Elements involved in light regulation of the parsley *chs* promoter: *cis*-acting nucleotide sequences and *trans*-acting factors

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Summary

In order to investigate the mechanism(s) of plant responses to short-wavelength light, the regulation of chalcone synthase (CHS) expression has been analyzed. CHS catalyzes the first committed enzymatic step of flavonoid biosynthesis and is encoded in parsley (*Petroselinum crispum*) by a single gene whose expression is tightly controlled at the transcriptional level. Light is the primary external stimulus regulating the activity of the *chs* gene in leaf epidermis as well as suspension-cultured parsley cells. Analysis of the *chs* promoter by *in vivo* footprinting revealed four short sequences, designated Boxes I, II, III, and IV, that displayed light-induced protein contacts. Transient expression experiments in parsley protoplasts demonstrated that the four sequences are functionally relevant components of the *chs* promoter. These *cis*-acting elements are arranged in two light-regulatory units which are about 50 bp in length (LRU 1 containing Boxes I and II, LRU 2 containing Boxes III and IV). Each of them was shown to be sufficient for light responsiveness. Point mutation experiments defined a critical nucleotide sequence of seven bases (5'-ACGTGGC-3') within Box II of LRU 1. This heptameric sequence is also present in a closely related form in Box III of LRU 2. Nuclear extracts from suspension-cultured parsley cells contain a set of proteins which recognize the heptamer and related sequences. We isolated three parsley cDNAs encoding proteins which specifically bind to the 5'-ACGTGGC-3' sequence. Related sequences recognized by these "common plant regulatory factors" (CPRF-1, 2 and 3) contain an ACGT core motif which is present in similar sequence contexts in many *cis*-acting elements. Such ACGT elements (ACEs) are also of functional significance in a variety of other plant promoters, where they are involved in abscisic acid regulation, tissue- and development-specific gene expression as well as light responsiveness of *rbc*S promoters. The deduced amino acid sequences of all three ACGT-binding proteins revealed conserved basic and leucine-zipper domains characteristic of bZIP-type DNA-binding proteins.

Key-words: gene regulation, transcription factors, DNA binding, bZIP proteins

Introduction

Throughout their development and life cycle, organisms are exposed to various biotic and abiotic stress factors. Plants have evolved an array of mechanisms to protect themselves against these stresses. As a defense response against the potential damaging effects of the abiotic stress factor UV light, plants accumulate UV-absorbing substances in the exposed tissue. The main compounds deposited are flavonoids, which absorb light of 230 to 380 nm. The basic biochemical structure of flavonoids is a chalcone scaffold which is synthesized in a stepwise condensation reaction catalyzed by chalcone synthase (CHS; Figure 1). This reaction is the first enzymatic step committed to the flavonoid-specific branch of phenylpropanoid metabolism (Hahlbrock et Grisebach, 1979).

Extensive studies using cell suspension cultures and intact leaves of parsley have revealed an increased synthesis of flavonoids in response to UV light, preceded by transient, coordinated expression of the enzymes catalyzing the formation of these compounds (Chappell et Hahlbrock, 1984). In leaves exposed to UV-containing white light, the vacuolar accumulation of flavonoids is restricted in a tissue-specific manner to epidermal cells (Schmelzer *et al.*, 1988). We now focus our interest on the regulatory mechanisms involved in light-dependent gene expression.

Methods and materials

Standard techniques

The basic molecular biology techniques were carried out according to (Sambrook *et al.*, 1989). Parsley cell suspension cultures, light treatment conditions, and protoplast preparation were as described (Block *et al.*, 1990).

Plasmid constructions

The constructs pBT 3'u1-2 and pBT 3'u1-4 were created by placing LRU 1 dimer and tetramer fragments between the *Bg/II* and *NheI* sites located in pBT-2 in the region 3' to the *nos* poly(A) addition site (Weißhaar *et al.*, 1991b). The dimer and tetramer fragments were prepared by *XbaI* and *BamHI* digestion of pucOL containing LRU 1 in two or four copies (Weißhaar *et al.*, 1991a). Plasmid puc061 contains the *chs* promoter/*uidA* fusion from construct 061, which is based on pRT99 (Schulze-Lefert *et al.*, 1989b), in the polylinker of puc9.

