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PLANT TRANSCRIPTION FACTOR STUDIES

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ABSTRACT

Major advances have been made in understanding the role of transcription factors in gene expression in yeast, Drosophila, and man. Transcription factor modification, synergistic events, protein-protein interactions, and chromatin structure have been successfully integrated into transcription factor studies in these organisms. While many putative transcription factors have been isolated from plants, most of them are only poorly characterized. This review summarizes examples where molecular biological techniques have been successfully employed to study plant transcription factors. The functional analysis of transcription factors is described as well as techniques for studying the interactions of transcription factors with other proteins and with DNA.

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INTRODUCTION

Regulated gene expression is one of the most complex activities in cells because it involves the integration of signal transduction pathways, the movement of proteins between cellular compartments, alterations in chromosome structure, RNA synthesis, and RNA processing (10). To understand plant growth and development at the molecular level, a detailed knowledge of the mechanisms of transcription is required. To achieve this, a comprehensive set of techniques and approaches must be assembled and used in a complementary manner. Here, using selected examples, systems are described that are presently available for studying transcription factors in vivo and in vitro. Techniques are outlined that allow the identification of transcription factor domains and of proteins that interact with transcription factors. In the closing section, techniques are summarized for studying the interactions between transcription factors and DNA.

TRANSCRIPTION STUDIES IN VIVO AND IN VITRO

Transient assays can provide prompt information about transcription factor function or their DNA-binding specificity. Test systems for plant transcription factors range from transient transformation of plant cells (Table 1) to in vitro transcription systems (107, 135) as well as transformation of yeast (49, 70, 73, 87, 88, 91, 92, 106, 122) and human cells (56, 97).

Transient Transformation of Plant Cells

Plant tissue can be transiently transformed by particle bombardment, electroporation, or polyethylene-glycol (PEG)/$CaCl_2$–mediated procedures. Table 1 summarizes transient assays from different plants that have been used for the study of plant transcription factors. For in vivo experiments, a DNA construct, directing the expression of the transcription factor of interest (the effector), is cotransfected with a suitable promoter/reporter construct (67). Depending on the nature of the transcription factor, reporter gene expression should be activated or repressed in its presence. Reporter gene activity can be measured within hours or days of transformation. To ensure reproducibility and to account for variations between individual transformations, a second reporter gene is usually cotransfected that is not affected by the transcription factor under study (67). In several cases, transcription factor action could be stimulated

Table 1 Summary of examples for transient assay systems that have been used for transcription factor studies

Method	Plant species	Plant material	Transcription factor	Reference
Particle bombardment	*Hordeum vulgare*	Aleurone	GAMyb, Viviparous1	41, 48, 103
	Nicotiana tabacum	Leaves	activating sequences	29
	Petunia hybrida	Floral organs	Lc, C1	83
	Phaseolus vulgaris	Cotyledons, leaves	PvAlf, ROM1, ROM2	8, 17, 18
	Zea mays	Cotyledons, endosperm	C1, R, Opaque2	9, 118
		Embryo, aleurone, embryogenic cellus	B, C1, Viviparous1	35, 36, 48, 54, 69, 86
			activating sequences	29
		A636, L6 maize suspension cells	B, C1, P, Opaque2	93, 132
Protoplast transformation	*Arabidopsis thaliana*	Leaf protoplasts	ATMYB2	119
	Nicotiana tabacum	Mesophyll protoplasts	HSF, PosF21, VSF-1, SPA, Myb.Ph3	3, 4, 106, 111, 112
		BY2	GAL4-fusions	74
	Zea mays	Black Mexican Sweet suspension cells	SPA	4
Electroporation	*Glycine max*	Cell culture	GBF1	97
	Daucus carota	WOO1C	GBF, GAL4-fusions	117
		Cell suspension culture	Viviparous1	71
	Nicotiana tabacum	Suspension culture (line XD)	GT-2	24
	Oriza sativa		OSH42, OSH44, OSH45	108
		Suspension culture (line Oc)	Osvp1, RITA-1	45, 52
	Zea mays	Black Mexican Sweet suspension cells	Opaque2, C1, R	9, 116
		Endosperm-derived suspension culture cells	Opaque2, Viviparous1	46, 54, 71, 116, 125

by the addition of phytohormones, elicitors, or other inducers of gene activity (25, 41, 46, 48, 54, 95, 102, 117, 125, 128).

The choice of assay can be crucial for accurate assessment of transcription factor action because the requirements for cofactors or posttranscriptional modifications should be considered. This was shown in the case of C1 and P, two maize Myb-like transcription factors. In vitro, both proteins can bind the same promoter element of the dehydroflavonol reductase (*A1*) promoter (58, 93, 114). But while P is sufficient to activate reporter gene expression from the *A1* promoter in a maize suspension culture system, the closely related C1 protein requires the presence of the Myc-like protein B (or R) for transcriptional activation. C1 cannot activate transcription in tissues unless either B or R is expressed (58, 93).

