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Expression of the tobacco β-1,3-glucanase gene, *PR-2d*, following induction of SAR with *Peronospora tabacina*

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Abstract

Systemic acquired resistance (SAR) is induced following inoculation of *Peronospora tabacina* sporangia into the stems of *Nicotiana tabacum* plants highly susceptible to the pathogen. Previous results have shown that accumulation of acidic β-1,3-glucanases (PR-2's) following induction of SAR by *P. tabacina* may contribute to resistance to *P. tabacina*. We showed that up-regulation of the *PR-2* gene, *PR-2d*, following stem inoculation with *P. tabacina*, is associated with SAR. Studies using plants transformed with GUS constructs containing the full length promoter from *PR-2d* or promoter deletions, provided evidence that a previously characterized regulatory element that is involved in response to salicylic acid (SA), may be involved in regulation of *PR-2d* following induction of SAR with *P. tabacina*. This work provides evidence that regulation of *PR-2* genes during *P. tabacina*-induced SAR may be similar to regulation of these genes during infection of *N*-gene tobacco by TMV or following exogenous application of SA, and provides further support for the role of SA in regulation of genes during *P. tabacina*-induced SAR.

Keywords: β 1,3-Glucanase; Downy mildew; Immunity; Oomycete; *Nicotiana tabacum*; Pathogenesis-related protein; *Peronospora tabacina*; Salicylic acid; Systemic acquired resistance; Tobacco blue mold

1. Introduction

Systemic acquired resistant (SAR) is a phenomenon in which a localized infection or other stress causes the plant to be resistant or even immune to further infections at a distance from the initial infection, thus demonstrating that plants susceptible to certain pathogens nevertheless have the genetic capacity to express effective resistance against those same pathogens. Classical studies of SAR demonstrate induction by necrotizing pathogens [13,37]. One way this can be accomplished is to inoculate a cultivar containing a resistance (R) gene to an avirulent pathogen [12]. One thoroughly-studied system involves *Nicotiana tabacum* carrying the R gene (*N*) against Tobacco Mosaic Virus (TMV). Inoculation of *N*-gene containing tobacco with TMV can result in SAR induction, increasing resistance to not only TMV but also allows resistance to pathogens the plant is otherwise susceptible to, including oomycetes [42]. However, induction in an otherwise susceptible and compatible interaction also can be accomplished, as has been demonstrated in cucumber—*Colletotrichum lagenarium* [19] or tobacco—*Peronospora tabacina* [7] systems. Extensive research into pathogen-induced SAR has demonstrated that increased resistance is associated with an accumulation of proteins, called pathogenesis-related (PR) proteins [48], and, in some plant systems, of over 20-fold increased accumulation of salicylic acid (SA) [26].

Cultivated tobacco is highly susceptible to *P. tabacina* and no known R-gene resistance is available in commercially desirable cultivars [14]. The leaf is a natural site of infection by air-borne sporangia (asexual spores) of the pathogen, resulting in the production of chlorotic lesions from which emerge huge numbers of sporangia [2,29]. Heavily infected leaves can wilt and die, and the pathogen

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sometimes goes systemic resulting in severe stunting of the plant [31]. Even highly susceptible cultivars of *N. tabacum* can be induced to resistance to *P. tabacina* by introduction of the pathogen into the plant stem by injecting sporangia just beneath the epidermis [5]. This treatment results in a rising necrotic lesion within the external phloem and cambium, with no detectable detriment to the plant, and with no hyphae or sporangia of the pathogen emerging [5]. The stem-injected ('induced') plants become progressively more refractory to foliar 'challenge' inoculation with *P. tabacina* and by 3 weeks following stem injection many plants thusly treated appear to be resistant [33]. If plants used have the *N* gene, inoculation of lower leaves with TMV causes SAR, which also protects against *P. tabacina* [28]. Using a cultivar of tobacco (KY14) carrying the *N* gene, assays showed that plants initially inoculated with TMV or with *P. tabacina* had increased activity of PR proteins, including β-1,3-glucanase (PR-2), and this increased activity was associated with resistance to challenge inoculation with *P. tabacina* [51]. In vitro evidence [27] and evidence provided by transgenic plants [54], suggest that PR-2's may have a role in protecting plants against fungal and oomycete pathogens. Extracellular secretion of β-1,3-glucanases following pathogen infection or SA treatment [41,45], may place them in proximity to the infecting agent and provides further support for their potential role in defense.

