

Inducibility of the CaDRRG-Pro promoter from chickpea by defense hormones and *Ascochyta rabiei* elicitors in stable transgenic tobacco as a model plant

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Abstract

Analysis of early responsive gene promoters provides tools for engineering plant disease resistance. *Ascochyta* blight, caused by the fungus *Ascochyta rabiei*, is a major constraint to chickpea cultivation. The *CaDRRG* gene was previously identified as an early responsive gene showing more than 11-fold increased expression within 3 hours of *A. rabiei* inoculation. In this study, the *CaDRRG-Pro* promoter was functionally characterized using stable transgenic tobacco plants. The binary vector pCaDRRG-Pro-Gi, containing a 772 bp *CaDRRG-Pro* fragment fused to an intron-containing *GUS* reporter gene, was transferred to *Agrobacterium tumefaciens*. Stable transgenic lines were generated, and a hierarchical GUS assay identified a high-expressing line (code 1-5) from 30 regenerated plantlets with 63% savings in assays. The promoter was rapidly induced by methyl jasmonate and salicylic acid, with detectable GUS activity at 1 hour and maximum induction within 6 hours, reaching levels comparable to the constitutive CaMV 35S promoter. The promoter also responded to crude mycelial extracts from *A. rabiei* pathotypes, with clear induction at 3 hours. Direct fungal inoculation resulted in delayed induction (120 hours) with vascular-associated expression patterns. No basal expression was observed in stable transgenic lines under non-induced conditions. The intron-containing *GUS* reporter prevented prokaryotic expression in *Agrobacterium*, ensuring specificity. These results provide functional validation of the *CaDRRG-Pro* promoter, demonstrating its rapid inducibility by defense hormones and fungal elicitors, and position it as a valuable tool for pathogen-inducible expression of resistance genes in chickpea breeding programs.

Keywords: *Ascochyta* blight, Hierarchical GUS assay, Methyl jasmonate, Pathogen-inducible promoter, Salicylic acid, Transgenic tobacco.

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Introduction

Analysis of early responsive gene (ERG) promoters to biotic stresses can contribute to a better understanding of the molecular mechanisms involved in plant defense systems against pathogens and has important applications in selecting appropriate strategies for developing resistant plants (Wang et al., 2025). Chickpea (*Cicer arietinum* L.) cultivation is severely constrained by Ascochyta blight, a devastating disease caused by the necrotrophic fungal pathogen *Ascochyta rabiei* (Foresto et al., 2023). The high genetic diversity of this pathogen, with multiple pathotypes exhibiting varying aggressiveness, can rapidly overcome the limited resistance found in current chickpea cultivars, leading to significant yield losses (Shahnaz et al., 2025). Understanding plant defense mechanisms at the molecular level can provide new strategies for developing chickpea cultivars resistant to this disease (Sane et al., 2025). Studying gene expression patterns during plant-pathogen interactions leads to the identification of early responsive genes (ERGs) that are induced within the first few hours after pathogen inoculation (Jaiswal et al., 2012).

In silico analysis of the upstream sequence of the *CaDRRG* gene confirmed the presence of known pathogen-inducible cis-acting regulatory elements, including W-box (binding site for WRKY transcription factors), AS-1, and G-box, within this promoter. Furthermore, by comparing the promoters of highly inducible genes, three potential novel regulatory elements, named New Box I, II, and III, were also identified in this region. Results from transient expression analysis of a 772 bp fragment of this promoter demonstrated that it significantly induced β -glucuronidase (GUS) reporter gene expression in response to treatment with crude extract of *A. rabiei* (Shokouhifar et al., 2025).

So far, the analysis of the *CaDRRG* gene promoter sequence has only been investigated using transient expression assays (Sadeghi et al., unpublished). Although transient expression methods such as agroinfiltration are valuable as rapid and low-cost tools for initial screening and providing preliminary evidence of promoter activity (Nosaki et al., 2021), they have several limitations and must be validated in stable transgenic plants under realistic conditions (Bélanger et al., 2024). A key limitation is the observation that *Agrobacterium* infiltration itself can induce plant defense responses, leading to artifactual promoter activation (Sinha et al., 2025). In transient expression systems, only a limited number of cells receive the expression cassette (Sindarovska, 2026), and they are only able to express the transgene as early as 3 hours and it could persist till 10 days (Komarova et al., 2010). Moreover, these cells receive a high copy number of the construct

(Szarzanowicz et al., 2025), leading to extremely high and potentially unrepresentative transgene expression (Akhtar et al., 2026) that may not reflect the true regulatory behavior of the promoter. In contrast, under stable transformation conditions, a limited number of transgene copies are integrated into the genome, and these copies are present consistently across all cells of the transgenic plant, allowing for more stable and comparable assessment of promoter responses to various inducing agents (Negrutiu et al., 1990; Hernandez-Garcia & Finer, 2014). Several critical parameters must be evaluated for a comprehensive characterization of any promoter, including assessment of basal expression level (Rushton et al., 2002), timing and magnitude of promoter induction (Li et al., 2023), and identification of plant tissues capable of expressing the promoter. It is evident that these evaluations cannot be accurately performed using transient expression systems alone (Villao-Uzho et al., 2023). The application of pathogen-inducible promoters is crucial for engineering durable disease resistance. Recent studies have demonstrated the utility of such promoters for developing disease-resistant crops without growth trade-offs (Wei et al., 2024; Lin et al., 2026) (Kauder et al., 2025; Zhu et al., 2026). Given the pronounced inducibility and specificity of the *CaDRRG* gene promoter in response to *A. rabiei*, this study aimed to evaluate its expression pattern following treatment with the defense-related hormones methyl jasmonate and salicylic acid. In future studies, the responsiveness of this promoter to a broader range of pathogenic organisms as well as beneficial symbiotic microorganisms should also be investigated to further elucidate its role in plant defense responses. This study specifically aimed to generate stable transgenic tobacco lines harboring the *CaDRRG-Pro:GUSi* cassette and evaluate the basal expression level and inducibility of the *CaDRRG-Pro* promoter in response to MeJA, SA, and *A. rabiei* elicitors.

Materials and Methods

Plant material and growth conditions

Nicotiana tabacum L. var. *Xanthi* and *N. benthamiana* were used. Seed sterilization, germination, and growth were performed as described (Mamarabadi et al., 2026).

Fungal pathogen

Ascochyta rabiei pathotypes PI and PVI were maintained on PDA at 24°C (Bahrabadi et al., 2025).

Vectors and strains

Vectors (pCaDRRG-Pro-Gi, pCAMBIA3301, pBI121) were obtained from Ferdowsi University Microorganisms Bank. They were previously constructed and described (Shokouhifar et al., 2025). pCaDRRG-Pro-Gi (11.26 kb) contains a 772 bp *CaDRRG-Pro* promoter + intron-containing *GUS* (*GUSi*); pCAMBIA3301 (11.32 kb) is constitutive positive control; pBI121 (14.76 kb) is prokaryotic expression control (Chen et al., 2003). *A. tumefaciens* LBA4404 was used for stable transformation.

