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Molecular Characterization of a Fruit-Preferential Thaumatin-Like Gene from Apple (*Malus domestica* cv. Fuji)

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Functioning of the thaumatin-like (TL) protein is known to be pathogenesis- or stress-related. Here we identified TL protein cDNA, from an apple- skin library, that was nearly identical to that of the *MdTL1* gene. Transcripts of this so-named gene, *MdTL1a*, were highly expressed in the fruit, but rarely in other tissue types. Expression was found in both the skin and the flesh of the fruit. We also examined the environmental or hormonal control of *MdTL1a* expression. Exposing the fruit to light caused this gene to be induced in the skin tissues. Accumulation of *MdTL1a* mRNA reached a peak between Days 1 and 5 after exposure. In the leaves, *MdTL1a* was induced by salicylic acid (SA), but was not significantly affected by any other stresses. Analysis of the *MdTL1a* genomic clone, λTL1, revealed that transcription of *MdTL1a* begins 53 bp upstream of the start codon. Sequence analysis of the ca. 1.0-kb *MdTL1a* promoter region has enabled us to predict that it has a stress-related *cis*-element, such as the ABA responsive element (ABRE), as well as a light-responsive GT-1 and I-box.

Keywords: apple, cDNA, fruit, stress, thaumatin-like protein

Thaumatin is a sweet-tasting protein synthesized in the cytoplasm of fruit cells of *Thaumatococcus daniellii* Benth., a tropical monocotyledon plant (van der Wel and Loeve, 1972; Richard et al., 1992). Thaumatin-like (TL) proteins have also been found in other plant organs, such as leaves and flowers (Uknes et al., 1992; Malehorn et al., 1994; Sassa and Hirano, 1998), but they are detected mainly in ripening fruit (Fils-Lycaon et al., 1996; Clendennen and May, 1997; Tattersall et al., 1997; Sassa and Hirano, 1998; Kim et al., 2002; Wang and Ng, 2002). In the cherry, TL proteins comprise up to 42% of the total soluble proteins in the mesocarp at the ripe stage (Fils-Lycaon et al., 1996). Expression of a grape TL gene, *VVTL1*, was specific to ripening fruit; accumulation of the VVTL1 protein occurred simultaneously with increased sugar content, suggesting that it may either be induced by sugar or act as a storage protein (Tattersall et al., 1997).

TL genes are regulated by environmental or hormonal factors. For example, tobacco osmotin mRNA was induced by ABA in cultured cells (Singh et al., 1989), and by salicylic acid (SA) and methyl jasmonate (MJ) in seedlings (Xu et al., 1994). In *Solanum commersonii*, an osmotin-like gene was induced by ABA, cold, and desiccation (Zhu et al., 1993). An apoplastic TL protein, AHCSP33, was induced by low temperatures in *Arachis hypogaea* (Dave and Mitra, 1998), while a TL protein in winter rye served as an antifreeze protein (Yu and Griffith, 1999, 2001). In the leaf tissues of rice, TL proteins was induced by jasmonic acid (JA) and UV light (Rakwal et al., 1999). Finally, TL transcripts have been shown to accumulate in response to JA, but not SA, in pepper fruits (Kim et al., 2002).

Genomic clones of TL have also been studied. In the tobacco PR-5 gene, *cis*-elements involved in tobacco mosaic virus-inducible expression were found (Albrecht et al., 1992). In a *Benincasa hispida* TL gene, *cis*-elements important for transcription such as TATA box and CAAT box were predicted (Shih et al., 2001). Sassa et al. (2002) have reported a pistil-specific TL promoter in the Japanese pear with motif III-related sequences that are found in the promoters of self-incompatibility genes.

In this study, we investigated how expression of the *MdTL1a* gene from apple is affected by treatments with light and SA. We also isolated an *MdTL1a* genomic clone in order to determine its transcription start site and to describe the putative *cis*-elements present in the promoter region.