Transient expression analysis

The transient expression assay was performed as described (Block *et al.*, 1990).

Genomic analysis

The experiments to determine the sequence of the parsley *cprF*-1 gene and its expression are described in Feldbrügge *et al.* (*manuscript in preparation*)

Results and discussion

In many plant species, CHS gene expression is strongly induced by more than one signal, e.g. floral development and light in *Petunia*, or elicitor and light in french bean and soybean (van Tunen et Mol, 1989). In cultured parsley cells, maximal CHS expression is UV light dependent, although blue light, red light, diurnal rhythm and developmental state of the tissue have additional modulating effects (Kreuzaler *et al.*,
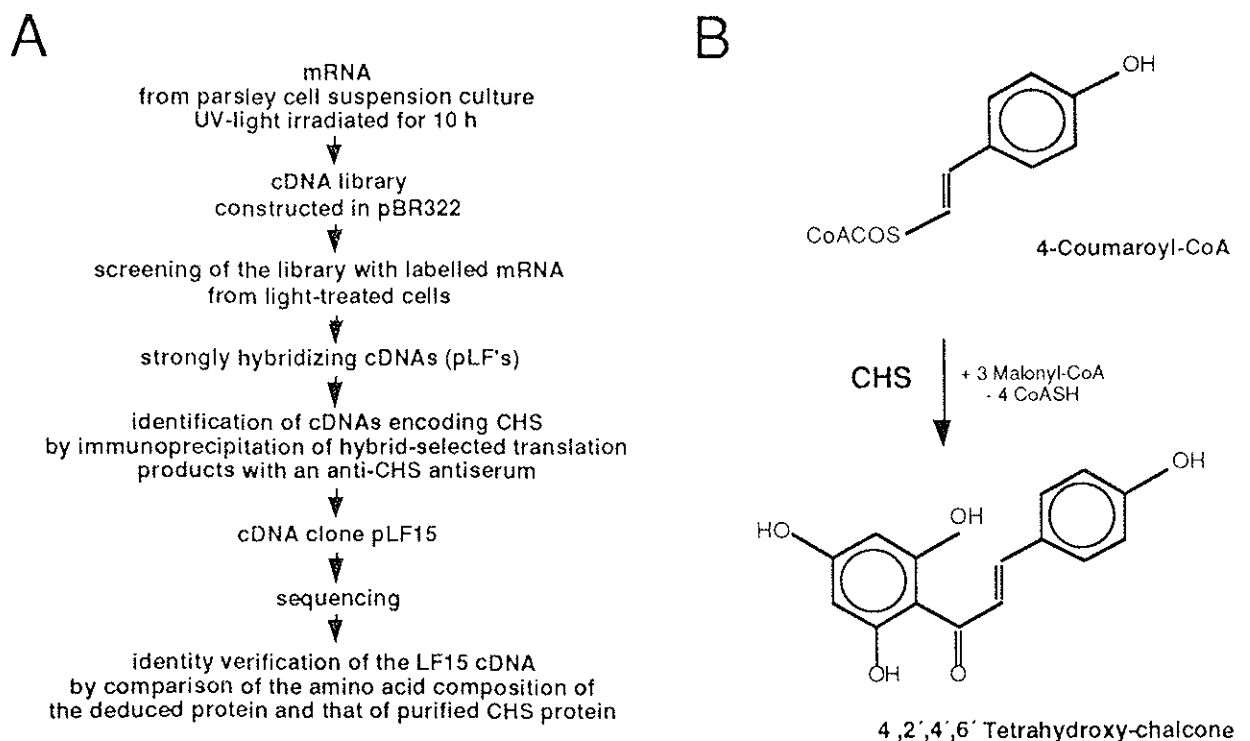
A

mRNA
from parsley cell suspension culture
UV-light irradiated for 10 h

↓

cDNA library
constructed in pBR322

↓

screening of the library with labelled mRNA
from light-treated cells

↓

strongly hybridizing cDNAs (pLF's)

↓

identification of cDNAs encoding CHS
by immunoprecipitation of hybrid-selected translation
products with an anti-CHS antiserum