Media

MS medium was prepared as described (Murashige & Skoog, 1962). RM (pre-culture/co-cultivation) contained MS salts, 30 g/L sucrose, 2 mg/L BAP, 0.2 mg/L IBA, agar (pH 5.8). SM1/SM2 contained RM + 200 mg/L cefotaxime. Rooting medium contained MS salts, 20 g/L sucrose, agar (pH 5.8). LB medium and induction buffer were prepared as described (Gallois & Marinho, 1995).

***Agrobacterium* transformation and PCR confirmation**

Vectors were introduced into LBA4404 using the freeze-thaw method (Weigel & Glazebrook, 2006). Colonies were selected on kanamycin (50 mg/L) and rifampicin (30 mg/L). Colony PCR was performed with specific primers (Shokouhifar et al., 2019): PSh4-F2/PSh4-R (1287 bp) and PKa-F/PSh4-R (640 bp) for pCaDRRG-Pro-Gi; PSh3-F/PSh4-R for pCAMBIA3301 (1213 bp) and pBI121 (1021 bp). Screening is shown in Fig. S1. Absence of prokaryotic GUS expression was confirmed as reported (Shokouhifar et al., 2019).

Leaf explant preparation and transformation

Leaves from 4-week-old *N. tabacum* var. Xanthi were surface-sterilized (1% NaOCl, 10 min), rinsed, cut into 1 cm² explants, and pre-cultured on RM for 3 days (25°C, 16/8 h photoperiod). Explants were immersed in *Agrobacterium* suspension (OD₆₀₀ = 1.0) for 20 min, blotted dry, and co-cultivated on RM for 3 days at 25°C in the dark.

Transgenic plantlet selection

Transgenic shoots were selected using the hierarchical GUS assay (GPS) (Mamarabadi et al., 2026). Plantlets were treated with 50 µM MeJA for 24 h (Sadeghi et al., unpublished). Leaf squares

(1×1 mm) from 30 plantlets were pooled in four rounds: (1) single bulk of 30; (2) 5 pools of 6; (3) 2 pools of 3; (4) individual screening. Untreated and non-transgenic controls were included. Only 11 assays vs. 30 conventional (63% reduction). Rooting, acclimatization, and seed collection followed Mamarabadi et al. (2026).

GUS histochemical assay

GUS activity was detected using the protocol of Jefferson et al. (1987). Leaf segments were incubated in X-Gluc solution (1 mg/mL X-Gluc, 100 mM phosphate buffer pH 7.0, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA), vacuum-infiltrated for 10 min, and incubated at 37°C for 16–24 h. Chlorophyll was removed by ethanol series. Blue coloration was examined under a stereomicroscope. Imaging was performed as described (Heidari et al., 2018; Huang et al., 2024). Non-transgenic and untreated samples were negative controls; pCAMBIA3301 was positive control.

Fungal elicitor and treatments

Mycelial extracts from PI and PVI were prepared as described (Nesrine et al., 2024). Mycelia from 10-day-old PDA cultures were homogenized in phosphate buffer, centrifuged, and supernatants stored at -80°C. For hierarchical screening, leaf squares were immersed in 50 μ M MeJA for 2 h. For induction analysis, detached leaves were sprayed with 500 μ L of 50 μ M MeJA or 2 mM SA in 0.01% Tween-20. Leaf discs were harvested at 1, 3, 6, and 24 h. For fungal elicitor treatment, detached leaves were sprayed with 500 μ L of crude mycelial extract from PI or PVI. For direct fungal inoculation, agar blocks (5×5 mm) with actively growing mycelium (PI or PVI) were placed on detached leaves; leaf discs were harvested at 1, 24, 36, and 120 h.

Molecular confirmation

Genomic DNA was extracted using CTAB (Doyle & Doyle, 1987). PCR was performed with PSh4-F2/PSh4-R (1287 bp). Two negative controls were included: C_1^- (regenerated plantlet not confirmed by GUS) and C_2^- (non-transgenic wild-type).

Results and Discussions

Transformation of LBA4404 strains with the pCaDRRG-Pro-Gi construct

The pCaDRRG-Pro-Gi binary vector, harboring the *CaDRRG-Pro* promoter fragment was previously constructed (Sadeghi et al., unpublished) and successfully transferred into *A. tumefaciens* strain LBA4404 using the heat shock method with high efficiency. Transformed colonies were selected on LB medium containing appropriate antibiotics (rifampicin and kanamycin). The complete screening process for selection of transformed colonies is presented in Supplementary Fig. S1, which shows the colony PCR screening of 20 independent colonies. Colonies that did not show amplification of the expected band served as additional negative controls, confirming that the strain and the PCR reactions themselves are not able to produce the expected band. Selected confirmed colonies were subsequently subjected to colony PCR using specific primer pairs designed to amplify distinct regions within the promoter-reporter cassette (Fig. 1A). This step was critical to ensure that only confirmed transformants were used for subsequent plant transformation experiments. The results of the colony PCR confirmation were presented in Fig. 1-B. The electrophoretic pattern showed a band within the 1000-1500 bp range of the DNA size marker that is consistent with the expected product size. A second primer pair named PKa-F/PSh4-R was also used to further confirm the transformation which amplifies a 640 bp fragment spanning the junction between the *CaDRRG-Pro* promoter and the *GUS* gene. This reaction also produced a single specific band of the expected size (Fig. 1-B, lanes 1-5). Amplification with the primer pair PSh4-F2/PSh4-R, which spans a region encompassing the *CaDRRG-Pro* promoter and the intron-containing *GUS* reporter gene, produced a single specific band of the expected 1287 bp (Fig. 1-B, lanes 6-9). The successful amplification of fragments with the expected sizes in all tested colonies confirmed the presence and integrity of the pCaDRRG-Pro-Gi construct in the *Agrobacterium* LBA4404 strain. From the confirmed colonies, one representative colony per construct was selected for subsequent plant transformation experiments.