Stem inoculation experiments of tobacco with *P. tabacina* sporangia have provided evidence that such treatments can protect the plant from infection by *P. tabacina* and pathogens similar to *P. tabacina*. The β-1,3-glucanases PR-2b and PR-2c were shown to accumulate in stem-inoculated plants, which were protected against a challenge infection [33]. Stem inoculation of a TMV susceptible cultivar (TN86) with *P. tabacina* showed a significant increase in glucanase activity in leaves over water-inoculated controls, and provided resistance against challenge inoculation with *P. tabacina* but not against TMV [51]. The *PR-2b* gene was shown to be transcriptionally regulated during TMV-induced SAR against challenge inoculation with *P. tabacina* [23]. Plants transformed with *PR-2b* under the control of a constitutive promoter, showed increased constitutive β-1,3-glucanase activity that was associated with increased resistance to *P. tabacina* and another oomycete, *Phytophthora parasitica*, while such constitutively-expressing plants, even those accumulating the highest levels of PR-2b, remained susceptible to three tobacco viruses [24]. Reduced levels of β-1,3-glucanase activity following *P. tabacina*-induced SAR of transgenic plants carrying an anti-sense construct of *PR-2b*, was associated with increased susceptibility to *P. tabacina* and *Ph. parasitica* [24]. Taken together, this body of evidence suggests that induction of SAR in susceptible tobacco via stem-inoculation with *P. tabacina* results in resistance to *P. tabacina* but not to tobacco viruses.

Beta-1,3-glucanases are a serologically related group of enzymes with similar activities [18]. They are regulated differentially in response to pathogen attack [6,48], in response to other stresses [30] and during development [6,21]. The class of β-1,3-glucanases that are acidic and extracellular, responding to pathogen infection (class IIa), was originally defined by the PR proteins, PR-2a, PR-2b and PR-2c (formerly known as PR-2, PR-N and PR-O, respectively; [18]). Genes encoding PR-2a, PR-2b, PR-2c, and a fourth β-1,3-glucanase gene, *PR-2d*, have high sequence identity; from approximately 91 to 97%, in the open reading frame [15,48].

Regulation of two *PR-2* genes, *PR-2b* [44] and *PR-2d* [15], has been analyzed by promoter and promoter deletion fusions with a bacterial β-glucuronidase (GUS) reporter gene. In this way, it has been determined that regions of the promoters of these genes contain regulatory elements that respond during development in healthy plants, following TMV infection in *N* gene tobacco, and following treatment of leaf disks with SA [15,44]. In healthy plants, expression was primarily in seedlings [15] and in floral organs of mature plants [15,44]. Following TMV inoculation, an *N*-gene containing line transformed with a *PR-2d* promoter construct initially exhibited increased GUS activity in tissues surrounding the inoculation site then later at further distances in the inoculated leaf, including along veins, and in uninoculated leaves [15]. These results suggest that *PR-2d* may exhibit expression patterns following TMV inoculation consistent with genes encoding class IIa β-1,3-glucanases.

Using 1 mM SA applied to leaf disks removed from transgenic plants containing different promoter deletion-GUS constructs of *PR-2d*, two regions were identified that contained SA-responsive elements: one, within 1047 bp upstream of the start site, that increased expression 2-fold, and a second, within 607 bp upstream of the start site that increased expression 3–4-fold over water controls [15,38]. Fine dissection of the 607 bp upstream region revealed a 43 bp element within 364 bp from the start site that responded to SA application with a 4–5-fold increase in GUS activity over that of a deletion construct containing 321 bp of the upstream sequence [38]. Within this 43 bp region is a 6 bp region that appears essential for SA induction and a 25 bp region that binds nuclear proteins from tobacco leaves [38]. Thus, the region from −364 to −321 bp of *PR-2d* has been identified as containing a SA-responsive element (SARE; [38]).

Our goals in this study were to determine expression patterns of the *PR-2* gene, *PR-2d*, during induction of SAR following *P. tabacina* stem inoculation and to identify promoter regions that may contain elements involved in regulation of this gene following *P. tabacina*-induced SAR. We report the expression of *PR-2d*, as measured by GUS activity of a transgene construct, following stem inoculation with *P. tabacina*, provide evidence that regulation of this gene is similar to regulation following SAR induced by

TMV inoculation, and following exogenous application of SA to leaf disks. Additionally, evidence is discussed suggesting that a region upstream from the previously characterized SARE in the promoter of *PR-2d* may carry a regulatory element that is involved in increasing transcription in response to inoculation by *P. tabacina*.

