Construction of an ABA-Responsive RUBY Reporter for Transient Expression in Nicotiana Leaves

Amedeo Leproni, 25 November 2025

Acknowledgements: *I thank Dr. Jiafu Tan for his supervision and guidance during this project, and for valuable discussions that informed its design and interpretation, as well as the Brockington group for hosting me.*

Abstract.

Early, reliable detection of plant stress can support improved crop management and reduce yield and quality losses. Drought is a major abiotic stress, with responses strongly mediated by abscisic acid (ABA) signalling, and ABA-responsive promoters enable transcriptional outputs linked to stress. The RUBY betalain cassette provides a visible reporter that can be assessed without specialised substrates or imaging hardware. Here, an ABA-responsive promoter derived from the Arabidopsis RD29A regulatory region was placed upstream of a RUBY reporter to evaluate a drought-responsive biosensor concept in transient expression.

RD29A promoter variants were PCR-amplified; an internal restriction site in one variant was addressed by splitting the promoter into fragments and introducing a disruptive mutation. Promoter fragments were assembled into a RUBY-compatible backbone by restriction digestion and ligation, propagated and verified in E. coli, and transferred into Agrobacterium by electroporation under antibiotic selection. Cultures were prepared for syringe agroinfiltration into Nicotiana leaves alongside a constitutive RUBY positive control.

Betalain pigmentation confirmed reporter expression, but no consistent increase in pigmentation was observed for the inducible construct relative to the control under ABA or cold treatments. Basal reporter signal across conditions suggested that infiltration-associated tissue perturbation may have activated stress signalling sufficient to drive promoter activity, limiting discrimination in a transient assay. The work established construct assembly and delivery and motivates follow-on testing strategies, including alternative assay designs and evaluation in stable transformants.

Table of contents

1. Introduction

Environmental stress constrains agricultural productivity by reducing yield and quality. Earlier and more direct measurement of stress can support improved crop management and reduce losses. Drought is among the most pervasive abiotic stresses affecting plants, and many responses to water deficit are mediated by ABA signalling, which coordinates transcriptional, stomatal, and metabolic adjustments.

Stress-inducible promoters are widely used to link signalling inputs to measurable transcriptional outputs. The Arabidopsis RD29A promoter is a commonly used stress-responsive regulatory element, with cis-acting motifs responsive to drought and cold and documented interplay between dehydration-responsive elements and ABA-responsive elements in controlling expression (Narusaka et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 1994).

Visible reporters can simplify screening by reducing instrumentation requirements. RUBY is a betalain biosynthesis cassette that produces red pigmentation in plant tissues, enabling noninvasive monitoring of transgene expression (He et al., 2020). Here, an RD29A-driven RUBY construct was built and tested via Agrobacterium-mediated transient expression in Nicotiana leaves, with the aim of assessing whether ABA or cold treatment produced a detectable increase in pigmentation relative to a constitutive RUBY control.

2. Materials and Methods

2.1. Construct design and genetic elements

An ABA-responsive reporter construct was designed in which an RD29A promoter variant drove a RUBY betalain cassette. Two promoter variants were used, referred to as 29A and 29B. Variant 29A contained an internal restriction site incompatible with the intended cloning strategy; it was therefore planned as a two-fragment assembly, with a point mutation introduced in the second fragment to disrupt the internal site while preserving promoter function. Variant 29B lacked this internal restriction site and was handled as a single PCR fragment.

The RUBY cassette comprised three betalain pathway genes expressed as a single unit, enabling visible red pigment production in plant tissue (He et al., 2020).

2.2. PCR amplification and gel electrophoresis

Three PCR reactions were set up using Phusion polymerase with the associated buffer system, dNTPs, template DNA, and primers:

- Reaction 1: 29as + 29aas
- Reaction 2: 29as1 + 29aas
- Reaction 3: 29bs + 29bas

Cycling conditions included annealing at 60 °C and extension for 30 s at 72 °C, consistent with amplification of approximately 1 kb products.

A 1.5% (w/v) agarose gel was prepared in TAE buffer, stained with ethidium bromide, cast with wells, and used to resolve PCR products mixed with loading dye prior to electrophoresis.

Figure 1. Agarose gel electrophoresis workflow for RD29A promoter PCR products: gel preparation and casting; separation of PCR products by electrophoresis; band visualisation by ethidium bromide fluorescence under green light prior to excision.

2.3. DNA purification and gel extraction

DNA was purified using a column-based protocol. Samples were mixed with isopropanol to facilitate binding, applied to the column by centrifugation, washed according to the kit workflow, and eluted into a clean tube for downstream cloning.

2.4. Restriction digestion, ligation, and cloning

Promoter fragments were assembled with the RUBY cassette and plasmid backbone using restriction digestion and ligation. For promoter 29A, the promoter was split into two fragments to avoid internal digestion; a mutation was incorporated to prevent reconstitution of the internal restriction site after ligation. Promoter 29B was cloned as a single fragment.

