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# Using transgenic modulation of protein synthesis and accumulation to probe protein signaling networks in *Arabidopsis thaliana*

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**Abbreviations:** RNAi, RNA interference; T-DNA, transfer-DNA; UAS, upstream-activating sequence

Deployment of new model species in the plant biology community requires the development and/or improvement of numerous genetic tools. Sequencing of the *Arabidopsis thaliana* genome opened up a new challenge of assigning biological function to each gene. As many genes exhibit spatiotemporal or other conditional regulation of biological processes, probing for gene function necessitates applications that can be geared toward temporal, spatial and quantitative functional analysis in vivo. The continuing quest to establish new platforms to examine plant gene function has resulted in the availability of numerous genomic and proteomic tools. Classical and more recent genome-wide experimental approaches include conventional mutagenesis, tagged DNA insertional mutagenesis, ectopic expression of transgenes, activation tagging, RNA interference and two-component transactivation systems. The utilization of these molecular tools has resulted in conclusive evidence for the existence of many genes, and expanded knowledge on gene structure and function. This review covers several molecular tools that have become increasingly useful in basic plant research. We discuss their advantages and limitations for probing cellular protein function while emphasizing the contributions made to lay the fundamental groundwork for genetic manipulation of crops using plant biotechnology.

## Overview

Analyses of numerous genetically modified plants generated in recent years have greatly expanded our knowledge of physiological processes and gene regulation mechanisms. Efficient tools for forward and reverse genetics are invaluable for determining protein function. One vital module in these molecular tools is the ability to carry out targeted analyses of gene function. Emergence of a new model species for basic research requires the development and/or improvement of a myriad of genetic and genomic tools and one model plant, *Arabidopsis thaliana,* has been utilized widely in basic research towards understanding biological and biochemical functions. In the past three decades, the Arabidopsis genetic system has undergone extensive manipulation to aid researchers in better understanding protein function and dissecting complex signaling networks. Arabidopsis has proven to be an exceptional model plant to study gene and/or protein function. A tractable genome and short generation time are two of the features that make it useful for basic plant research. Considering the ongoing quest to identify the biological function of all of the genes of Arabidopsis, in this review we collectively assess some of the molecular tools that are commonly used in determining functions of effectors in complex protein signaling networks.

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**Approaches to study plant gene function.** The availability of efficient and cost-effective genetic tools that are applicable to different plant species is essential for functional gene analysis. As a result of the Arabidopsis Genome Sequencing Project, the daunting task of determining the functions of all genes in Arabidopsis was initiated. Genomic and proteomic tools are useful for the initial step in genome-wide screening for mutants and selection of genes with desirable traits for subsequent application of knowledge to other plant species. Therefore, mutant analyses, ectopic expression, mis-expression, overexpression and RNA interference (RNAi), have become effective tools to study numerous biological functions of proteins in planta.

A common genetic approach to the study of protein function is mutational analysis. Classic loss-of-function mutations generated through random mutagenesis mediated by chemical mutagens and radiation aid in dissecting gene functions in genetic pathways. However, a handicap of loss-of-function mutant screens is that they are not effective in identifying functionally redundant genes or genes whose loss of function results in early embryonic or gametophytic lethality.[1] A more recent tool in mutational analysis is the construction of DNA insertion mutants. This approach requires transgenic generation of populations of DNA insertion mutants, mapping the disrupted genes through screening, characterizing the mutations and cloning the target genes.[2] A limitation of classic and DNA insertion loss-of-function mutants is that they can reveal only global effects of gene function.

Constitutive mis-expression or overexpression of transgenes has been successful for elucidating gene function in many species, but not all species can be transformed, nor can all genes be expressed in a constitutive fashion in planta. Examples are genes encoding proteins with highly detrimental or lethal dominant-negative phenotypes that lead to drastic changes in crucial metabolic pathways leading to growth and developmental defects (reviewed in ref. 3). Transfer-DNA (T-DNA) mutagenesis is frequently associated with loss-of-function or hypomorphic mutations. However, T-DNA mutagenesis can be manipulated to generate gain-of-function alleles by activation tagging. Commonly, T-DNA vectors that contain multiple copies of transcriptional enhancers from the cauliflower mosaic virus (CaMV) 35S gene have been constructed to transform Arabidopsis plants through Agrobacterium-mediated transformation.[4] Upon integration into the genome, the enhancers stimulate the transcription of a gene and cause its ectopic expression.[1] Through activation tagging, novel gene functions have been discovered, even though constitutive mis-expression has the drawback of not being able to limit transgene expression to discrete tissues, organs or developmental stages.[5] In particular, the mis-expression of genes that cause lethality or strong pleiotropic effects cannot be accomplished through activation tagging.[6] Another concern of activation tagging is whether the gain-of-function phenotype of a mutant reflects the normal biological role of a gene. Notably, the CaMV 35S enhancers can lead to enhancement of endogenous gene expression and the resultant phenotype, as opposed to ectopic overexpression or mis-expression driven by native promoters, which would be more likely to reflect the normal role of the activated gene.[1]

Transgenic approaches such as overexpression, ectopic expression, mis-expression and RNAi have become effective strategies in functional genomics and proteomics in Arabidopsis. These technologies can be implemented easily for a single gene using specific constructs that direct its down or upregulation under constitutive, tissue-specific or inducible conditions.[6] Developmental processes and differentiation of distinct cell types depend on the regulation of gene expression in space and time. Most systems that manipulate gene expression allow control in one dimension, space or time. Molecular tools that allow spatial and/or temporal control of gene expression are vital for the accurate elucidation of gene and protein function. Furthermore, to understand the functions of proteins encoded by critical genes, which if mutated could be severely detrimental to plant growth and survival, one solution has been to use tissue- and/or organ-specific promoters to limit the expression and the activity of transgenes to particular tissues or organs. Such systems that are currently available have advantages as well as limitations. One limitation is that tissue- and organ-specific promoters are active during the process of regenerating transgenic plants. Tissue-specific expression also restricts the scope of the analysis to a few cell types and does not always allow the appropriate tissues to be studied.[7] In cases where promoters respond to external inducers (i.e., inducible promoters) the expression of the transgenes can be controlled temporally and/or spatially. Techniques such as those described above require establishment of stable and independent transgenic lines whose growth and propagation impose extensive time, labor and space requirements. Development of enhancer trap and two-component transactivation systems has allowed more carefully controlled expression and has been used to overcome some of the limitations of spatial-specific, promoter-driven mis-expression strategies.

Advances in inducible mis-expression technologies, spatial and temporal regulation of transgenes and RNAi are anticipated to facilitate more fine-tuned functional analyses of endogenous and exogenous genes, revealing new roles for genes that act at multiple stages in the plant life cycle. Such analyses are expected to assist the development of new, improved crop varieties, as plant biotechnology depends on the manipulation of genomes to induce desirable traits, while mitigating detrimental traits, in commercial crops. Basic research on Arabidopsis promotes our understanding of the molecular and cellular bases of complex protein signaling networks; this review covers some examples, advantages and limitations of spatial-specific and inducible mis-expression systems, RNAi, enhancer trap-induced gene expression and two-component transactivation systems that have increasingly gained popularity and efficacy in recent years (**Table 1**).

