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The Use of a Dexamethasone-inducible System to Synchronize *Xa21* Expression to Study Rice Immunity

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[Abstract] Inducible gene expression systems offer researchers the opportunity to synchronize target gene expression at particular developmental stages and in particular tissues. The glucocorticoid receptor (GR), a vertebrate steroid receptor, has been well adopted for this purpose in plants. To generate steroid-inducible plants, a construct of GAL4-binding domain-VP16 activation domain-GR fusion (GVG) with the target gene under the control of upstream activation sequence (UAS) has been developed and extensively used in plant research.

Immune receptors perceive conserved molecular patterns secreted by pathogens and initiate robust immune responses. The rice immune receptor, *XA21*, recognizes a molecular pattern highly conserved in all sequenced genomes of *Xanthomonas*, and confers robust resistance to *X. oryzae* pv. *oryzae* (*Xoo*). However, identifying genes downstream of XA21 has been hindered because of the restrained lesion and thus limited defense response region in the plants expressing *Xa21*. Inducible expression allows for a synchronized immune response across a large amount of rice tissue, well suited for studying XA21-mediated immunity by genome-wide approaches such as transcriptomics and proteomics. In this protocol, we describe the use of this GVG system to synchronize *Xa21* expression.

Materials and Reagents

1. Wild-type rice seeds (*Oryza sativa* ssp. japonica cv. Kitaake)
2. Transgenic rice seeds containing *pTA7002::Myc::Xa21* (Park *et al*., 2012)
3. Transgenic rice seeds containing *Ubi::Myc::Xa21* (Park *et al*., 2010)
4. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*; Philippines race 6, strain PXO99Az)
5. Dexamethasone (Sigma-Aldrich, catalog number: D1756)
6. Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, catalog number: D128-1)
7. Tween-20 (Bio-Rad Laboratories, catalog number: 170-6531)
8. Sterile H_2O (Milli-Q)
9. TRIzol (Life Technologies, Invitrogen™, catalog number: 15596-026)
10. M-MLV reverse transcriptase (Life Technologies, Invitrogen™, catalog number: 28025-013)
11. SsoFastEvaGreenSupermix (Bio-Rad Laboratories, catalog number: 172-5203)

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12. Peptone sucrose agar (PSA) solid media containing 20 µg/ml cephalexin (MP Biomedicals, catalog number: 02150585) (see Recipes)
13. Dexamethasone (see Recipes)
14. Greenhouse rice growing conditions (see Recipes)
15. Walk-in growth chamber rice growing conditions (see Recipes)

Equipment

1. Spray bottle (550 ml) (any supplier) for dexamethasone foliar spray
2. 1.5 ml Eppendorf tube (any supplier)
3. Surgical scissors (sharp/sharp, straight, 5 ½ inch or similar) for *Xoo* clipping inoculation
4. 5 ½ inch square disposable pots
5. Supertub (24 inch x 36 inch x 8 inch) (Mac Court Products, model: ST3608 or similar)
6. Scale suitable for measurements down to 0.0001 g (any manufacturer)
7. Spectrophotometer suitable for taking optical density measurements at 600 nm (any manufacturer)
8. Growth chamber (14 h light and 10 h dark photoperiod with 28 °C temperature) (any manufacturer) for rice seed germination
9. Incubation chamber (28 °C) (any manufacturer) for *Xoo* preparation
10. Greenhouse capable of temperature and humidity control for growing rice plants
11. Walk-in growth chamber (conviron or equivalent) for *Xoo* inoculation and dexamethasone treatment
12. qPCR machine (Bio-Rad Laboratories, model: CFX96 Real-Time PCR)

Procedure

A. Rice growth conditions

1. Sow 12-20 rice seeds on filter paper in a petri dish (10 cm diameter) with 10 ml of sterile water and place it in an incubation chamber for one week.
2. Transplant one-week old seedlings into 5 ½ inch pots (three seedlings per pot) and grow them in the green house for approximately another five weeks. For irrigation, keep individual pots in supertubs filled with fertilized water until four weeks then continue filling super tub with reverse osmosis water.