MATERIALS AND METHODS

Plant Material and Bacterial Strains

Apples (*Malus* × *domestica* Borkh. cv. Fuji) were sampled at the National Horticultural Research Institute (NHRI,

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Suwon, Korea). To study light-responsive gene expression in the skin, young fruits on the tree were encased in double-layered paper bags beginning four weeks after pollination (WAP), or 35 days after full bloom. After 15 weeks of maturation, the bags were taken away and the fruits were harvested 0, 1, 5, 11, and 20 days after bag removal (DABR). For our 40 DABR treatment, the fruit that had been harvested at 20 DABR was kept for another 20 d at room temperature (RT) in an NHRI storehouse. The skin and flesh tissues were separated by peeling the fruit with a knife, followed by immediate placement in liquid nitrogen. For the wounding experiments, leaves were cut into pieces that were then floated on deionized water at RT for 3 h. For our chemical treatments, the wounded leaf fragments were floated for 3 h on water containing 4 mM ethephon, 10 μM MJ, or 1 mM SA. Treatment with UV light involved transferring detached branches (with their leaves) to water, followed by irradiation (1.2 Wm^{-2} s^{-1}) for 30 min. The exposed branches were then incubated in the dark for 3 h. Two *Escherichia coli* strains, XL1-Blue MRF and XL1-Blue MRA, were used according to procedures in the Stratagene manual.

Screening of Genomic Library

Phages from a genomic library were screened by plaque hybridization, using the *MdTL1a* probe labeled with [α-^{32}P] dCTP. After tertiary screening, positive plaques were isolated and the restriction fragment of the recombinant clone was subcloned into the pBluescript SK(–) vector (Stratagene).

DNA Sequence Analysis

DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977), using a Thermo Sequenase Cycle Sequencing kit (Amersham) and an autosequencer (ABI). The sequences were compared with those in the database via the BLAST program (Altschul et al., 1990). DNASIS and PROSIS software (Hitachi) were used for the sequence analysis.

Northern Analysis

Total RNA was isolated according to the guanidium thiocyanate (GTC)-CsCl method, as described by Wang and Vodkin (1994). For the northern analysis, 10 or 30 micrograms of total RNA were denatured and resolved by electrophoresis through a 1.3% agarose gel, then blotted onto nylon membranes and hybridized with ^{32}P-labeled DNA (Sambrook et al., 1989). We performed the hybridization for 16 to 24 h at 42°C. Afterward, the membranes were washed in a 0.1 to 1.0× SSC, 0.1% SDS solution at RT, depending on the background. Hybridization signals were detected either on Hyperfilm™ MP film (Amersham) or with a BAS phosphoimage analyzer (BAS 1500, Fuji).

Primer Extension Analysis

Primers were labeled by ^{32}P in a 10 μL labeling solution [1× Kinase buffer, 20 pmol of primer, 30 μCi (γ-^{32}P) ATP, and 10 units T4 polynucleotide kinase]. The reaction was incubated 30 min at 37°C and terminated by heating to 90°C for 2 min. About 5×10^5 cpm of labeled primer in an annealing solution (0.9× AMV Primer extension buffer and 8 μg of total RNA) was extended according to manufacturer's instructions (Promega). The extended cDNA was collected by centrifugation, and the pellet was dissolved in 4 μL of nuclease-free water and 4 μL of loading buffer, then denatured at 90°C for 10 min. A 3.5 μL sample was analyzed on a 6% polyacrylamide sequencing gel. Signals were detected using the BAS phosphoimage analyzer.

RESULTS AND DISCUSSION

Sequence Analysis of *P19* from Apple Skin Library

An Expressed Sequence Tag (EST), *P19*, was obtained during EST analysis (Sung et al., 1998). Our nucleotide sequence analysis of *P19* revealed that the clone was 945 bp long, with an open reading frame of 747 bp, 25 bp of a 5' untranslated region (UTR), 154 bp of 3' UTR, and a poly (A)+ tail. This nucleotide sequence was found to be nearly identical to that of *MdTL1* cDNA (Oh et al., 2000) if nine gaps were given to the alignment between the *P19* and the *MdTL1* sequences in the 5' region (Fig. 1). This shift generated four amino-acid differences between the P19 and MdTL1 proteins in their signal peptide regions of the proteins as predicted according to von Heijne's (1983). Two base pairs of *P19* in the 3' UTR region also differed from those of *MdTL1*.