## Tissue- and Organ-Specific, Promoter-Driven Gene Expression

Tissue- and organ-specific promoters enable researchers to drive the expression of a transgene in a distinct subset of cells, tissues or organs. Spatial-specific regulation of transgene expression has been successfully utilized in many studies that were aimed at further understanding the functions of proteins during the life cycle of Arabidopsis. Proper onset and fine tuning of developmental transitions and adaptive processes requires detection of external, environmental cues. For example, plants detect the presence or absence of light, but also the spectral quality, quantity, directionality and periodicity (reviewed in ref. 8). Plants sense and adapt to light through light-absorbing molecules called photoreceptors (reviewed in ref. 8–11). Through photoreceptor mutant analyses, it is known that far red-light-absorbing phytochrome A and blue light-absorbing cryptochrome 2 (cry2) are involved in perceiving long-day photoperiods and thus control flowering (reviewed in ref. 8). Although promoter-fusion studies indicate that these photoreceptors are found in tissues and organs throughout plants,[12] a wealth of prior studies has confirmed that light perception in leaves is associated with the photoperiodic induction of flowering (reviewed in ref. 13). Transgenic Arabidopsis lines where the *CRY2-GFP* gene fusion was expressed under the control of organ- or tissue-specific promoters (e.g., *CAB*, mesophyll specific; *SUC2*, vascular bundle specific; *ML1* and *CER6*, epidermis specific) in a *cry2*-deficient mutant background have been used to study the site of light perception by cry2 at the molecular level in the regulation of flowering.[14] Only cry2-GFP accumulation in vascular bundles was able to rescue the late flowering phenotype of a *cry2* mutant, suggesting that the site of cry2 photoperception that regulates flowering is localized in vascular bundles. Phytochromes are red- and far red-light-absorbing photoreceptors.[15] Studies with stable transgenic lines displaying mesophyll-specific and

**Table 1.** Experimental approaches for modulating gene expression and protein accumulation in planta

| Approach | Utility | Advantages | Disadvantages |
|---|---|---|---|
| Mutagen-induced mutagenesis | Induced mutations using mutagens, including chemicals or radiation | Allows recovery of point mutations (i.e., missense or nonsense), which can provide important insight into gene function; Can result in dominant-negative mutations, which have proven useful for novel insight into gene function; Useful for all species, including those that cannot be transformed. | Mapping untagged mutations is laborious; Can result in multiple genetic mutations in a single individual making it difficult to definitively associate disrupted gene function with specific phenotypes. |
| T-DNA insertional mutagenesis | Random insertion of transfer DNA (T-DNA) resulting in tagged insertional mutants | Gene tagging with known insertion sequence allows easier recovery of disrupted genes; If insertion sequence has a selectable marker, single insertion mutants can be identified. | Not useful for inactivating multiple, tandemly arrayed genes; Not useful for studying genes of essential function; Cannot be used for species that cannot be transformed. |
| Activation tagging | Randomized genomic insertion of transcriptional enhancers resulting in ectopic gene expression | Allows analyses of gain-of-function phenotypes; Genes are tagged and thus easier to identify. | Gain-of-function phenotypes associated with activation tagging may not reflect normal biological gene function; Not useful for lethal genes or those associated with strong pleiotropic effects; Cannot be used for species that cannot be transformed. |
| Constitutive gene expression | Constitutive mis- or overexpression of genes of interest | Allows complementation studies using null mutants; Allows gain-of-function analyses. | May not reflect normal biological function; Not useful for lethal genes or those associated with strong pleiotropic effects; Cannot be used for species that cannot be transformed; Can result in co-suppression of some genes. |
| Promoter-driven gene expression | Using tissue- or organ-specific promoters, or inducible promoters, selective expression of transgenes can be accomplished | Allows spatial- or temporal-specific expression or expression at specific developmental stages; Allows inducible expression, which can be used for the study of lethal genes. | Use of specific promoters is limited to isolated and characterized promoters; Requires generation, isolation and characterization of several independent transformants; Cannot be used for species that cannot be transformed. |
| RNA Interference (RNAi)-mediated inhibition of gene expression | Constitutive or selective suppression of gene expression based on sequence-specific RNA targeting and turnover | Allows robust suppression of gene expression in cell culture or in whole organisms; Can be useful for suppression of multiple, tandemly arrayed genes or genes closely related in sequence. | Can result in suppression of closely related genes; Cannot be used for species that cannot be transformed. |
| Enhancer trap-driven gene expression | Random insertion of a transgene with a minimal promoter results in expression of a gene of interest driven by endogenous genomic enhancers | Allows spatial- or temporal-specific gene expression that depends on genomic enhancers rather than isolated, characterized promoters. | Cannot be used for species that cannot be transformed. |
| Two-component transactivation systems (e.g., GAL4-UAS or pOp6/LhG4 systems) | Random insertion of a promoter-driven transgene or a transgene with a minimal promoter driven by endogenous genomic enhancers results in expression of a transcriptional activator that in turn drives expression of a target gene in progeny resulting from a cross between activator line and a line carrying a transactivatable target gene | Allows silent maintenance of a target transgene until expression is activated by the combined action of two distinct components; Can be used for temporal- and/or spatial-specific or inducible control of gene expression. | Cannot be used for species that cannot be transformed. |

meristem-specific phytochrome-chromophore deficiencies have revealed that localized pools of phytochromes can regulate distinct physiological responses and established the efficacy of a novel tissue- and organ-specific, promoter-based molecular technique to investigate sites of light perception.[16]

Additional studies investigating the tissue-specific function of specific proteins that impact light-dependent growth and development in Arabidopsis have also emerged.[17] Tissue-specific expression of *SPA1*, a gene that encodes a repressor of photomorphogenesis, indicated that SPA1 has distinct functions in discrete

tissues.[17] In Arabidopsis, phloem-specific SPA1 functions in the regulation of etiolation in dark-grown seedlings and in the regulation of photoperiodic flowering in light-grown plants.[17] By comparison, both phloem- and mesophyll-localized expression of *SPA1* is required to regulate light-dependent leaf expansion.[17]