2. Materials and methods

2.1. Plants and plant lines

Lines of *N. tabacum* cv. Xanthi nc transformed with the *PR-2d* promoter and promoter deletion constructs, were a kind and generous gift of D. Klessig and J. Shaw [15,38]. Lines containing the *PR-2d* promoter or promoter fragments fused with the GUS reporter gene are designated as the full-length promoter ('−1706') and as deletion constructs carrying 1462, −1047, 607, 364, 321 or 171 bp upstream of the start site of *PR-2d* ('−1462', '1047', '−607', '−364', '−321', and '−171', respectively). Other plant lines used were *N. tabacum* cultivars KY14, Xanthi nc, and Xanthi nc transformed with the *NahG* gene [10]. Seeds of KY14 were sown onto soil and all other lines were sown onto Murashige and Skoog basal medium with Gamborg's vitamins (Sigma–Aldrich, St. Louis), 3% sucrose (Sigma–Aldrich) and 0.7% Phytagar (Invitrogen Life Technologies, Carlsbad, CA). For transformed lines (containing p*PR-2d*::GUS constructs or *NahG*) 300 μg/mL kanamycin (Sigma–Aldrich) was added to tissue culture medium. Germinated seedlings were transferred to soil in 8″ pots and maintained in the greenhouse.

2.2. Maintenance of P. tabacina, sporulation of infected leaf tissue, and preparation of P. tabacina sporangia for inoculations

P. tabacina, an obligate parasite, was maintained on *N. tabacum* cv. KY14 plants by spraying 4 week old plants with a suspension of sporangia and placing plants in a negative pressure Hepa-filtered walk-in growth chamber (PGC Scientific, Parameter Generation and Control, Black Mountain, NC) at approximately 20–22 °C with 8 h light. When chlorosis began to develop, 4–6 days post inoculation, individual leaves or entire plants were sprayed with water and placed in resealable bags with moistened paper towels in an unlighted incubator for individual leaves or in covered storage boxes (115 L, Sterilite brand purchased from Walmart, Lexington, KY) inside the walk-in growth chamber, to induce sporulation. The following day, sporangia were removed by spraying plants with water and gently rubbing the leaf surface with a rubber scraper. To continue cultivation of the pathogen, the suspension was collected and sprayed onto uninfected 4-week-old plants.

For experimental inoculations, collection of sporangia was conducted using sterile purified water and sterilized instruments. Trichomes and other plant parts and larger *P. tabacina* hyphal fragments were excluded from the sporangial suspension by filtration through cheese cloth. The spore suspension was further purified by filtration through a 0.22 μm Steriflip filter (Millipore, Bedford, MA), three times. A small volume of sterile distilled water was added to resuspend the sporangia, which were quantified and the appropriate dilution was prepared.

For assessment of sporulation of wild type Xanthi and *NahG* plants, the fourth or fifth leaf from the top of each plant was detached and placed in conditions, as above, for sporulation. The following day, a 1.1 cm diameter disks was removed from the center of each leaf quarter, using a #8 cork borer, for a total of four disks per leaf. Leaf disks were placed in tubes in which water was added and sporangia dislodged from the disk. Sporangial suspensions were diluted as necessary, and numbers of sporangia per cm^2 for each plant was assessed.

2.3. P. tabacina spray inoculations

For *P. tabacina* infection assays, 11 week old plants from each transformed line containing either the −1706 (two different lines), −607, −364 or −321 constructs were ordered by height then numbered, with the tallest plant as number 1. Leaf disks were removed and stored as described above. Odd-numbered plants were sprayed until run-off (approximately 50 mL) with a suspension of *P. tabacina* sporangia (4×10^4 sporangia/mL) while even-numbered plants were sprayed until run-off with sterile purified water ('mock-challenged'). Plants were covered with 20 gal., black garbage bags and placed into the walk-in growth chamber. Bags were removed the following day and plants were scored 6 days following spraying for symptoms: necrosis and/or chlorosis on any leaves was scored as positive, while no visible symptoms on all leaves was scored as negative. At this time, leaf disks were collected and stored at −80 °C.