↓

cDNA clone pLF15

↓

sequencing

↓

identity verification of the LF15 cDNA
by comparison of the amino acid composition of
the deduced protein and that of purified CHS protein

B

4-Coumaroyl-CoA

CHS | + 3 Malonyl-CoA
- 4 CoASH

4,2',4',6' Tetrahydroxy-chalcone

Figure 1: Scheme for the cloning procedure of the parsley *chs* cDNA (A) and a part of the flavonoid biosynthetic pathway (B).
The diagram (A) summarizes the experimental steps from the source of mRNA to the identification of the cDNA encoding CHS (Kreuzaler *et al.*, 1983; Reimold *et al.*, 1983). Part (B) shows the condensation reaction resulting in the chalcone scaffold which is catalyzed by CHS.

1983; Ohl *et al.*, 1989). Cloning of cDNAs encoding CHS (Figure 1; Reimold *et al.*, 1983) permitted experiments to determine the kinetics of *chs* mRNA accumulation in cultured parsley cells (Kreuzaler *et al.*, 1983) as well as leaves (Schmelzer *et al.*, 1988). Importantly, the kinetics of CHS induction in the *in planta* studies paralleled those obtained with cell suspension cultures. The light-induced increase in CHS mRNA was shown to be under transcriptional control. As a basis for promoter analysis, the parsley *chs* gene was cloned and sequenced (Herrmann *et al.*, 1988; Figure 2).

Genomic footprinting to the parsley *chs* promoter

To gain insight into the mechanisms governing light-regulated gene expression, experiments were carried out to identify regulatory sequences which are necessary and sufficient for the light-dependent activation of the parsley *chs* promoter. The method of choice was the *in vivo* footprinting technique (Church et Gilbert, 1984). Using suspension-cultured parsley cells, the *chs* promoter region from +40 to -615 (Figure 2) was analyzed for the appearance of light-induced *in vivo* footprints. Four short sequences showing differential reactivity to dimethylsulfat in dark-grown versus UV-irradiated cells were detected (Schulze-Lefert *et al.*, 1989a; Schulze-Lefert *et al.*, 1989b). These differences were taken as indications of light-inducible protein/DNA interactions, and the four regions defined by the outermost residues showing altered reactivity *in vivo* were designated Boxes I, II, III, and IV. Subsequent experiments showed that Boxes II and III belong to a large family of *cis*-acting elements with an ACGT core sequence. We now refer to these elements as ACEs (ACGT-elements; Box II is referred to as ACE[chsII] and Box III as ACE[chsIII], see below).
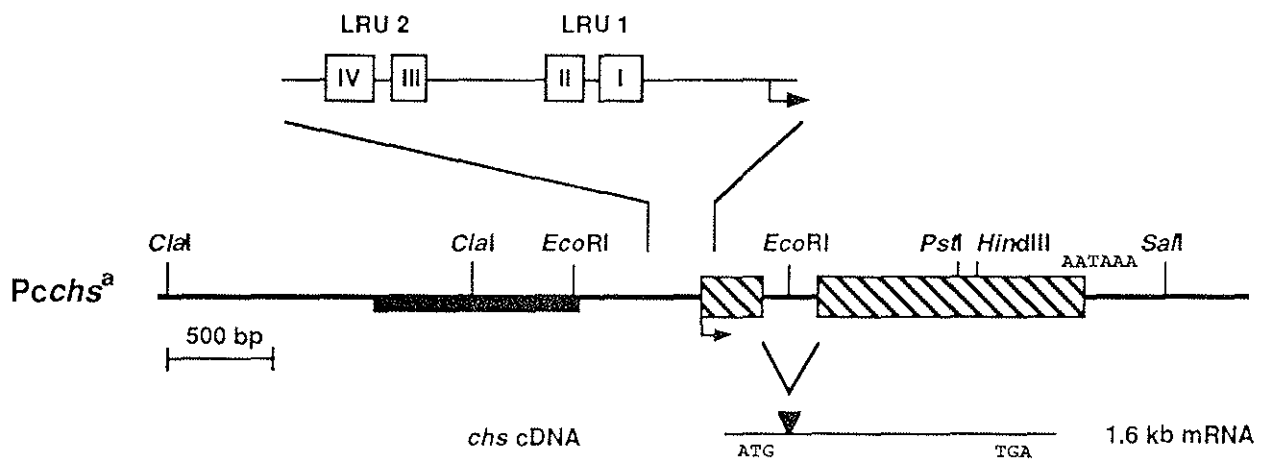