During development, leaves of dicotyledonous plants undergo a gradual transition from sink to source status; therefore, establishing and maintaining phloem pressure is central to plant growth.[18] *AtSUC2* in Arabidopsis (1) encodes a phloem-localized sucrose/proton symporter involved in photoassimilate transport from source to sink tissues, (2) plays a central role in coordinating the demands of sink tissues with the output capacity of source leaves and (3) is active in maintaining phloem hydrostatic pressure during changes in plant-water balance.[18,19] To elucidate the role of AtSUC2 in whole plant carbon partitioning, the promoter of the *GALACTINOL SYNTHASE* gene from *Cucumis melo* was fused to *AtSUC2* cDNA to target its expression to collection phloem (allows the entry of nutrients to the phloem network in source tissues) in an *Atsuc2* mutant background.[19] The results from this report suggested that AtSUC2 is not required for efflux in the transport and release of phloem, but its retrieval function (acceptance of nutrients by recipient cells) likely participates in fine tuning whole-plant carbon partitioning.[19] Because the expression of *AtSUC2* and activity of AtSUC2 are regulated, both positively and negatively, by developmental (sink to source transition) and environmental cues (i.e., light, diurnal changes, photoassimilate levels, turgor pressure, drought and osmotic stress and hormones), in a follow-up study to further understand its regulation, *AtSUC2* was expressed under the regulatory control of two phloem-specific promoters [a promoter element from Commelina Yellow Mottle Virus (CoYMV) and *rolC* promoter from *Agrobacterium rhizogenes*] in an *Atsuc2* mutant background.[18] Conclusions from this study suggest that (1) strong, phloem-localized *AtSUC2* expression is sufficient for efficient photoassimilate transport and (2) expressing *AtSUC2* from promoters that aid in efficient phloem transport but are subject to regulatory cascades different from the endogenous sucrose/proton symporter genes has implications for biotechnology, especially in manipulating carbon partitioning as desired.[18]

Even though targeted expression of transgenes using tissue- and organ-specific promoters has been promising in providing additional insight into protein function, a current limitation is the availability of cloned and characterized promoters that can direct gene expression in a targeted manner. The use of tissue-specific promoters across species, even those that are closely related, can impose changes or loss of transgene expression due to the lack of essential trans-acting factors in the recipient plant species. The specificity of transgene expression also could be lost due to the presence of novel trans-acting factors that interact with the promoter cis-elements.[2] Moreover, this tool requires the establishment of stable, independent transgenic lines whose growth, propagation and analyses impose extensive time, labor and space requirements. Some of the aforementioned limitations can be overcome through inducible systems, which add another dimension to the regulation of transgene expression, allowing temporal and quantitative control of transferred genes in vivo. Induction systems with spatial, temporal and quantitative control of transgenes are applicable in many areas of basic and applied biology, including the study of gene function, cell lineage ablation experiments, enhanced synthesis of recombinant proteins and expression of commercially valuable traits.[20]

## Inducible Promoter-Driven Gene Expression

Use of promoters that respond to inducers allows fine-tuned manipulation of transgene expression. This molecular technique has become an attractive tool to maintain a transgene silently in the absence of the inducer, allowing transgene expression to be regulated by the presence of a specific, inductive signal under defined conditions. With appropriate chemical inducers (i.e., tetracycline, dexamethasone, 17-β-oestradiol and ethanol) and/or physiological cues (i.e., heat or cold), transgene activity can be controlled spatially, limiting the expression to particular cells, tissues or organs of interest; temporally, determining the effects at distinct developmental stages; and quantitatively, analyzing gene dosage responses in complementation experiments (reviewed in ref. 3).

Inducible expression systems rely on either endogenous or exogenous elements to control transgene activity; the ideal system should have very low activity in the absence of the inducer. Activity of the system should increase and decrease quickly and significantly in direct correlation with the amount of inducer to which the system is exposed, in vivo as well as in vitro, and with neither pleiotropic effects on endogenous gene expression nor toxic effects on plant metabolism.[20] Because most if not all genes in plants respond to external (i.e., heat, cold, light, wounding) and internal (i.e., hormones) cues that affect gene expression, such cues may not serve as the best candidates in vivo. Regulatory sequences that are responsive to chemical treatment, such as promoters and enhancers, are attractive because techniques based on such sequences depend upon the cloning of the responsive promoter upstream of the coding region of the gene of interest. However, a disadvantage of this approach is that native genes controlled by these regulatory sequences may also be induced upon addition of the chemical regulator.[20] Thus, it is important to choose an inducer that affects a set of genes that does not interfere with normal growth and development. Notably, four such chemical types—elicitors, safeners, wound signals and compounds that activate genes in systemic acquired resistance—have been widely used in the construction of inducible expression systems in planta.[20] Promoters that respond to otherwise inactive chemical inducers[20] have added to the molecular tools available for analyzing gene and protein function in vivo and manipulating desirable traits in plants, including crops.

In plant biotechnological applications, most commercially important crops, as well as non-crop plants, are regenerated via somatic embryogenesis,[5] which is useful for mass asexual propagation or somatic cloning in plants. The underlying signal transduction pathway—i.e., the molecular mechanism involved in the transition of a vegetative cell to an embryogenic competent cell—remains largely unexplored.[21] A study designed to gain understanding of the complex molecular process of induction of somatic

embryogenesis in Arabidopsis is based on an estradiol-inducible system. WUSCHEL (WUS) is essential for meristem identity of shoot and floral meristems, i.e., to maintain their structural and functional integrity,[22] and also induces shoot stem cell activity and developmental plasticity in the root meristem.[21] To dissect the signaling pathway active during somatic embryogenesis, an estradiol-inducible *XVE-WUS* transgene was expressed in wild-type Arabidopsis plants. These transgenic plants are phenocopies of *pga6-1* and *pga6-2* gain-of-function mutant phenotypes having a high vegetative-to-embryogenic transition, which suggests that WUS and newly identified Plant Growth Activator 6 (PGA6) play a key role during embryogenesis, presumably by promoting the vegetative-to-embryogenic transition and/or maintaining the identity of the embryonic stem cells.[5] In the event that a gain-of-function mutation causes growth defects or seedling lethality, the use of an inducible promoter-driven mis-expression system has an added advantage, i.e., allowing transgene activation upon application of the inducer at a specific time and elimination of its activity once the inducer is removed.[23]

An ethanol-inducible gene switch system has been used to study carbon metabolism[23] and stomatal development[24] based on the *alc* regulon of the fungus *Aspergillus nidulans*. Several factors made this system promising: no plant transcription factor was known to interfere at the *alcA* promoter, levels of natural ethanol in the plants are extremely low, and ethanol is a small organic inducer with relatively low phytotoxicity.[23] These attributes have made the use of this inducible system favorable for many different plant species, including Arabidopsis,[25] Tobacco,[23] tomato,[26] Populus[27] and *Catharanthus roseus*.[28] However, the ethanol-inducible system is limited by the volatile nature of the inducer, which can cause unwanted gene activation in adjacent plants, phytotoxicity on the induced plant, and activation by endogenous inducers under low-oxygen conditions.[29] These drawbacks complicate the use of an ethanol-inducible system to produce distinct sectors of induced and uninduced gene expression that are essential to determine the cell autonomy of a phenotype. Some other inducible systems, based on nonvolatile inducers, have proven to be leaky[23] or are unable to activate accurately inducer-dependent T-DNAs randomly inserted in the genome.[6]

Another option to overcome the above limitations is the use of heat-shock promoters that can achieve relatively high-level expression in many cell types. In a study of gene function in epidermal cell polarity, a heat shock-inducible promoter from the Arabidopsis *HSP18.2* gene was fused to a gene encoding ADP-ribosylation factor1 (ARF1).[30] Six identical *ARF1* genes are ubiquitously expressed and single loss-of-function mutants in these genes reveal no obvious developmental phenotypes due to gene redundancy, thereby precluding conventional genetic dissection of ARF1 functions.[30] However, a study using transgenic Arabidopsis lines expressing heat shock-inducible, promoter-driven *ARF1* reported a successful dissection of downstream signaling networks involved in local and cell-specific aspects of epidermal cell polarity.[30] Another study reported the use of a heat shock-inducible construct to study the function of the *ACL* gene in internode elongation in Arabidopsis.[31] Even though heat-shock promoters have been successful in many studies, one of the disadvantages is that subsequent analyses have to be performed at a temperature around 37°C and plants can be subjected to heat stress. Therefore, carefully controlled growth conditions become a necessity. Another general concern of heat-shock inducible systems is induction of endogenous heat shock proteins or related endogenous responses that could potentially complicate analysis of results.