Activator studies require the target promoter to be silent or of low activity in the absence of ectopic effector. It is difficult to test stress- or wounding-induced promoters in transient assay systems, because these promoters can be activated during tissue preparation (90, 91). The tissue from where the effector originates is generally not suitable for transient assays because it contains endogenous transcription factor. Consequently, the ideal test tissue would be derived from a mutant where the transcription factor is not expressed. This strategy has been employed in studies of the maize transcription factors C1, P, R, B, and Viviparous1 (VP1). The complex developmental and interactive regulation of these proteins was elucidated using transient transformation experiments in mutant maize tissue and also in different suspension cultures of known genetic composition (46, 48, 71, 125). It was shown that the VP1 protein is a transcriptional activator of the anthocyanin regulator C1 (46) and the seed maturation–associated EM protein (71). Application of abscisic acid to the VP1 transient assays led to a synergistic increase in the activation of EM (71) but had only an additive effect on the expression of C1 (46). During seed maturation, VP1 represses the expression of the seed germination–specific α-amylase gene, and overexpression of VP1 during seed germination can even reverse the activating effects of gibberellic acid on α-amylase gene expression (48).

Yeast as an Alternative Assay

Studies in yeast can provide important information about plant transcription factors and can be useful in defining their DNA-binding specificity. These studies are possible because certain classes of transcriptional regulators activate transcription through activation domains that can mediate transcriptional activation in plants and in yeast, e.g. acidic activation domains (73, 91, 92, 106). Other classes of activation domains, e.g. the glutamine-rich class, are not active in yeast (82) and cannot be productively modeled in this heterologous host.

For the study of Myb-like transcription factors, DNA-binding specificity and activation potential have been characterized from a range of promoter elements for genes of the phenylpropanoid biosynthetic pathway (73, 91, 92, 106). Activation studies in yeast were used to confirm results obtained from a random binding site selection (RBSS) with Myb.Ph3 from petunia (106). Myb.Ph3, but not mutant forms deficient in DNA binding, could activate reporter gene expression from reporter constructs bearing consensus binding sites derived from RBSS, thus confirming its DNA-binding specificity. The same binding sites could be identified in a number of chalcone synthase promoters from various plant species, and it was shown that Myb.Ph3 can transcriptionally activate expression from a petunia chalcone synthase (*chsJ*) promoter in tobacco protoplasts (106).

The activity of the cauliflower mosaic virus 35S (CaMV 35S) promoter was the subject of several studies in yeast. This promoter is repressed in *Saccharomyces cerevisiae* but can be activated under nitrogen-limiting conditions and by cAMP. The regulatory elements of the CaMV 35S promoter involved in this activation were mapped to the *as*-1 element, which contains binding sites for the bZIP transcription factor TGA1 (87). Subsequently, it was shown that co-expression of TGA1 and a reporter gene regulated by either the CaMV 35S promoter or consensus *as*-1 elements can confer high levels of transcriptional activation in yeast cells (88, 122).

An interference between plant and endogenous yeast activators was observed in studies with the maize bZIP protein OPAQUE-2 (O2). O2 recognizes promoter elements that confer high levels of endosperm-specific storage protein expression in maize. Its cognate promoter elements share high sequence homology to the DNA-binding sites of the yeast bZIP transcription factor GCN4. Although it had been demonstrated in yeast that O2 can recognize and activate transcription from the yeast GCN4 DNA-binding site (70) and from the related plant promoter elements (49, 99), the results with the plant promoter elements also indicated that the activator function depended on an intact yeast GCN4 protein. O2 could activate transcription in a *gcn*4^{-} yeast mutant background only to a minor extent (49, 70); it was postulated that heterodimerization between maize O2 and yeast GCN4 is required for the formation of an active transcription factor (49).

In Vitro Transcription Assays

In vitro transcription is a powerful tool for studying general and activated transcription and in defining the requirements for initiation, elongation, and termination. In vitro transcription offers a number of advantages over in vivo studies in that transcription from the desired template can be studied using heterologously expressed or biochemically purified transcription factors. The lack of

reliable plant in vitro transcription systems in the past has meant that to date only one heterologously expressed plant transcription factor has been studied in in vitro assays. There, it could be shown in a wheat germ–based system that the tobacco bZIP protein TGA1a activates transcription by increasing the number of active pre-initiation complexes (129).

Several attempts have been made to establish reliable and reproducible in vitro transcription systems for plants using a variety of nuclear and cell extracts (107, 135). The most promising transcription system makes use of nuclear extracts from tobacco BY-2 cells (30, 31, 51, 131), a rapidly dividing cell culture. The BY-2 system supports transcription of RNA polymerase I– (31), RNA polymerase II– (30), and RNA polymerase III–dependent genes (30, 131). Accurate initiation of transcription was reported in all cases. Primer extension is generally used for the detection of transcript, but in the case of the $tRNA^{Ser}$ analysis it was possible to detect the transcript directly by the addition of radioactively labeled nucleotides to the transcription reaction (131). In the most intriguing report, transcription from the light-inducible tomato *Rbc*S promoter, which is not active in BY-2 nuclear extracts, is restored by the addition of leaf nuclear extract from light-grown tomatoes (30). This demonstrated that plant in vitro systems can be used to study plant-specific regulatory mechanisms by complementation and that the addition of transcription factors and cofactors can mediate transcriptional activation in a nonresponsive extract. A second in vitro transcription system is based on whole cell extracts derived from rice or tobacco. Using this system, it has been demonstrated that accurate transcription can be initiated from a rice phenylalanine ammonia-lyase gene and from a tobacco sesquiterpene cyclase gene promoter (136, 137). The main test for both in vitro transcription systems will be whether they can be reproduced independently in a number of laboratories.

