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Strategies for Performing Dynamic Gene Perturbation Experiments in Flowers

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[Abstract] Dissecting the gene regulatory networks (GRNs) underlying developmental processes is a central goal in biology. The characterization of the GRNs underlying flower development has received considerable attention, however, novel approaches are required to reveal temporal and spatial aspects of these GRNs. Here, we provide an overview of the options available to perform dynamic gene perturbations to identify downstream response genes at specific stages of development in the flowers of *Arabidopsis thaliana*.

[Introduction] Gene activity perturbation followed by expression analysis of downstream targets represents a powerful method to understand gene function and dissect regulatory hierarchies. The transcriptomes of "static" mutant lines (*e.g.,* caused by point mutations, transfer-DNA insertions, or constitutive expression) are often compared with those of wild type counterparts, which can confound the biological interpretation of the expression data. For example, a null mutant (noted *ag-1*) of the transcription factor AGAMOUS (AG) lacks stamens and carpels, which are replaced by sepals and petals (Figure 1A and 1B) (Bowman *et al.*, 1989). A comparison of the transcriptomes of *ag-1* flowers with wild-type flowers may lead to the conclusion that AG regulates the expression of genes controlling the specification of mature tissues in the anther and gynoecium. This interpretation is inappropriate, as AG is required for the specification of these organ-types (Bowman *et al.*, 1989; Yanofsky *et al.*, 1990). Therefore, the cause of the expression differences observed may be a result of tissue biases (Wellmer *et al.*, 2004). Dynamic perturbations circumvent these and other issues: i) the tissues harvested will be morphologically identical, as tissue from "mock-treated" and inducer-treated plants would normally be harvested within a 24 h time-frame, ii) the response of downstream targets can be measured in the range of minutes and hours, which can be useful to infer direct and indirect interactions, iii) the development-dependent functions of the regulators of interest can be dissected (O'Maoileidigh *et al.*, 2015).

Flowers of the model plant *Arabidopsis thaliana* are minute, particularly at early stages of development. Furthermore, they are initiated in a sequential manner such that no two flowers are at the exact same stage of development on any given inflorescence (Smyth *et al.*, 1990). Until recently, these traits have inhibited investigations into the molecular mechanisms underlying the earliest stages of flower development. There are many strategies available to isolate tissue from young flower buds including laser capture microdissection and fluorescent

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activated cell sorting (Birnbaum *et al.*, 2003; Mantegazza *et al.*, 2014; Wuest and Grossniklaus, 2014). We prefer to use a floral induction system (FIS) to isolate flowers at specific stages of development, as it is a user friendly, low-cost approach (O'Maoileidigh *et al.*, 2015; Wellmer *et al.*, 2006; O'Maoileidigh and Wellmer, 2014). This system is based on the reintroduction of APETALA1 (AP1) activity into the *apetala1-1 cauliflower-1* (*ap1-1 cal-1*) background *via* either a rat glucocorticoid (Wellmer *et al.*, 2006; O'Maoileidigh and Wellmer, 2014; DS *et al.*, 2013) or a mouse androgen (O'Maoileidigh *et al.*, 2015) receptor ligand binding domain (noted GR and AR, respectively) fusion with AP1. Once the FIS *ap1-1 cal-1* inflorescences are treated with dexamethasone or dihydrogentestosterone (DHT, 5α-androstan-17β-ol-3-one), respectively, AP1 translocates to the nucleus to regulate transcription, which leads to the formation of many flowers on a single inflorescence that are at similar stages of development (Wellmer *et al.*, 2006; O'Maoileidigh and Wellmer, 2014).

We have shown that stage-specific flowers can be reliably harvested from early to late stages of development (Wellmer *et al.*, 2006; Ryan *et al.*, 2015). We have also demonstrated that these FISs can be combined with two-component inducible systems that facilitate dynamic perturbations (O'Maoileidigh *et al.*, 2015; O'Maoileidigh *et al.*, 2013; Wuest *et al.*, 2012). We have generated FISs that are responsive to dexamethasone and DHT, which we have combined with the ethanol-responsive AlcApro/AlcR and the OPpro/GR-LhG4 dexamethasone-responsive two-component systems, respectively, to express artificial microRNAs (amiRNAs) that perturb gene activity. We have successfully used these lines to assess the stage-specific functions of genes of interest (O'Maoileidigh *et al.*, 2015; O'Maoileidigh *et al.*, 2013; Wuest *et al.*, 2012). Notably, we also observed that these two-component inducible systems can influence the expression of the *A. thaliana* genome independently of the intended amiRNA-mediated perturbation (O'Maoileidigh *et al.*, 2015). Therefore, we developed and described several control measures that must be taken in order to account for these experimental artifacts (O'Maoileidigh *et al.*, 2015).