The best-characterized, plant-based inducible system involves a tetracycline-inducible promoter developed for tobacco (that provides temporal regulation of gene expression in tobacco cell cultures[32]). This system has been successfully utilized with cell culture, though its usefulness and reliability for spatial control of gene expression in the various tissues of whole plants is less clear.[7] It appears that the tetracycline chemical switch is not effective in Arabidopsis and is leaky when the genes of interest are lethal in planta.[23] A few studies have combined the benefits of tissue-specific promoters with the use of inducers to drive inducible, tissue-specific gene expression (reviewed in ref. 33). Despite the efficient use of targeted transgene expression with spatial-specific and inducible promoters in Arabidopsis, they are not entirely adequate in some studies. The reason is that the tool is relatively inefficient (high background and/or only modest induction), depends on sustained gene repression or relies on the application of chemical inducers at concentrations that may be toxic to plants.[20] In addition to mutational analyses and expression of transgenes, an RNA-based reverse genetics approach is currently in use for studies of protein and gene function in plants.

## RNAi Interference

RNAi is a remarkable experimental tool that regulates gene expression based on sequence-specific targeting and turnover of RNA molecules, which would otherwise be translated to produce protein. RNA silencing was first observed in transgenic Petunia plants as cosuppression,[34] leading to silencing of expression from integrated genes and restricting molecular applications that necessitated high-level transgene expression. With an increased understanding of the molecular basis of RNAi, it has become a plant engineering tool with enormous potential to control gene expression. Several mechanisms result in RNAi, and sequence specificity of RNAi-based gene activation allows silencing of individual genes as well as several genes simultaneously.[35,36] These properties make it an invaluable technique in functional genomics and in gene function validation. Silencing can be achieved constitutively or in a targeted manner, with the latter being very useful in determining gene function. Successful reports on tissue-specific and/or cell type-specific RNAi have been presented in many model organisms, namely, in *Caenorhabditis elegans*,[37] mice,[38] Drosophila,[39] Arabidopsis,[40,41] and *Brassica napus*.[40]

Some recent studies have been focused on manipulating floral organs to improve photosynthetic efficiency.[42] During analyses of flowering time in *B. napus*, researchers determined that bright-yellow flowers significantly reduce the amount of photosynthetically active radiation that reaches the leaf canopy, thereby accelerating leaf and bract senescence.[40] To gain better understanding of genes associated with floral organ identity and

heritability of floral organ variants, gene silencing technology was implemented to silence B-type MADS-box floral identity genes in a second whorl-specific manner in Arabidopsis and *B. napus*.[40] The results from this study indicate that transgenic plants have the ability to produce male fertile flowers, which exhibit conversion of petals to sepals in Arabidopsis and petals to sepaloid petals in *B. napus*.[40] Because organ conversion is preferable over the removal of petals to avoid interfering with insect pollination, this study confirmed that organ-specific silencing of B-type MADS-box genes can result in novel floral organ variants in both species that were stable in subsequent generations,[40] revealing a strategy to manipulate organ identity in a desirable manner.

To achieve temporal and quantitative regulation of RNAi, RNA-mediated silencing can be chemically induced. Inducible RNAi systems maintain the silencing construct inactively in the absence of the inducer to avoid spurious gene silencing and, upon inducer addition, silencing is induced rapidly across the whole plant or tissues of interest to achieve repression of target genes. An important feature of such a system is reversibility after the removal of the inducer.[41] An inducible RNAi system has been constructed using a dexamethasone-inducible pOp6/LhG4 promoter[43] and pHELLSGATE GATEWAY™ gene silencing vectors.[41] In the latter report, stable transgenic lines with the inducible gene silencing system have displayed efficient silencing of two genes encoding phytoene desaturase and luciferase, and recovery of transcript levels was observed upon removal of the inducers. Heat shock-[44] and ethanol-inducible[45] RNAi technologies have also been described in plants. Inducible RNA-based silencing opens the possibility for knocking down the expression of gene products at specific developmental stages or in specific tissues. The capacity to manipulate gene expression through inducible RNAi allows the dissection of gene functions related to complex pleiotropic phenotypes in loss-of function mutants or stable RNAi lines.

RNAi can be engineered as an efficient method for silencing genes in cultured cells[38] and can be manipulated to study protein function in isolated protoplasts,[46] thereby allowing rapid functional genetic screens and circumventing the maintenance of stable RNAi transgenic lines. Protoplasts can be isolated from various plant tissues, making the study of cell type-specific processes feasible. Moreover, cellular transport mechanisms and sub-cellular localization of proteins of interest can be determined through the implementation of RNAi in protoplasts. An example for efficient use of RNAi in protoplasts is the reduction of transcript and polypeptide levels of γ-glutamylcysteine synthase, a key enzyme in glutathione biosynthesis, by transient double-stranded (ds) RNAi, causing a drastic reduction in glutathione content in Arabidopsis protoplasts.[46] The reduction of γ-glutamylcysteine synthase activity through RNAi was comparable to *cad2-1* (a mutant in the gene encoding γ-glutamylcysteine synthase, *ECS1*).[47] Protoplasts can be isolated from tissues as young as 14 days old instead of 1 month old,[46] thus avoiding the need to maintain stable transgenic RNAi lines. Additionally, the use of RNAi in isolated protoplasts complements current genetic tools by allowing fast, cost- and space-effective initial screening and selection of genes for later in planta studies. In summary, RNAi at the whole-plant level and/or in isolated protoplasts is useful to silence a single gene or a family of related genes. In cases where genes within a gene family are functionally redundant, RNA-based silencing can effectively knock down the transcripts encoding the protein of all family members, which is otherwise difficult using classic mutational analysis. Silencing mechanisms can be experimentally induced with chemicals and unwanted RNA silencing can be alleviated using viral suppressor technology or mutants harboring mutations in the gene encoding key players in the RNAi mechanism.[36] Because the silencing mechanism can be manipulated, RNAi has a wide range of practical applications in biotechnology.

Despite a plethora of molecular tools available for use in plants, many of these molecular genetic approaches are not efficiently applied to genes that regulate primary growth and developmental processes and reproduction. The main drawback is that changing the expression of such vital genes can negatively impact plant growth, propagation and survival. In such cases, the generation and maintenance of useful transgenic plants may not be cost-effective. Additionally, numerous environmental factors such as light and molecules such as phytohormones have distinct effects on growth and development in different tissues and organs.[13,14,16,48] Thus, it is vital to establish an applicable system for successful spatial regulation of transgene expression that is not hampered by intervention or imposition of abiotic stress.