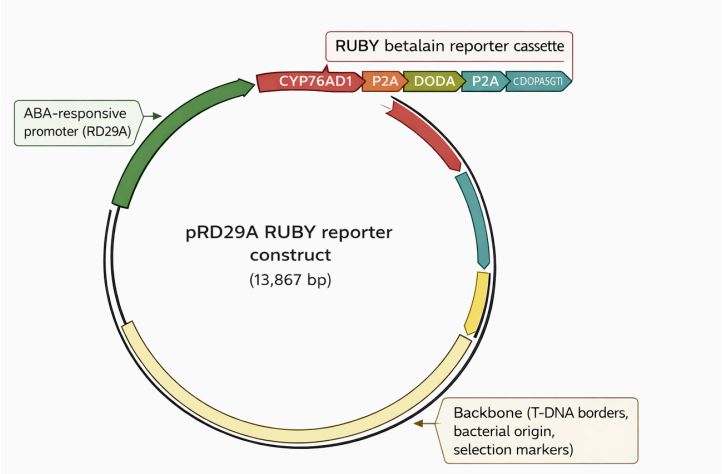

Figure 2. Plasmid map of the pRD29A-RUBY reporter construct used for agroinfiltration. The construct contains an RD29A-derived promoter upstream of the RUBY cassette, within T-DNA borders for Agrobacterium-mediated delivery.

2.5. *E. coli* transformation and screening

Chemically competent E. coli were thawed on ice and transformed with 5 µL ligation mixture. Cells were heat-shocked at 42 °C for 1 min, incubated on ice for 5 min, recovered in LB, and plated on selective agar. Colonies were screened by colony PCR and plasmid preparation, with sequence verification performed for selected clones. An initially selected 29A clone was identified as incorrect during screening, whereas the 29B construct was confirmed as correct. Additional colonies were retained as backups.

2.6. *Agrobacterium* transformation and selection

Verified plasmid DNA was transformed into Agrobacterium by electroporation. Electrocompetent cells were prepared in 10% glycerol. Plasmid DNA (3 µL) was mixed with 50 µL cells in an electroporation cuvette, pulsed, and recovered in liquid medium before plating. Recovery cultures (100 µL) were plated on selective agar containing gentamicin and spectinomycin to select for the strain background and plasmid backbone, respectively. Colonies were grown on selective media for subsequent use.

2.7. Glycerol stock preparation

Agrobacterium glycerol stocks were prepared at approximately 10% (v/v) glycerol and stored at -70 °C.

2.8. Plant material and agro-infiltration

Nicotiana plants approximately one month old were used. Infiltration cultures were prepared from Agrobacterium carrying the 29B pRD29A-RUBY construct and a constitutive RUBY positive control (CK). Syringe infiltration was performed using needleless syringes. For within-leaf comparison, the inducible construct and constitutive control were infiltrated into opposite sides of the same leaf.

After infiltration, plants were returned to standard growth conditions for 2 days to allow transient expression to establish prior to treatment. During this period, visible RUBY pigmentation appeared in regions infiltrated with the constitutive control.

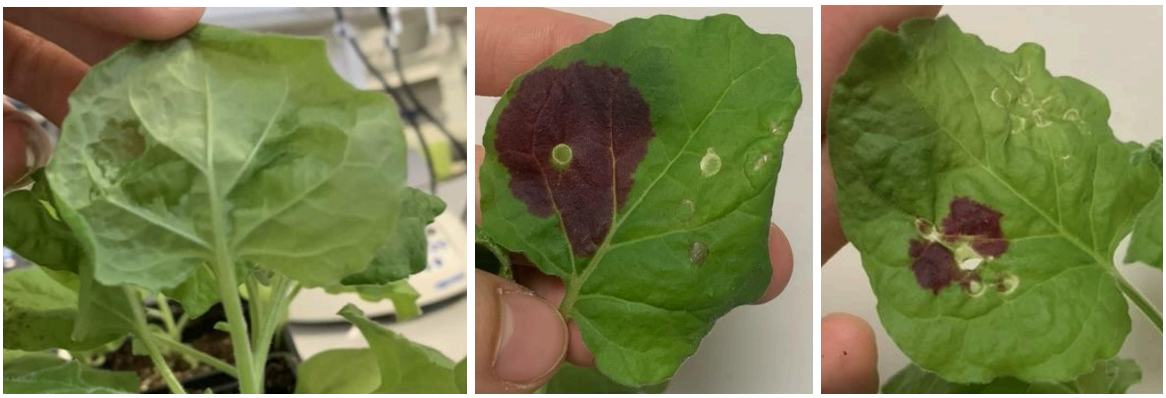

Figure 3. Representative Nicotiana leaves after agroinfiltration and prior to stress treatment: immediate post-infiltration appearance; visible pigmentation in constitutive control regions during the establishment period; no clear induction of the RD29A-driven reporter prior to treatment.

2.9. Hormone and cold treatments

An ABA solution diluted 1:10 in distilled water was applied by brush to a subset of infiltrated plants 2 to 3 days after infiltration. In parallel, selected plants were placed in a refrigerator for cold treatment, while others remained under standard growth conditions as untreated controls. Plants were maintained for a further 3 to 4 days prior to assessment of reporter activity.