TRANSCRIPTION FACTOR ANALYSIS IN PLANTA

In the absence of genetic analysis, identification of transcription factor target genes is one of the most demanding tasks in transcription factor studies. The high conservation between transcription factors has led to the identification of many orphan transcription factors of unknown function. Where mutants are available, downstream genes can be securely identified using genetic analysis and differential display or related techniques.

Two methods have principally been used to study the role of transcription factors in plants: overexpression and antisense technology. Overexpression, where a gene is expressed from a high-level constitutive or tissue-specific promoter in transgenic plants, can produce either plants that accumulate high levels of transcription factor or knock-out plants through inactivation of the transgene

and/or the endogenous gene by cosuppression (5, 94, 115). Antisense technology, where an RNA is expressed that is complementary to a target mRNA, is used to suppress expression of the endogenous transcription factor gene (11, 81, 121). Both approaches can cause lethal or strong pleiotropic effects in transgenic plants that cannot easily be differentiated from the desired phenotypes (94, 115). Overexpression and inactivation of the transgene do not necessarily occur in all plant cells and the extent of overexpression or cosuppression is difficult to assess. Transgene inactivation by antisense requires homology over as little as 50 bp, which makes it difficult to generate antisense plants for highly conserved transcription factors such as the class of MADS-box or the Myb-like transcription factors (11). High-level expression of a transcription factor in a plant cell might favor the binding of the transcription factor to low affinity binding sites and the activation of gene expression from noncognate promoters. In addition, it is difficult to assess whether genes, which are upregulated in an overexpressing line, are directly upregulated by the transcription factor under study or indirectly as a consequence of the expression of other genes.

An alternative technique, which allows the identification of mutants in specific genes, employs the polymerase chain reaction to screen large populations of plants containing T-DNA or transposon insertions (61). This approach is currently being used for the identification of mutant *Myb* loci in Arabidopsis (62), a family of transcription factors that comprises around 100 members in this plant species. The outcome of this research should provide valuable information regarding the role of the many different Myb proteins in Arabidopsis and their functional and genetic redundancy.

Inducible Gene Expression

Inducible gene expression is used to avoid problems associated with overexpression. It allows the temporal, spatial, and quantitative control of gene expression in a mutant for the transcription factor or in a heterologous host plant or tissue (33). To prevent interference from endogenous plant genes, inducible systems are based on nonplant components. In uninduced conditions, the transgene is not expressed or not active, should not interfere with normal plant development, and therefore should not cause pleiotropic effects. Gene activation or expression should occur rapidly after induction and be stable for a defined period of time. The inducing signal should be readily perceived, taken up, distributed within the plant, and be active in the whole plant or a tissue of interest in a dosage-dependent manner.

Several inducible gene expression systems have been developed for plants that fulfill one or several of these criteria (33). To study plant transcription factors, posttranslational induction makes use of animal steroid–inducible receptors (6, 7, 65, 95, 105). The steroid-binding domain of the glucocorticoid

receptor is fused to a plant transcription factor. The transcription factor accumulates in the inactive unliganded state (79, 80). It is thought that the unliganded hormone-binding domain represses nuclear localization, DNA binding, and perhaps other activities of the transcription factor. After induction by application of a steroid, repression is relieved, and active protein can rapidly enter the nucleus and exert its transcription factor function. Glucocorticoid receptor fusion proteins were first tested in transiently transformed tobacco protoplasts, and induction was achieved using the glucocorticoid derivatives dexamethasone and progesterone (95). A glucocorticoid-responsive GAL4-VP16 fusion protein has been used to induce the activation of a luciferase reporter gene in transgenic Arabidopsis and tobacco plants, either by growing the plants on nutrient agar containing dexamethasone or by spraying the plants with the inducing compound (6). Induction of the target gene was dosage-dependent and could be observed within 1 h after application of the chemical. Four different glucocorticoid derivatives were tested for their induction levels and sustainability of induction. In this and other studies, glucocorticoids had no visible impact on wild-type plants.

The maize regulatory protein R was studied using a glucocorticoid-based system by inducing its overexpression in the Arabidopsis mutant transparent testa glabra (*ttg*) (65); *ttg* mutant Arabidopsis plants lack trichomes, anthocyanins, and seed coat pigment and produce excess root hairs. Production of trichomes and anthocyanins was restored by overexpression of the maize transcription factor R in a constitutive and inducible manner. Trichomes started to form 24 h after immersion of plants in inducing solution. The number of trichomes formed on a leaf and anthocyanin accumulation in plants depended on the concentration of the glucocorticoid.