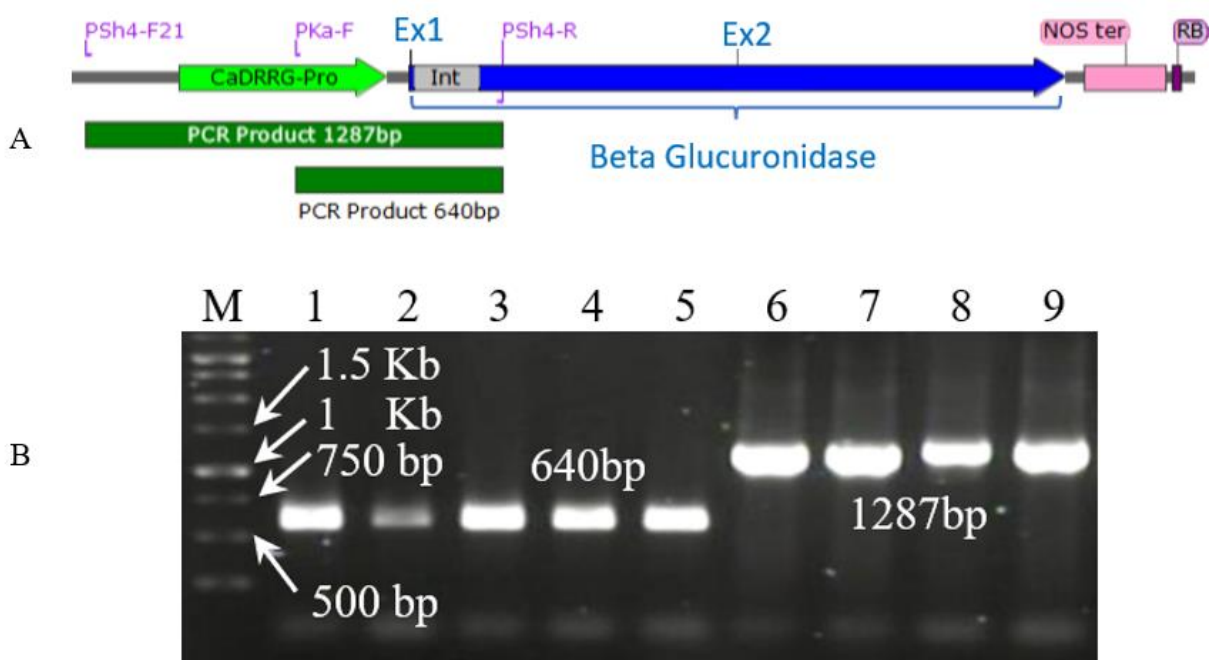


Fig. 1. Schematic representation of the *CaDRRG-Pro* promoter-reporter cassette (*CaDRRG-Pro:GUSi*), which has been constructed by Shokouhifar et al., (2025), showing the binding positions of specific primer pairs PSh4-F2/PSh4-R and PKa-F/PSh4-R and the expected amplicon sizes (A). Agarose gel electrophoresis of colony PCR products from *Agrobacterium* LBA4404 colonies transformed with the p*CaDRRG-Pro-Gi* construct (B). Lanes 1-5: PCR products using primer pair PKa-F/PSh4-R showing the expected 640 bp band. Lanes 6-9: PCR products using primer pair PSh4-F2/PSh4-R showing the expected 1287 bp band. M: 1 kb DNA size marker (CinnaGen). *CaDRRG-Pro*: 700 bp upstream sequence of the chickpea *CaDRRG* gene; Ex1, Int, and Ex2: first exon, intron, and second exon of the β -glucuronidase coding sequence, respectively; *Nos ter*: nopaline synthase transcription terminator; RB: right border of the T-DNA region in the p*CaDRRG-Pro-Gi* construct. More data concerning tested colonies are presented in supplementary data Fig. S1.

Confirmation of absence of prokaryotic GUS expression in transformed LBA4404 strains

To confirm that any observed GUS activity originated from plant cells rather than bacterial contamination, histochemical GUS assays were performed directly on *Agrobacterium* LBA4404 colonies molecularly confirmed to carry the p*CaDRRG-Pro-Gi* construct (Fig. 1). The results are presented in Fig. 2. As shown in the Fig. 2. No blue coloration was observed in non-transformed *Agrobacterium* (Fig. 2, C⁻), colonies harboring p*CaDRRG-Pro-Gi* (Fig. 2, A) and pCambia3301 (Fig. 3, B). In contrast, *Agrobacterium* transformed with the intron-less *GUS* vector pBI121 (Fig. 3, C⁺) exhibited intense blue coloration, confirming the functionality of the GUS assay and the ability of *Agrobacterium* to express an intron-less reporter. The absence of GUS activity in intron-containing constructs confirms that the intron prevents prokaryotic

expression, regardless of promoter recognition (Ohta *et al.*, 1990). Thus, any blue coloration observed in subsequent plant analyses originates from eukaryotic expression following proper splicing of the intron-containing *GUS* transcript.

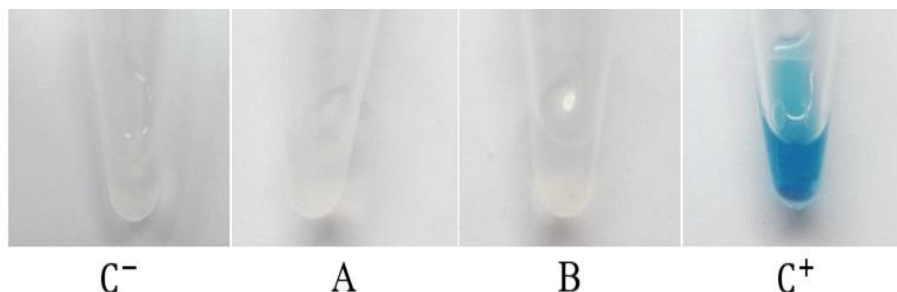


Fig. 2. Histochemical GUS assay of *Agrobacterium* LBA4404 strains transformed with intron-containing and intron-less *GUS* constructs. (A) pCaDRRG-Pro-Gi (intron-containing *GUS*). (B) pCambia3301 (intron-containing *GUS*). (C+) pBI121 (intron-less *GUS*; positive control). (C-) Non-transformed *Agrobacterium* (negative control). Absence of blue in (A) and (B) confirms no prokaryotic expression; blue in (C+) confirms assay functionality.

Generation of stable transgenic tobacco lines and selection of high-expressing transgenic plantlets using hierarchical GUS assay

Stable transformation of tobacco leaf explants was performed using *A. tumefaciens* LBA4404 as described (Mamarabadi *et al.*, 2026). The pCaDRRG-Pro-Gi construct was obtained from Ferdowsi University Microorganisms Bank (Shokouhifar *et al.*, 2025). Transgenic plantlets were selected using the hierarchical GUS assay (GPS) as described (Mamarabadi *et al.*, 2026). Since the *CaDRRG-Pro* promoter is MeJA-inducible (Sadeghi *et al.*, unpublished), all regenerated plantlets were treated with 50 μ M MeJA for 24 h before GUS assay (Fig. 3).

Among 30 regenerated plantlets, seven exhibited GUS activity in response to MeJA, while 23 showed no blue coloration (Fig. 3B, MeJA-basal expression), similar to non-transgenic controls (Fig. S2). Two of the seven positive plantlets showed expression levels comparable to the constitutive pCambia3301 control (Fig. 3, C+). The plantlet 1-5, showing the highest expression, was selected for further analysis. The GPS method proved efficient for marker-independent screening, relying solely on functional GUS expression without bar gene as herbicide selection marker. The 23% transformation efficiency (7/30) is consistent with tobacco *Agrobacterium*-mediated transformation reports (Azizi-Dargahlou and Pouresmaeil, 2024). Variable GUS intensity among positive lines likely reflects differences in transgene copy number or integration site effects (Vaucheret *et al.*, 1998; Butaye *et al.*, 2005).

