Overexpression analysis of plant transcription factors

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Transcription factors (TFs) play important roles in plant development and its response to the environment. A variety of reverse genetics tools have been developed to study TF function, the two most commonly used ones being knockout and overexpression. Because of the unique characteristics and modes of action of TFs, the overexpression strategy has been particularly effective in revealing TF function. Thus, a number of overexpression-based methodologies — constitutive expression, tissue-specific expression, chemically inducible expression and overexpression of modified TFs – have been developed and are used in the analysis of TF function.

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Abbreviations

AG	AGAMOUS
AP3	APETALA3
bHLH	basic helix–loop–helix
CBF4	C-BOX BINDING FACTOR4
CYC	cycloheximide
DEX	dexamethasone
GL1	GLABRA1
GR	glucocorticoid receptor
PI	PISTILLATA
RNAi	RNA interference
SEP3	SEPALLATA3
STM	SHOOT MERISTEMLESS
TF	transcription factor

Introduction

Transcription factors (TFs) are sequence-specific DNA-binding proteins that are capable of activating and/or repressing transcription. They are largely responsible for the selectivity in gene regulation, and are often expressed in a tissue-specific, developmental-stage-specific, or stimulus-dependent manner. As many biological processes in plants are regulated at the level of transcription, not surprisingly, the evolution of many morphological traits during the domestication of plants has been associated with changes in TFs [1] or their regulation [2]. Therefore, understanding TF function is an important step towards understanding plant development and evolution.

With the completion of the *Arabidopsis* genome sequence, the identities of the complete set of *Arabidopsis* TFs are now available, and all of the TFs within a plant species can be functionally characterized for the first time [3–5]. Because most TFs are grouped into families according to their well-conserved DNA-binding domains [3], the genes that encode them can be identified easily from the genome sequence, making them particularly amenable for genomics research. As a result, a variety of reverse genetics tools have been developed to study TF function, the two most commonly used ones being knockout and overexpression.

In this review, I first compare and contrast the knockout and overexpression techniques, and illustrate how the unique characteristics and modes of action of TFs make the overexpression strategy particularly effective. I then summarize the various overexpression-based methodologies — constitutive expression, tissue-specific expression, chemically inducible expression and overexpression of modified TFs — and the rationales behind them to illustrate how these techniques are used to analyze TF function.

Functional genomics of TFs: knockout versus overexpression

Traditionally, geneticists have relied on mutants (mostly knockouts or knockdowns) to study gene function. With the complete genome sequences of many organisms now available, it has become possible to undertake systematic and genome-wide analysis of gene function. Recently, large-scale knockout analyses of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* have been carried out using insertion mutagenesis and RNA interference (RNAi), respectively [6•,7•]. These studies have provided a wealth of information that was unimaginable just a few years ago. One of the key observations made during these experiments was that only a low percentage of mutants showed a phenotype. For example, 86% of the 19 427 predicted *C. elegans* genes were knocked out but only 10% of the resulting knockout mutants revealed any phenotype [7•]. Furthermore, genes that are present as a single copy in the genome are more than twice as likely to have an RNAi phenotype than those that are present in more than one copy (31% versus 12%, respectively). This observation led Kamath *et al.* [7•] to speculate that recently duplicated paralogs are at least partially redundant and thus responsible for the lower phenotype frequency.

Similar observations are beginning to emerge from studies that are based on plants. For example, although estimates suggest that the *Arabidopsis* genome has been

saturated with T-DNA and transposable element insertions, relatively few informative knockouts that provide a clue to gene function have been reported [8]. It is possible that the lack of phenotypes in knockouts could be explained by our inability to detect small phenotypic changes. Functional redundancy due to the extensive duplication of the *Arabidopsis* genome is also likely [9,10]. The redundancy problem can be especially acute for TFs because they are, in general, members of large gene families that often include closely related genes [3]. In several cases, knockout of multiple TFs has been necessary to produce informative phenotypes [11–13].

Overexpression offers an alternative and complementary strategy to knockout/knockdown analysis that is less affected by functional redundancy. In yeast, the large-scale overexpression of random genes has proven to be useful in identifying numerous genes that are involved in controlling the cell cycle [14]. The functions of these genes have been difficult or impossible to uncover using the recessive knockout approach. In plants, there are several recent examples in which knockouts, in these cases knockouts of TF genes, failed to generate informative phenotypes. Gene functions were revealed only when the genes were overexpressed [15–18]. Even when the knockout mutant has a clear informative phenotype, overexpression can still be valuable because it can generate completely unexpected phenotypes [19] and thus shed light on aspects of TF function that would be missed otherwise [20,21]. There are not yet enough direct data to allow us to compare and contrast the knockout and overexpression strategies, but the emerging theme is that both of these complementary strategies should be used to fulfill the tremendous potential that functional genomics has to offer.