   Notes:

   a. *Water level should be maintained near the soil surface but not higher to prevent green algae from covering the soil in the pots.*
   b. *A maximum capacity of 24 pots is possible per supertub, but 12 pots are optimum for these experiments to allow spacing between pots.*
3. When the flag leaf is fully extended but before panicle emergence (approximately six

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weeks after germination) transfer plants to walk-in growth chamber for *Xoo* inoculation and dexamethasone treatment.

Notes:

a. *Allow a minimum of three days for plants to equilibrate to chamber conditions before inoculation.*
b. *Roots often grow out of the bottom of the pots. Take care to minimize root damage while transferring plants to walk-in chambers.*

B. Rice inoculation with *Xoo*

1. Two days before transferring plants to the walk-in growth chamber, transfer 30 µl of *Xoo* from -80 □ C to a PSA solid media containing cephalexin and incubate at 28 □ C for three days until a biofilm is formed.
   Note: If needed, after biofilm formation Xoo can be stored at 4 □ C until ready to proceed, but should not be stored for more than two weeks.
2. Two days before inoculating rice plants, subculture approximately 1 cm diameter of *Xoo* from the original PSA solid media containing cephalexin to a new PSA solid media containing cephalexin and incubate at 28 □ C for an additional two days until biofilm has formed.
3. On the day of inoculation, suspend *Xoo* from the most recent PSA solid media containing cephalexin in sterile H_2O to OD_{600} nm=0.5.
4. Inoculate rice leaves by dipping scissor tips into the *Xoo* suspension and cutting the leaf approximately 2-3 cm away from the leaf tip of the 1st and 2nd leaves, or just 2nd leaf. Expected bacterial load immediately after inoculation is approximately 1 x 10^5 cfu/ml (Song *et al.*, 1995).
   Note: For a non-inoculated control, rice leaves should be clipped with scissors dipped into water alone.
5. Allow five to seven days after inoculation for *Xoo* to spread from the inoculated region and form a more even distribution across the rice leaves.
   Note: Five to seven days after inoculation was selected because based on previous experiments (Song et al., 1995), Xoo was shown to be completing log growth phase during that period of time. After seven days, disease symptoms begin to be more visibly pronounced.

C. Dexamethasone foliar spray

1. Prepare 30 µM dexamethasone + 0.01% Tween-20 fresh (400 ml) in 550 ml spray bottle on the day of foliar application.
2. Spray 200 ml of dexamethasone per supertub from a distance of 12-24 inches away from plants, walking around tub as you spray and moving in an up and down motion to ensure spray is being applied evenly to all portions of the plants.
   Notes:

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a. *Avoid bumping or disturbing the plants at this step or dexamethasone solution will slide off the leaves.*
b. *If walk-in growth chamber has a strong circulating fan, plants should be placed in a less windy area within the growth chamber for dexamethasone application as the fans will cause dexamethasone to evaporate too quickly and dexamethasone uptake may not be as uniform. Plants can be returned after dexamethasone solution is no longer visible on the surface of leaves (after 1 to 2 h).*

3. Harvest tissues at the desired time points after dexamethasone treatment. To harvest tissue, 3 to 5 cm at the inoculated leaf tip were cut with sterilized scissors and immediately frozen in liquid nitrogen.

