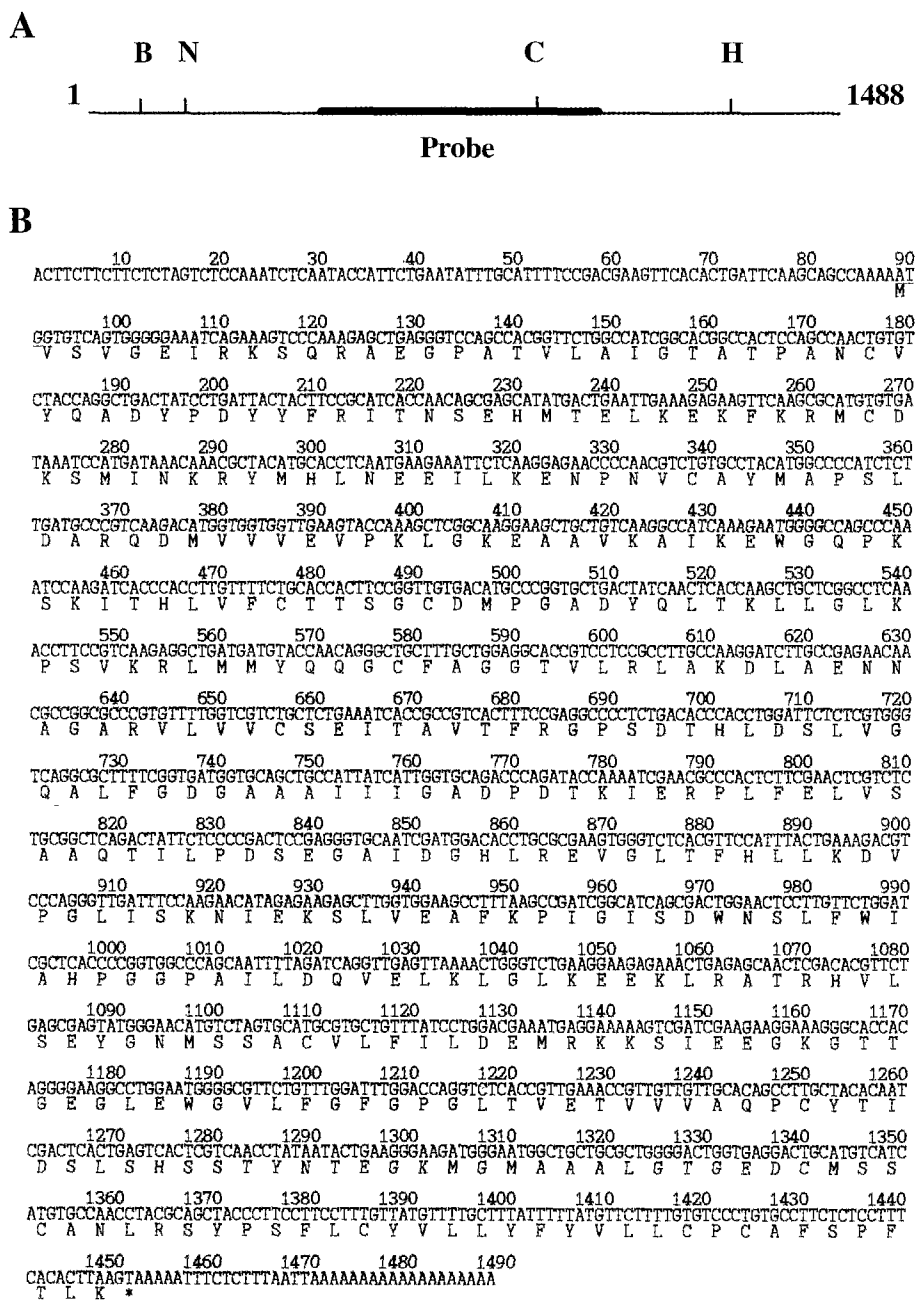


Figure 1. (A) Restriction enzyme map analysis of chalcone synthase (CHS) cDNA clone. The position of the hybridization probes generated by PCR are presented by solid bar. B, *Bstx*I; C, *Cla*I; H, *Hinc*II; N, *Not*I. (B) Nucleotide sequence of cloned CHS gene and deduced amino acid sequence. Asterisk indicates the stop codon.