Caveat of TF overexpression

The function of any TF can be defined at one level as the transcriptional network (or TF regulon) it controls. However, this analysis only addresses the TF's function mechanistically, and does not necessarily get to the real 'biological function' of the TF. For example, even though the drought-inducible TF C-BOX BINDING FACTOR4 (CBF4) largely regulates the same regulon as the cold-inducible TFs CBF1–3 (JZ Zhang, unpublished data), the 'biological function' of CBF4 (drought adaptation) is distinct from that of CBF1–3 (cold acclimation) [22•]. A similar example is WEREWOLF (WER) and GLABRA1's (GL1's) roles in root hair and trichome development, respectively [23]. In both of these examples, the 'biological functions' of the genes depend on the tissue specificity and/or their specific responses to environmental stimuli. Therefore, to understand the real role that a TF plays during plant development, overexpression data need to be interpreted in conjunction with other supporting data, such as the expression patterns of the TF, the interactions of the TF with other proteins and the phenotype of the knockout mutants.

Owing in part to the particular mode of action of TFs, which distinguishes them from most other proteins, there are other limitations to the overexpression strategy. Overexpression phenotypes, which usually (but not always) result from gain-of-function, can include two types: hypermorphs and neomorphs. Hypermorphs (high form) are overexpressors in which the introduced gene confers the same function as the endogenous gene but with higher activity. In neomorphs (new form), the introduced protein (because of its abundance or inappropriate tissue and/or developmental-stage context) confers a new function that is not present in wildtype. Therefore, despite the many advantages of the overexpression strategy and its numerous successes (Table 1), one of its limitations is the possibility of creating neomorphic alleles.

Neomorphic alleles of TF genes are commonly created by squelching. The transactivation domains within many TFs often interact with other components of transcription, thus squelching is defined as the repression of transcription of otherwise unrelated genes by sequestering limiting components (such as co-activators) that are required for transcription elsewhere [24,25]. The same process could also enhance the transcription of otherwise unrelated genes by sequestering repressors of transcription. Therefore, a gene whose expression depends upon the availability of co-factors could be influenced, positively or negatively, by the expression of a broad range of unrelated TFs. Consequently, one needs to be cautious when interpreting phenotypes that are caused by the overexpression of TF genes; transcriptional controls that are independent of DNA-binding should always be considered. It should be noted that certain neomorphs can be useful. For example, antimorphs (or dominant negative neomorphs) are overexpressed mutant TFs that can prevent the function of a normal endogenous protein (Table 1; see following section).

Strategies for TF overexpression

Overexpression of complete TFs using a strong constitutive promoter

In the most common form of gain-of-function TF alleles, the TF genes are overexpressed under a strong constitutive promoter, such as the cauliflower mosaic virus 35S promoter. The overwhelming majority of overexpression studies published recently have utilized the 35S promoter (Table 1). This strategy has been used successfully in numerous cases in which the overexpression phenotypes, together with other complementary data, are unambiguous in assigning TF function. Overexpression using this strategy often creates hypermorphic alleles, and thus the phenotype directly implies the endogenous function of the gene. For example, when overexpressed, many TFs are sufficient by themselves to confer dramatic phenotypes, such as abiotic stress tolerance [22•,26–32], disease resistance [33–35], the formation of ectopic somatic embryos ([36,37]; Figure 1), ectopic trichome formation

Table 1

Plant TFs whose function has been analyzed by transgenic approaches since 2001.