In the following protocol, we provide technical advice for implementing dynamic perturbation strategies during flower development and the establishment of the experimental design. As a guide, we briefly discuss the perturbation strategies implemented to understand the functions of the homeotic gene *AG*, including our published and unpublished results, as well as data from the literature.

Materials and Reagents

1. 20 cm x 32 cm x 50 cm trays with plastic lids [Romberg & Sohn, catalog number: 51221K (trays) and 74051K (lids)]
2. 50 ml centrifuge tubes (Sigma-Aldrich, catalog number: CLS430829-500E)
3. Disposable pasteur pipettes (Thermo Fisher Scientific, catalog number: 12837625)
4. Mouse AR coding sequence [obtained from the vector pBJ36-mAR (O'Maoileidigh *et al.*, 2015)]

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Note: Only the ligand-binding domain was fused to AP1.

5. Rat GR coding sequence [obtained from the vector pBJ36-GR (O'Maoileidigh *et al.*, 2013)

   Note: Only the ligand-binding domain was fused to AP1.
6. SRDX domain fusions [produced using the vector p35SSRDXG (Mitsuda *et al.*, 2011)]

   Note: The SRDX amino acid sequence is LDLELRLGFA (Ikeda and Ohme-Takagi, 2009).
7. The WUSB coding sequence [obtained from the vector p35SWUSB (Ikeda *et al.*, 2009)]

   Note: The WUSB amino acid sequence is HRRTLPLFPMHGED (Ikeda et al., 2009).
8. VP16 domain fusions [produced using the vector p35SVP16 (Mitsuda *et al.*, 2011)]

   Note: Genbank accession KM486811 for VP16 activation domain sequence in a cloning vector.
9. AlcA sequence [obtained from AlcApro-pBJ36 (Leibfried *et al.*, 2005)]
10. AlcR coding sequence [obtained from 35Spro:AlcR-pML-BART (Leibfried *et al.*, 2005)]
11. GR-LhG4 coding sequence [obtained from 35Spro:GR-LhG4-pML-BART (Wuest *et al.*, 2012)]
12. OPpro sequence [obtained from 6xOPpro-pBJ36 (Wuest *et al.*, 2012)]
13. Floral induction system (FIS) [Various versions of the FISs are available from Dr. Frank Wellmer [1-3]]
14. CONTROL-amiRNA sequence [obtained from pBJ36-AlcApro:CTRL-amiRNA (O'Maoileidigh *et al.*, 2015)]
15. GR antibody [obtained from Santa Cruz (sc-1002) (Kaufmann *et al.*, 2010)]
16. Dexamethasone (Sigma-Aldrich, catalog number: D4902)
17. α-Androstan-17β-ol-3-one (DHT) (Sigma-Aldrich, catalog number: A8380)
18. General reagents for molecular cloning and expression analysis
19. Liquid nitrogen
20. 10 mM dexamethasone stock solution (see Recipes)
21. 100 mM DHT stock solution (see Recipes)
22. GR activation solution (see Recipes)
23. AR activation solution (see Recipes)

Equipment

1. Sharp Forceps (Sigma-Aldrich, catalog number: T5790)
2. Dissection microscope
3. General equipment for molecular cloning and expression analysis

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Procedure

A. Gain-of-function-mediated dynamic perturbation

The use of inducible gain-of-function strategies to understand regulatory hierarchies can be extremely informative. For example, Gomez-Mena *et al*. (2005) overexpressed an AG-GR fusion under the control of the Cauliflower Mosaic Virus (CMV) 35S promoter (Benfey and Chua, 1990), to identify targets of AG (Gomez-Mena *et al.*, 2005). Many of the genes they identified as differentially expressed in response to AG activation were shown to be direct targets (O'Maoileidigh *et al.*, 2013; Gomez-Mena *et al.*, 2005). Ito *et al*. (2004) also used a 35Spro:AG-GR line to perform perturbation experiments in the presence of the protein biosynthesis inhibitor cycloheximide, which was used to infer the direct regulation of the *SPOROCYTELESS* gene by AG. In addition, (Ito *et al*., 2007) introduced the 35Spro:AG-GR transgene into the *ag-1* background and determined the temporal requirements for AG activity, particularly during stamen development, using an AG dosage-dependent rescue assay (Ito *et al.*, 2004). Although these gain-of-function-mediated perturbation strategies are extremely useful, they can also produce artifactual results, as the activity of the protein of interest may behave differently due to its altered spatio-temporal pattern of expression (Smith *et al.*, 2011).