In another example, the Arabidopsis gene *Constans* (*Co*) was expressed as a glucocorticoid fusion in a *co* mutant background (105). CO is a protein with homology to the GATA class of transcription factors, and mutations in CO delay flowering under long day conditions but have almost no effect under short days. The onset of flowering is CO dosage–dependent. After induction by dexamethasone, Arabidopsis plants carrying a CO-glucocorticoid transgene flowered earlier than the untransformed *co* mutant plant. Promotion of flowering could be observed at any stage in plant development, from early in germination until the time when the mutant plant would form flowers. Transcripts of the floral meristem-identity gene *Leafy* and of *Terminal Flower* could be detected by in situ hybridization 24 h after CO induction, indicating that these genes are downstream of CO.

Using inducible expression of transcription factors in combination with differential display should make a large contribution to the identification of target genes activated by a transcription factor. To date, at least two reports exist where this approach has been successful (16, 89).

A Viral Vector for the Ectopic Expression of Transcription Factors

Studying the interaction between transcription factors and their promoter binding sites is difficult. Ideally, it requires the generation of independent transgenic lines carrying either the reporter or the effector gene. These lines are crossed to study the effects of the transcription factor. This problem was circumvented in one study by using a potato virus X (PVX)-based vector (14, 90). Leaves from transgenic tobacco plants carrying different versions of the bean phenylalanine ammonia-lyase 2 (*PAL2*) promoter regulating a GUS reporter gene were inoculated with a PVX-construct expressing Myb305 (90). Myb305 from snapdragon is a putative homologue of the tobacco transcriptional activator that regulates the *PAL2* promoter in tobacco petals (91). Ectopic expression of Myb305 using PVX produced high concentrations of transcription factor in the infected tissue and resulted in the expression of the GUS reporter gene in plants carrying the wild-type but not a mutant *PAL2* promoter element (90). The relative instability of the vectors and the restricted host range of the virus may, however, limit its use.

TRANSCRIPTION FACTOR DOMAINS

Traditionally, transcription factors have been described as modular proteins containing a variety of domains for DNA binding, activation, binding of signaling molecules, and interaction with other proteins. Nuclear localization motifs regulate the import of transcription factors into the nucleus. Modularity permits the combinations of different domains to form transcription factors with discrete functions from a relatively small number of components.

Gene Fusions for the Identification of Protein Domains

DNA-binding domains are usually highly conserved and can often be identified from the primary amino acid sequence. In contrast, activation domains are not so conserved and can only be classified by their overall amino acid composition as being rich in acidic, glutamine, or proline residues (113).

Fusion of a putative activation domain to a known DNA-binding domain can define an activation domain even for a transcription factor with no defined target promoters. The DNA-binding domain of the yeast transcriptional activator GAL4 is most frequently chosen to “host” the putative activation domain. GAL4-transcription factor fusion constructs can be tested in transient assays for the activation of GAL4 responsive reporter genes. GAL4 fusions have been used to identify the activation domains of the Arabidopsis G-box binding factor GBF1 (97); the maize activators O2 (118), C1 (35), and VP1 (71); the bean protein PvAlf (8); and the group of alternatively spliced rice transcription factors OSH42, OSH44, and OSH45 (108). Although there are two reports

where the GAL4 DNA-binding domain alone could confer reporter gene activation (74, 98), generally this has not posed a problem. Addition of GAL4 binding sites to a minimal promoter increased the background activity of the reporter construct in the GBF1 study, but this was negligible when compared to the expression levels obtained with the activator fusion proteins (97).

Auxin-responsive elements (AuxREs) of the soybean *GH3* promoter are required but not sufficient for auxin-inducible gene expression (117). Promoter sequences flanking the AuxREs were constitutively active when the AuxRE element was deleted or mutated. Promoters containing the AuxRE element and its flanking sequences were silent, but gene expression could be activated through the addition of auxins. Using a composite promoter containing GAL4 binding sites and an auxin-responsive element (AuxRE), the heterologous transcriptional activator GAL4-cRel can only activate transcription from this promoter when it is derepressed by the addition of auxin (117).

Not all DNA-binding proteins are transcriptional activators. To show that a DNA-binding protein can recognize a putative promoter target sequence, fusions of the DNA-binding protein to a strong activation domain can be tested. The activation domains of the herpes simplex virus protein VP16 and the yeast activator GAL4 both act as strong activation domains in plants. VP16 fusions have been used to show DNA recognition of the parsley bZIP protein CPRF1 (common plant regulatory factor 1) (32) and the maize regulator VP1 (71). CPRF1 could not activate a GUS reporter gene on its own but yielded high levels of activation once fused to VP16 (32). When the VP16 activation domain was used to replace the endogenous activation domain of VP1, the resulting VP1/VP16 fusion protein was significantly less active than the original VP1 protein but was still a strong transcriptional activator (71). In the case of C1, the endogenous activation domain was replaced by the activation domain of GAL4. Activation by the C1-GAL4(AD) fusion protein from the *Bronze1* target promoter was still dependent on the presence of the Myc-like cofactor B-Peru, suggesting that B-Peru interacts with C1 at its DNA-binding domain (35).