Name (family)	Promoter	Function category	Result/conclusion of overexpression study	Reference(s)
Overexpression of unmodified TFs				
LEP (AP2)	35S	Development	Overexpression results in an increase in the number of xylem cells, but knockout has no phenotype.	[15]
LMADS2 or EgMADS1 (MADS)	35S	Development	Lily and *Eustoma grandiflorum* MADS genes function when overexpressed in transgenic *Arabidopsis* plants to cause homeotic conversion of sepals and petals.	[81]
Oskn2 or Oskn3 (HD)	35S	Development	Overexpression of these two closely related genes results in similar, but not identical, phenotypic effects on panicle branching, internode elongation and leaf patterning in transgenic rice.	[82]
BBM (AP2)	35S, UbB1	Development	Overexpression is sufficient for the spontaneous formation of somatic embryos.	[36]
LEC2 (ABI3/VP1)	35S	Development	Overexpression is sufficient to induce ectopic embryo formation on seedlings.	[37]
ZmMADS3 (MADS)	pAct1 (rice actin)	Development	Overexpression affects plant height and male spikelet development in maize, but the precise function of the gene can not be determined by the overexpression phenotype.	[83]
KAN (GARP)	35S	Development	Overexpressors have no well-defined adaxial and abaxial domains, supporting the conclusion that KAN promotes abaxial identity. In addition, overexpression provides clues to indicate that KAN may be involved in regulating peripheral identity in the embryo.	[84]
AtMYB23 (MYB)	35S	Development	Overexpression is sufficient to induce ectopic trichome formation.	[38]
ATHB13 (HD)	35S	Development	Overexpression leads to an overall plant phenotype and pattern of gene expression that are indicative of ATHB13's role in sugar sensing.	[85]
AGL24 (MADS)	35S	Development	Overexpressors are early flowering whereas mutants are late flowering. AGL24 and SOC1 upregulate each other in transgenic plants.	[46]
OsTB1 (bHLH)	Rice actin	Development	Knockout results in enhanced lateral branching, whereas overexpression results in reduced lateral branching.	[42]
ATHB-8 (HD)	35S	Development	Overexpression increases the production of xylem tissue in stems and promotes vascular differentiation, even though null *athb-8* mutants have no observable phenotype.	[16]
CCA1 (MYB)	35S	Development	Overexpression of CCA1 demonstrates that a functioning circadian system is important for plant viability.	[86]
Oshox1 (HD)	35S	Hormone action	Overexpression of Oshox1 in transgenic rice enhances polar auxin transport and the plant's sensitivity to auxin.	[87]
ABI5 (bZIP)	35S	Hormone action	Overexpression results in increased sensitivity to ABA and sugar, whereas mutants are ABA insensitive.	[43,44,88]
CnABI3 (ABI3/VP1)	35S	Hormone action	Overexpression of a yellow cedar ABI3 homolog in transgenic tobacco plants shows that this TF has a role that is similar to that of the ABI3-like proteins of angiosperms.	[89]
HAT2 (HD)	35S	Hormone action	Overexpressors have characteristics of auxin-overproducing plants in shoots, but less auxin sensitivity in roots. Thus, HAT2 plays opposite roles in shoots and roots.	[90]
ESR1 (AP2)	35S	Hormone action	A cDNA library was transformed into *Arabidopsis* roots and plants that had cytokinin-independent shoot formation were selected. ESR1 regulates the induction of shoot regeneration.	[91]
TGA5 (bZIP)	35S	Biotic stress	TGA5 interacts with NIM1/NPR1 and confers SAR-independent resistance of *Arabidopsis* to *Peronospora parasitica*.	[33]
AtWRKY18 (WRKY)	35S	Biotic stress	Overexpressors are more resistant to *Pseudomonas syringae*.	[34]

Table 1 Continued

Name (family)	Promoter	Function category	Result/conclusion of overexpression study	Reference(s)
Pti4,5,6 (AP2)	35S	Biotic stress	Overexpression of tomato genes in *Arabidopsis* results in the upregulation of defense genes and disease resistance.	[35,92]
ICE1 (bHLH)	35S	Abiotic stress	ICE1 regulates CBF3 and confers freezing tolerance when overexpressed.	[32]
CBFs (AP2)	35S	Abiotic stress	The *CBF* genes are sufficient to cause increased freezing and drought tolerance when overexpressed.	[22•,26,27,31,93]
ABF3/4 (bZIP)	35S	Abiotic stress	Overexpressors show growth retardation and increased drought tolerance and ABA, salt, and glucose sensitivity.	[28]
Tsi1 (AP2)	35S	Abiotic and biotic	Overexpression of the tobacco salt-induced gene *Tsi1* in tobacco plants results in improved tolerance of salt and pathogens.	[29]
SCOF-1 (Zn finger)	35S	Abiotic stress	Overexpression of SCOF-1 results in freezing-tolerant *Arabidopsis* plants and chilling-tolerant tobacco plants. SCOF-1 does not bind to DNA directly but interacts with an ABRE element through another TF.	[30]
Sn (bHLH)	35S	Biochemistry	The maize gene *SN*, which is involved in the control of anthocyanin biosynthesis, can transactivate both the anthocyanin and condensed tannin pathways in *Lotus*.	[41]
FaMYB1 (MYB)	35S	Biochemistry	Overexpression of strawberry FaMYB1 in tobacco decreases the biosynthesis of anthocyanins and flavonol quercetin, thus it is a repressor of their biosynthetic pathways.	[39]
TT2 (MYB)	35S	Biochemistry	Overexpression of TT2 induces the expression of BAN and proanthocyanidin accumulation.	[40]
Overexpression of multiple TFs				
AP1, PI, AP3, AG, SEP3 (MADS)	35S	Development	Triple and quadruple transgenes are sufficient to transform leaf organs into flower organs. These transgenics provide clear *in vivo* evidence of tertiary and quaternary protein interactions.	[56–58]
AP1 and SEP3 (MADS)	35S	Development	The early flowering phenotype of 35S::AP1 is enhanced by 35S::SEP3.	[55]
E2Fa and Dpa	35S	Development	Although either TF works by itself to some extent, both are necessary to induce ectopic cell division, enhanced endo-duplication, and the upregulation of S-phase genes.	[53]
AtMYC (bHLH) and AtMYB2 (MYB)	35S	Abiotic stress	Overexpression of both genes results in improved stress tolerance.	[51]
LC (bHLH) and C1 (MYB)	35S and/or fruit-specific E8	Biochemistry	Both *LC* and *C1* genes from maize are required to activate downstream genes and to upregulate flavanoid biosynthesis in transgenic tomato.	[54]
Overexpression of modified TFs				
LFY:VP16 (Leafy)	35S	Development	The activation domain of VP16 is used because it eliminates the requirement for other co-factors. A dominant-negative allele of *LFY* represses shoot identity genes.	[62]
RF2a (bZIP)	35S	Development	A dominant-negative version of RF2a that contains only the DNA-binding domain and the leucine-zipper region affects the development of transgenic tobacco plants.	[61]
TGA2 (bZIP)	35S	Hormone action	The *tag2* mutant has no phenotype. A dominant-negative version of TGA2, which contains the NPR1-interaction domain but no DNA-binding or transactivation domains, has the same phenotype as the *npr1* mutant, demonstrating that TGA2 interacts with NPR1.	[17,18]
GAI (GRAS)	35S and maize ubiquitin	Hormone action	Overexpression of either *gai* (constitutively active allele) or *GAI* (wildtype allele) results in dwarfism in transgenic rice, validating the de-repressible repressor model of GAI function.	[59]
RGL1 (GRAS)	35S	Hormone action	A dominant gain-of-function RGL1 (constitutively active) has been created by deletion of the DELLA motif, indicating that RGL1 (like GAI) is a negative regulator of GA responses.	[60]