To perform a gain-of-function-mediated dynamic perturbation experiment, the protein of interest can simply be expressed from a promoter of interest in combination with the glucocorticoid (GR) or androgen (AR) receptor fusion technique (O'Maoileidigh *et al.*, 2015; Yamaguchi *et al.*, 2015; Sun *et al.*, 2009). Alternatively, the chemically inducible two-component promoter systems OPpro/GR-LhG4 or AlcApro/AlcR can be employed (Roslan *et al.*, 2001; Deveaux *et al.*, 2003; Craft *et al.*, 2005). Use of these promoter systems would not require modification of the protein of interest, which simplifies the design process relative to the GR/AR fusion techniques and eliminates the possibility that the GR/AR domain will interfere with protein function.

1. Fuse the coding region of your gene of interest to the coding region of the GR/AR ligand binding domain (Notes 1-2). Be sure to preserve the open reading frames of the gene of interest and the GR/AR domain. Place the gene fusion downstream of a promoter of choice (Note 3) in a binary vector. Alternatively, utilize a two-component inducible system (see above and Note 4).
2. Transform wild-type or mutant plants (Note 5) with the inducible transgene by floral dip and identify transformants using the appropriate selection technique (Clough and Bent, 1998).
3. Discard first generation (T_1) plants displaying phenotypes in the absence of the inducing agent.
4. Treat the inflorescences of the remaining first generation plants with the appropriate inducer (Note 6).

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5. Phenotype the flowers at least every second day after treatment. A gradient of phenotypes would be expected, depending on the stage of a given flower when it was treated (Note 7).
6. Alternatively, the plants can also be characterized on a molecular level to select candidates for further analysis (Notes 8-9). However, the phenotype produced after activating the transgene should be carefully examined.
7. Select several independent lines and, if using the GR/AR fusion technique, perform Western blotting analysis, using an anti-GR or anti-AR antibody, in the second generation to determine the level of expression of the fusion protein.
8. Select lines that produce full-length proteins, desirable phenotypes and/or desirable mRNA levels. Isolate lines that are homozygous for the transgene.
9. At this point, an inducible perturbation experiment can be initiated. However, it may be difficult to dissect the stage-specific functions of the factor of interest on a molecular level. Therefore, we recommend crossing the transgenic lines with the appropriate floral induction system.

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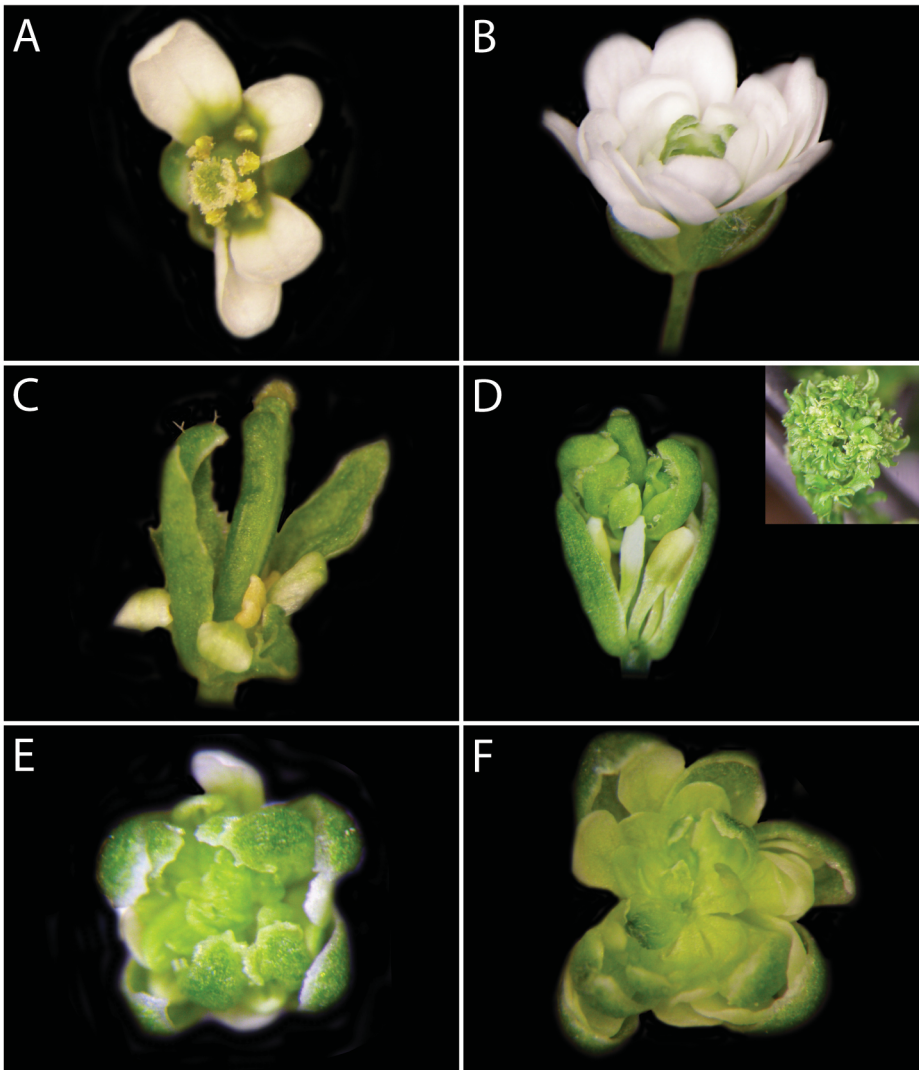