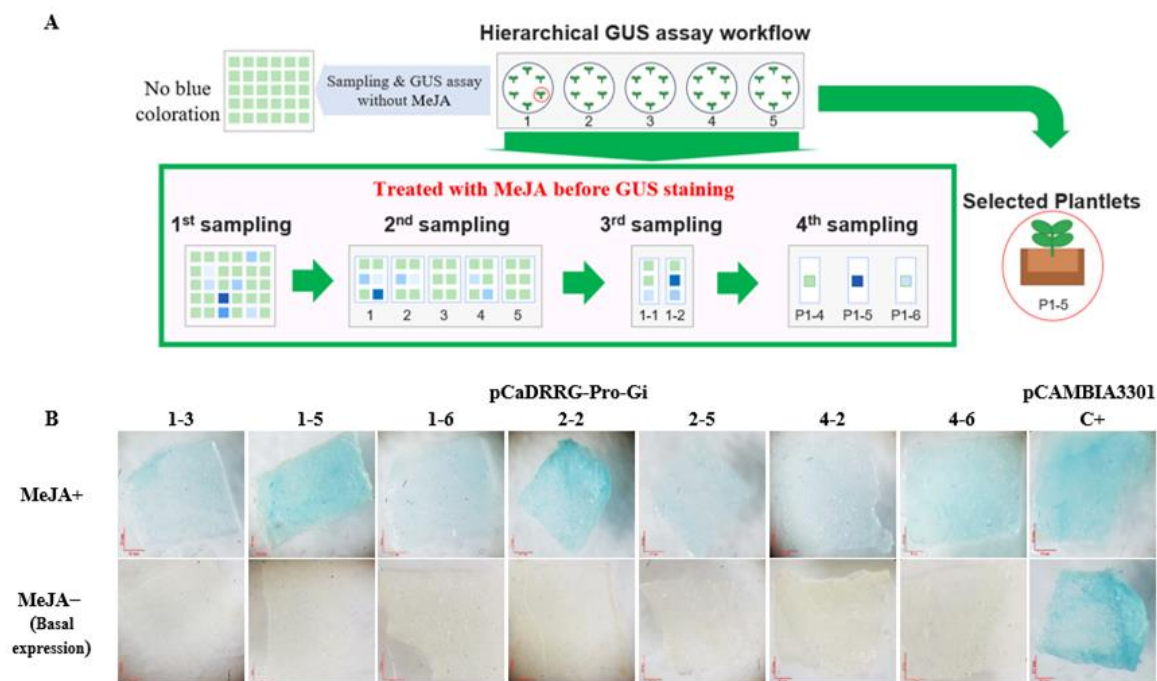


Fig. 3. Hierarchical GUS assay (GPS) for screening transgenic tobacco plantlets harboring the pCaDRRG-Pro-Gi construct following MeJA treatment. (A) Schematic diagram of the four-round hierarchical pooling strategy. (B) Representative photographs of leaf squares (1×1 mm) from regenerated shoots after GUS staining. Upper row: MeJA-treated (24 h). Lower row: untreated (basal expression control). C⁺: pCAMBIA3301 positive control (constitutive GUS). Red arrow indicates selected line 1-5. The hierarchical assay required only 11 GUS assays (63% reduction) vs. 30 conventional.

Molecular confirmation of transgenic plants and basal expression analysis

To confirm the presence of the *CaDRRG-Pro* promoter fragment in the selected transgenic plantlets, molecular analysis was performed using specific primers. Three plantlets named 1-3, 1-5 and 1-6 which previously exhibited GUS activity (Fig. 3), were subjected to PCR analysis using the primer pair PSh4-F2/PSh4-R, which amplifies a specific fragment spanning the *CaDRRG-Pro* promoter and the intron-containing *GUS* reporter gene. The results are presented in Fig. 4. Amplification of the expected 1287 bp band was observed in all three plantlets (Fig. 4, lanes 1-3). This confirmed that each of the three plantlets, which had previously shown β -glucuronidase enzyme activity and blue coloration in leaf segments following GUS assay, indeed harbored the template region corresponding to the primer binding sites.

Two negative controls were included to ensure the specificity of the PCR reaction. C₁⁻ represents a regenerated plantlet that was not confirmed by the GUS assay, used to check whether the target region could be amplified in plantlets regenerated from the transformation procedure but lacking

the transgene. C_2^- represents non-transgenic wild-type tobacco plants, used to confirm that the target fragment does not exist endogenously in the tobacco genome. Both negative controls showed no amplification, confirming the specificity of the PCR reaction and the absence of the *CaDRRG-Pro* promoter fragment in non-transgenic and non-confirmed regenerated plantlets. These results also demonstrate that if the selection of transgenic lines were based solely on PCR analysis, plants with lower expression levels such as plantlets 1-3 and 1-6 would still have been selected, despite their lower GUS activity compared to plantlet 1-5 (Fig. 3). This limitation of PCR-based screening has been well discussed in the hierarchical GUS assay method (Mamarabadi et al., 2026), where it was shown that PCR confirms the presence of the transgene but provides no information about its expression level. Therefore, the combination of functional GUS screening followed by molecular confirmation provides a more reliable strategy for selecting high-expressing transgenic lines. To confirm the absence of basal GUS expression in the selected transgenic line prior to induction experiments, leaf samples from the potted T0 plantlets were assayed for GUS activity without any hormonal treatment (Supplementary Fig. S2). As shown in Fig. S2, the selected pCaDRRG-Pro-Gi transgenic plantlet (code 1-5) showed no blue coloration in the absence of MeJA treatment, confirming the absence of detectable basal expression. In contrast, transgenic plants harboring pCAMBIA3301 (constitutive GUS expression) and pBI121 (intron-less GUS) from previous studies (Shokouhifar et al., 2025) showed clear blue coloration, serving as positive controls for GUS activity. Non-transgenic regenerated plantlets that were not confirmed by GUS or PCR screening showed no blue coloration, confirming the absence of endogenous GUS activity in tobacco tissues. Among the three molecularly confirmed plantlets, plantlet 1-5, which exhibited the highest GUS expression level in the hierarchical screening (Fig. 3-B), was selected for further analysis. Following rooting, the plantlet was transferred to culture medium according to the protocol described in the hierarchical GUS assay method (Mamarabadi et al., 2026). After the acclimatization phase under plastic cover, the plantlet was maintained under controlled conditions in a growth chamber at 24°C with a photoperiod of 14 hours light and 10 hours darkness. The plantlet was maintained until the end of its growth cycle for seed production, and seeds were collected for future studies.