Table 1 Continued

Name (family)	Promoter	Function category	Result/conclusion of overexpression study	Reference(s)
Tissue-specific expression				
SHR (SCR)	SCR and 35S	Development	Overexpression of SHR by the 35S promoter led to chaotic division patterns in the root meristem. Overexpression of SHR driven by the SCR promoter leads to increased cell layers that exhibit a high degree of radial symmetry, facilitating subsequent analysis. Also, the cell-specific expression of SHR has helped to address questions regarding protein movements.	[64,65]
DEF (MADS) and GLO (MADS)	L1 meristematic layer-specific promoter, AFI	Development	To assess the contribution of the epidermis to the control of petal and stamen organ identity, it is necessary to express genes ectopically in the epidermal layer only. The epidermal 'B function' autonomously controls the differentiation of *Antirrhinum* petal epidermis cell types, but cannot control the cell division and specification of sub-epidermal cells that have petal identity. Epidermal 'B function' in *Arabidopsis*, however, can control most epidermal and sub-epidermal differentiation events in petals and stamens.	[67]
WUS (HD)	LFY and AP3	Development	Because the flowers of *wus* mutant do not show homeotic organ transformations, it is not clear whether WUS directly regulates the expression of flower homeotic genes. As a test of WUS's ability to activate AG, two complimentary promoters were used to direct WUS expression. The ectopic formation of stamens and carpels, organs that are specified by AG, in the transgenic plants is the first evidence that WUS is a regulator of AG.	[66]
WUS (HD)	AG and AP3	Development	Tissue-specific expression of WUS reveals the feedback loop between WUS and AG. WUS induces AG, which in turn represses WUS.	[69]
SUP (Zn finger)	AP1	Development	AP1::SUP suppresses petal and stamen development.	[70]
SUP (Zn finger) or CRC (YABBY)	INO	Development	INO::SUP phenocopies *ino* mutations, whereas INO::CRC overcomes the inhibitory effects of SUP, indicating that SUP suppresses INO expression at least partly through the coding region of INO.	[72]
WUS and STM (HD)	35S, ANT, or CLV1	Development	WUS and STM were expressed under a variety of promoters, both in the wildtype background and in mutant backgrounds, to demonstrate the respective roles that WUS and STM play in shoot apical meristems. Glucocorticoid-inducible expression of WUS was used to circumvent the deleterious effects caused by constitutive overexpression of WUS.	[68]
HvGAMYB (MYB)	Multiple copies of locus	Development	An interesting strategy has been used to study the overexpression of this TF that involves inserting multiple copies of the same gene in the genome. Overexpression of the *HvGAMYB* gene in transgenic barley results in anther phenotypes that include male sterility.	[74]
Inducible TF activity				
R (bHLH)	35S	Development	Inducible expression of an *R* gene (a *GL3* homolog) results in the complete rescue of the 35S::CPC *gl* phenotype but not the 35S::TRY phenotype.	[21]
WUS (HD)	ANT	Development	Inducible WUS expression is used because ANT::WUS plants show meristem defects and fail to develop beyond the seedling stage. Inducible WUS expression demonstrates that WUS is sufficient to induce integument initiation.	[71]
LFY (LEAFY)	35S	Development	Glucocorticoid-inducible LFY, together with AP1, regulates the expression of AP3. CYC was used to inhibit the expression of secondary targets.	[80]
ATHB-2 (HD)	35S	Development	A fusion comprising the activation domain of VP16 and the hormone-binding domain of the glucocorticoid receptor and ATHB-2, in addition to the overexpression of the endogenous gene, was used to reveal the negative auto-regulation of the *ATHB-2* gene.	[94]