Figure 1. Perturbation of AG activity. A. A wild-type Landsberg *erecta* flower; B. An *ag-1* flower; C. A flower from a plant containing a 35Spro:AG-SRDX transgene (*unpublished*); D. A flower from a plant containing a 35Spro:AG-WUSB transgene (*unpublished*). Inset: An inflorescence from an independent line containing the 35Spro:AG-WUSB transgene (*unpublished*); E. A flower from a plant containing a 35Spro:RNAi-AG transgene (*unpublished*); F. A flower from a plant containing a 35Spro:AG-4-amiRNA transgene (O'Maoileidigh *et al.*, 2013).

B. Transcriptional effector domain-mediated dynamic perturbation

The function of the protein of interest can be modified by fusing it to a transcriptional repressor domain. For example, overexpression of a fusion between AG and an ERF-associated amphiphilic repression (EAR) motif from the protein SUPERMAN (SRDX) was shown to phenocopy a strong *ag* mutant phenotype (Mitsuda *et al.*, 2006). We independently generated a 35Spro:AG-SRDX fusion that was designed to produce an identical protein to the published version in Mitsuda *et al.* (2006) (Figure 1C), however, we

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did not observe any strong *ag*-like phenotypes. We also overexpressed a fusion of AG with the WUSB repression domain from the transcription factor WUSCHEL in plants (Ikeda *et al.*, 2009) and, in this case, did observe *ag*-like phenotypes (Figure 1D). However, we also observed plants with leaf-like floral organs (Figure 1D, inset). The reasons behind these discrepancies are unclear to us, however, they highlight the usefulness of generating control lines that constitutively express the chimeric gene prior to generating an inducible version.

The gene of interest can also be expressed as fusion with a transcriptional activation domain. To our knowledge, such domains have not been fused with AG, however, they have been successfully used to understand the functions of other important floral regulators (*e.g.,* Parcy *et al.*, 1998). As with the gain-of-function experiments, the overexpression of the chimeric protein may alter its behavior.

To perform this type of perturbation, the protein of interest can be fused to a transcriptional activation [*e.g.,* VP16 (Triezenberg *et al.*, 1988; Cousens *et al.*, 1989)] or repression domain [*e.g.,* SRDX (Hiratsu *et al.*, 2002)]. This should result in the activation or repression of all genes that the protein associates with, respectively (Note 10). The choice of an activation or repression domain would be weighted based on the known activities of the protein of interest and on the presence of endogenous transcriptional effector domains in the protein of interest.

1. Fuse the protein of interest to the transcriptional effector domain of choice so that the reading frame of the gene and domain remain intact. The chosen domain should be positioned so that it is unlikely to interfere with the activity of the protein of interest (Notes 1-2). To generate a non-inducible version of the fusion protein, place this fusion gene downstream of a chosen promoter (Note 11) in a binary vector.
2. Transform wild-type plants with the fusion gene by floral dip (Clough and Bent, 1998) and identify transformants using the appropriate selection technique (Miki and McHugh, 2004).
3. Characterize the phenotypes of the resulting transgenic plants. If they match expectations, proceed to step B4 (Note 12).
4. To make an inducible version, introduce the fusion gene into the dexamethasone or ethanol-responsive two-component promoter systems (Note 4).
5. Transform wild-type plants with inducible transgene by floral dip (Clough and Bent, 1998) and identify transformants using the appropriate selection technique (Miki and McHugh, 2004).
6. Discard T_1 plants displaying phenotypes in the absence of the inducing agent.
7. Treat the inflorescences of the remaining T_1 plants with the appropriate inducer (Note 6).
8. Phenotype the flowers periodically after treatment. A gradient of phenotypes would be expected, depending on the stage of a given flower when it was treated (Note 7).