Nuclear Localization of Transcription Factors

The import of transcription factors from the cytoplasm into the nucleus is a necessary and important step in posttranslational control (64). For small proteins (<40–60 kDa), this import could take place by diffusion, but most proteins require nuclear localization signals (NLSs) for selective import into the nucleus (26, 84). These NLSs can be recognized by transporter proteins that shuttle between the cytoplasm and the nucleus or by import receptors located at nuclear pores (26, 64, 84). NLSs have been described as rich in arginine and lysine residues and have been classified into three groups: those

resembling the SV40 large T type antigen NLS (PKKKRKV), those resembling the NLS of the yeast mating type factor Matα2 (KIPIK), and NLSs with a bipartite structure, which are usually a combination of two basic protein regions separated by approximately 10 amino acids (e.g. nucleoplasmin SPPKAV**KR**PAATKKAGQA**KKKK**LDKEDES) (26, 84).

The nuclear localization signals in a range of plant transcription factors have been identified mainly in transient transformation assays using GUS-fusion constructs (1, 72, 104, 120, 124). After histochemical staining of transformed tissue, GUS enzymatic activity is identified in the cytoplasm or the nucleus. C- and N-terminal OPAQUE-2 (O2)/GUS reporter gene fusions showed that O2 possesses two NLSs, designated NLS A (SV40-like motif) and NLS B (bipartite motif) as observed in stable tobacco transformants and in transiently transformed onion epidermis cells (124). Both NLSs conferred nuclear localization to the O2/GUS protein independently, with NLS B being more efficient than NLS A. The nuclear localization function of the bipartite NLS B is located at the basic domain of the bZIP domain of O2, which is involved in DNA binding. That the nuclear localization function in O2 is independent from its ability to bind DNA was demonstrated with an O2 mutant deficient in DNA binding (123). More recently, the presence of NLS-binding proteins (NBPs) for the O2 NLS B peptide at the nuclear pores of tobacco cells has been suggested (47).

The MADS-box transcription factors APETALA3 (AP3) and PISTILLATA (PI) belong to the B class of organ identity genes and are involved in the formation of petals and pistils in Arabidopsis (130). Several lines of evidence suggested that AP3 and PI form a heterodimer (37, 85, 130). Using C- and N-terminal GUS fusions, it was found that nuclear import of AP3 and PI was mutually interdependent (72). Expression of AP3/GUS or PI/GUS fusion proteins alone resulted in cytoplasmic localization of the fusion proteins. However, when one fusion protein was expressed together with the other transcription factor in its native form, nuclear localization of the GUS fusion protein was observed. Primary results from transiently transformed onion epidermis cells were confirmed by stable Arabidopsis transformation.

The use of green fluorescent protein (GFP) as a reporter gene for nuclear localization could allow the observation of nuclear import and export as a dynamic process. Attempts to use GFP for nuclear localization studies have, however, only been successful when a full-length transcription factor was fused to GFP (110). GFP fused to short NLS sequences appears to diffuse freely in and out of the nucleus, perhaps because of its small size (39). To circumvent this, GFP and GUS were jointly fused to an NLS for nuclear localization studies (39).

Apart from the use of reporter genes, a number of immunochemical techniques have been employed to study nuclear import of transcription factors.

The cellular distribution of the tomato heat shock proteins HSFA1 and HSFA2 was studied in tobacco protoplasts using immunofluorescence (68). While under stable temperature conditions, the heat shock factors appeared to be present in the cytoplasm and the nucleus, a clear shift in distribution from the cytoplasm to the nucleus could be observed after temperature stress. Light-dependent transport of the G-box binding factor (GBF) to the nucleus was examined using an in vitro GBF antibody cotranslocation assay (44). The rationale of these experiments was that only under conditions that cause the nuclear import of GBF would the cotransported GBF antibody also be present in the nucleus. Therefore, dark-grown evacuolated parsley protoplasts were incubated with antisera against GBF and subsequently irradiated by light. Nuclear extracts were prepared and the presence of antibody was evaluated by western blot analysis. Antibody was detected in the nuclear fraction only after light treatment, indicating that the transport of GBF from the cytoplasm to the nucleus was light-dependent. Conditions reported to inhibit nuclear import, e.g. addition of lectins and decrease of the incubation temperatures, could significantly decrease GBF import into the nucleus.

PROTEIN-PROTEIN INTERACTIONS

Regulation of transcription factors through heterodimerization (63) or modifications by protein kinases have been shown to be important in transcription factor activation or inactivation and in determining their DNA-binding specificity (50, 55).

Protein-Protein Interaction Studies In Vivo

Traditionally, biochemical techniques such as immunoprecipitation (37, 85, 132) or expression library screening with radiochemically labeled proteins (132) were used to identify protein-protein interactions. The yeast two-hybrid system can now be used for finding proteins that interact with a protein of interest (15, 19, 28). The protein of interest is translationally fused to the DNA-binding domain of the yeast transcription factor GAL4 or the bacterial repressor LexA in a yeast expression vector. This "bait" construct is cotransformed into yeast with "prey" constructs bearing translational fusions that combine either a known protein or a cDNA expression library with a strong activation domain. If the expressed bait and prey proteins can interact, they reconstitute a functional transcription factor recognized as reporter gene activation from a promoter bearing GAL4 or LexA binding sites (15, 19, 28).