2.4. P. tabacina-induced SAR bioassays

Plants used in SAR bioassays were planted and maintained in the greenhouse for approximately 2 months prior to stem inoculation and for 3 weeks following stem inoculation. Plants were maintained in warm temperatures (as high as 33 °C; [5]) and individual plants were given adequate space throughout the 12 week period; SAR bioassays were not feasible in growth chambers (Funnell and Schardl, unpublished). Six (assay A) or seven (assays B and C) lines were planted for each experiment. SAR bioassays were conducted from July through December only (1999 and 2000), to prevent the unlikely event of initiating infection of susceptible plants in nearby seedling greenhouses or tobacco fields by escaping sporangia during the other months.

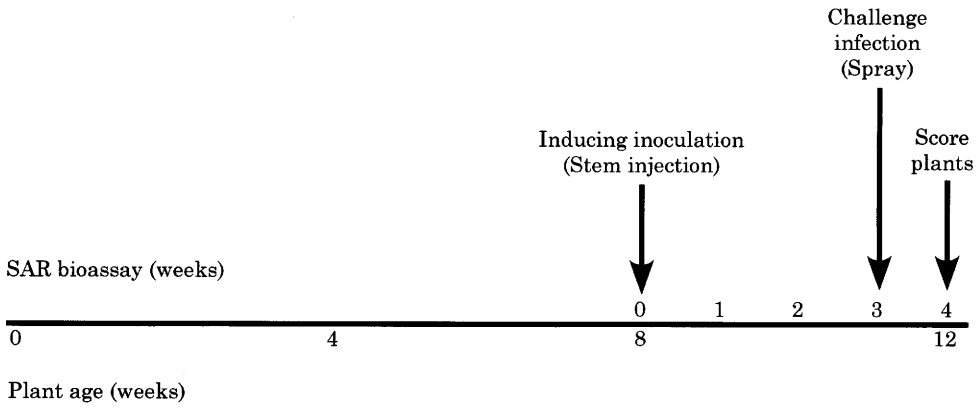


Fig. 1. Timing of stem inoculations and challenge inoculations. Timeline illustrating time points for inoculations and indicating nomenclature used throughout text.

One millilitre of a suspension of *P. tabacina* sporangia (1×10^6/mL) or sterile purified water was injected into plants, just under the plant epidermis, at about 2.5 cm above the soil line, using a tuberculin syringe [40]. Plants containing p*PR-2d*::GUS constructs were stem-inoculated 8 weeks following sowing (Fig. 1), while *NahG* plants and untransformed control plants, were inoculated after 9 weeks. Stem-inoculated plants were checked daily and the occasional plant exhibiting symptoms or signs of infection on leaves was removed and destroyed. Plants were challenge-inoculated 3 weeks following stem-inoculation (Fig. 1) by spraying spores over the entire plant, as described above. For assay #1, plants carrying −1706 (one sporangia-inoculated plant, four water-inoculated plants), −1462 (two sporangia-inoculated plants, three water inoculated plants), −607 (two sporangia-inoculated plants, three water inoculated plants), −364 (two sporangia-inoculated plants, three water inoculated plants), −321 (two sporangia-inoculated plants, three water inoculated plants) or −171 (two sporangia-inoculated plants, three water inoculated plants) GUS constructs were inoculated; for assay #2, plants carrying −1706, (two sporangia-inoculated plants, two water inoculated plants), −1462 (one sporangia- inoculated plant, three water inoculated plants), −1047 (two each sporangia-inoculated and water-inoculated plants), −607 (two each sporangia-inoculated and water-inoculated plants), −364 (two sporangia-inoculated plants, three water-inoculated plants), −321 (two sporangia-inoculated plants, two water inoculated plants), or −171 (one each sporangia-inoculated and water-inoculated plant) GUS constructs were inoculated; for assay #3, plants carrying −1706 (two sporangia-inoculated plants, three water-inoculated plants), −1462 (two sporangia-inoculated plants, three water-inoculated plants), −1047 (two sporangia-inoculated plants, three water-inoculated plants), −607 (two each sporangia-inoculated and water-inoculated plants), −364 (three each sporangia-inoculated and water-inoculated plants), −321 (three each sporangia-inoculated and water-inoculated plants) or −171 (one sporangia-inoculated plant, two water-inoculated plants) GUS constructs were inoculated. Four to eight plants were mock-challenged for each assay. Symptoms were assessed 1 week following challenge inoculation (Fig. 1). Plants exhibiting symptoms of *P. tabacina* infection, mild chlorosis (assigned the value of '1'), moderate to severe chlorosis (assigned the value of '2'), or necrosis with or without chlorosis (assigned the value of '3'), were considered positive for infection, while no visible symptoms on all leaves (assigned the value of '0') was scored as negative for infection.