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9. Alternatively, the plants can also be characterized on a molecular level to select candidates for further analysis (Notes 8-9). However, the phenotype produced after activating the transgene should be carefully examined.
10. Select plants that produce desirable phenotypes and/or desirable mRNA levels and isolate lines that are homozygous for the transgene.
11. At this point, an inducible perturbation experiment can be initiated. However, it may be difficult to dissect the stage-specific functions of the factor of interest on a molecular level. Therefore, we recommend crossing the transgenic lines with the appropriate floral induction system.

C. Loss-of-function-mediated dynamic perturbation

The use of loss-of-function-mediated perturbation strategies offers several advantages over the perturbation strategies described above. In this case, the activity of the factor of interest would be removed *via* small RNAs. Therefore, the caveats associated with overexpression of the factor of interest, or a modified version, would not apply. The options available to perform this type of perturbation include the production of short interfering RNAs from a double-stranded RNA (dsRNA) precursor (termed RNA interference, RNAi) or artificial microRNAs (amiRNAs) (Ossowski *et al.*, 2008; Schwab *et al.*, 2006). The RNAi strategy relies on expressing a dsRNA that is homologous to a large portion of the factor of interest. This results in the production of many short RNA molecules, whereas amiRNAs rely on the production of a single type of small RNA to target a specific transcript (Schwab *et al.*, 2006). Notably, RNAi-mediated perturbation is thought to lead to more off-targets than amiRNA-mediated perturbation (Ossowski *et al.*, 2008; Schwab *et al.*, 2006).

We previously screened seven independent amiRNA constructs before identifying an amiRNA that efficiently targets the *AG* mRNA, which, when overexpressed, can recapitulate a strong *ag* null phenotype (Figure 1F) (O'Maoileidigh *et al.*, 2013). We also successfully perturbed AG activity with an RNAi construct, however, the phenotype was somewhat weaker than the phenotype observed for the functional amiRNA (Figure 1E). Furthermore, the proportion of first generation plants that displayed a phenotype in the RNAi lines (~8%) was much lower than the proportion of lines that displayed a phenotype in the amiRNA lines (100%). Given this, and the potential off-target effects associated with RNAi-mediated perturbation, our recommendation would be to utilize amiRNAs to perturb gene activity.

To perform a loss-of-function-mediated dynamic perturbation experiment, the chemically inducible two-component promoter systems described above can be used. To demonstrate functionality, we recommend making stable transformants that constitutively express the RNAi/amiRNA of interest prior to making an inducible version (Note 13).

1. Design an RNAi (Note 14) or amiRNA (Note 15) construct and place it under the control of a promoter of choice in a binary vector.

   Notes:

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a. The materials and the cloning procedure to generate RNAi constructs are described in Eamens and Waterhouse (2011).
b. The cloning procedure to generate amiRNAs is described by Schwab *et al*. (2006) while the appropriate vectors can be obtained through wmd3.weigelworld.org.

2. Transform wild-type plants with the construct by floral dip (Clough and Bent, 1998) and identify transformants using the appropriate selection technique (Miki and McHugh, 2004).
3. If the phenotypes of the transgenic plants match expectations, proceed to step C4 (Note 12). Alternatively, if the phenotype of a corresponding null mutant is unknown, the mRNA levels of the gene of interest can be determined.
4. To allow an inducible knockdown of gene activities, introduce the RNAi/amiRNA sequence into the dexamethasone or ethanol responsive two-component promoter systems (Note 4).
5. Transform wild-type plants with inducible transgene by floral dip (Clough and Bent, 1998) and identify transformants using the appropriate selection technique (Miki and McHugh, 2004).
6. Discard T_1 plants displaying phenotypes in the absence of the inducing agent.
7. Treat the inflorescences of the remaining first generation plants with the appropriate inducer (Note 6).
8. Phenotype the flowers periodically after treatment. A gradient of phenotypes would be expected, depending on the stage of a given flower when it was treated (Note 16).
9. Alternatively, the plants can be characterized on a molecular level to select candidates for further analysis (Notes 8-9).
12. Select plants that produce desirable phenotypes and/or desirable mRNA levels (Note 17) and isolate lines that are homozygous for the transgene.
13. At this point, an inducible perturbation experiment can be initiated. However, it may be difficult to dissect the stage-specific functions of the factor of interest on a molecular level. Therefore, we recommend crossing the transgenic lines with the appropriate floral induction system.