## Enhancer-Trap and Two-Component Transactivation Systems

The limited number of well-characterized promoters and corresponding expression patterns restricts the cell types and developmental processes that can be targeted. Limitations of tissue-, organ-specific or inducible promoter-driven transgene expression and RNAi can be overcome through the utilization of enhancer-trap-based gene induction or inactivation or two-component transcription activation systems based on promoter- or enhancer-trap strategies.

**Enhancer-trap systems.** Through the utilization of enhancer-trap systems, distinct expression patterns can be achieved in a localized manner, which makes it an effective tool to determine sites of signal perception in physiological processes (e.g., photoperception) that have spatial-specific aspects. Use of the Cauliflower mosaic virus (CaMV) 35S minimal promoter-based enhancer-trap system to express phyB-GFP in a phytochrome B (*phyB*) mutant of Arabidopsis revealed that *phyB* expression in the mesophyll, but not in vascular bundles, suppresses the expression of a key flowering regulator, *FLOWERING LOCUS T* (*FT*), in vascular bundles.[49] This finding indicates that a novel inter-tissue signaling mechanism occurs between mesophyll and vascular bundles, making it a critical step in the regulation of flowering by the photoreceptor phyB.[49]

Enhancer-trap approaches have also been used to identify expression patterns of genes. In this approach, reporter genes under the control of minimal promoters are transformed into host plants to identify genes regulated by external cues, e.g., circadian-regulated genes[50] or pathogen infection-induced genes.[51]

Such systems have also been used to identify tissue- or organ-specific expression of genes in a range of plant species.[52-55]

**Two-component transactivation systems.** For two-component transactivation systems, genes of particular interest can be introduced into the Arabidopsis genome under the control of a heterologous upstream-activating sequence (UAS).[56] The parental UAS-transgenic line is maintained independently and the gene of interest will not be expressed until it is crossed with an activator line that expresses the corresponding transcription factor or transcriptional activator. The activator line contains a gene encoding a transcriptional activator under control of a minimal promoter. In this system, the gene of interest is expressed only in a subset of cells in which the transcription factor is expressed, and this depends on native genomic enhancers that drive the expression of the transcription factor. The effect of transgene expression on plant growth and development can be analyzed in the F3 progeny. Two-component transactivation systems circumvent the necessity to maintain and genotype multiple stable transgenic lines, which is tedious and labor intensive.

*GAL4/UAS two component transactivation.* A two-component, enhancer-trap mis-expression system based on the yeast Gal4 transcription factor has been used successfully in Drosophila to study regulatory mechanisms during embryonic development,[57] and in both Arabidopsis[58] and rice.[59] A more stringent plant regulatory system based on promoter/enhancer-trap activation can be achieved through the use of transcription factors with sequence-specific DNA-binding activities that are not normally found in plants.[7] Transgenic GAL4-enhancer trap lines are T-DNA insertion lines with diverse expression patterns of the yeast GAL4 transcription factor and its expression depends on the presence of native genomic enhancer sequences. The GAL4- responsive *mGFP5* gene marks the expression pattern mediated by genomic enhancers in green fluorescence.[58,60] The GAL4-enhancer trapping system overcomes the limited availability of cloned and characterized promoters by using native genomic enhancers within a host genome.[2]

The mechanisms of lateral root development have been studied using an effective GAL4/UAS two-component system. Laplaze et al.[58] screened a population of Arabidopsis GAL4-GFP enhancer trap lines and selected lines with GAL4 expression in root xylem pole pericycle cells (i.e., cells competent to become lateral root founder cells, line J0121). These authors initiated a study to investigate the molecular and cellular bases of lateral root development in genetic ablation experiments by targeting the expression of a toxin-encoding gene (i.e., Diphtheria toxin chain A, *DTA*).[58] J0121-directed transactivation of the toxin gene indicated that only xylem-pole pericycle cells can form lateral roots.[58] In a follow-up study, Parizot et al.[61] reported that there are two distinct pericycle cell types and confirmed that the cell specification between them occurs in early development with vascular tissue determination. Genetic crosses between transgenic lines with a UAS-linked *IPT* (isopentenyltransferase) transgene and xylem-pole pericycle cell or lateral root primordia-specific *GAL4-GFP* expression demonstrated that xylem pole pericycle cells are sensitive to cytokinins, whereas early lateral root primordia are not.[62] The physiological consequences of this difference were that endogenous levels of cytokinins in lateral root founder cells limit lateral root formation and, in xylem pole pericycle cells, cytokinins are able to disrupt lateral root formation.[62]

An ability to perform non-invasive measurements of cell-specific changes in molecules in vivo is an additional advantage of the GAL4/UAS enhancer trap system. To gain insight into low-temperature-induced calcium ion signaling in guard cells, an enhancer-trap line with guard cell-specific *GFP* expression was utilized to obtain cell-specific aequorin expression.[63] Temperature-dependent accumulation of aequorin, a recombinant bioluminescent reporter of [$Ca^{2+}$], in guard cells of Arabidopsis indicated that the circadian clock can modulate cold-induced $Ca^{2+}$ signals.[63] The GAL4/UAS system has been combined with RNAi technology to investigate cell-specific inactivation of particular genes as well. In an investigation of the chlorophyll synthase gene (*CS*) in Arabidopsis, an enhancer-trap approach was taken to express an RNAi construct specifically in cells surrounding the plant veins.[64] In this study, a cell-specific reduction of *CS* expression in veins and vein-proximal cells was associated with lower photosynthetic capacity and reduced growth and leaf senescence.[64] The GAL4-GFP system has also been combined with an inducible system for spatial and temporal control of gene expression.[65] In this system, researchers transformed GAL4/UAS enhancer-trap lines with an ethanol-inducible system under UAS control.[65] Thus, in the cells producing GAL4 protein, the UAS-driven, ethanol responsive gene could be temporally turned on by ethanol treatment.[65] Despite many reports of successful utilization of GAL4/UAS transactivation systems in planta,[58,59] a limitation is that the DNA-binding domain of GAL4 possesses intrinsic sensitivity to UAS methylation, especially in plants, thereby making GAL4-based expression systems less effective in some cases.[66]

*LhG4/pOp two-component transactivation.* Moore et al.[7] initiated work to combine the high-affinity DNA-binding activity of a mutant form of the *lac* repressor from *Escherichia coli* with a transcription activation domain from the yeast GAL4 protein to produce an effective chimeric transcriptional activator for plants called LhG4. The LhG4/pOp two-component transactivation system is based on the *lac* operon and pOp promoter that consists of two *lac* operators cloned upstream of a CaMV 35S minimal promoter.[6] The LhG4/pOp system may be used to study toxic or deleterious gene products, to coordinate the expression of multiple gene products, or to restrict transgene phenotypes to the F1 generation.[6] The LhG4 system complements inducible expression systems that offer temporal control of gene expression in tissues that can be treated with inducing chemicals. Furthermore, as the *lac* repressor is derived from *Escherichia coli,* it is unlikely that plants harbor genes that are regulated by a similar DNA-binding activity. It also seems unlikely that LhG4 will bind fortuitously near an endogenous plant gene and cause its ectopic expression, a potential problem with other systems.[7] A chemically inducible and spatially controlled expression system may be obtained by regulating LhG4 by fusing it to the ligand-binding domain of the rat glucocorticoid receptor, allowing an additional level of stringency to mediate transgene expression.[7] The LhG4/pOp system has been used successfully to regulate the activity of genes

involved in meristem maintenance,[67] organ polarity,[68] cytokinin metabolism,[69,70] and to study embryogenesis in Arabidopsis.[71]