Plants were sampled immediately following stem inoculation, at 1 and 2 weeks following stem inoculation, immediately following challenge inoculation (3 weeks following stem inoculation), and 1 week following challenge inoculation and after assessment of symptoms (Fig. 1). At each sampling, a 6 cm diameter disk was excised from the third fully expanded leaf from top of plant. Disks from each line and the same treatment were combined and stored at −80 °C.

2.5. Assessment of β-glucuronidase activity

Proteins were extracted from collected plant tissue as previously described [11], concentration was determined using Bradford solution (Bio-Rad Laboratories, Hercules, CA) and 100 μg of protein was assayed. GUS activity [pmol 7-hydroxy-4-methylcoumarin (MU)/min/mg protein] was determined as previously described (Gallagher, 1992) using the Versafluor Fluorometer (Bio-Rad). For each assay, 50 μl of a commercial preparation of β-1,3-glucuronidase in glycerol (Sigma–Aldrich), diluted to 10^{-5} in extraction buffer, was included as a control. GUS controls for analysis of infection assays averaged approximately 620 pmol MU/min/mg protein for infection assays, and 540 pmol MU/min/mg protein for SAR bioassays. GUS activities from experimental samples were standardized using GUS controls as indicated.

Since the purpose of analysis of GUS was to assess expression by the *PR-2d* promoter constructs following *P. tabacina*-induced SAR, only GUS activity in *P. tabacina*-inoculated plants that, by visual expression, were not

infected or had only mild necrosis following challenge with *P. tabacina*, and having water-inoculated plants that were infected, are used in the analyses of promoter regions.

2.6. Statistical analyses

For GUS analysis of activity following the *P. tabacina* infection assay, measurements were analyzed utilizing Proc GLM (SAS, Cary, NC) firstly comparing water- versus *P. tabacina*-sprayed plants and each construct, and all combinations of treatment and construct; and secondly, comparing constructs of *P. tabacina*-sprayed plants. Among the main effects (construct and treatment) and their interaction, there was significance at $P \leq 0.05$. Because differences observed between constructs prior to spraying may be due to up-regulation of the GUS gene during development [15], the water controls were discounted when conducting analysis of GUS activity between constructs 6 days following treatment.

For SAR bioassays, Proc GLM (SAS) was used to compare numbers of infected plants between assays and between stem inoculation treatments. Due to no significant treatment X assay interactions, assays were pooled for further analysis. Analysis of variance (Proc GLM; SAS) was used for conducting comparisons among stem inoculation treatments and constructs. For the purpose of this analysis, symptoms were scored using the following numbering system: plants exhibiting no visible symptoms were given the value '0', plants exhibiting mild chlorosis on one or a few leaves were assigned the value '1', plants exhibiting moderate to severe chlorosis on most or all leaves were assigned the value '2', and plants exhibiting necrosis over most or all of the plant, with or without chlorosis, were assigned the value of '3'.

Comparisons were considered significant if P values were less than or equal to 0.05.

2.7. Nucleotide database searches

Database searches of *PR-2d* nucleotides upstream of nucleotide −364 were conducted utilizing discontinuous MegaBLAST [25] and FASTA [34]. Promoters from plant nuclear genes that exhibited similarities with *PR-2d* were included in the results.