D. Inducible perturbation followed by gene expression analysis and phenotypic analysis

Gene expression profiling using dynamic perturbations with whole inflorescences

It is possible to perform an inducible gene perturbation experiment using whole inflorescences, however, no temporal information will be available from these data. In fact, the RNA populations isolated are likely to be dominated by RNA from older flowers (Wellmer *et al.*, 2004).

1. Grow plants so that they bolt in a relatively synchronous manner (Note 18).
2. Divide plants into two populations: those that will be treated with the inducer and those that will be treated with a mock solution. The same strategy would be applied to any control plants being used.

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3. Treat the inflorescence to activate expression of the dynamic perturbation transgene (Note 6).
4. Harvest approximately 20 inflorescences per sample from a few hours to days after initiating the dynamic perturbation using liquid nitrogen to keep the samples frozen. Extract RNA and perform qRT-PCRs to measure the responsiveness of i) the effector molecule (*e.g.,* amiRNA precursor, protein fusion) and ii) a gene (or several genes) that will be affected by the induction. These data will allow the assessment of the kinetics of the knockdown, which will inform the subsequent experimental design (Note 19).
5. Select the time at which the tissue will be harvested after the perturbation construct is activated.

Figure 2. Response of AP1pro:AP1-GR *ap1 cal* inflorescence to dexamethasone treatment. A. An untreated inflorescence-like meristem. B-C. An inflorescence meristem 5 days (B) and 8 days (C) after treatment with a solution containing dexamethasone.

6. Repeat steps 1-3 in Section D, above.
7. Harvest approximately 20 inflorescences at chosen time-points using liquid nitrogen to keep the samples frozen.
8. Process tissue for molecular analysis.

Gene expression profiling in a stage-specific manner using dynamic perturbations

Combining dynamic perturbation strategies with the FIS, which facilitates the collection of flowers at similar stages of development, affords the user with a low-cost, easy to use approach to identify stage-specific functions of their gene of interest (Figure 2A-C). The inducible transgene of choice can be crossed with the appropriate FIS or can be transformed directly into the FIS (Notes 20-21).

1. Isolate the inducible transgene in a suitable FIS background (Note 20).
2. Grow plants so that they bolt in a relatively synchronous manner (Note 18).
3. Induce synchronous flowering by locally treating the *ap1-1 cal-1* inflorescences (Note 22) (O'Maoileidigh *et al.*, 2015).

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4. Divide plants into two populations: those that will be treated with the inducer and those that will be treated with a mock solution. The same strategy would be applied to any control plants being used.
5. Treat the inflorescence to activate expression of the dynamic perturbation transgene (Note 6).
6. Harvest whole flowers (Note 23) from approximately 20 plants per sample from hours to days after initiating the dynamic perturbation using liquid nitrogen to keep the samples frozen. Extract RNA and perform qRT-PCRs to measure the responsiveness of i) the effector molecule (*e.g.,* amiRNA precursor, protein fusion) and ii) candidate gene(s) that will be affected by the perturbation to the inducer. These data will allow the assessment of the kinetics of the knockdown, which will inform the subsequent experimental design (Note 19).
7. To perform the perturbation experiment, select the time-points of flower development you wish to investigate using Table 1.
8. Select the time at which the tissue will be harvested after the perturbation construct is activated.
9. Repeat steps 1-4 in Section D, above.
10. Harvest whole flowers from approximately 20 plants at the chosen time-points from inducer-treated and mock-treated inflorescences using liquid nitrogen to keep the samples frozen.
11. Process tissue for molecular analysis.

Table 1. Correlation of days after activation of synchronous flowering in the FIS with stages of flower development. After day 7-8, the flowers developing from the inflorescence are no longer synchronized. At this point, however, it is possible to distinguish them morphologically. The table indicates approximations of the developmental stages that can be harvested on the indicated day after initiation of synchronous flowering using the FIS. These approximations are based on the flower stages described in Smyth *et al*. (1990) and growth of the plants in continuous light.

Stage	0	2	3	4	5 - 6	6 - 7	8	9 - 10	11 - 12	13
Days after initiation of synchronous flowering	0	1	2	3	4	5	7	9	11	13

Phenotyping flowers after a dynamic gene perturbation

1. Grow plants so that they bolt in a relatively synchronous manner (Note 18).
2. Activate the inducible promoter system with the appropriate inducer (Note 6).