5′-GTTCTTG(G/A)AAATCA (A/G)CCCTGGG-3′. The PCR fragment was subcloned in pGEM®-T Easy Vector (Promega) and sequenced using automatic sequencer (ABI377) for identification. Three clones selected from the cDNA library screening using the $^{32}$P-labeled CHS PCR product were rescued by *in vivo* excision. The analysis of nucleotide and amino acid sequence alignments were carried out with the CLUSTAL W program.

*Southern hybridization.* Genomic DNA was purified from grape suspension cells grown in dark. Ten micrograms of genomic DNA digested with various enzymes were separated by electrophoresis on 0.8% agarose gel and then transferred to $N^+$-nylon membrane. Hybridization was carried out in 6 ×SSC, 1% sarcosin, 1% dextran sulfate and 1% non-fatmilk at 65℃. Probes of CHS and STS gene were prepared using the random priming system. All membranes were washed at high stringency (0.2 ×SSC, 0.1% SDS at 65℃).

*Northern hybridization.* Total RNA was extracted from grape suspension cells as described by Sparvoli *et al.* [4] Twenty micrograms of total RNA were separated on a formaldehyde gel and transferred on membrane. Hybridization was carried out in 6 × SSC, 1% sarcosin, 1% dextran sulfate, 1% non-fatmilk and 50% formamide at 42℃ with $^{32}$P-labeled grape CHS or STS cDNA clone. Filters were deprobed by boiling with 0.2 ×SSC, 0.1% SDS and rehybridized with 28S grape rRNA.

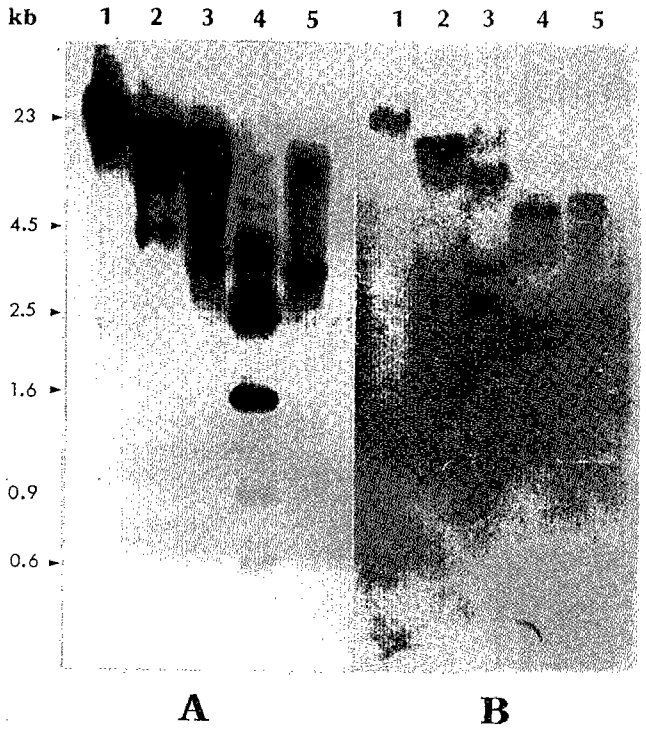


Figure 2. Southern analysis of grape genomic DNA digested with four different restriction enzymes. Lanes 1, uncut ; Lane 2, *Bam*HI ; Lane 3, *Eco*RI ; Lane 4, *Hind* III; Lane 5, *Sac* II. The membranes were hybridized with the $^{32}$P-labeled STS (A) and CHS (B) cDNA clone.

## RESULTS AND DISCUSSION

*Cloning and characterization of CHS and STS genes in grape suspension cells.*

The PCR fragment of the CHS gene in grape was used to screening the cDNA library constructed with grape suspension cells. Three positive clones were selected and each clone was rescued by *in vivo* excision. Two (1.4 and 1.2Kb) of three clones were identified as chalcone synthase and the longest clone (VCHS) was contained an open reading frame consisted of 454 amino acids (Fig. 1). The other (0.9Kb) was identified as a partial stilbene synthase (STS). The nucleotide similarity of VCHS scored 75% for STS (*V. vinifea*), 87% for VvCHS (*V. vinifea*) and 78% for UV inducible CHS (*B. pendula*).

In order to investigate the copy number of CHS and STS in grape, 10ug genomic DNA were digested with various restriction enzymes (*Bam*HI, *Eco*RI, *Hind* Ⅲ and *Sac*I) and then transferred to nylon membranes. Both CHS and STS seemed to be encoded by mutigene family (Fig. 2).