Figure 2: Diagram of the parsley *chs* gene.
Some structural features of the chalcone synthase gene are shown, including positions of relevant restriction enzyme recognition sequences. Striped boxes indicate the positions of the two exons, the bent arrow indicates the major transcription start site designated +1 as reference for nucleotide positions in the *chs* gene. The region marked with a black bar represents a transposon-like insertion found in one of the two alleles analyzed (Pc*chs*[a]; Herrmann *et al.*, 1988). In the enlargement of a part of the promoter the locations of the *cis*-acting elements defined by *in vivo* footprinting (Schulze-Lefert *et al.*, 1989b) are given. The two light responsive units (LRU 1 and 2) are indicated. In the lower part the *chs* mRNA, with the position of the spliced intron, is indicated

The four boxes are functionally relevant *cis*-acting sequences

Parsley protoplasts retain the responsiveness of previously dark-grown, suspension-cultured cells to light. Thus, chimaeric gene constructs can be introduced into parsley protoplasts to analyze the effects of promoter manipulations on the light-dependent expression of a reporter gene, e.g. the *uid*A gene coding for β-D-glucuronidase (GUS). This transient expression system allowed the detailed analysis of the parsley *chs* promoter in the homologous system. Chimaeric *chs* promoter constructions containing sequences up to -615 that were translationally fused to the reporter gene respond to

light qualitatively in the same fashion as does the endogenous *chs* gene. Serial 5' deletions operationally defined sequences from -226 to +147 as the minimal light-responsive promoter containing Box I and ACE[chsII] (Box II; Schulze-Lefert *et al.*, 1989b). By the introduction of clustered point mutations into Box I and ACE[chsII] it was shown that mutation of either sequence element resulted in the loss of light responsiveness. Therefore, both Box I and ACE[chsII] are *cis*-acting elements necessary for the light response in· the context of the minimal *chs* promoter (Schulze-Lefert *et al.*, 1989b). Since both sequences had to be intact and located at a certain distance from each other for promoter activity (Block *et al.*, 1990), they were defined together as a light-responsive *cis*-acting unit (LRU 1).

LRU 1 is sufficient for light-dependent *chs* gene expression

After demonstrating that LRU 1 is necessary for light responsiveness in the context of the CHS promoter, the next question was if "gain of function" experiments could show that LRU 1 is also sufficient for light inducibility. To answer this question, a synthetic oligonucleotide comprising the complete sequence of LRU 1 was cloned in both orientations in front of a deleted 35S promoter. The results obtained with the oligonucleotide-containing constructs showed that in either orientation LRU 1 was able to confer light responsiveness on a heterologous transcription start site (Weißhaar *et al.*, 1991a). Therefore, we concluded that LRU 1 is not only necessary, but also sufficient for directing light-dependent expression in the homologous system. The orientation-independent activity of LRU 1 encouraged us to also test another property of enhancer sequences, namely distance-independent action. LRU 1 was inserted 3' to the *uidA* open reading frame, which is equal to about 2.5 kb upstream from the transcription start site on the circular plasmid, in two and four copies. These constructs showed no increase of GUS enzyme activity after light treatment of transfected protoplasts (Figure 3). In conclusion, LRU 1 behaves in terms of orientation independence and modular structure like an enhancer, but does not function over a long distance.

construct	specific GUS activity (μkat x kg^{-1})		fold induction
	dark	light	
pBT 2	2.13/1.80	0.97/0.85	-
pBT Δ	0.46/0.53	0.33/0.35	-
puc 061 (minimal promoter)	4.70/4.82	34.12/37.60	7.6
pBT u1-2	2.72/5.75	17.37/42.83	7.0
pBT 3' u1-2	0.97/2.65	0.87/1.40	-
pBT 3' u1-4	0.97/3.00	1.25/2.28	-

Figure 3: GUS activity data from constructs containing LRU 1 3' to the *uidA* coding region. Names of promoter/*uidA* fusions are listed on the left and the results from a selected experiment performed with two parallel samples for each construct are shown. On the right the calculated fold induction is indicated. The amount of fluorescent 4-methylumbelliferone formed is given as specific activity (μkat) of GUS enzyme per kg protein and is taken as a measure for promoter activity. Light-dependent increase of transcription is only observed for the *chs* minimal promoter (puc061) and the LRU 1 construct (pBT u1-2) containing a dimer close to start site. As additional controls the basic construction with only the CaMV 35S promoter up to -46 (pBT 2) and the vector without eukaryotic promoter sequences (pBT Δ) are included.