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3. Dissect flowers at anthesis in the following days and phenotype them as desired. Continue this process until the flowers have produced the strongest mutant-like phenotype and the flowers return to a wild-type state.
4. Correlate the phenotype you observed at anthesis with the stage at which the transgene was activated (Note 24).
5. Compile a stage-dependent phenotyping series to compare with expression profiling data.

E. Controls

We outline the controls below that can be used for each perturbation strategy. In addition, we outline controls that are required for the inducible systems themselves. These latter controls are extremely important, as we have previously characterized the effects of these inducible systems on the transcriptome of *A. thaliana* (O'Maoileidigh *et al.*, 2015).

Transcriptional effector-mediated perturbations: Essential sequences in the transcriptional effector domain of choice can be mutated to produce a functionally inactive transcriptional effector domain [*e.g.,* mSRDX (Hiratsu *et al.*, 2004) or mVP16 (Cress and Triezenberg, 1991)]. These inactive domains can be fused to the factor of interest and expressed in an identical way to the primary experimental line. The protein-mEAR fusion should behave as a gain-of-function line. Therefore, it may also be desirable to generate an equivalent gain-of-function line that lacks an EAR motif as a control.

amiRNA-mediated perturbation: We have previously described a CONTROL-amiRNA sequence that is not predicted to target any annotated *A. thaliana* transcript (O'Maoileidigh *et al.*, 2015). Therefore, this CONTROL-amiRNA can be expressed in an identical manner to the primary experimental line to control for expression or phenotypic artifacts. An additional control includes generating an amiRNA-resistant version of the factor of interest by introducing synonymous mutations in the amiRNA target site of the corresponding gene. This would require the generation of an equivalent mutant plant whose phenotype is restored by an amiRNA-resistant factor.

AlcR and GR-LhG4 transcription factors: These chemically responsive transcription factors have been shown to influence the expression of the *A. thaliana* transcriptome (O'Maoileidigh *et al.*, 2015). Therefore, an essential control is to transform plants with T-DNAs that contain the AlcApro/AlcR or OPpro/GR-LhG4 cassettes and identify plants that express the transcription factors to a similar level to the primary experimental line.

Treatment controls: All treatment controls should be partnered with a “mock” treatment. This mock treatment solution should consist of an identical mix of the solvents and surfactants used but lacking the active ingredient. An additional control includes the treatment of a wild-type line, or an equivalent line, with the active solution.

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Notes

1. For example, the GR domain was fused to the C-terminus of AG since the DNA binding domain of AG is present at the N-terminus (Ito *et al*., 2004). It is possible that several versions with the tag of interest in different positions may need to be made.
2. Often short linkers of approximately 6-10 amino acids are placed between the protein of interest and the domain being fused to it (Sabourin *et al.*, 2007). This may reduce the likelihood of unwanted negative interactions. A repeat of nine alanine amino acids or a mix of glycine and alanine are commonly used in plants (Tian *et al.*, 2004; Heisler *et al.*, 2005).
3. Often the CMV 35S promoter is used (Benfey and Chua, 1990), however, it may not drive expression sufficiently in the tissues of interest. The promoter of *UBIQUITIN10* is frequently used as an alternative for constitutive expression (Grefen *et al.*, 2010). Promoters that drive expression only in certain tissues can also be used. This latter option may be desirable, especially if the inducible transgene is being expressed in its mutant background, as it may reduce the occurrence of false positives.
4. The promoters upstream of the AlcR and GR-LhG4 chimeric transcription factors can be altered to suit the experimental design.
5. Mutant plants can be useful in this case in order to show that the fusion protein can restore wild-type activity, as in the case of 35Spro:AG-GR *ag-1* plants (Ito *et al.*, 2004). The use of wild-type plants to identify a gain-of-function phenotype is also possible, however, it may be more difficult to interpret.
6. The concentration of dexamethasone or DHT to use may need to be optimized. As little as 50 nM DHT has been used to activate gene expression (Sun *et al.*, 2009). Ten µM dexamethasone is commonly used, however, the use of a lower concentration may be desirable (Yamaguchi *et al.*, 2015). Silwett L-77 should be used at a concentration of 0.015 % (v/v) to improve the uptake of the chemical. We have used ethanol vapor to activate the AlcApro/AlcR system (O'Maoileidigh *et al.*, 2013; Wuest *et al.*, 2012). Briefly, we enclosed plants in sealed containers (18 cm x 32 cm x 50 cm) with two 50 ml tubes containing 10 ml 100% ethanol for a range of times (O'Maoileidigh *et al.*, 2013; Wuest *et al.*, 2012).
7. This was elegantly shown by Ito *et al*. (2007), who used this strategy to dissect the stage-specific activities of AG. Flowers of 35Spro:AG-GR *ag-1* plants that were treated with dexamethasone at early stages of development produced mature flowers containing carpels and stamens whereas flowers treated at later contained carpelloid sepals and stamenoid petals (Ito *et al.*, 2004).
8. If using the GR/AR fusion technique, the mRNA levels of the fusion gene can be determined. If using a two-component inducible system, the mRNA levels of the gene of interest can be determined after treatment with the inducer. Alternatively, the mRNA