Although P19 is not identical to MdTL1, we named it *MdTL1a*, based on its near-perfect identity in the UTR region and its high homology with MdTL1. The size of the deduced MdTL1a protein was the same as that of MdTL1. Southern blot analysis revealed that *MdTL1a* is composed of a small gene family (data not shown), with a hybridization pattern identical to that reported by Oh et al. (2000). Self-incompatibility causes

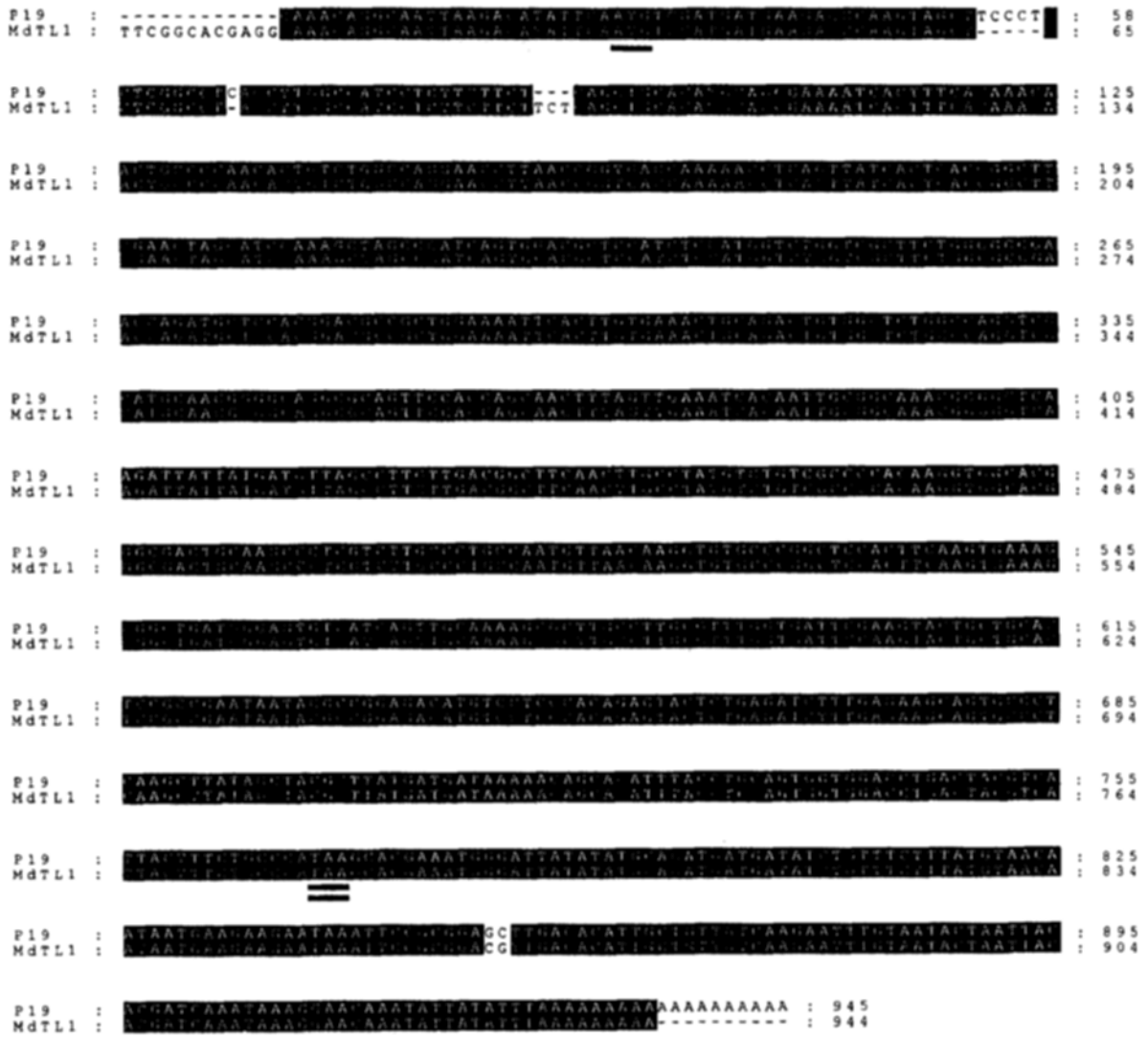

Figure 1. Nucleotide sequence alignment of *P19* (*MdTL1a*) and *MdTL1* cDNA. Numbers indicate the position of nucleotide. Identical residues are highlighted in black. Start codon and termination codon are marked by underline and double underlines, respectively.