Representative data

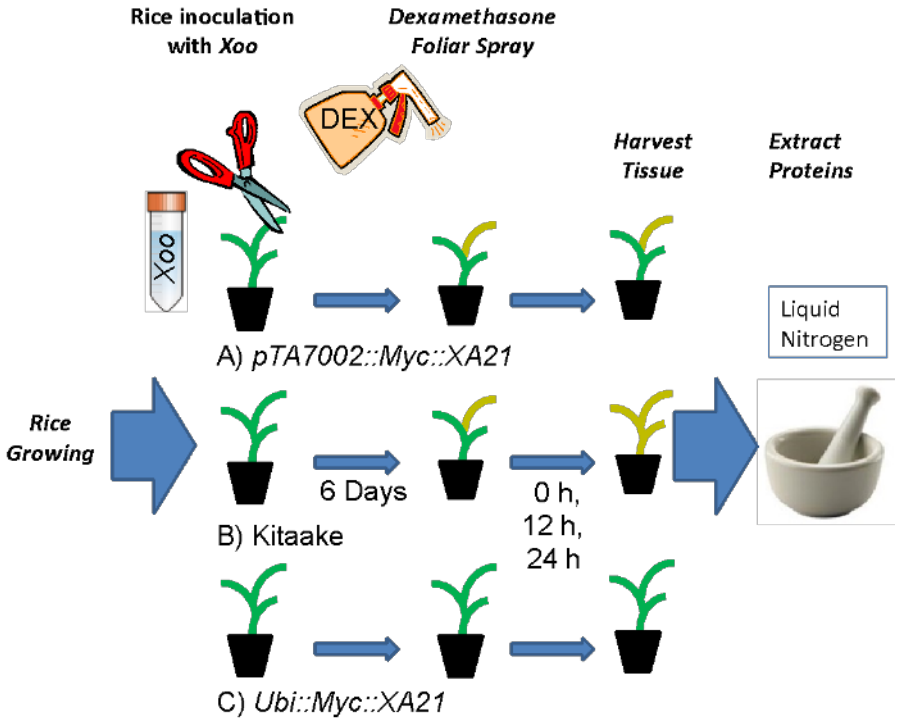

Figure 1. Experimental design using the dexamethasone-inducible system. Six-week old rice plants are inoculated with *Xoo*. Before dexamethasone treatment, *pTA7002::Myc::XA21* plants (A) do not express XA21 and phenocopy the susceptible Kitaake plants (B). Six days after inoculation, dexamethasone is applied to rice plants and XA21 begins to be expressed, inducing a robust immune response. Leaf tissue is harvested at 0 h, 12 h, and 24 h after dexamethasone application and frozen in liquid nitrogen for downstream applications. *Ubi::Myc::XA21* plants(C) overexpressing under the control of the maize ubiquitin promoter (Park *et al.*, 2010) are resistant to *Xoo* and used as a positive control here.

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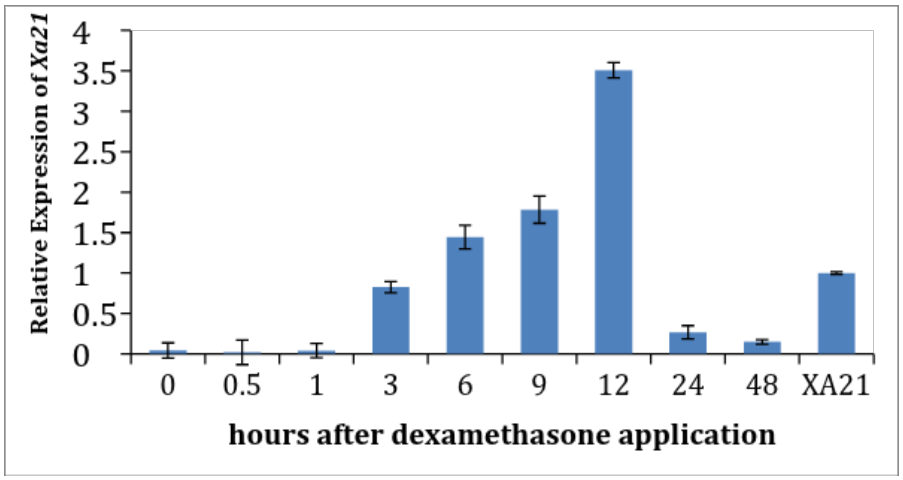

Figure 2. Expression of *Xa21* after dexamethasone application. *pTA7002::Myc::XA21* rice leaves were harvested at the indicated time points after application of dexamethasone. RNA was extracted using TRIzol with standard protocol; cDNA used for quantitative PCR was transcribed with M-MLV reverse transcriptase; and quantitative PCR was performed with SsoFastEvaGreenSupermix on a PCR machine. The gene expression of *Xa21* was normalized using the rice ubiquitin gene (LOC_Os06g46770) as an internal control, and the expression level in the *Ubi::Myc::XA21* plants (labeled as XA21) was set as 1.0.