Prior studies with spatial-specific promoters have added to our understanding of meristem development in Arabidopsis.[5,21,22] As previously mentioned, WUS protein is required for stem cell identity, whereas the *CLAVATA 1, 2* and *3* (*CLV1*, *2*, *3*) genes promote organ initiation.[68] The LhG4 transcription factor was expressed under control of the *CLV1* promoter in the activator line and was crossed to the target line with a pOp-*WUS::GUS* construct, resulting in *WUS* expression in apical, lateral cells and in cells at the periphery of the shoot meristem. Analysis of progeny resulting from the genetic cross aided in revealing a regulatory feedback loop between *WUS* and *CLV* activities in late stages of embryogenesis in Arabidopsis.[68]

Kuderova et al. employed the pOp/LhGR system to induce ectopic *ipt* (isopentenyltransferase) overexpression with a glucocorticoid, dexamethasone, to obtain temporal regulation of *ipt* and study the inhibitory effect of *ipt*-dependent cytokinin enhancement on root growth. This study showed that mechanisms involved in the maintenance of cytokinin homeostasis by cytokinin glycosylation might differ in a temporal manner. The group learned how differences in the specificity of inactivation of cytokinins by glucosylation might influence the developmental-specific sensitivity of the immature root meristems of Arabidopsis.[70] Mis-expression of the homeodomain gene BEL1-like homeodomain 1 (*BLH1*) in the embryo sac by the pOp/LhGR transactivation system showed that the cell-fate switch of synergid to egg cell in Arabidopsis *eostre* mutant embryo sacs is mediated by *BLH1*.[71]

Two-component transactivation systems can be further manipulated to achieve conditional transactivation, allowing temporal as well as spatial ectopic gene expression. Developing a system that allows localized, conditional gene induction within sectors of the plant exposed to the inducer would make possible an investigation into protein function in two dimensions. One limitation of two-component transactivation systems that could occur in very rare cases would be the integration of the transgene at the same locus in the enhancer-trap parent and the UAS parent. However, the occurrence of transgene integration to the same locus within the 120-Mb genome of Arabidopsis is very low, making two-component systems efficient for study of protein function.

## Summary and Perspectives

Genomic and proteomic tools are fundamental in genome-wide assessment of gene and protein function. Widely accepted and extensively used plant biology molecular tools are mutant analyses, ectopic expression, mis-expression, overexpression and RNAi. Even though each technique has its own advantages and limitations, a suitable strategy or system must be selected to address the specific goals of individual projects. Whereas a number of tools allow tissue-, organ- or temporal-specific regulation of gene expression that aids investigations into the roles of specific genes in distinct aspects of biological or metabolic processes, additional methods for such analyses are emerging. The use of laser-capture microdissection (LCM), which results in the isolation of relatively pure pools of cells[72] and can be used in combination with genomic (e.g., microarray) and proteomic methodologies, is gaining in popularity and providing novel insight into cell- and tissue-specific aspects of biological processes in plants (reviewed in ref. 73–79). Furthermore, tested and reproducible methods for regulating tandemly arrayed genes, members of gene families that exhibit closely related sequences and targeted gene deletion or modification (e.g., homologous recombination) are still atypical and infrequently used in plants. Improvements to RNAi will contribute to the analyses of tandemly arrayed genes,[80] a notoriously difficult biological problem. Improved methods for gene targeting via homologous recombination in plants are emerging,[81-85] as are new methods for gene targeting, including the use of zinc-finger nucleases to modify genes in a directed fashion.[86-88] In the latter protocol, zinc-finger nucleases are used to modify genes based on their ability to create double-strand DNA breaks at sequence-specific sites.[86-88] The continued development of genetic tools that will add to our comprehensive understanding of biological, metabolic, growth and developmental processes in *Arabidopsis thaliana* is essential for improving our general knowledge of plants, as well as aiding in subsequent application of knowledge to crop plants for successful manipulation of their genomes in order to increase crop yield and quality.


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### References


1. Weigel D, Ahn JH, Blazquez MA, Borevitz JO, Christensen SK, Fankhauser C, et al. Activation tagging in Arabidopsis. Plant Physiol 2000; 122:1003-13.
2. Wu K, Tian L, Zhou C, Brown D, Miki B. Repression of gene expression by Arabidopsis HD2 histone deacetylases. Plant J 2003; 34:241-7.
3. Corrado G, Karali M. Inducible gene expression systems and plant biotechnology. Biotechnol Adv 2009; 27:733-43.
4. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 1998; 16:735-43.
5. Zuo J, Niu QW, Frugis G, Chua NH. The *WUSCHEL* gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 2002; 30:349-59.
6. Baroux C, Blanvillain R, Betts H, Batoko H, Craft J, Martinez A, et al. Predictable activation of tissue-specific expression from a single gene locus using the pOp/LhG4 transactivation system in Arabidopsis. Plant Biotechnol J 2005; 3:91-101.
7. Moore I, Galweiler L, Grosskopf D, Schell J, Palme K. A transcription activation system for regulated gene expression in transgenic plants. Proc Natl Acad Sci USA 1998; 95:376-81.
8. Franklin KA, Quail PH. Phytochrome functions in Arabidopsis development. J Exp Bot 2010 ;61:11-24.
9. Franklin KA, Whitelam GC. Light signals, phytochromes and cross-talk with other environmental cues. J Exp Bot 2004; 55:271-6.
10. Neff MM, Fankhauser C, Chory J. Light: an indicator of time and place. Genes Dev 2000; 14:257-71.
11. Kami C, Lorrain S, Hornitschek P, Fankhauser C. Light-regulated plant growth and development. Curr Top Dev Biol 2010; 91:29-66.
12. Toth R, Kevei E, Hall A, Millar AJ, Nagy F, Kozma-Bognar L. Circadian clock-regulated expression of phytochrome and cryptochrome genes in Arabidopsis. Plant Physiol 2001; 127:1607-16.
13. Zeevaart JA. Florigen coming of age after 70 years. Plant Cell 2006; 18:1783-9.