many of the *Malus* species to be cross-pollinating plants. Therefore, trees grown from seeds are frequently interspecific hybrids, as in the case of *Malus domestica* (Brown, 1975; Korban and Skirvin, 1984). The minor variations observed between *MdTL1* and *MdTL1a* might have been due to their originating from different parental species.

Northern Blot Analysis of *MdTL1a*

We used RNA blot analysis to study the expression of *MdTL1a* in various organs and tissues (Fig. 2). Low level transcripts were detected in mature floral buds, but not in young floral buds, young leaves, or seedlings (Fig. 2). Neither was *MdTL1a* detectable in the roots (data not shown). Levels of *MdTL1a* mRNA were high in the fruit at both 10 and 20 weeks after pollination (WAP). Interestingly, the transcript was present in the skin as well as in the flesh of mature fruit. These results are consistent with those of Oh et al. (2000), who reported that *MdTL1* mRNA accumulated in the fruit at the beginning of the growth phase with cell expansion, and reached the maximum level at the end of that phase.

Induction of *MdTL1a* by Light in Skin Tissues

Because *MdTL1a* was expressed in skin tissues, we

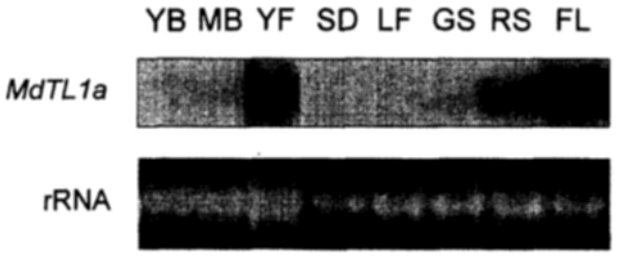

Figure 2. Northern analysis of *MdTL1a*. Ten μg of total RNA was loaded on each lane and hybridized with full-length *MdTL1a* cDNA. The amount of RNA loading on each lane was shown by EtBr-staining of rRNA. YB, young floral buds; MB, mature floral buds; YF, young fruit of 10 WAP; SD, seedlings; LF, young leaves; GS, green skin at 0 DABR; RS, red skin at 5 DABR; FL, flesh at 5 DABR.

decided to further analyze its light response. At 4 WAP (early June), apple fruits were double-bagged to protect them from light exposure for 15 weeks (until late September). After the bags were removed, we examined the expression of *MdTL1a* in the fruit skin tissues (Fig. 3A). Although the bagged fruits were fully mature by this stage, their skin color had remained yellow to green.