Functional redundancy in the parsley *chs* promoter

The light-responsive unit (LRU 1) within the minimal promoter generated lower GUS activity when compared with the longer construct containing all sequences up to -615. The presence of ACE[chsIII] and Box IV, which were defined by the *in vivo* footprinting experiments upstream of LRU 1, motivated the construction of *chs* promoter-*uidA* fusions carrying these upstream elements in the context of various other parts of the *chs* promoter (Schulze-Lefert *et al.*, 1989a). The results demonstrate that a second light-responsive sequence is contained in the *chs* promoter consisting of ACE[chsIII] and Box IV. In analogy to LRU 1 ACE[chsIII] and Box IV were designated LRU 2. When LRU 2 was tested in "gain of function" experiments similar to those described above for LRU 1, it also behaved as a regulatory sequence necessary and sufficient for light induction (Weißhaar *et al.*, *manuscript in preparation*). Taken together, these and other data (Block *et al.*, 1990) demonstrate that the expression of the parsley *chs* gene in response to light is regulated by at least two separable light-responsive *cis*-acting units.

Single base substitutions within ACE[chsII](BoxII) define a functional core of 7 nucleotides

Detailed analysis by site-directed mutagenesis of ACE[chsII] in the context of the *chs* minimal promoter defined a functional core of seven nucleotides, 5'-ACGTGGC-3'. The asymmetry of nucleotides surrounding the ACE palindrome may be mandatory for the proper interaction with Box I, the other (known) *cis*-acting element in LRU 1 required for light responsiveness. Extensive comparative studies recently demonstrated that nucleotide differences outside the ACGT symmetry center have a strong impact on protein/DNA interaction at the ACE (Schindler *et al.*, 1992b; Williams *et al.*, 1992).

A family of related *cis*-acting elements contains an ACGT core

It seems to be more the rule than the exception that similar *cis*-acting elements are present in a variety of promoters which are regulated by diverse stimuli. A prominent example in mammalian systems is an element with the consensus $^T/_C\,^T/_G$ACGTCA known as CRE (cAMP responsive element) or ATF site (see, e.g. Flint et Jones, 1991). In the case of ACE[chsII] (5'-CCACGTGGCC-3'), similar sequences have been found in many other promoters from several plant species which respond to different kinds of stimuli (Schulze-Lefert *et al.*, 1989b). Sequence comparison (summarized in Figure 4) of well defined *cis*-acting elements with known functional importance revealed the existence of a family of plant *cis*-acting elements (Guiltinan *et al.*, 1990; Weißhaar *et al.*, 1991a) related to a conserved sequence found in promoters of *rbcS* genes (Giuliano *et al.*, 1988). Mutational analysis (Block *et al.*, 1990) and *in vitro* binding studies using plant nuclear extracts carried out in several laboratories (Armstrong *et al.*, 1992; Schindler *et al.*, 1992a; Williams *et al.*, 1992) demonstrated that the ACGT sequence present in these elements is of pivotal importance. We define an ACE as a promoter sequence that fulfills the following criteria: an ACGT symmetry center, sequence similarity to Box II, recognition by nuclear factors (CPRFs, see below), and established relevance in promoter function.

Regulatory factors binding to ACGT elements are common to many plant species

Nuclear extracts from many plant species were shown to contain factors able to recognize ACEs (Giuliano *et al.*, 1988; Bouchez *et al.*, 1989; Lam *et al.*, 1989; Mikami *et al.*, 1989; Staiger *et al.*, 1989; DeLisle et Ferl, 1990; Guiltinan *et al.*, 1990). Often, a complex pattern of protein/DNA interactions detected by electrophoretic mobility shift assays (EMSA) indicates that several different nuclear proteins from a given nuclear extract are able to bind a distinct ACE (Armstrong *et al.*, 1992). We concluded that ACEs are recognized by multiple factors from various plant species. We refer to these factors as (nuclear) CPRFs (see below).