Table 1 Continued

Name (family)	Promoter	Function category	Result/conclusion of overexpression study	Reference(s)
ARR1 (MYB)	35S	Hormone action	Overexpressors are hypersensitive to cytokinin, whereas knockouts are hyposensitive. Overexpression of the dominant form (constitutively active without the hormone sensor) produces the same phenotype as the wildtype overexpressor treated with cytokinin. This indicates that the signal-receiver domain of ARR1 suppresses its function in the absence of cytokinin, and that the cytokinin signal releases this suppression. To establish a direct link between the ARR1 TF and the downstream gene *ARR6*, 35S::ARR1deltaDDK::GR was used to generate plants whose ARR1 function is induced by DEX instead of cytokinin. DEX treatment results in an increase of ARR6 transcript even in the presence of CYC. In fact, ARR6 transcript levels are higher and lower in BA-treated overexpression and knockout plants, respectively, indicating that ARR1 directly activates the transcription of ARR6 in response to cytokinin.	[45]

Abbreviations: ABA, abscisic acid; ABF3, ABRE-BINDING FACTOR3; ABI3, ABA-*INSENSITIVE3*; ABRE, ABA-RESPONSIVE ELEMENT; AFI, *Antirrhinum* FIDDLEHEAD; AGL24, AGAMOUS-LIKE24; ANT, AINTEGUMENTA; ARR1, *Arabidopsis*-RESPONSE REGULATOR1; ATHB13, *Arabidopsis thaliana* HOMEOBOX13; BA, 6-benzylaminopurine; BAN, BANYULS (Anthocyanin in seed coat); BBM, BABY BOOM; C1, COLORED ALEURONE1; CCA1, CIRCADIAN CLOCK ASSOCIATED1; CLV1, CLAVATA1; CRC, CRABS CLAW; DEF, DEFICIENS; Dpa, *Arabidopsis* Dp homolog a; E2Fa, *Arabidopsis* E2F homolog a; ESR1, ENHANCER OF SHOOT REGENERATION1; GAI, GIBBERELLIC ACID INSENSITIVE; GARP, GOLDEN2-, ARR- and PSR1-like genes; GLO, GLOBOSA; GRAS, GAI-, RGA-, SCR-like genes; HAT2, homeobox of *Arabidopsis thaliana*2; HD, homeodomain; ICE1, INDUCER OF CBF1; INO, INNER NO OUTER; KAN, KANADI; KN, KNOTTED; LC, RED LEAF COLOR; LEC2, LEAFY COTYLEDON2; LEP, LEAFY PETIOLE; LFY, LEAFY; NIM1, NON-INDUCIBLE IMMUNITY1; NPR1, NON-EXPRESSION OF PR GENES1; OsHOX1, *Orzya sativa* HOMEOBOX PROTEIN1; OsTB1, *Orzya sativa* TEOSINTE BRANCHED1; PTI, PTO-INTERACTING PROTEIN; R, resistance protein; RF2A, RF2A TF; RGA, REPRESSOR OF GA1–3; RGL1, RGA-LIKE1; SAR, systemic acquired resistance; SCOF-1, SOYBEAN COLD-INDUCIBLE FACTOR-1; SCR, SCARECROW; SHR, SHORT ROOT; SN, SCUTELLAR NODE COLOR; SOC1, SUPPRESSOR OF OVER-EXPRESSION OF CO; SUP, SUPERMAN; TGA5, TGACG BINDING FACTOR5; TRY, TRIPTYCHON; TSI1, TOBACCO STRESS-INDUCED GENE1; TT2, TRANSPARENT TESTA2; VP1, VIVIPAROUS1; WUS, WUSCHEL.

([38]; Figure 1), or changes in biochemical composition ([39–41]; Table 1). It can be particularly reassuring to confirm that the correct TF function has been identified when the overexpression phenotype is the opposite of the knockout phenotype [42–46], although it is still not clear how common this is.