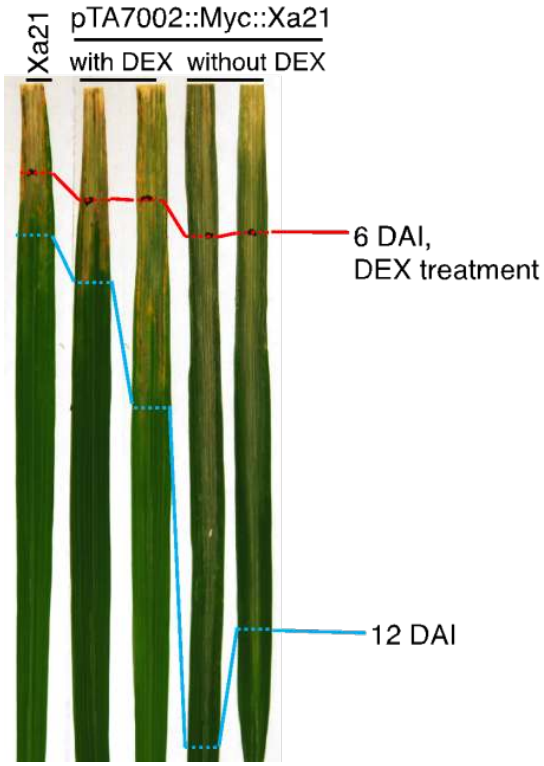

Figure 3. Dexamethasone treatment inhibits the elongation of lesion lengths in *pTA7002::Myc::XA21* plants. Six week old rice plants were inoculated with *Xoo*. At six days after inoculation (6 DAI) disease progression was measured (red line) before dexamethasone (DEX) was applied to the plants. Until 6 DAI, *pTA7002::Myc::XA21* plants did not express *Xa21* and the observed variation in disease development between with DEX and without DEX

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treated plants was not statistically significant. Disease progression was allowed to continue until 12 days after inoculation (12 DAI, 6 days after the DEX treatment), and lesion length was measured as indicated (blue line). *pTA7002::Myc::XA21* plants treated with dexamethasone showed significantly less disease progression compared to non-dexamethasone treated plants indicating that DEX treatment was successful in inducing expression of *Xa21* and that XA21-mediated immunity was triggered.

Notes

1. "Leaky" expression in the absence of hormone has previously been reported in chemical-inducible systems (Padidam, 2003; Zuo and Chua, 2000). In our system, about half of the independently transformed rice lines displayed weak or moderate leaky expression (Park *et al.*, 2012). Therefore, it is critical to identify the transgenic lines that display the least leaky expression while maintaining highly inducible expression. To confirm non-leaky expression of *Xa21*, selected lines should be fully susceptible to *Xoo* in the absence of DEX and resistant to *Xoo* after DEX is applied. Additionally, *Xa21* gene expression and protein abundance should be low or not detectable by quantitative RT-PCR and western blot analysis before DEX treatment and should be induced by DEX application (Park *et al.*, 2012).
2. To ensure dexamethasone treatment is successful, inoculate additional plants and maintain without harvesting leaves to measure lesions 12 to 14 days after inoculation.
3. In our experience, when we used separate walk-in growth chambers for dexamethasone and non-dexamethasone treatments, we observed variation in *Xoo* lesion lengths between Kitaake (dexamethasone treated) and dexamethasone-inducible XA21 plants (non-dexamethasone induced) even when identical settings were applied to both walk-in growth chambers (data not shown). Therefore, we recommend limiting experiments to a single growth chamber in which dexamethasone is applied to all plants and Kitaake wild type rice are used as controls.
4. Plants were transferred to walk-in growth chambers for inoculation with *Xoo* due to University biosafety protocols. The authors suspect that *Xoo* inoculation and dexamethasone application should be successful in the greenhouse as well, although these conditions were not tested.
5. In our pre-experiments, we combined a 30 µM dexamethasone foliar application with 10 µM dexamethasone irrigation. However we didn't see additional *Xa21* expression compared to foliar application alone (data not shown).