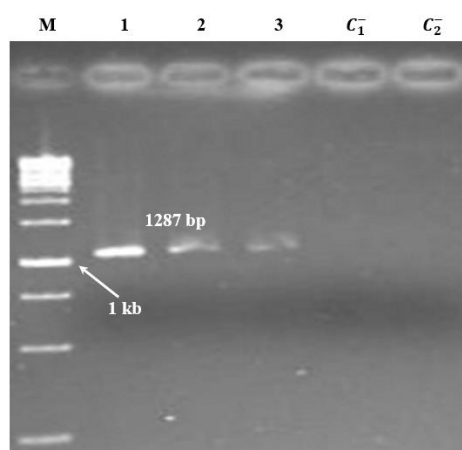


Fig. 4. Molecular confirmation of transgenic plantlets expressing β -glucuronidase enzyme selected at different stages of the hierarchical GUS assay. PCR amplification was performed using specific primers PSh4-F2/PSh4-R, which amplify an expected 1287 bp fragment. Lanes 1-3: Transgenic plantlets codes 1-3, 1-5 and 1-6, respectively, as previously identified in Fig. 3-B. M: 1 kb DNA size marker. C₁⁻: Regenerated plantlet not confirmed by GUS assay, used to check whether the target region could be amplified in plantlets regenerated from the transformation procedure but lacking the transgene. C₂⁻: non-transgenic wild-type tobacco plant, used to confirm that the target fragment does not exist endogenously in the tobacco genome. The presence of the expected band in all three transgenic plantlets confirms that they harbor the *CaDRRG-Pro* promoter fragment, while the absence of amplification in both negative controls confirm the specificity of the PCR reaction.

Expression pattern of the *CaDRRG-Pro* promoter in response to methyl jasmonate and salicylic acid

To investigate the response kinetics of the *CaDRRG-Pro* promoter to defense hormones, two leaves from the selected transgenic line (code 1-5) were detached and treated separately with 500 μ L of 2 mM salicylic acid (SA) and 500 μ L of 50 μ M methyl jasmonate (MeJA) on the leaf surface. The leaves were maintained in Petri dishes, and leaf segments were sampled at 1, 3, 6, and 24 hours after treatment for histochemical GUS assay (Fig. 5).

The results clearly demonstrated that blue coloration resulting from β -glucuronidase enzyme activity was visible in leaf tissue treated with both hormones as early as 1 hour after treatment (Fig. 5). The intensity of blue coloration increased progressively over time in both treatments. Although precise quantitative comparison of expression levels requires fluorometric GUS assays, the histochemical data showed noticeable differences in expression intensity up to 6 hours post-treatment. The difference between 6 hours and 24 hours was not considerable based on histochemical observation, suggesting that maximum induction levels are achieved within 6 hours. However, precise quantitative comparison of expression levels requires fluorometric GUS assays.

These results indicate that the *CaDRRG-Pro* promoter is induced by both MeJA and SA. This finding suggests the presence of cis-acting elements associated with both MeJA- and SA-mediated defense pathways within the 700 bp promoter fragment. Consequently, this promoter can potentially be influenced by both induced systemic resistance (ISR) and systemic acquired resistance (SAR) pathways. The absence of basal GUS expression in untreated leaf samples harboring pCaDRRG-Pro-Gi construct and non-transgenic plants were confirmed as shown in Supplementary Fig. S2.

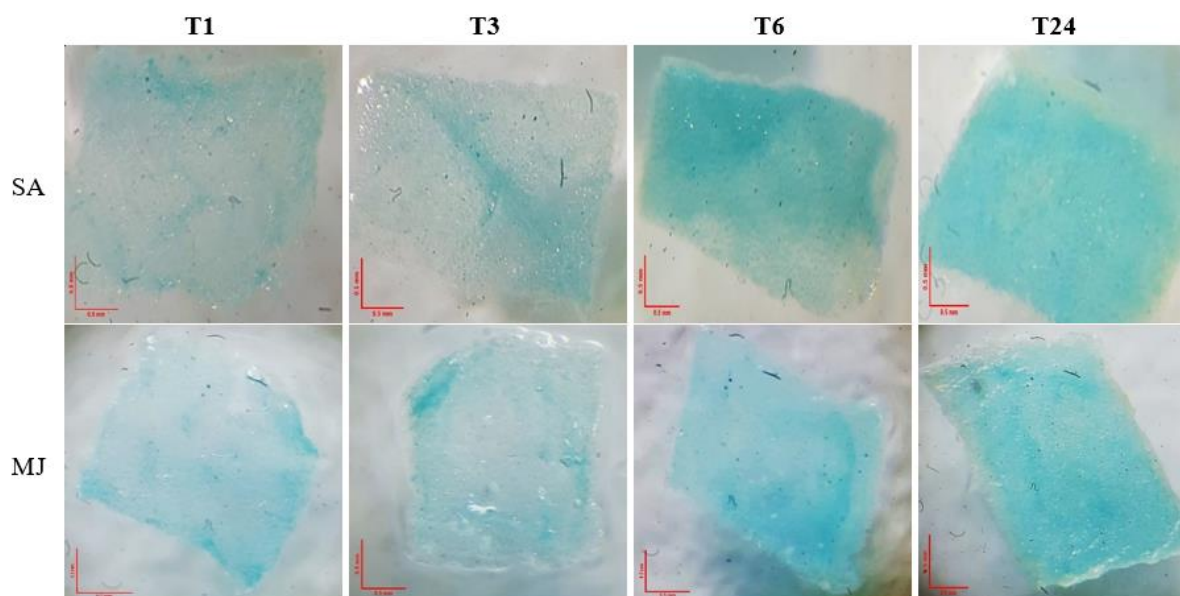


Fig. 5. Histochemical GUS assay of transgenic tobacco leaves harboring the pCaDRRG-Pro-Gi construct (Line code 1-5) at 1, 3, 6, and 24 hours (T1, T3, T6, T24) after spraying with 500 μ L of 2 mM salicylic acid (SA) or 500 μ L of 50 μ M methyl jasmonate (MeJA) on detached leaves, according to the protocol described by Sadeghi et al. (unpublished). Blue coloration indicates β -glucuronidase enzyme activity resulting from *CaDRRG-Pro* promoter induction. Scale bar = 0.5 mm.

Rapid GUS activity (1-hour post-treatment) confirms the early responsive nature of the *CaDRRG-Pro* promoter, consistent with the 11-fold increase in *CaDRRG* transcript within 3 hours of *A. rabiei* inoculation (Jaiswal et al., 2012). The progressive increase in GUS activity from 1 to 6 hours, followed by a plateau between 6 and 24 hours, suggests that the promoter reaches maximum activation within 6 hours of hormone treatment. This temporal pattern is typical of promoters containing multiple cis-acting elements that act synergistically to amplify the transcriptional response (Rushton et al., 2002; Venter, 2007).

A significant finding of this study is that the *CaDRRG-Pro* promoter is induced by both methyl jasmonate and salicylic acid. This dual responsiveness is notable because these two phytohormones typically mediate distinct defense pathways. SA is primarily associated with systemic acquired resistance (SAR) against biotrophic and hemibiotrophic pathogens, while JA/MeJA is associated with ISR against necrotrophic pathogens and herbivorous insects (Zhang *et al.*, 2025, Glazebrook, 2005, Hönig *et al.*, 2023). The dual responsiveness of the *CaDRRG-Pro* promoter suggests that the 700 bp upstream region contains cis-acting elements that are recognized by transcription factors from both signaling pathways. Based on the *in silico* analysis of this promoter (Shokouhifar *et al.*, 2025), several candidate cis-acting elements like W-box, AS-1 and G-box may explain this dual inducibility.