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levels of the AlcR/GR-LhG4 genes can be determined, which should correlate with the expression output of the gene of interest.

9. If selecting plants based on expression levels, it may be desirable to identify plants with strong, intermediate and weak expression levels of the transgene as the effects of extremely high expression can have negative consequences for downstream applications.
10. The protein of interest does not necessarily have to be a transcription factor, however, it must be localized in proximity to DNA.
11. The chimeric protein may not function as expected and the screening of inducible versions can be laborious. Therefore, the production of a constitutively expressed version is advised. Promoters that drive expression only in certain tissues can also be used.
12. In order to interpret the phenotype produced, it may be necessary to identify null mutants or gain-of-function mutants of the same gene.
13. Multiple amiRNAs may need to be screened, therefore, the expression of these amiRNAs from a constitutive promoter to select a functional amiRNA is advised.
14. The region cloned to generate a dsRNA construct should not be homologous to other annotated genes.
15. We prefer to select amiRNAs from the WMD3 platform (wmd3.weigelworld.org) so that the gene body is tiled with different amiRNAs. This is because the secondary structure of the target mRNA may interfere with amiRNA function.
16. Strong expression of the AlcR or GR-LhG4 transcription factors exacerbate non-specific effects. Therefore, a balance between the level of the knockdown and the expression of the chemically-responsive transcription factor should be reached.
17. We rotate our plants in the growth chambers to minimize effects of differing temperature and light gradients that might be present.
18. The depletion and recovery of the mRNA of the target gene in response to the activation of the RNAi/amiRNA construct will dictate the time at which tissue will be harvested. The optimal time to collect tissue would be when the mRNA levels of the target gene are lowest, however, protein abundance of the target may also need to be measured.
19. There are dexamethasone and DHT-responsive FISs available. The AlcApro/AlcR two-component system is compatible with either, however, the OPpro/GR-LhG4 system is compatible only with the DHT-responsive FIS.
20. There are versions of the FIS that are resistant to glufosinate ('BASTA') and kanamycin treatments, respectively (O'Maoileidigh *et al.*, 2015). Therefore, these plants can be transformed with a T-DNA containing the inducible transgene of interest with a compatible selection marker.
21. If the recovery of the mRNA levels of the targeted factor is rapid, then multiple or continuous treatments may be necessary prior to see strong mutant phenotypes.

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22. The AP1pro:AP1-GR *ap1 cal* FIS can be activated by treating the inflorescences with a solution containing 10 μM dexamethasone, 0.015% (v/v) Silwett L-77. The AP1pro:AP1-AR *ap1 cal* FIS can be activated by treating the inflorescences with a solution containing from 500 μM DHT (higher concentrations can be used), 0.015% (v/v) Silwett L-77.
23. The flowers at early stages should be scraped from the surface of the inflorescence with sharp forceps rather than harvesting the entire inflorescence (O'Maoileidigh and Wellmer, 2014).
24. Smyth *et al*. (1990) characterized the development of *A. thaliana* flowers in detail, which includes the approximate timings of each developmental stage. The length of these stages can be correlated with the perturbation series generated using the inducible perturbation line.

Recipes

1. 10 mM dexamethasone stock solution
   Resuspend dexamethasone powder in 100% EtOH to make a 10 mM stock
   Stored at -20 °C
2. 100 mM 5α-Androstan-17β-ol-3-one (DHT) stock solution
   Resuspend DHT in 100% EtOH to make a 100 mM stock
   Stored at -20 °C
3. GR activation solution
   10 μM dexamethasone
   0.015% (v/v) Silwet L-77
   0.1% (v/v) ethanol
4. AR activation solution
   500 μM DHT
   0.015% (v/v) Silwet L-77
   0.5% (v/v) ethanol

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