14. Endo M, Mochizuki N, Suzuki T, Nagatani A. CRYPTOCHROME2 in vascular bundles regulates flowering in Arabidopsis. Plant Cell 2007; 19:84-93.
15. Franklin KA, Whitelam GC. Phytochromes and shade-avoidance responses in plants. Ann Bot 2005; 96:169-75.
16. Warnasooriya SN, Montgomery BL. Detection of spatial-specific phytochrome responses using targeted expression of biliverdin reductase in Arabidopsis. Plant Physiol 2009; 149:424-33.
17. Ranjan A, Fiene G, Fackendahl P, Hoecker U. The Arabidopsis repressor of light signaling SPA1 acts in the phloem to regulate seedling de-etiolation, leaf expansion and flowering time. Development 2011; 138:1851-62.
18. Srivastava AC, Ganesan S, Ismail IO, Ayre BG. Effective carbon partitioning driven by exotic phloem-specific regulatory elements fused to the *Arabidopsis thaliana AtSUC2* sucrose-proton symporter gene. BMC Plant Biol 2009; 9:7.
19. Srivastava AC, Ganesan S, Ismail IO, Ayre BG. Functional characterization of the Arabidopsis AtSUC2 Sucrose/$H^+$ symporter by tissue-specific complementation reveals an essential role in phloem loading but not in long-distance transport. Plant Physiol 2008; 148:200-11.
20. Gatz C. Chemical control of gene expression. Annu Rev Plant Physiol Plant Mol Biol 1997; 48:89-108.
21. Gallois JL, Nora FR, Mizukami Y, Sablowski R. WUSCHEL induces shoot stem cell activity and developmental plasticity in the root meristem. Genes Dev 2004; 18:375-80.
22. Laux T, Mayer KF, Berger J, Jurgens G. The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development 1996; 122:87-96.
23. Caddick MX, Greenland AJ, Jepson I, Krause KP, Qu N, Riddell KV, et al. An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nat Biotechnol 1998; 16:177-80.
24. Xiong TC, Hann CM, Chambers JP, Surget M, Ng CK. An inducible, modular system for spatio-temporal control of gene expression in stomatal guard cells. J Exp Bot 2009; 60:4129-36.
25. Roslan HA, Salter MG, Wood CD, White MR, Croft KP, Robson F, et al. Characterization of the ethanol-inducible *alc* gene-expression system in *Arabidopsis thaliana*. Plant J 2001; 28:225-35.
26. Garoosi GA, Salter MG, Caddick MX, Tomsett AB. Characterization of the ethanol-inducible *alc* gene expression system in tomato. J Exp Bot 2005; 56:1635-42.
27. Filichkin SA, Meilan R, Busov VB, Ma C, Brunner AM, Strauss SH. Alcohol-inducible gene expression in transgenic Populus. Plant cell reports 2006; 25:660-7.
28. Peebles CA, Gibson SI, Shanks JV, San KY. Characterization of an ethanol-inducible promoter system in *Catharanthus roseus* hairy roots. Biotechnology progress 2007; 23:1258-60.
29. Roslan HA, Salter MG, Wood CD, White MR, Croft KP, Robson F, et al. Characterization of the ethanol-inducible alc gene-expression system in *Arabidopsis thaliana*. Plant J 2001; 28:225-35.
30. Xu J, Scheres B. Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. Plant Cell 2005; 17:525-36.
31. Hanzawa Y, Takahashi T, Michael AJ, Burtin D, Long D, Pineiro M, et al. *ACAULIS5*, an Arabidopsis gene required for stem elongation, encodes a spermine synthase. EMBO J 2000; 19:4248-56.
32. Gatz C, Frohberg C, Wendenburg R. Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. Plant J 1992; 2:397-404.
33. Maizel A, Weigel D. Temporally and spatially controlled induction of gene expression in *Arabidopsis thaliana*. Plant J 2004; 38:164-71.
34. Jorgensen R. Altered gene expression in plants due to trans interactions between homologous genes. Trends Biotechnol 1990; 8:340-4.
35. Baulcombe D. RNA silencing in plants. Nature 2004; 431:356-63.
36. Vance V, Vaucheret H. RNA silencing in plants—defense and counterdefense. Science 2001; 292:2277-80.
37. Qadota H, Inoue M, Hikita T, Koppen M, Hardin JD, Amano M, et al. Establishment of a tissue-specific RNAi system in *C. elegans*. Gene 2007; 400:166-73.
38. Rao MK, Wilkinson MF. Tissue-specific and cell type-specific RNA interference in vivo. Nat Protoc 2006; 1:1494-501.
39. Schulz JG, David G, Hassan BA. A novel method for tissue-specific RNAi rescue in Drosophila. Nucleic Acids Res 2009; 37:93.
40. Byzova M, Verduyn C, De Brouwer D, De Block M. Transforming petals into sepaloid organs in Arabidopsis and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner. Planta 2004; 218:379-87.
41. Wielopolska A, Townley H, Moore I, Waterhouse P, Helliwell C. A high-throughput inducible RNAi vector for plants. Plant Biotechnol J 2005; 3:583-90.
42. Jiang L, Becker HC. Inheritance of apetalous flowers in a mutant of oilseed rape. Crop Sci 2003; 43:508-10.
43. Craft J, Samalova M, Baroux C, Townley H, Martinez A, Jepson I, et al. New pOp/LhG4 vectors for stringent glucocorticoid-dependent transgene expression in Arabidopsis. Plant J 2005; 41:899-918.
44. Masclaux F, Charpenteau M, Takahashi T, Pont-Lezica R, Galaud JP. Gene silencing using a heat-inducible RNAi system in Arabidopsis. Biochem Biophys Res Commun 2004; 321:364-9.
45. Linka N, Theodoulou FL, Haslam RP, Linka M, Napier JA, Neuhaus HE, et al. Peroxisomal ATP import is essential for seedling development in *Arabidopsis thaliana*. Plant Cell 2008; 20:3241-57.
46. Zhai Z, Sooksa-nguan T, Vatamaniuk OK. Establishing RNA interference as a reverse-genetic approach for gene functional analysis in protoplasts. Plant Physiol 2009; 149:642-52.
47. Cobbett CS, May MJ, Howden R, Rolls B. The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. Plant J 1998; 16:73-8.
48. Montgomery BL. Right place, right time: Spatiotemporal light regulation of plant growth and development. Plant Signal Behav 2008; 3:1053-60.
49. Endo M, Nakamura S, Araki T, Mochizuki N, Nagatani A. Phytochrome B in the mesophyll delays flowering by suppressing *FLOWERING LOCUS T* expression in Arabidopsis vascular bundles. Plant Cell 2005; 17:1941-52.
50. Michael TP, McClung CR. Enhancer trapping reveals widespread circadian clock transcriptional control in Arabidopsis. Plant Physiol 2003; 132:629-39.
51. Fridborg I, Williams A, Yang A, MacFarlane S, Coutts K, Angell S. Enhancer trapping identifies *TRI*, an Arabidopsis gene upregulated by pathogen infection. Mol Plant Microbe Interact 2004; 17:1086-94.
52. Campisi L, Yang Y, Yi Y, Heilig E, Herman B, Cassista AJ, et al. Generation of enhancer trap lines in Arabidopsis and characterization of expression patterns in the inflorescence. Plant J 1999; 17:699-707.
53. Groover A, Fontana JR, Dupper G, Ma C, Martienssen R, Strauss S, et al. Gene and enhancer trap tagging of vascular-expressed genes in poplar trees. Plant Physiol 2004; 134:1742-51.
54. Peng H, Huang H, Yang Y, Zhai Y, Wu J, Huang D, et al. Functional analysis of GUS expression patterns and T-DNA integration characteristics in rice enhancer trap lines. Plant Sci 2005; 168:1571-9.
55. Vijaybhaskar V, Subbiah V, Kaur J, Vijayakumari P, Siddiqi I. Identification of a root-specific glycosyltransferase from Arabidopsis and characterization of its promoter. J Biosci 2008; 33:185-93.
56. Haseloff J. GFP variants for multispectral imaging of living cells. Methods Cell Biol 1999; 58:139-51.
57. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 1993; 118:401-15.
58. Laplaze L, Parizot B, Baker A, Ricaud L, Martiniere A, Auguy F, et al. GAL4-GFP enhancer trap lines for genetic manipulation of lateral root development in *Arabidopsis thaliana*. J Exp Bot 2005; 56:2433-42.
59. Johnson AA, Hibberd JM, Gay C, Essah PA, Haseloff J, Tester M, et al. Spatial control of transgene expression in rice (*Oryza sativa* L.) using the GAL4 enhancer trapping system. Plant J 2005; 41:779-89.
60. Haseloff J. GFP variants for multispectral imaging of living cells. Methods Cell Biol 1999; 58:139-51.
61. Parizot B, Laplaze L, Ricaud L, Boucheron-Dubuisson E, Bayle V, Bonke M, et al. Diarch symmetry of the vascular bundle in Arabidopsis root encompasses the pericycle and is reflected in distich lateral root initiation. Plant Physiol 2008; 146:140-8.
62. Laplaze L, Benkova E, Casimiro I, Maes L, Vanneste S, Swarup R, et al. Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell 2007; 19:3889-900.
63. Dodd AN, Jakobsen MK, Baker AJ, Telzerow A, Hou SW, Laplaze L, et al. Time of day modulates low-temperature Ca signals in Arabidopsis. Plant J 2006; 48:962-73.
64. Janacek SH, Trenkamp S, Palmer B, Brown NJ, Parsley K, Stanley S, et al. Photosynthesis in cells around veins of the C3 plant *Arabidopsis thaliana* is important for both the shikimate pathway and leaf senescence as well as contributing to plant fitness. Plant J 2009; 59:329-43.
65. Jia H, Van Loock B, Liao M, Verbelen JP, Vissenberg K. Combination of the ALCR/alcA ethanol switch and GAL4/VP16-UAS enhancer trap system enables spatial and temporal control of transgene expression in Arabidopsis. Plant Biotechnol J 2007; 5:477-82.
66. Gälweiler L, Conlan RS, Mader P, Palme K, Moore I. Technical advance: the DNA-binding activity of gal4 is inhibited by methylation of the gal4 binding site in plant chromatin. Plant J 2000; 23:143-57.
67. Schoof H, Lenhard M, Haecker A, Mayer KF, Jurgens G, Laux T. The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. Cell 2000; 100:635-44.
68. Schoof H, Lenhard M, Haecker A, Mayer KF, Jurgens G, Laux T. The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. Cell 2000; 100:635-44.
69. Guo J, Hu X, Duan R. Interactive effects of cytokinins, light and sucrose on the phenotypes and the syntheses of anthocyanins and lignins in cytokinin overproducing transgenic Arabidopsis. J Plant Growth Regul 2005; 24:93-101.
70. Kuderova A, Urbankova I, Valkova M, Malbeck J, Brzobohaty B, Nemethova D, et al. Effects of conditional IPT-dependent cytokinin overproduction on root architecture of Arabidopsis seedlings. Plant Cell Physiol 2008; 49:570-82.
71. Pagnussat GC, Yu HJ, Sundaresan V. Cell-fate switch of synergid to egg cell in Arabidopsis *eostre* mutant embryo sacs arises from misexpression of the *BEL1*-like homeodomain gene *BLH1*. Plant Cell 2007; 19:3578-92.
72. Simone NL, Bonner RF, Gillespie JW, Emmert-Buck MR, Liotta LA. Laser-capture microdissection: opening the microscopic frontier to molecular analysis. Trends Genet 1998; 14:272-6.
73. Cai S, Lashbrook CC. Laser capture microdissection of plant cells from tape-transferred paraffin sections promotes recovery of structurally intact RNA for global gene profiling. Plant J 2006; 48:628-37.
74. Casson SA, Spencer MWB, Lindsey K. Laser-capture microdissection to study global transcriptional changes during plant embryogenesis. In: Suárez MF, Bozhkov PV, Eds. Plant Embryogenesis. Totowa, NJ: Humana Press 2008; 111-20.