The two-hybrid assay faces some potential difficulties in that many transcriptional activators, e.g. those belonging to the class of acidic activators, are also transcriptional activators in yeast (34, 73, 91, 106). This will lead to

auto-activating bait proteins where reporter gene expression is activated in the absence of a prey construct. Nevertheless, many plant transcription factors do not activate transcription in yeast, and in several cases it has been possible to use the two-hybrid system for the identification of proteins that interact with transcription factors (23, 27, 34, 78, 93, 100).

The interaction between the maize Myc-like factor B and the Myb-like factor C1 was studied with the two-hybrid assay (34, 93). Fusions of both proteins to the GAL4 DNA-binding (DBD) and activation (AD) domains were prepared, but the GAL4(DBD)/C1 fusion strongly activated reporter gene expression in yeast (34). In contrast, GAL4(DBD) fusions of B and a fragment of B did not function as activators. When the GAL4(DBD)/B fusions were cotransformed with expression constructs encoding C1 fused to the GAL4(AD) or even C1 alone, high levels of reporter gene activity were obtained with the bait construct containing the B fragment but not with the full-length B protein. This suggested a physical interaction between B and C1 and also that C1 confers transcription activation potential to the B/C1 heterodimer (34). The fact that the B/C1 interaction could only be observed with a fragment of B and not with the full-length protein highlights that proteins can be misfolded or that interacting domains are not always displayed properly in the context of protein fusions. In a further study in yeast, it was shown that a deletion mutant of C1, C1-I, which is deficient in DNA binding, can still interact with the B protein, indicating that interaction with B and DNA binding are independent functions of C1(93). These findings were also of interest because C1 has similar DNA-binding specificity to the Myb-like regulator P, which regulates phlobaphene synthesis (40, 93). Both proteins can bind to the *A1* promoter in vitro. In vivo P alone can activate *A1* but C1 requires co-expression of B or R to activate transcription in plant cells. C1 alone can act as a strong transcriptional activator in yeast when fused to the GAL4 DNA-binding domain (40, 93). Therefore, it has been suggested that B relieves an inhibitory masking of the C1 activation domain by the C1 protein itself (93).

Several new transcription factors have been identified in a two-hybrid screen using a flower-specific cDNA expression library with the floral homeotic MADS-box transcription factors PLENA, DEFICIENS, and GLOBOSA from *Antirrhinum majus* as bait (23). Using northern blotting analysis and in situ hybridization, it was confirmed that the cDNAs encoding interacting domains were expressed in the same tissues as the bait constructs. The screen with DEFICIENS detected only GLOBOSA cDNAs, and reciprocally the screen with GLOBOSA detected only DEFICIENS cDNAs, suggesting that both transcription factors interact exclusively with each other. This screen also confirmed biochemical data obtained from the Arabidopsis homologues APETALA3 and PISTILLATA, which suggested that these proteins also form a heterodimer (37, 85). SQUAMOSA and three novel MADS-box factors were identified

with the PLENA construct. The interaction with SQUAMOSA was the most interesting because SQUAMOSA is also expressed in tissues where PLENA cannot be detected. This indicated that SQUAMOSA might also exert a function that is independent from the interaction with PLENA, perhaps by interaction with other MADS-box factors. The novel MADS-box factors, DEFH42 and DEFH200, have putative orthologues in petunia and tomato, FBP2 and TM5, and studies in these plants underline their role in PLENA action (5, 81). A third MADS-box factor, DEFH49, showed high homology to the Arabidopsis protein AGAMOUS-LIKE 2 but had a different expression pattern from the Arabidopsis protein.

Posttranslational Modifications

Many stimuli that affect gene expression also activate protein kinases. Some transcription factors are directly regulated by phosphorylation (50, 55) at three levels: import of transcription factors or associated proteins into the nucleus, enhancement or repression of the DNA-binding affinity, and positive or negative regulation of the activation potential. Regulation at several distinct levels could be achieved by phosphorylation at different sites by different protein kinases.