Assessment of *CaDRRG* promoter inducibility in response to treatment with two *A. rabiei* pathotypes with different levels of aggressiveness

The pathotype PVI has been previously reported to exhibit higher aggressiveness compared to pathotype PI (Shokouhifar *et al.*, 2003). To assess whether the *CaDRRG-Pro* promoter responds differentially to these two pathotypes, detached leaves from the transgenic line (code 1-5) were sprayed with 500 μ L of crude mycelial extract from each pathotype, and leaf segments were sampled at 1, 3, 6, and 24 hours after treatment for histochemical GUS assay (Fig. 6).

The results showed that 1 hour after spraying with pathotype extracts (Fig. 6, T1), a very faint blue background was observable in leaf tissue for both pathotypes (Fig. 6, row PI and row PVI, column T1). The response was largely similar between the two pathotypes at this early time point. At 3 hours post-spraying (Fig. 6, T3), blue coloration was clearly visible to the naked eye in leaf segments treated with both pathotypes. No significant difference was observed between the two pathotypes at this time point, although the intensity was obviously higher compared to the T1 samples. At 6 hours post-spraying (Fig. 6, T6), the intensity of blue coloration showed a relative increase compared to the 3-hour time point for both pathotypes. At 24 hours post-spraying (Fig. 6, T24), complete and uniform blue coloration was observed throughout the stained leaf segments. The blue color appeared as very fine but completely uniform dots across the entire leaf segment. This intensity was substantially higher compared to earlier time points. No significant differences were observed between the two fungal pathotypes at any of the time points examined. The absence of basal GUS expression in untreated leaf samples was confirmed in Supplementary Fig. S2.

To further investigate whether cellular-level differences exist in the inducibility of tissues in response to treatment with different fungal extracts, samples from the 24-hour time point for both pathotypes were examined microscopically at 10 $\times$ magnification (Fig. 7). As shown in Fig. 7, blue spots resulting from β -glucuronidase enzyme activity were observed within cells. Although both samples exhibited intracellular blue spots, a very slight difference in the abundance of blue spots could be observed in leaf samples treated with pathotype PVI (Fig. 7, PVI-T24) compared to those treated with pathotype PI (Fig. 7, PI-T24). However, this difference was extremely minor and not statistically reliable. Untreated leaf samples from the same transgenic line showed no visible blue coloration (Fig. 7, Untreated).

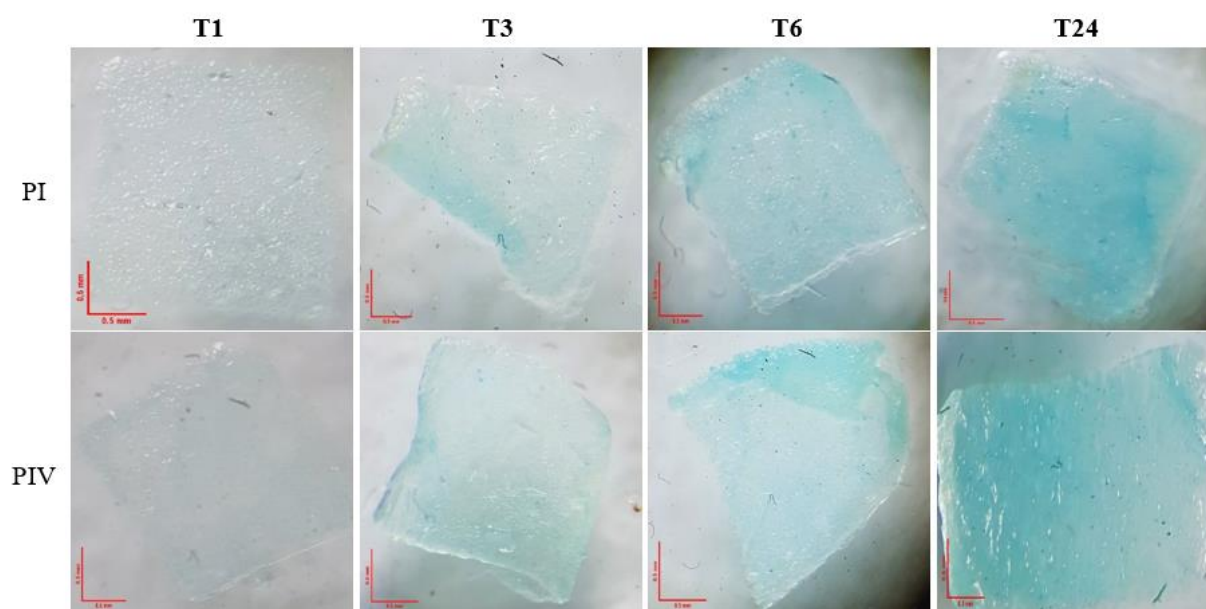


Fig. 6. Histochemical GUS assay of transgenic tobacco leaves (line 1-5) harboring the pCaDRRG-Pro-Gi construct at 1, 3, 6, and 24 hours (T1, T3, T6, T24) after spraying with 500 μ L of crude mycelial extracts from *Ascochyta rabiei* pathotypes PI and PVI on detached leaves from transgenic tobacco plant code 1-5. Images were captured using a stereomicroscope at 4 $\times$ magnification. Scale bar = 2 mm.

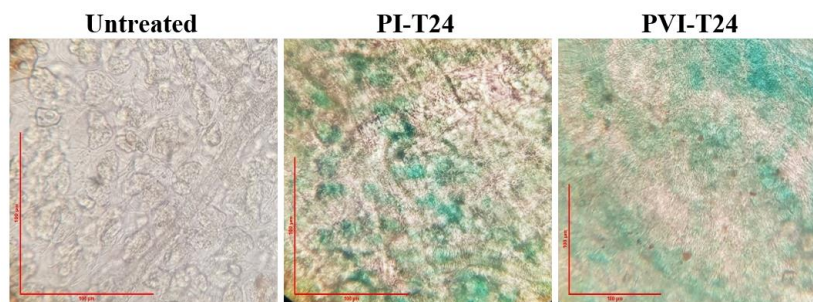


Fig. 7. Microscopic image (10× magnification) of leaf samples from transgenic tobacco plant code 1-5 harboring the pCaDRRG-Pro-Gi construct 24 hours after spraying with crude mycelial extracts from *Ascochyta rabiei* pathotypes PI and PVI (PI-T24 and PVI-T24, respectively), showing blue spots corresponding to β -glucuronidase enzyme activity following histochemical GUS assay. Untreated leaf samples from the same transgenic line showed no visible blue coloration, confirming the absence of basal GUS expression at the cellular level (Untreated). Scale bar = 0.5 mm.