75. Kerk NM, Ceserani T, Tausta SL, Sussex IM, Nelson TM. Laser capture microdissection of cells from plant tissues. Plant Physiol 2003; 132:27-35.
76. Klink V, Overall C, Alkharouf N, MacDonald M, Matthews B. Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean (*Glycine max*) roots infected by the soybean cyst nematode (*Heterodera glycines*). Planta 2007; 226:1389-409.
77. Nakazono M, Qiu F, Borsuk LA, Schnable PS. Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: Identification of genes expressed differentially in epidermal cells or vascular tissues of Maize. Plant Cell 2003; 15:583-96.
78. Tauris B, Borg S, Gregersen PL, Holm PB. A roadmap for zinc trafficking in the developing barley grain based on laser capture microdissection and gene expression profiling. J Exp Bot 2009; 60:1333-47.
79. Wu Y, Llewellyn D, White R, Ruggiero K, Al-Ghazi Y, Dennis E. Laser capture microdissection and cDNA microarrays used to generate gene expression profiles of the rapidly expanding fibre initial cells on the surface of cotton ovules. Planta 2007; 226:1475-90.
80. Jander G, Barth C. Tandem gene arrays: a challenge for functional genomics. Trends Plant Sci 2007; 12:203-10.
81. Cotsaftis O, Guiderdoni E. Enhancing gene targeting efficiency in higher plants: rice is on the move. Transgenic Res 2005; 14:1-14.
82. Iida S, Terada R. Modification of endogenous natural genes by gene targeting in rice and other higher plants. Plant Mol Biol 2005; 59:205-19.
83. Shaked H, Melamed-Bessudo C, Levy AA. High-frequency gene targeting in Arabidopsis plants expressing the yeast RAD54 gene. Proc Natl Acad Sci USA 2005; 102:12265-9.
84. Terada R, Johzuka-Hisatomi Y, Saitoh M, Asao H, Iida S. Gene targeting by homologous recombination as a biotechnological tool for rice functional genomics. Plant Physiol 2007; 144:846-56.
85. Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S. Efficient gene targeting by homologous recombination in rice. Nat Biotech 2002; 20:1030-4.
86. Wright DA, Townsend JA, Winfrey RJ Jr, Irwin PA, Rajagopal J, Lonosky PM, et al. High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J 2005; 44:693-705.
87. Townsend JA, Wright DA, Winfrey RJ, Fu F, Maeder ML, Joung JK, et al. High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 2009; 459:442-5.
88. Zhang F, Maeder ML, Unger-Wallace E, Hoshaw JP, Reyon D, Christian M, et al. High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. Proc Natl Acad Sci USA 2010; 107:12028-33.