Casein kinase II (CKII) is hypothesized to be involved in central cellular functions such as cell division and growth, gene expression, and DNA replication (50). Several casein kinases have been identified from plants (38, 59, 60, 133) that potentially participate in the regulation of signal transduction pathways by counteracting the activity of protein phosphatases. It has been shown that the DNA-binding activity of the Arabidopsis GBF1, which interacts with a conserved element in the promoter of several light-inducible genes, is regulated by phosphorylation (59, 60). Purified nuclear CKII from broccoli (60) and a recombinant and reconstituted CKII from Arabidopsis (59) have been used to phosphorylate GBF1 and enhance its DNA-binding activity in an ATP- or GTP-dependent manner. DNA-binding activity was lost upon treatment with calf alkaline phosphatase and could then be rescued by rephosphorylation with CKII (60). In a different study on GBFs, cytosolic and nuclear protein extracts from parsley were tested for their ability to bind to the G-box (44). Upon dephosphorylation with alkaline phosphatase, DNA-binding activity in both fractions was almost completely lost. By adding ATP to the protein extracts, G-box binding activity could be restored in the cytosolic fraction but not in the nuclear fraction, suggesting the presence of a protein kinase in the cytosol that can regulate GBF DNA-binding activity. A different study revealed that the maize transcription factor O2 is phosphorylated in vivo and can be phosphorylated in vitro by CKII (20). Only nonphosphorylated and hypophosphorylated forms could bind target DNA sequences. The different phosphorylated forms of O2 were separated by isoelectric focusing. When these gels were blotted and

probed with an O2 target DNA sequence, no DNA binding was observed for the phosphorylated forms unless the protein blot had been treated with potato acidic phosphatase. It was also shown that the profile of phosphorylated to hypophosphorylated forms changed diurnally, suggesting that storage protein synthesis might be slowed down at night and that this event is regulated by phosphorylation of O2 (20).

DNA-PROTEIN INTERACTIONS

Most promoters contain an array of *cis*-elements that can be recognized by transcription factors. Promoter studies are usually initiated by fusing promoter regions to a reporter gene and assaying reporter gene activity in transiently or stably transformed plants. Defined promoter regions can then be further characterized by electrophoretic mobility shift assays (EMSAs) where short promoter fragments are used in binding reactions with a nuclear protein extract or a heterologously expressed DNA-binding protein. More detailed analysis can be carried out using footprinting techniques or random binding site selection (RBSS), which allow the identification of bases important for transcription factor binding.

Electrophoretic Mobility Shift Assay

EMSA, the most widely applied technique for studying DNA-protein interactions, is based on the ability of a DNA-binding protein to alter the mobility of DNA in a nondenaturing acrylamide gel (12). EMSA is a reliable and simple technique for demonstrating the specific binding of a protein to a particular DNA sequence, but it provides only limited information about the bases that are directly involved in protein binding. Using antibodies, it is possible to confirm the identity of a protein present in a DNA-protein complex because the addition of the antibody to the binding reaction can supershift the complex or inhibit complex formation (17, 73, 91).

EMSAs can be used for the identification of DNA-binding domains. Several lines of evidence suggested the presence of multiple DNA-binding domains in the rice transcription factor GT-2, which can recognize three different GT-boxes in the rice phytochrome A gene promoter (24). Using a range of truncated GT-2 polypeptides, the boundaries of two minimal DNA-binding domains could be defined by EMSA. These two DNA-binding domains bound preferentially to the GT-2 and the GT-3 box respectively, and their binding specificity was further enhanced by flanking peptide sequences (75).

EMSAs have also been used to study the ability of DNA-binding proteins to form homo- and heterodimers, e.g. in the case of the Arabidopsis bZIPs GBF1, GBF2, and GBF3 (96). While addition of one GBF to the binding

reaction resulted in a single band representing the homodimer, addition of a second GBF led to the formation of two or three bands representing homo- and heterodimeric complexes. For each GBF protein, a different potential for homo- and heterodimer formation was observed.

The effect of covalent modifications on the DNA-binding ability of a protein can also be studied by EMSA. Phosphorylation by CKII of the bZIP protein GBF1 was shown to stimulate its DNA-binding activity (59), while it has been reported for other transcription factors that their DNA-binding ability can be enhanced by dephosphorylation (22, 73).

DNA Footprinting Techniques

Footprinting techniques have been used extensively to determine transcription factor binding sites on promoter fragments. Footprinting methods are based on either chemical modification and subsequent cleavage of the promoter DNA or on DNase I treatment (2, 127). When the resulting DNA fragments are separated on denaturing acrylamide gels, residues protected by the protein are identified as missing or underrepresented bands.

In vitro footprinting with DNase I exploits the ability of DNase I to nick randomly either strand of DNA. Treatment with DNase I can take place before (interference) or after (protection) protein binding, but the latter technique is far more frequently used. DNase I footprinting was used to delineate the binding sites for the bean repressor proteins ROM1 and ROM2 (17, 18). ROM1 and ROM2 antagonize the activating function of the bean VP1-homologue PvAlf in the promoters of the storage protein genes phytohemagglutinin and β-phaseolin. Using recombinant proteins, it was shown that the bZIPs ROM1 and ROM2 recognize almost identical elements in the promoters of both target genes, but expression studies indicate that they do so at different times during seed maturation. While ROM1 expression precedes the onset of phytohemagglutinin and β-phaseolin expression (18), ROM2 expression coincides with a decrease in the transcription of the storage proteins at later stages of seed maturation (17).