The observation that GUS activity was detectable as early as 1 hour after treatment with crude mycelial extracts from both pathotypes demonstrates that the *CaDRRG-Pro* promoter responds rapidly to fungal elicitors. This rapid induction is consistent with the early responsive nature of the native *CaDRRG* gene from which this promoter was derived, which showed an 11-fold increase in transcript accumulation within 3 hours of *A. rabiei* inoculation (Jaiswal et al., 2012). The progressive increase in GUS activity from 1 to 24 hours suggests sustained promoter activation following initial recognition of pathogen-associated molecular patterns (PAMPs). No significant difference was observed between the two *A. rabiei* pathotypes (PI and PVI) in their ability to induce the *CaDRRG-Pro* promoter, despite previous reports that PVI exhibits higher aggressiveness on chickpea compared to PI (Shokouhifar et al., 2003). Several factors may explain this observation. First, continuous *in vitro* subculturing of these pathotypes over extended periods may have resulted in the loss or downregulation of many genes involved in pathogenicity and virulence. It is well documented that prolonged culture on artificial media can lead to attenuation of virulence in fungal pathogens, including *A. rabiei* (Bahrabadi et al., 2025). This phenomenon occurs because selective pressures present during host infection are absent *in vitro* conditions, allows strains with reduced virulence factor expression to proliferate.

Second, the inability to detect differential induction may be due to the fact that the factors determining aggressiveness differences between pathotypes may not be involved in activating the defense pathways that regulate the *CaDRRG-Pro* promoter. Third, and most significantly, tobacco is a non-host for *A. rabiei*. The defense signaling pathways activated in tobacco in response to *A.*

rabiei elicitors may not fully recapitulate the specific responses that occur in chickpea, the natural host (Oh & Choi, 2022). Consequently, the differential recognition of pathotype-specific elicitors that may exist in chickpea might not be preserved in this heterologous system. This highlights the importance of validating promoter inducibility in the native host species.

The conservation of defense signaling pathways across plant species is well documented. Studies have shown that defense signaling components are evolutionarily conserved from bryophytes to angiosperms (Agorio et al., 2024). For example, the promoter of *PgDI* (defensin 1) from white spruce (*Picea glauca*) maintained its expression pattern in *Arabidopsis thaliana*, demonstrating that signaling pathways are conserved between gymnosperms and angiosperms (Germain et al., 2012). Similarly, core components of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) are shared across the plant kingdom (Ramírez-Zavaleta et al., 2022).

The conservation of NPR1-mediated and WRKY-mediated defense signaling pathways has been demonstrated across diverse plant species, with central hubs involved in signaling being shared between PTI and ETI responses (Zhong et al., 2025). This evolutionary conservation explains why a promoter from chickpea can respond to fungal elicitors in a tobacco heterologous system.

Direct interaction of *A. rabiei* pathotypes with transgenic tobacco leaves harboring pCaDRRG-Pro-Gi

To investigate the effect of direct fungal mycelial growth on the induction of the *CaDRRG-Pro* promoter, fresh mycelial cultures of both low-virulent (PI) and high-virulent (PVI) pathotypes of *A. rabiei* were prepared on PDA medium (Fig. 8, A and B). After approximately 10 days of growth, agar blocks containing actively growing mycelium were excised from the colony margin. A fully expanded leaf (the fourth leaf from the apical bud) of the transgenic tobacco line (code 1-5) was detached, washed with distilled water, dried on sterile filter paper, and placed in a Petri dish containing sterile moist filter paper. The fungal agar block was placed in the center of the leaf (Fig. 8, C). Leaf segments were sampled approximately 1 cm from the fungal block placement site at 1-, 24-, 36- and 120-hours post-inoculation for histochemical GUS assay.

The results showed that no blue coloration was observed in samples up to 24 hours post-inoculation. At 36 hours after inoculation, very limited and weak blue spots were detected (data not shown). Samples at 120 hours after inoculation clearly exhibited blue coloration (Fig. 8, D-I). However, the blue coloration was not uniform across all cells; rather, it was more intense along

distinct vein patterns. The temporal appearance of blue coloration was similar between the aggressive pathotype (PVI; Fig. 8, rows G-I) and the weak pathotype (PI; Fig. 8, rows D-F), with both showing blue coloration at 120 hours post-inoculation (Fig. 8, T120, F and I). The absence of basal GUS expression in untreated leaf samples was confirmed in Supplementary Fig. S2.

Microscope examination of these samples confirmed that no blue coloration was visible at 1 hour and 24 hours post-inoculation for either pathotype. However, at 120 hours post-inoculation, blue spots were clearly visible at the cellular level and along vascular margins for pathotypes PI and PVI (Fig. 8, panels F and I).

The observation of vascular-associated GUS expression in this study is similar to findings reported by Heise et al. (2002), who showed that the *AtCMPG1* promoter, from which the F element was derived, is induced in vascular tissues in response to fungal elicitors. This tissue-specific expression pattern may be advantageous for resistance to vascular pathogens like *Fusarium* and *Verticillium* species, but may also be relevant for *A. rabiei*, which can systemically colonize chickpea through vascular tissues.

Moreover, the detached leaf assay used in this experiment, while convenient for controlled inoculation, has some limitations. Detached leaves are under physiological stress due to water loss and reduced nutrient availability, which may affect both fungal growth and plant defense responses. Additionally, systemic defense signaling that would occur in intact plants is absent in detached leaves. Future experiments using whole-plant inoculations would provide a more accurate assessment of promoter inducibility under natural infection conditions. The comparison of induction kinetics between the three treatment methods used in this study was summarized in the table 1.