Recipes

1. Peptone sucrose agar (PSA) solid media containing cephalexin (1 L)
   Preparation of cephalexin stock: Dissolve cephalexin hydrate to 10 mg/ml in 50%

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ethanol and store 2 ml aliquots at -20 °C until ready to prepare media

Dissolve 10 g peptone, 10 g sucrose and 1 g of L-glutamic acid in 900 ml in sterile H_2O

Adjust the pH of the medium to 7.0 using 1 N NaOH and bring volume up to 1 L

Add 16 g agar and autoclave to sterilize

Allow solution to cool to 55 °C, and add 2 ml of stock cephalexin to a final concentration of 20 µg/ml

2. Dexamethasone, 30 µM solution containing 0.01% (w/v) Tween-20 (400 ml)

   For two supertubs, dissolve 0.0048 g of dexamethasone into 400 µl DMSO in a 1.5 ml eppendorf tube

   After dissolving, add it into 400 ml of sterile H_2O containing 40 µl of tween-20
3. Greenhouse rice growing conditions

   Greenhouse (26-28 °C light, 18-20 °C dark, 40-60% humidity)

   From April to September, natural light

   From October to March, light supplemented with 1,000 W metal halide bulbs from 6 am to10 pm (14 h light and 10 h dark photoperiod)
4. Walk-in growth chamber rice growing conditions

   Walk-in growth chamber (14 h light, 28 °C, 80% humidity; 10 h dark, 24 °C, 85% humidity)
5. Fertilized water composition

   Fertilized water kept at EC: 0.8-1

   Theoretical breakdown of PPM when injected at 1:200:

N	173.58
P	45.39
K	165.64
Ca	60.05
Mg	38.88
S	146.42
Fe	5.23
Cu	0.18
B	0.4
Mn	1.20
Mo	0.05
Zn	0.37

Acknowledgments

This protocol was modified from Park *et al.* (2012).

This research was supported by the National Institute of Health (NIH, GM55962), the National Science Foundation (NSF, IOS-0817738), and the Monsanto's Beachell-Borlaug International Scholars Program.

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Appendix

1. Nucleotide sequence of *Myc::Xa21*. The underlined region defines the N-terminal Myc tag.The Myc tag was inserted in domain B of the *Xa21* gene following the putative signal peptide (domain A) as previously described (Park *et al.*, 2010).

atgatatcactcccattattgctcttcgtcctgttgttctctgcgctgctgctctgcccttcaagcagtgacgacgatggtgatg
ctgccggcgacgaactcgcgctgctctctttcaagtcatccctgctataccaggggggccagtcgctggcatcttggaa
cacgtccggccacggccagcactgcacatgggtgggtgttgtgtcggccgccgccgccgccggcacccacacag
ggtgGAGCAAAAGCTGAACCCACAACACACGCCGGCGGCGGCGGCGGCCGTGG
GTGTGTCCCACCTCGTTTTCGACTTTTCTGAGGAGGATCTGCTGGAGAAGCACA
GGCTGgtgaagctgctgctgcgctcctccaacctgtccgggatcatctcgccgtcgctcggcaacctgtccttcctca
gggagctggacctcggcgacaactacctctccggcgagataccaccggagctcagccgtctcagcaggcttcagct
gctggagctgagcgataactccatccaagggagcatccccgcggccattggagcatgcaccaagttgacatcgctag
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gattatcaggagctataccttcatcactggggcagctcagcagtctattgactatgaatttgggacagaacaatctaagt
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cctaacaaattgctccaaattacaaacattgaacttgggagaaaataacctgggggagttcttcctaattcgttttccaa
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aaggtgggagccttgaattctga

2. *pTA7002::Myc::Xa21* construct map. SP signal peptide, RB right border, 35S cauliflower mosaic virus 35S promoter, GVG GAL4-binding domain, VP16 activation domain–glucocorticoid receptor fusion, E9 poly (A) addition sequence of the ribulosebisphosphate carboxylase small subunit (rbcS-E9), NOS-P nopaline synthase promoter, NOS-T nopaline synthase terminator; 6×UAS six copies of the GAL4 upstream activating sequence and the −46 to +1 region of the 35S promoter, 3A poly (A) addition sequence of the pea rbcS-3A, LB left border.

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