Figure 3 is showed that CHS and STS genes have high similarities in amino acid sequences. Especially, the amino acid sequence of VCHS were longer than any other CHS and STS as 454 residues. Tropf *et al.* [16] proposed that STS gene was thought to have evolved independently from CHS at least three times in seed plant evolution.

*The effects on CHS and STS expression of white light and UV-B.*

To investigate the expression of CHS and STS genes by lights irradiation in the grape suspension cells, we performed a northern analysis for each treatment. CHS was not expressed in dark (data not shown) and very weak in white light irradiated cells, but was high between 32h and 64h in grape suspension cells irradiated with UV-B (Fig. 4). The expression of STS was also very low under white light, but high in cells irradiated UV-B for 32h although degraded in 64h. UV-B irradiation caused severe damage in plant. [17,18] CHS is responsible for the defence mechanism of UV-B, wounding and pathogen attacking. [2,17] Isoflavonoid compounds contained phytoalexin properties were demonstrated that their formations have a role as an indicator of UV stress in bean.19 Yalpani *et al.* [3] suggest that UV light and ozon can activate the defense signal pathway of biotic or abiotic stress. The expression of CHS and STS in UV-B irradiated grape suspension cells also can act as defense role against UV-B stress.

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VCHS      MVSVGEIRKSQRAEGPATVLAIGTATPANCVYQADYPDYYFRITNSEHMTELKEKFKRMCDKSMINKRYMHLNEEILKENPNVCAYMAPS  90
VvCHS     MVSVAEIRKAQRAEGPATVLAIGTATPANCVYQADYPDYYFRITNSEHMTELKEKFKRMCEKSMINKRYMHLTEEILKENPNVCAYMAPS  90
BpCHS     MASVEEIRKAQRAHGPATVLAIGTATPSNCITQADYPDYYFRITKSDHMTELKEKFKRMCDKSMIKKRYMYLNEEILNENPNMCAYMAPS  90
VSTSY     MASVEEIRNAQRAKGPATILAIGTATPDHCVYQSDYADYYFRVTKSEHMTALKKKFNRICDKSMIKKRYIHLTEEMLEEHPNIGAYMAPS  90
VvSTSY    MASVEEIRNAQRAKGPATILAIGTATPDHCVYQSDYADFYFRVTKSEHMTALKKKFNRICDKSMIKKRYIHLTEEMLEEHPNIGAYMAPS  90
          * ** ***  *** **** ********  *  * ** * *** * * *** ** ** * * **** ***  * ** * * **  *******

VCHS      LDARQDMVVVEVPKLGKEAAVKAIKEWGQPKSKITHLVFCTTSGCDMPGADYQLTKLLGLKPSVKRLMMYQQGCFAGGTVLRLAKDLAEN 180
VvCHS     LDARQDMVVVEVPKLGKEAAAKAIKEWGQPKSKITHLVFCTTSGVDMPGADYQLTKLLGLKPSVKRLMMYQQGCFAGGTVLRLAKDLAEN 180
BpCHS     LDARQTIVVVEVPKLGKEAATKAIKEWGQPKSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAEN 180
VSTSY     LNIRQEIITAEVPKLGKEAALKALKEWGQPKSKITHLVFCTTSGVEMPGADYKLANLLGLEPSVRRVMLDQQGCYAGGTVLRTAKDLAEN 180
VvSTSY    LNIRQEIITAEVPKLGKEAALKALKEWGQPKSKITHLVFCTTSGVEMPGADYKLANLLGLEPSVRRVMLYHQGCYAGGTVLRTAKDLAEN 180
          *  **     ********** ** ********************  ******* *  **** *** * *   *** ******* *******

VCHS      NAGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAIIIGADPDTKIERPLFELVSAAQTILPDSEGAIDGHLREVGLTFHLLKD 270
VvCHS     NAGSRVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAVIIGADPDTKIELPLFELVSAAQTILPDSEGAIDGHLREVGLTFHLLKD 270
BpCHS     NKGARVLVVCSEITAVTFRGPTDTHLDSLVGQALFGDGAAAVIVGADPDTSVERPLFELISAAQTILPDSDGAIDGHLREVGLTFHLLKD 270
VSTSY     NAGARVLVVCSESTVVTFRGPSEDALDSLVGQALFGDGSAAVIVGSDPDISIERPLFQLVSAAQTFIPNSAGAIAGNLREVGLTFHLWPN 270
VvSTSY    NAGARVLVVCSEITVVTFRGPSEDALDSLVGQALFGDGSAAVIVGSDPDISIERPLFQLVSAAQTFIPNSAGAIAGNLREVGLTFHLWPN 270
          * * ******** * *******    ************** ** * * ***   * *** * *****  * * *** * **********