During the last three years, cDNAs encoding plant DNA-binding proteins which specifically recognize ACEs have been cloned in several laboratories (Katagiri *et al.*, 1989; Tabata *et al.*, 1989; Guiltinan *et al.*, 1990; Singh *et al.*, 1990; Lohmer *et al.*, 1991;

ACE^{chsII}
Block et al., (1990)

CC ACGTGGC C

G-box
Giuliano et al. (1988)

aCACGTGGCa

Em1a element
Marcotte et al. (1989)

ACGTGGCg

hex **motif**
Mikami et al. (1987)

tgACGTGGCC

ocs **element**
Bouchez et al. (1989)

aaACGTaagcgcttACGTac

as-1 element
Lam et al. (1989)

ctgACGTaagggatgACGcac

OLE^{cprF1}
Feldbrügge et al. (in preparation)

gaCACGTGttcgatgACGTGGtac

CRE/ATF site
Kerppola and Curran (1991)

tgACGTca

Figure 4: Comparison of *cis*-acting elements with ACGT cores.
The boxed region in ACE^{chsII} indicates the functional important nucleotides defined by point mutation experiments (Block *et al.*, 1990). In case of the *ocs*-like element from the *cprF*-1 gene (OLE^{cprF1}) the functional relevance has not yet been directly demonstrated.

Oeda *et al.*, 1991; Weißhaar *et al.*, 1991a; Schindler *et al.*, 1992a). In the parsley system, we have concentrated on the identification of proteins which interact with sequences critical for light regulation of the parsley CHS promoter. This approach resulted in the cloning of cDNAs encoding three putative transcription factors which interact with ACE^{chsII} and ACE^{chsIII} *in vitro*. These putative transcription factors were designated Common Plant Regulatory Factors (CPRF-1, CPRF-2, and CPRF-3) because of the common occurrence of similar binding activities and because of their (possible) involvement in the activity of the widely distributed ACE family of regulatory sequences. Of the three parsley factors cloned, CPRF-1 appeared to be a good candidate for involvement in the light-induced transcription of the *chs* gene. CPRF-1 mRNA accumulates in response to irradiation more rapidly than *chs* mRNA (Weißhaar *et al.*, 1991a; Figure 5). A problem with this hypothesis is that in parsley, as well as in other systems, no clear-cut changes in *in vitro* DNA/protein interactions were detected after application of the stimulus of interest *in vivo* (Armstrong *et al.*, 1992). One expla-
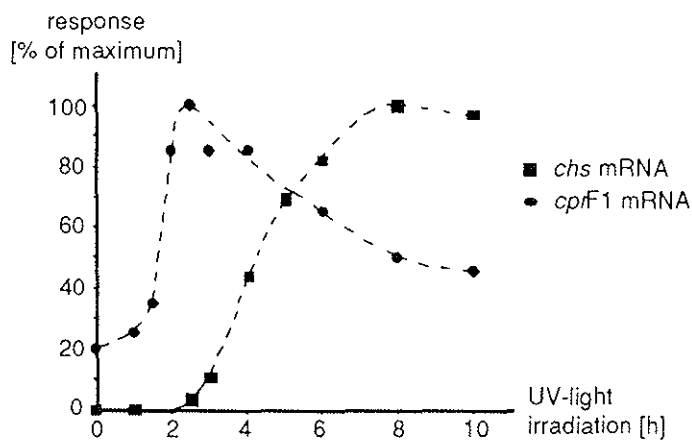

response [% of maximum]

■ *chs* mRNA
● *cprF*1 mRNA

UV-light irradiation [h]

Figure 5: Comparison of the relative changes of *chs* and *cprF*-1 mRNA levels in suspension-cultured parsley cells under UV light irradiation.
Samples of 10µg total RNA, isolated at the time-points indicated (hours of constant illumination), were electrophoretically size separated and transferred to a nylon membrane. After hybridization to the *cprF*-1 probe the membrane was subsequently reprobed with a parsley *chs* cDNA fragment. Results were quantitated by scanning of the autoradiograms (Weißhaar *et al.*, 1991a) with a 2202 Ultroscan Laser Densitometer. The broken line indicates the anticipated time course of mRNA accumulation.

nation in parsley could be the sequestering or inactivation of ACE^chsII-binding factors in the dark *in vivo*, possibly through differential modification, which may be lost upon isolation of nuclear extracts. Another explanation could lie in the formation of heterodimers with non-induced factors thereby masking the amount of newly synthesized protein although different regulatory qualities are created.