Overexpression of multiple TFs

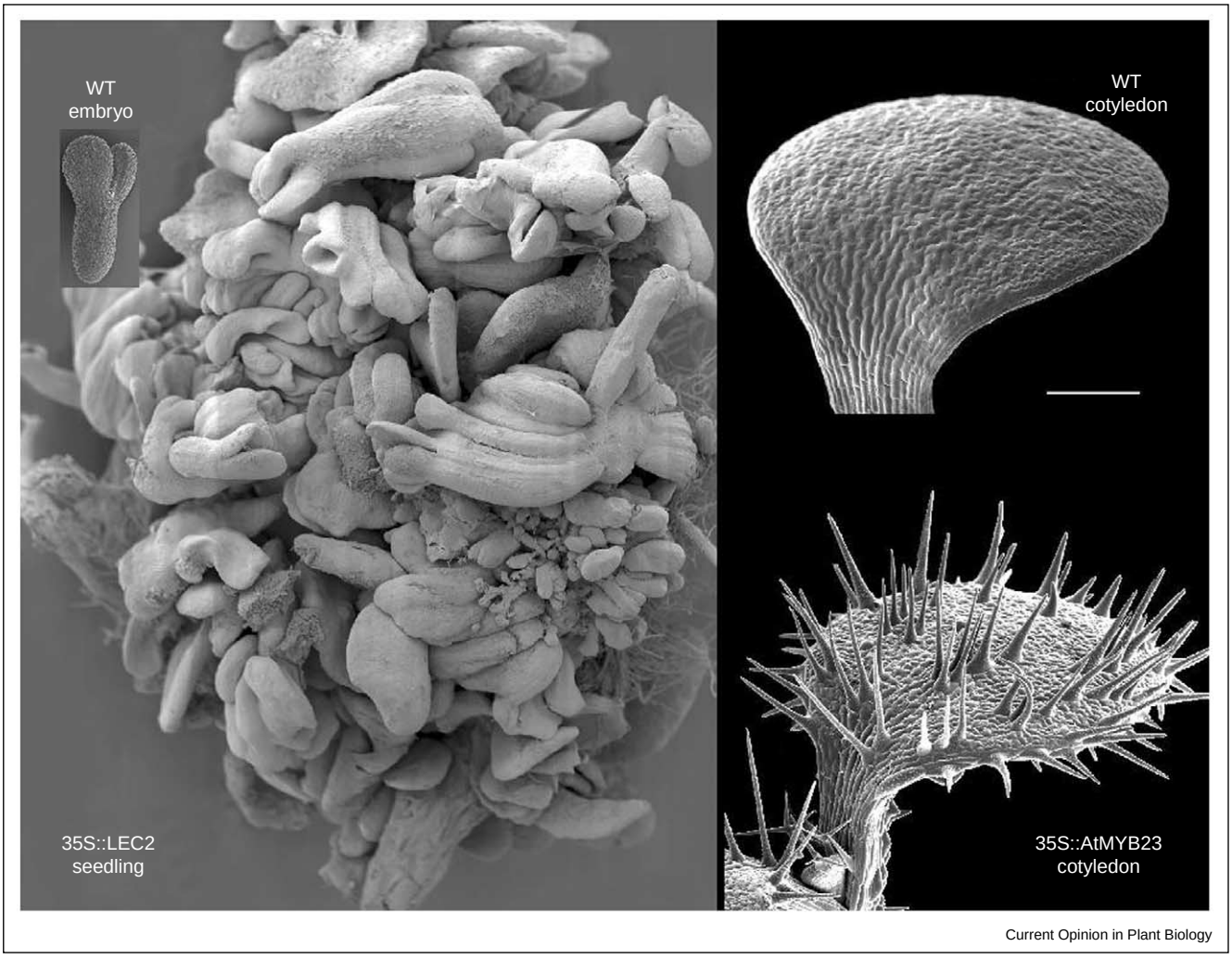
It is becoming increasingly clear that combinatorial control of gene expression, which allows the creation of regulatory diversity by a limited number of TFs, is a common mechanism in eukaryotic cells. There are several examples in which TFs are known to interact directly. These interacting TFs can be either members of the same family (such as the MADS-domain proteins) [47,48] or members of different families (such as basic helix–loop–helix [bHLH] and MYB families) [23,49–51]. The fact that TFs usually function interdependently rather than alone [52] is possibly one of the major reasons why the overexpression of some TFs does not result in a phenotype. Therefore, to obtain instructive phenotypes, it can be necessary to overexpress multiple TFs together in the same plant ([51,53,54]; Table 1).