VCHS      VPGLISKNIEKSLVEAFKPIGISDWNSLFWIAHPGGPAILDQVELKLGLKEEKLRATRHVLSEYGNMSSACVLFILDEMRKKSIEEGKGT 360
VvCHS     VPGLISKNIEKSLVEAFTPIGISDWNSLFWIAHPGGPAILDQVELKLGLKEEKLRATRHVLSEYGNMSSACVLFILDEMRKKSIEEGKGS 360
BpCHS     VPGIISKNIEKSLAEAFAPLGISDWNSLFWIAHPGGPAILDQVESKLGLKEEKLRATRHVLSEYGNMSSACVLFILDEMRRNSLEGGKVT 360
VSTSY     VPTLISENIEKCLTQAFDPLGISDWNSLFWIAHPGGPAILDAVEAKLNLDKKKLEATRHVLSEYGNMSSACVLFILDEMRKKSLKGERAT 360
VvSTSY    VPTLISENIEKCLTQAFDPLGISDWNSLFWIAHPGGPAILDAVEAKLNLDKKKLEATRHVLSEYGNMSSACVLFILDEMRKKSLKGERAT 360
          **  ** **** *  ** * ********************** ** ** *  ** ************************** *

VCHS      TGEGLEWGVLFGFGPGLTVETVVVAQPCYTIDSLSHSSTYNTEGKMGMAAALGTGEDCMSSCANLRSYPSFLCYVLLYFYVLLCPCAFSP 450
VvCHS     TGEGLEWGVLFGFGPGLTVETVVL-HS---VSAPAAH---------------------------------------------------- 393
BpCHS     TGEGLEWGVLFGFGPGLTVETVVLHSVP-VPVEASH---------------------------------------------------- 395
VSTSY     TGEGLDWGVLFGFGPGLTIETVVLHSIPMVTN-------------------------------------------------------- 392
VvSTSY    TGEGLDWGVLFGFGPGLTIETVVLHSIPMVTN-------------------------------------------------------- 392
          ***** ************* ****

VCHS      FTLK                                                                                      454
VvCHS
BpCHS
VSTSY
VvSTSY
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Figure 3. Multiple alignment of deduced amino acid sequences of CHS and STS genes. Dashes indicate gaps introduced to maximize alignment. Identical amino acids are shown with asterisk. Alignment of amino acid was arranged with the CLUSTAL W program. *V. vinifera* ; vCHS (AF020709), VvCHS (X75969), VSTSY (S63221), VvSTSY (X76892), *B. pendula* ; BpCHS (Y11022).

*Induction of CHS and STS by JA treatment.*

CHS and STS genes were highly induced by JA treatment (Fig. 5). In JA treated cells STS was quickly induced without lag time, but CHS expression was needed more than 2h. STS transcription was initiated soon after JA treatment, but its espression seemed to be down regulated with the induction of CHS transcription. It has been reported that the treatment of JA and its precusors in plant caused accumulation of proteinase inhibitor genes as well as wound response genes (CHS, vspB, sbPRP). [7] In grape cell culture the transcription of CHS and STS was also accelerated by JA treatment. These results indicate that JA act as direct or indirect signal for anthocyanin accumulation, wounding or pathogen attack.