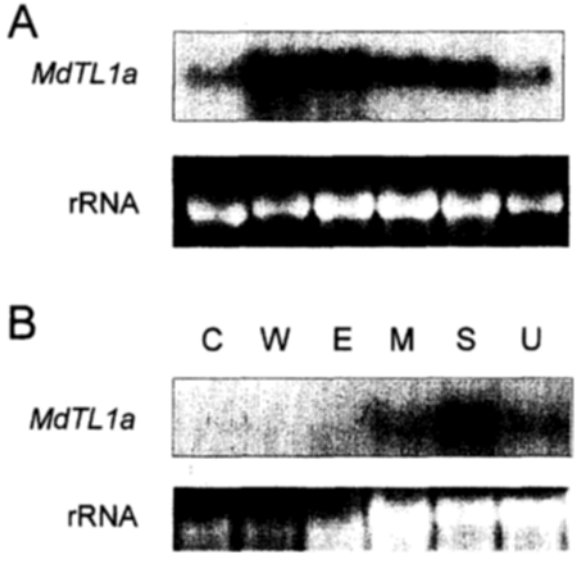

Figure 3. Environmental regulation of *MdTL1a* expression. **A.** Accumulation of *MdTL1a* mRNA in skin tissue, as induced by sunlight. Ten μg of total RNA was loaded on each lane and hybridized with *MdTL1a* probe. The amount of RNA loading on each lane was shown by EtBr-staining of rRNA. 0, 0 DABR; 1, 1 DABR; 5, 5 DABR; 11, 11 DABR; 20, 20 DABR; 40, 40 DABR. **B.** Induction of *MdTL1a* mRNA in leaves by various stimuli. Thirty μg of total RNA was loaded on each lane and hybridized with *MdTL1a* probe. The amount of RNA loading on each lane was shown by EtBr-staining of rRNA. C, control; W, wounding; E, 4 mM ethephon; M, 10 μM MJ; S, 1 mM SA; U, 1.2 $Wm^{-2}s^{-1}$ UV.

Transcript of the *MdTL1a* gene, which was already detectable at this stage, rapidly increased at 1 DABR. This expression continued until 5 DABR, then decreased. By 40 DABR, its level was reduced to the basal level at 0 DABR.

Although Tattersall et al. (1997) have suggested that the grape TL gene, *VVTL1*, may be induced by sugar, this is not clear yet. Furthermore, Song (1999) has shown that, in Fuji apple, sugar content increases gradually 40 d after full bloom. Therefore, we excluded the possibility that *MdTL1a* may be induced by sucrose. Based on these results, we propose that the gene is induced by light exposure on the skin tissues. To our knowledge, this is the first demonstration of tissue-specific light induction of a *TL* gene.

Expression of *MdTL1a* by Environmental Factors

It has been reported that *TL* genes can be induced by such environmental factors as wounding and SA (Uknes et al., 1992; Kim et al., 2002). Therefore, we examined whether this was true for *MdTL1a* transcripts in our apple leaves. Although expression was not detected in the controls, treatment with SA did induce *MdTL1a*. Other treatments, however, did not significantly increase transcript levels. Uknes et al. (1992) have shown that SA can induce *PR-5* transcripts in the leaves of *Arabidopsis*. Recently, it has been shown that pepper *TL* gene transcripts in ripe fruit are significantly accumulated in response to JA but not to either SA or ethephon (Kim et al., 2002). In that study, wounding the fruit and leaves also caused levels to increase. Because TL genes comprise a gene family, these data suggest that the *MdTL1a* gene may be regulated differentially by specific factors in various tissue types.

Isolation and Characterization of *MdTL1a* Genomic Clone

To further characterize the light- and SA-responsive expression of *MdTL1a*, we screened its genomic clone. About 3×10^5 phages of the 'Fuji' genomic library were plaque-hybridized with the *MdTL1a* probe. After tertiary screening, 10 recombinant plaques were obtained, which were divided into three groups based on their restriction maps (data not shown). Through partial DNA-sequencing analysis, we identified a 5.6 kb clone, λTL1, that corresponded with the *MdTL1a* cDNA in both its 5' and 3' UTRs. The restriction map of the *TL* genomic region of λTL1 is shown in Figure 4A. Partial sequencing of λTL1 revealed that it contains an intron similar to that of a TL gene from the Japanese pear (Sassa et al.,

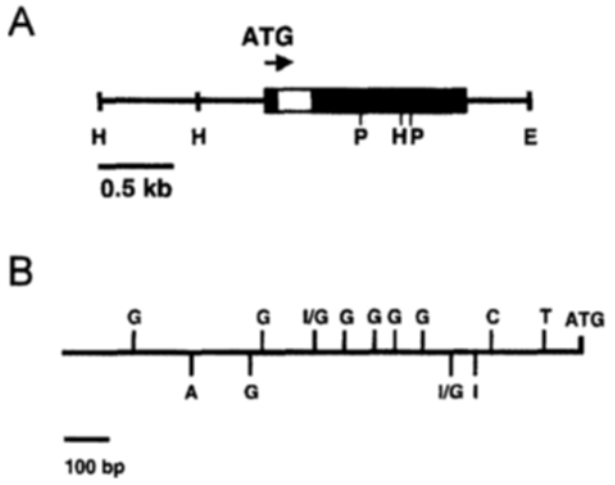

Figure 4. Restriction map of the *TL* genomic region of λTL1 (**A**) and diagrammatic representation of the putative *TL* promoter (**B**). Coding regions are shown in black bars with intron as a white bar. ATG and the arrow represent the start codon and the direction of translation, respectively. E, EcoRI; H, HindIII; P, PstI. Putative binding sites for transcription factors are indicated. T, TATA box; C, CAAT box; I, I box; G, GT-1; A, ABRE.