Overexpression of multiple TFs in the same transgenic plants can also be used to study interactions between two TFs [55] — or more complicated interactions, such as tertiary and quaternary protein interactions — by the overexpression of more than one TF at the same time [56–58]. For example, the overexpression of three TFs (PISTILLATA [PI]/APETALA3 [AP3]/AP1 or PI/AP3/SEPALLATA3 [SEP3]) is sufficient to transform leaves into petaloid organs. The overexpression of four TFs (PI/AP3/SEP3/AGAMOUS [AG]) is sufficient to transform leaves into staminoid organs, whereas the overexpression of any single TF alone was insufficient [56–58]. Unfortunately, despite such successes, the number of examples in which phenotypes have resulted from the overexpression of multiple TF in plants is still small. Many TF interactions are known in other organisms, and so it is expected that the number of similar interactions known to occur in plants will increase.

Overexpression of modified TFs – dominant gain-of-function transgenics or dominant negatives

One of the key characteristics of TFs is their modularity, whereby domains, such as the DNA-binding domain or the transcription activation (or repression) domain, can function independently of each other. This modularity makes it possible to modify TFs to create dominant gain-of-function or dominant negative alleles. Dominant gain-of-function alleles can be created by deleting the

Figure 1



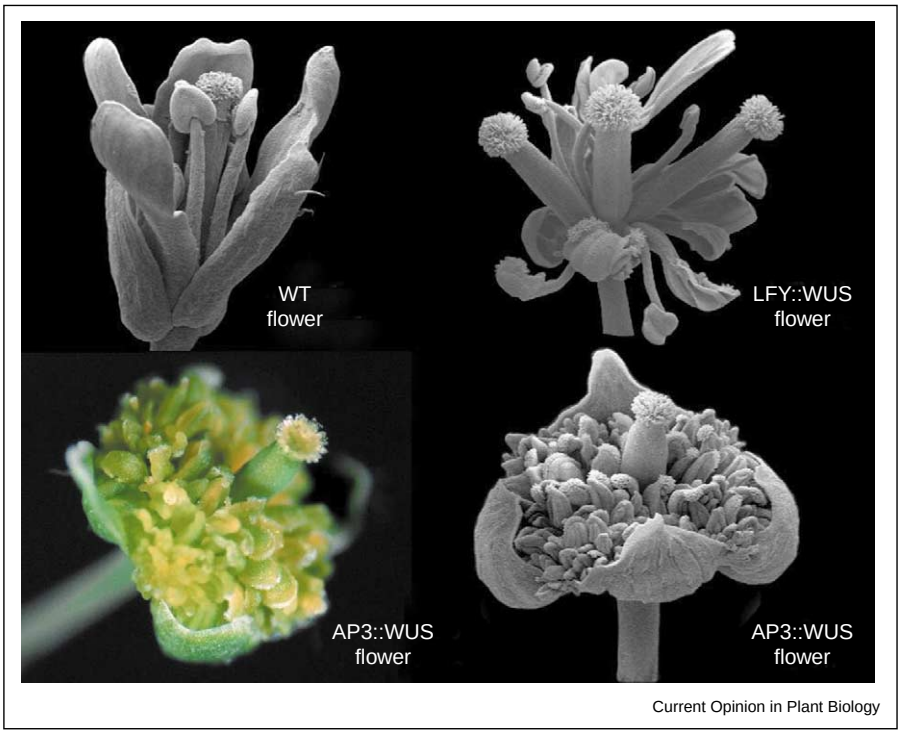
Overexpression of the single TFs LEAFY COTYLEDON2 [LEC2] and AtMYB23 under a strong constitutive promoter is sufficient for the ectopic formation of embryos and trichomes, respectively [37,38]. Photographs provided by Sandra Stone and Helmut Baumlein, and published with permission from National Academy of Sciences and Academic Press, respectively. WT, wildtype.

regulatory domain from TFs, thereby making them constitutively active [45,59,60]. Dominant negative alleles of TFs can be obtained by overexpressing a TF that contains only the DNA-binding domain [61], a TF from which the DNA-binding domain has been deleted [17,18], or a TF with a heterologous transcription activation domain [62]. Another method for generating dominant negative alleles of a TF is to fuse a repressor domain, such as the engrailed (en) repressor domain from *Drosophila*, to the TF of interest [63]. This technique has been tested using four TFs that belong to two different families, the homeodomain family (SHOOT MERISTEMLESS [STM] and KNOTTED-LIKE *Arabidopsis thaliana*1 [KNAT1]) and the MADS family (AP3 and PI). In the study, more than 75% of the original transformants resulted in *trans*-dominant phenocopies of the well-known loss-of-function phenotypes of *stm*, *ap3*/*pi*, or *brevipedicellus* with few neomorphic phenotypes [63].

Tissue-specific expression

Because many TFs are key regulators of developmental and biochemical processes, their constitutive overexpression can sometimes result in phenotypes that are difficult to interpret [64]. To obtain instructive phenotypes, it is often necessary to express the TFs in very specific tissues and/or during specific developmental stages ([65–70]; Figure 2; Table 1). It can be particularly informative to express TF genes under a tissue-specific promoter in the mutant background [71,72]. Transforming plants with a TF gene together with its own regulatory sequences is a particularly interesting form of tissue-specific expression that can increase the copy number of a particular TF

Figure 2



Tissue-specific expression of the TF WUSCHEL [WUS] results in supernumerary floral organs [66]. Photos provided by Jan Lohmann, and published with the permission of Cell Press. LFY, LEAFY.