In a number of cases, it has been possible to link chromatin structure and DNA binding using footprinting techniques. In the case of the wheat bZIP Em-binding protein (EmBP-1), Em promoter DNA was assembled in vitro with nucleosome cores from wheat and HeLa cells (76). It was shown that EmBP-1 binding is reduced in packaged DNA compared with naked DNA and that the position of the binding site within the nucleosome contributes to the extent of DNA binding. Although this study showed that histone octamers inhibit DNA binding by EmBP-1, linker histone 1 (which is not a component of the histone octamer complex) can enhance DNA binding of EmBP-1 in EMSAs (101).

Dimethyl sulfate (DMS) (91, 96, 97, 126), diethyl pyrocarbonate (DEPC) (40, 126), or 1,10-phenanthroline-copper (53, 57, 126) are used in chemical footprinting. Treatment with the chemical agents modifies specific nucleotides, and subsequent piperidine treatment breaks the DNA strand at the site of modification. The different modifying reagents react with different nucleotides, and therefore the choice of reagent depends on the base composition of the DNA fragment under study. A combination of three different footprinting techniques has been used to delineate the DNA-binding sites of the Arabidopsis Myb-like transcription factor CCA1 of the light-harvesting chlorophyll *a*/*b* protein gene (126). Only limited information about the cognate binding sites of CCA1 was obtained from DMS and DEPC footprints because of their AT-rich nature, as DMS and DEPC modify only guanine and adenine residues. 1,10-phenanthroline footprinting, however, which can modify every base, allowed the delineation of the CCA1-binding site (126).

Protein-DNA interactions over extensive promoter fragments can be studied in vivo in the context of intact, transcriptionally active chromatin using footprinting techniques. In vivo footprinting allows the detection of temporal and spatial interactions between DNA-binding proteins and their cognate elements. In vivo footprinting was used to confirm binding of the Arabidopsis bZIP transcription factor GBF3 to the G-box of the alcohol dehydrogenase promoter (66). Comparison of the footprints detected in vivo and in vitro suggests that GBF3 binds to the protected site. The extended sequence homology between different members of the bZIP family over their DNA-binding domain results, however, in similar DNA-binding signatures in vitro and makes it difficult to discriminate between interactions of different GBFs with a particular promoter element.

While the standard in vivo footprinting techniques are generally carried out using cell cultures, in vivo footprinting coupled with ligation-mediated polymerase chain reaction (LM-PCR) allows the analysis of transcription factors in the tissues from where they originate, when a suitable cell culture is not available (42, 43). In this technique, linkers are ligated to cleaved genomic DNA fragments that can then be used to amplify the resulting DNA fragments by PCR (42). Using LM-PCR, it was shown that the endosperm box of a wheat glutenin promoter is protected during the early stages of endosperm development in wheat grains (43). This observation was consistent with results that show that this element confers endosperm-specific expression in transgenic tobacco (21). The footprint was detected only in endosperm tissue. It was shown that the endosperm box consists of two distinct sites that are occupied sequentially by different proteins just before glutenin expression reaches its maximum levels (43).

Random Binding Site Selection

The range and degree of the DNA-binding specificity of a transcription factor can be determined by random binding site selection (RBSS). RBSS is based on the amplification of specific protein binding sites from a pool of randomized DNA sequences (77). This technique is particularly useful for defining differential DNA binding and the role of sequences flanking a core consensus site for different members of the same transcription factor family. It is difficult to interpret the results from RBSS when nothing is known about the cognate binding site of the transcription factor.

RBSS has been used successfully to determine the binding specificity of Arabidopsis GBF1 (97), which has been cloned by means of its binding to the tomato *RbcS*-3A G-box-like element (A**CACGTG**G) (96). GBF1 was also shown to bind to the "perfect" G-box (C**CACGTG**G) in the promoters of Arabidopsis *RbcS*-1A and alcohol dehydrogenase, and of parsley chalcone synthase, and to several other G-box–like elements, which raised the question of its DNA-binding specificity. RBSS, together with EMSAs, revealed that only sequences that contained the core motif ACGTG were bound by GBF1 with high affinity. In addition, specific bases flanking the G-box core motif were found to be required for high-affinity binding. Substitutions of bases in the core motif resulted in low-affinity binding (97).

RBSS has been employed to define binding specificity in the case of the maize Myb-like proteins P and C1, which regulate the expression of several genes in the flavonoid biosynthetic pathway (58, 93, 114). Both C1 and P recognize the same binding site ($CC^{T}/_{A}CC$) in the *A1* promoter (93). RBSS with C1 and P revealed that C1 has a more diverse binding spectrum than P, suggesting that the broader DNA-binding specificity of C1 results in an increased number of target promoter sites (93).

PERSPECTIVES

In this review, techniques were described that can be employed for transcription factors studies in plants. However, it is apparent that certain aspects of regulated gene expression cannot as yet be studied by the techniques currently available. Major advances are needed in the development of reliable in vitro systems and protein purification procedures from plants. This should allow the investigation of processes that are downstream of transcription factor–promoter recognition, such as the mechanisms of transcriptional activation and repression, and transcription factor–mediated changes in chromatin structure. Several recent reports describe also the integration of signal transduction cascades and gene expression (13, 109, 134). This research together with other approaches will soon provide us with an accurate understanding of the function of plant transcription factors.

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