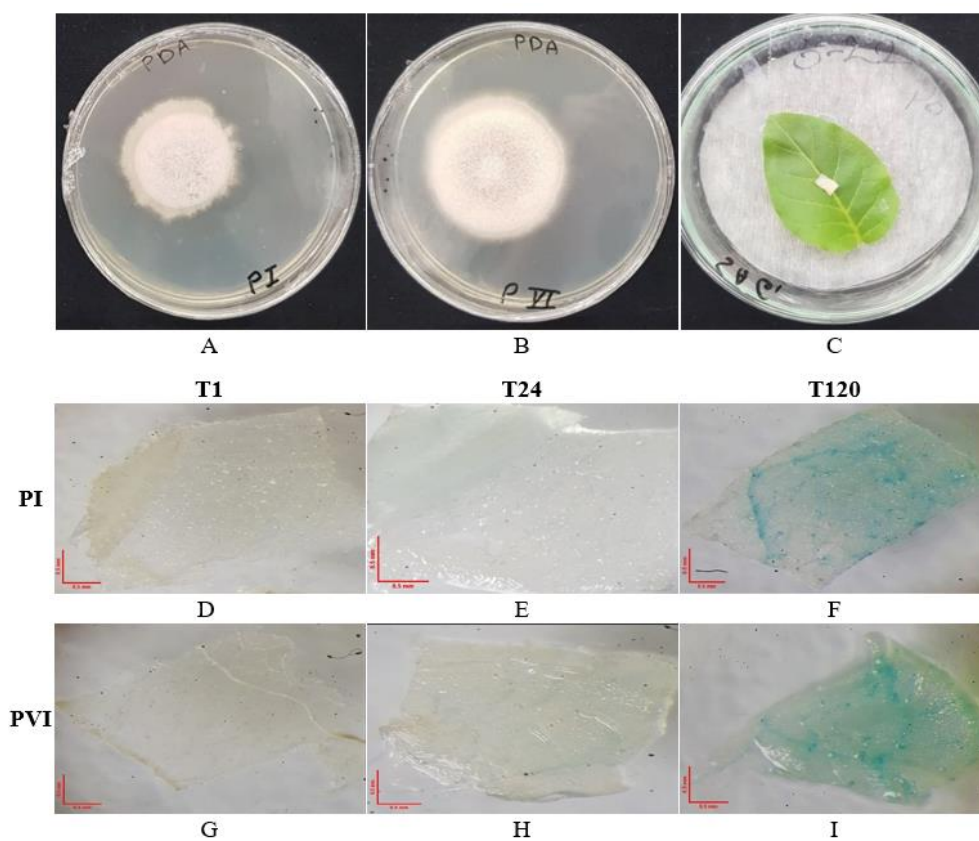


Fig. 8. (A, B) Mycelial cultures of *Ascochyta rabiei* pathotypes PI and PVI after 10 days of growth on PDA medium. (C) Direct inoculation method showing placement of an agar block containing actively growing fungal mycelium on a detached leaf of transgenic tobacco line 1-5 harboring the pCaDRRG-Pro-Gi construct. (D-I) Results of histochemical GUS assay on leaf segments sampled approximately 1 cm from the fungal block placement site at 1 hour (T1), 24 hours (T24), and 120 hours (T120) post-inoculation with pathotype PI (D, E, F) and pathotype PVI (G, H, I). Images were captured using a stereomicroscope at 4× magnification. Scale bar = 2 mm.

Table1. Comparison of induction kinetics of the *CaDRRG-Pro* promoter in response to different treatments.

Treatment	First detectable GUS activity	Maximum induction
MeJA/SA (hormones)	1 hour	6-24 hours
Crude mycelial extract	1 hour (faint)	24 hours
Direct fungal inoculation	36 hours (faint)	>120 hours

The differences in induction kinetics between these treatment methods highlight the importance of considering the experimental context when interpreting promoter activity data. Hormone treatments provide the most rapid and uniform induction because the signaling molecules are directly applied and readily absorbed by plant tissues. Crude extracts contain pre-formed elicitors that are immediately available for recognition. Direct fungal inoculation, however, requires active fungal growth and production of elicitors, resulting in delayed and heterogeneous induction.

Conclusions

This study successfully characterized the functional properties of the *CaDRRG-Pro* promoter using stable transgenic tobacco lines. The findings confirm that the promoter is rapidly induced by methyl jasmonate and salicylic acid, responds to *A. rabiei* elicitors, and exhibits no detectable basal expression in the absence of induction. The hierarchical GUS assay (GPS) proved to be an efficient, marker-independent screening method, reducing the number of assays by 63%. The strong induced expression of this promoter, its minimal basal activity, and its dual responsiveness to MeJA and SA position it as a valuable tool for pathogen-inducible expression of resistance genes in chickpea breeding programs. Future studies should prioritize the stable transformation of chickpea with the *CaDRRG-Pro:GUSi* cassette to evaluate promoter inducibility in the native host and to determine whether differential induction by *A. rabiei* pathotypes occurs in the natural host-pathogen context.

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القابذیری پروموتور CaDRRG-Pro گیاه نخود توسط هورمون‌های دفاعی و الیسیتورهای قارچ
*Ascochyta rabiei** در گیاه تراریخته پایدار تنباکو به عنوان گیاه مدل

سارا نعیمی، مجتبی معمرآبادی، و فرهاد شکوهی فر

چکیده

تحلیل پروموتورهای ژن‌های با پاسخ سریع، ابزارهایی را برای مهندسی مقاومت گیاه در برابر بیماری‌ها فراهم می‌کند. بیماری سوختگی آسکوکیتا (*Ascochyta blight*) که توسط قارچ *Ascochyta rabiei** ایجاد می‌شود، یکی از موانع اصلی در کشت نخود است. ژن CaDRRG پیش‌تر به عنوان ژنی با پاسخ سریع شناسایی شده بود که طی ۳ ساعت پس از مایه‌کوبی با *A. rabiei**, افزایش بیش از ۱۱ برابر در میزان بیان نشان می‌داد. در این مطالعه، عملکرد پروموتور CaDRRG-Pro با استفاده از گیاهان تراریخته پایدار تنباکو مورد ارزیابی قرار گرفت. وکتور دوتایی pCaDRRG-Pro-Gi، حاوی قطعه ۷۷۲ جفت‌بازی CaDRRG-Pro متصل به یک ژن گزارشگر GUS (دارای اینترون)، به باکتری *Agrobacterium tumefaciens** منتقل شد. لاین‌های تراریخته پایدار تولید شدند و با استفاده از یک روش سنجش سلسله‌مراتبی GUS، یک لاین با بیان بالا (کد ۵-۱) از میان ۳۰ گیاهچه باززایی‌شده شناسایی گردید که موجب صرفه‌جویی ۶۳ درصدی در انجام آزمایش‌ها شد. این پروموتور به سرعت توسط متیل‌جاسمونات و اسید سالیسیلیک القا شد؛ به طوری که فعالیت GUS پس از یک ساعت قابل تشخیص بود و حداکثر میزان القا در عرض ۶ ساعت حاصل شد و به سطحی قابل مقایسه با پروموتور همواره‌فعال CaMV 35S (constitutive) رسید. این پروموتور همچنین به عصاره‌های خام میسلیمی پاتوتیپ‌های *A. rabiei** پاسخ داد و القای آشکاری را در زمان ۳ ساعت نشان داد. مایه‌کوبی مستقیم با قارچ منجر به القای با تأخیر (۱۲۰ ساعت) و الگوهای بیان مرتبط با بافت آوندی شد. در شرایط عدم القا، هیچ‌گونه بیان پایه‌ای در لاین‌های تراریخته پایدار مشاهده نشد. وجود اینترون در ژن گزارشگر GUS از بیان پروکاریوتی در *Agrobacterium** جلوگیری کرد و بدین ترتیب اختصاصیت عملکرد آن تضمین گردید. این نتایج، تأییدی بر عملکرد پروموتور CaDRRG-Pro است و قابلیت القای سریع آن توسط هورمون‌های دفاعی و الیسیتورهای قارچی را نشان می‌دهد؛ ویژگی‌هایی که این پروموتور را به ابزاری ارزشمند برای بیان ژن‌های مقاومت (تحت کنترل عوامل بیماری‌زا) در برنامه‌های به‌نژادی نخود تبدیل می‌کند.