*Synergistic effect of CHS and STS gene expression by the treatments of JA with white light or UV-B.*

When JA supplemented in suspension cells of grape, the CHS and STS was induced (Fig. 5). Especially, STS was quickly expressed within 2h. We previously reported that the anthocyanin accumulation increased by simultaneous treatment of JA and white light or UV-B. [4] Figure 5 shows

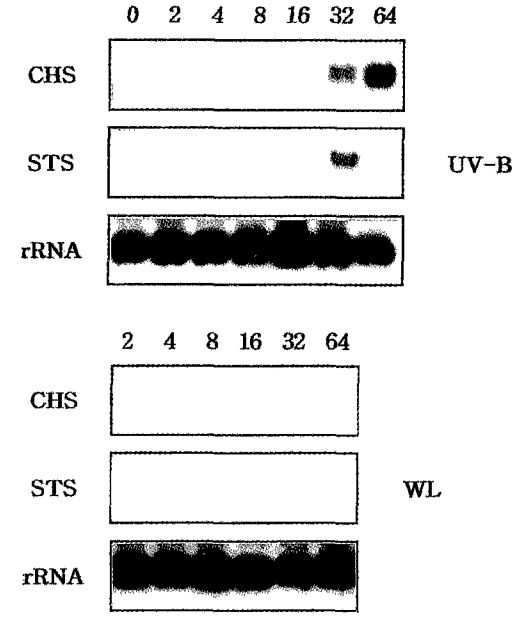


Figure 4. Effects of UV-B and white light on the CHS and STS gene expression. Grape cells grown for three days in the dark were cultured at each time (hours) in 3.5 $Wm^{-2}sec^{-1}$ white fluorescent light or 0.25 $Wm^{-2}$ $sec^{-1}$ UV-B light. Cells were harvested for total RNA isolation. WL : White fluorescent light, UV-B : ultraviolet-B.

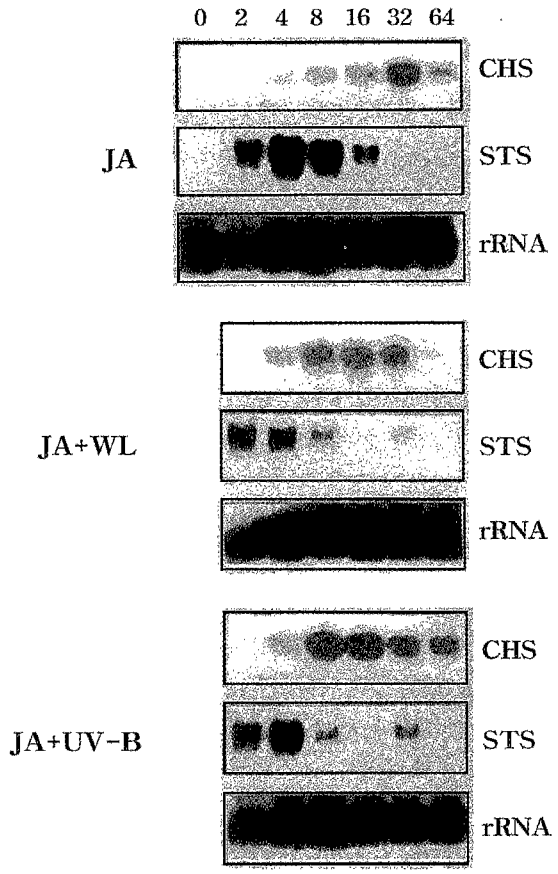


Figure 5. The expression of CHS and STS genes in various treatments (JA, JA and UV-B, JA and white light). Cells grown for three days in dark condition were treated with JA and then cultured for indicated time in 3.5$Wm^{-2}sec^{-1}$ white fluorescent light or 0.25 $Wm^{-2}sec^{-1}$ UV-B light. The treated cells were harvested for total RNA extraction. WL : White fluorescent light, UV-B : ultraviolet-B.

that CHS expression seemed to be accelerated by simultaneous treatment of JA with UV-B. But STS expression was rapidly reduced and observed in very low level at 16h. STS transcription was rapidly decreased in white light than UV-B. These results are indicated that the response against JA and light is controlled by different mechanisms. CHS expression was promoted by JA and light treatment, and was synergistically expressed at the simultaneous treatment of UV-B (Fig. 5).

There are many reports that CHS and STS showed an extensive simility in nucleotides and amino acids sequences, and a close relationship in their pathway. [20,21,22] In transgenic plants using the STS gene it was shown that STS transcription affects the CHS expression and disease resistance. [12] Fischer *et al.* [23] suggested that overexpression of STS alters flower pigment and male sterility by decreasing endogeneous gene. In grape suspension cells CHS and STS genes also have a high similarity at the nucleotides sequence and the amino acid residues, and the expressions of two genes were induced or stimulated by JA treatment and upon light exposure. Above results are indicated that CHS and STS have different response mechanisms against the various environmental stresses.

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