Deduced amino acid sequences of CRRFs reveal bZIP regions

It is of particular significance that all three parsley CPRFs contain the leucine zipper DNA-binding motif (Landschulz *et al.*, 1988). Outside of the bZIP regions (Vinson *et al.*, 1989) the three CPRF proteins are not significantly similar to each other, nor to other sequences available in the databases. Nevertheless, certain general properties such as high proline content, known from other regulatory proteins are also found in CPRF-1 (Weißhaar *et al.*, 1991a). The bZIP motif is characterized by a conserved region of basic amino acids located immediately N-terminal to a region containing hydrophobic residues (Figure 6). These hydrophobic amino acids show a 4-3 spacing with a strong preference for leucines at every seventh position. Amino acids in two basic regions are believed to contact the DNA when brought together by the leucine zipper which serves as a dimerization interface (Busch et Sassone-Corsi, 1990). Comparison of the sequences of the CPRF bZIP domains with those of other higher plant DNA-binding proteins described to date revealed a particularly high degree of conservation within the DNA-contacting basic region. A sequence of 14 amino acids in the basic region is nearly identical between nine of the plant DNA-binding proteins, including parsley CPRF-1 and CPRF-3, and is highly conserved in the other six. Conserved leucine

Figure 6: Comparison of DNA-binding and dimerization regions of bZIP proteins.
The region chosen for comparison was selected according to the smallest GCN4 fragment with DNA-binding activity which is shown at the bottom. The leucine zipper sequences are given in smaller characters except for every seventh amino acid. Positions of the 4-3 hydrophobic repeat are highlighted in bold. The number on the left gives the position of the first amino acid shown according to the reference listed on the right. Amino acids positions identical in at least five of the nine higher plant proteins which recognize ACEs of the CACGTG subfamily (upper group) are indicated by an asterisk in all plant sequences shown except for the top line. Under these circumstances the GBF3 sequence is always in accordance with the majority and was therefore chosen as "consensus".

residues are found at regular intervals, flanked by other aminoacids conserved at some of the intervening positions in the repeat. We take these data as another indication of the existence of a class of bZIP regulatory factors, all containing a related basic DNA-contacting domain mediating the interaction with different *cis*-acting elements of the ACE family.

Features of CPRF binding to DNA and selective dimerization

As predicted on the basis of extensive studies performed on mammalian and yeast bZIP proteins (reviewed in Johnson et McKnight, 1989; Busch et Sassone-Corsi, 1990), deletion analysis of the bZIP domains of the parsley CPRF-1, 2, and 3 (Armstrong *et al.*, 1992) showed that these domains are crucial for DNA binding and, by implication, for dimerization (compare Singh *et al.*, 1990; Tabata *et al.*, 1991; Schindler *et al.*, 1992a). Direct evidence for homo- and heterodimerization of CPRF-1, 2, and 3 was obtained by cotranslation of different polypeptide derivatives and subsequent EMSA. These experiments demonstrated that parsley CPRFs in particular, and probably plant bZIP proteins in general, selectively form DNA-binding heterodimers (Armstrong *et al.*, 1992).

Heterodimerization between different CPRFs (in the sense of ACE-binding factors according to our definition) leading to novel binding activities seems probable. We suggest that non-promiscuous dimer formation among nuclear CPRFs produces a large pool of factors capable of interacting with ACEs *in vivo*. The formation of CPRF homo- and heterodimers and their binding profiles to ACEs has its parallel in animal ATF/CREB and JUN/FOS factor families (Flint et Jones, 1991; Lamb et McKnight, 1991) and may also be a general phenomenon in plants.

Acknowledgments

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