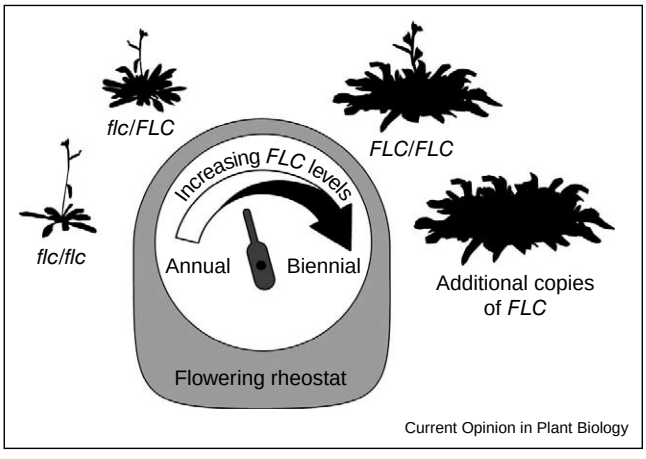
[73,74]. This approach has been elegantly demonstrated in the case of the floral inhibitor FLOWERING LOCUS C (FLC) ([73]; Figure 3).

Interestingly, many TF-coding regions have equivalent functions: their expression specificity and context define the roles that they play in plant development or their response to environment [22•,23]. Furthermore, some of the most important quantitative trait loci (QTLs) implicated in the domestication of crops are linked to changes in the expression pattern of regulatory genes (such as TFs) rather than to changes in their coding regions [2,75,76]. Expressing TFs (and other regulatory genes) under tissue-, developmental-stage-, or environmental-condition-specific promoters is expected to generate numerous novel phenotypes that could not be produced otherwise.

Inducible TF activity

In addition to tissue-specific expression, chemically inducible TF activity can also be used to study the functions of TFs that, when ectopically expressed, dramatically impact plant development and create uninformative phenotypes [68,71]. The modularity of TFs allows the construction of chimeric TFs whose activity can be controlled by chemicals. This has been achieved by fusing the TFs with the ligand-binding domain of the mammalian glucocorticoid receptor (GR). The GR binds to the heat shock protein hsp90 and prevents the chimeric TF from entering the nucleus, whereas dexamethasone

Figure 3



Example of the overexpression of a complete TF locus. FLC delays flowering in a rheostat-like manner [73]. Figure provided by Rick Amasino, and published with permission from Blackwell Science Ltd.

(DEX) releases the chimeric TF from hsp90 binding, thereby allowing its import into the nucleus.

Chemically inducible TF activity has implications that go far beyond the above examples. As the function of any TF can be defined at one level as the TF regulon it controls, one could not claim to have completely understood the function of any TF without knowing which genes it directly and indirectly regulates. Simple overexpression of TFs provides a very useful tool with which TF regulons can be studied, and in many cases can provide sufficient information [77]. Because the cascades of gene expression that are affected by the overexpression of TFs can be complex, however, it is sometimes difficult to distinguish between the TF's direct targets and the targets of its downstream genes (i.e. the secondary or tertiary targets). With the advent of microarray technology, which makes it possible to analyze the expression of tens of thousands of genes at any one time, it becomes particularly desirable to limit the complexity of the mRNA population to only the direct targets of the TF of interest. This can be achieved by translational inhibition using cycloheximide (CYC). For example, if CYC is applied in conjunction with DEX to plants that contain a DEX-inducible TF, the chimeric TF will only be able to activate the transcription of its most immediate target genes and not of indirectly regulated genes that are further downstream. This technique has been used successfully in identifying the proximal target genes of several TFs that regulate floral organs, flowering time, and cytokinin action [45,78–80]. With microarray technology becoming less expensive and more accessible, this technique should add another important tool to the ever-expanding tool kit for TF functional genomics.

Conclusions

With the sequence of the *Arabidopsis* and rice genomes completed and the sequencing of several other plant genomes in progress, the systematic analysis of transcriptional regulation is advancing. Overexpression of TFs in transgenic plants, in conjunction with mutant analysis and global transcript profiling, will continue to play a unique role in this endeavor. Comprehensive analysis of TF function by various overexpression strategies is currently being undertaken in both the public and the private sectors to provide an understanding of the function of each individual TF. The functional genomics analysis of TFs should represent the first step toward an understanding of the 'wiring diagram' of plant transcriptional networks in different species, and thus plant development and evolution.

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