Figure 4. Stress treatments applied after agroinfiltration: ABA application by brush; cold treatment by refrigeration; untreated controls maintained under growth conditions.

3. Results

Leaves were infiltrated with the 29B pRD29A-RUBY construct and a constitutive RUBY control on opposite sides of the same leaf. During the first 2 to 3 days post-infiltration, pigmentation was consistently observed in constitutive control regions, confirming successful delivery and expression. No distinct pigmentation attributable to the RD29A-driven construct was observed prior to treatment.

After 3 to 4 days under treatment conditions, betalain pigmentation was observed in RD29A-RUBY infiltration regions across all groups, including ABA-treated plants, cold-treated plants, and untreated controls. No consistent qualitative difference in pigmentation intensity was observed between treatment groups, and no clear treatment-dependent increase relative to the constitutive control was apparent. Pigmentation varied across infiltrated regions and plants, ranging from strong, well-defined patches to weaker, diffuse splotches.

Figure 5. Nicotiana leaf maintained under standard growth conditions. Opposite sides were infiltrated with RD29A-RUBY (left) and constitutive RUBY control (right). Pigmentation is evident in the control region, while the RD29A-RUBY region shows lower-intensity signal under non-treated conditions.

4. Discussion

This study established an end-to-end workflow for assembling an RD29A-driven RUBY reporter and deploying it in Nicotiana leaves via Agrobacterium-mediated transient expression. A key design constraint in promoter variant 29A, the presence of an internal restriction site, was addressed by two-fragment assembly with a disruptive mutation to prevent internal digestion during cloning. Promoter variant 29B lacked this constraint and was therefore more straightforward to clone, verify, and deploy for infiltration.

Functionally, the construct produced visible betalain pigmentation in infiltrated tissue, confirming that delivery and expression in planta were successful. However, the primary objective was to detect stress-dependent induction of the RD29A promoter, evidenced by increased pigmentation under ABA or cold treatment relative to untreated conditions and relative to the constitutive control. Under the conditions tested, a clear treatment-specific increase in RD29A-driven pigmentation was not observed. Instead, RD29A-RUBY pigmentation became detectable across treatments, including untreated controls, suggesting basal activation during the transient assay.

A plausible explanation is that syringe infiltration and associated tissue perturbation activated stress signalling pathways that overlap with RD29A regulatory inputs. RD29A integrates multiple stress signals through ABA-dependent and ABA-independent elements (Narusaka et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 1994), and transient delivery can couple promoter exposure to local stress at the infiltration site. The observed variability in pigmentation is also consistent with known sources of variation in agroinfiltration efficiency and tissue response (Zhang et al., 2020).

These results indicate that syringe-based transient assays may have limited discriminative power for stress-inducible promoters when delivery introduces a confounding stress component. Follow-on experiments should aim to decouple delivery from induction. Options include stable transformation to enable controlled induction without acute wounding at the time of measurement, or modified transient designs that minimise mechanical damage and standardise timing and readout to better resolve basal versus inducible activity.

5. Conclusion

An RD29A promoter-driven RUBY reporter construct was assembled, verified in E. coli, transferred into Agrobacterium, and delivered into Nicotiana leaves alongside a constitutive RUBY control. Visible betalain pigmentation confirmed that reporter expression in planta was achievable via agroinfiltration. Under the conditions tested, RD29A-driven pigmentation was detectable across treatment groups, including untreated controls, and did not show a strong treatment-specific increase relative to the constitutive control. The most likely explanation is basal activation of RD29A regulatory logic during the transient infiltration assay, limiting the ability to isolate ABA-specific induction. The workflow and observations provide a foundation for follow-on experiments that separate delivery effects from stress induction, including stable transformation or transient assay modifications that reduce wounding and improve temporal resolution of induction.

6. References

He, Y., Zhang, T., Sun, H., and Zhao, Y. (2020). A reporter for noninvasively monitoring gene expression and plant transformation. Horticulture Research.

Narusaka, Y., Nakashima, K., Shinwari, Z.K., Sakuma, Y., Furihata, T., Abe, H., Narusaka, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of the Arabidopsis rd29A gene in response to ABA. The Plant Journal.

Polturak, G., Heinig, U., Grossman, N., Battat, M., Leshkowitz, D., Malitsky, S., Rogachev, I., and Aharoni, A. (2016). Elucidation of the first committed step in betalain biosynthesis enables heterologous engineering of betalain pigments in plants. New Phytologist.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). A novel cis-acting element involved in responsiveness to dehydration, low temperature, or high salt stress in Arabidopsis. The Plant Cell.

Zhang, Y., Su, J., Duan, S., Ao, Y., Dai, J., Liu, J., Wang, P., Li, Y., Liu, B., Feng, D., Wang, J., and Wang, H. (2020). A highly efficient Agrobacterium-mediated method for transient gene expression in plants.