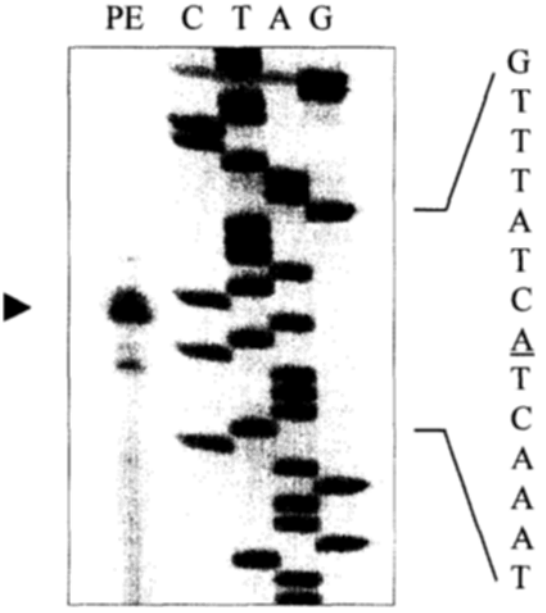

Figure 5. Primer extension analysis of *MdTL1a* transcript. An end-labeled primer, TLP2 (5'-ACTTGGCTCTTCATCATC-3'), was annealed to RNA from flesh tissues. HindIII/PstI fragment (1.1 kb) of λTL1 was sequenced using TLP2 and run in parallel with the primer extension product (PE). Lanes C, T, A, and G represent the termination dideoxynucleotide on each lane. A major primer extension product corresponding to the nucleotide 'A' located 53 bp upstream of the translation start site is underlined.

2002). This intron has consensus GT and AG sequences at the 5' and 3' ends, respectively.

Determination of the Transcription Start Site of *MdTL1a* and Analysis of the Upstream Sequence

We performed primer-extension analysis and found that the predicted transcription start site of the *MdTL1a* gene was at point 'A', which is 53 bp upstream of the translation start codon (Fig. 5). An approximately 1.2 kb 5'-flanking sequence of λTL1 was analyzed by nucleotide sequencing. A CAAT box and a TATA box sequence were predicted at -149 and -33 of the transcription start point, respectively (Fig. 4B). The I box and GT-1 box present in many light-regulated promoters (Terzaghi and Cashmore, 1995) were also found in our *MdTL1a* promoter region. Therefore, we assume that the *cis*-elements present in the *TL* promoter are responsible for light-responsive expression of the *MdTL1a* gene.

Because TL genes or proteins might also be regulated by stimuli other than light, we searched for other putative *cis*-elements. Although expression of *MdTL1a* was induced by SA (Fig. 3B), we could find no SA-responsive as-1-like elements (Qin et al., 1994) in the promoter region. This suggests that other SA-responsive elements are present in the promoter. Previous reports of a low temperature-induced apoplastic TL protein in *A. hypogaea* (Dave and Mitra, 1998) and an antifreeze TL protein from winter rye (Yu and Griffith, 1999, 2001) support the involvement of TL proteins in low temperature responses. Furthermore, tobacco osmotin transcripts were induced by ABA, a known stress hormone (Singh et al., 1989). Therefore, we searched the promoter region of our *MdTL1a* gene for the presence of low temperature-related *cis*-elements. Neither the cold-responsive C repeat/drought-responsive element (CRT/DRE) nor a sequence resembling the G-box-1 (Baker et al., 1994; Dolferus et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994) was found, although a G-box core sequence, also known as an ABA-responsive element (ABRE), was identified 0.8 kb upstream of the transcription start site (Fig. 4B). Nevertheless, further investigation is required to determine whether expression of *MdTL1a* is indeed regulated by the putative *cis*-elements found in this study.

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