3. Results

3.1. GUS activity of plant tissue from lines carrying pPR-2d:GUS constructs following infection by P. tabacina

To be certain the promoter of *PR-2d* responds to infection by *P. tabacina* sporangia, as measured by GUS activity, 11 week old plants (the age at which SAR bioassay plants are challenged; see Fig. 1) from lines containing GUS constructs with either the full-length promoter from *PR-2d* ('−1706'; two transformant lines) or deletions (one line each containing constructs '−607'; '−364'; or '−321') were sprayed either with water or with *P. tabacina* sporangia. Plants were scored for symptoms after 6 days. All plants sprayed with sporangia exhibited symptoms typical of *P. tabacina* infection: chlorosis and/or necrosis on lower leaves of all plants and on upper leaves of most plants (17 of 18 *P. tabacina*-sprayed plants). All 17 plants sprayed with water lacked visible symptoms or signs of infection. Leaf tissue was collected immediately prior to spraying and 6 days following spray treatment. GUS activities were analyzed in two groups (both treatments of each line in the same group) and were normalized according to the activities of the GUS control in each group. Mean GUS activity of plants prior to spraying was 121.0 (±113.1) for plants carrying construct −1706, 26.8 (±16.2) for plants carrying construct −607, 6.3 (±4.8) for plants carrying construct −364 and 0.9 (±1.2) for plants carrying construct −321. (Fig. 2) shows mean GUS activities, with positive standard deviations, as determined for tissue collected from plants 6 days following each spray treatment and containing each construct (see Section 2.3). There was plant-to-plant variation within each line and treatment. Nonetheless, statistically significant differences were demonstrated: GUS activity of plants carrying the −1706 construct exhibited significantly different ($P \leq 0.05$) activity from those carrying the −321 construct, indicating that the *PR-2d* promoter constructs exhibited a differential response to treatment with *P. tabacina*.

3.2. Analysis of lines transformed with pPR-2d: GUS constructs following P. tabacina stem-inoculation and after challenge infection

3.2.1. Response of stem-inoculated plants to challenge infection with spores of P. tabacina

P. tabacina-induced SAR bioassays were conducted on *N. tabacum* cv. Xanthi nc lines transformed with

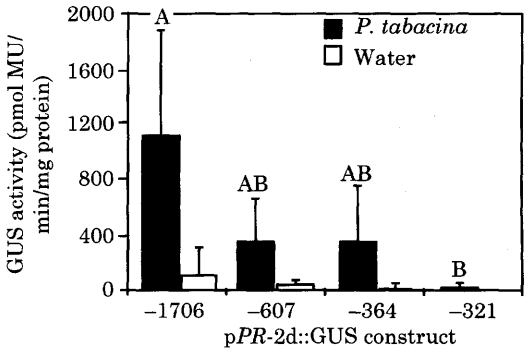


Fig. 2. GUS activity of plants carrying p*PR-2d*::GUS constructs following spray inoculations with *P. tabacina* sporangia or with water. Means of GUS activity (pmol MU/min/mg protein) for plants sprayed with a suspension of *P. tabacina* sporangia or with water at 6 days following treatment. Activity in samples was assessed in two groups and normalized using activity resulting from assaying of a commercially prepared β-glucuronidase enzyme in each group (Section 2.5). Positive standard deviations are indicated. Proc GLM groupings for *P. tabacina*-sprayed plants are shown. Bars with differing letters are statistically different ($P \leq 0.05$).

p*PR-2d*::GUS constructs. Plants were stem inoculated with either *P. tabacina* sporangia or with water then, 3 weeks following stem inoculation, were challenged by spraying leaves with *P. tabacina* sporangia (see Fig. 1). A few plants from each assay were challenged with water ('mock'). One week following challenge inoculation, plants were assessed for visible symptoms. Of the 36 *P. tabacina*-stem inoculated plants that were subsequently challenged with *P. tabacina* sporangia, 26 plants had no visible symptoms (each assigned the value of '0' for the purpose of statistical analysis of symptoms), four plants had mild chlorosis (each assigned the value of '1'), two plants exhibited moderate to severe chlorosis (each assigned the value of '2') and two plants were necrotic, with or without chlorosis, over most or all of the plant (each assigned the value of '3'). Out of 37 plants stem-inoculated with water then challenged with *P. tabacina* sporangia, four plants exhibited no symptoms, one plant had mild chlorosis, 18 plants had moderate to severe chlorosis and 14 plants had necrosis with or without chlorosis (scoring of symptoms as above). Assay effects were significant ($P=0.002$) but assay X treatment interactions were not. Therefore, effects were pooled across assays. The number of *P. tabacina*-stem-inoculated plants exhibiting symptoms (approximately 28%) was significantly ($P\leq 0.05$) less than the number of symptomatic water-inoculated plants (approximately 89%) 1 week following challenge with *P. tabacina* (Table 1). When the assigned symptom values were included in the analysis, the mean symptom value for *P. tabacina*-challenged plants stem inoculated with sporangia (0.53 ± 0.97) was highly significantly less ($P<0.0001$) than that of *P. tabacina*-challenged plants that had been stem inoculated with water (2.14 ± 0.92). Response of plant lines transformed with different constructs to challenge was not significant and analysis indicated that constructs did not interact with treatments (water- versus *P. tabacina*-inoculation) differently. All mock-challenged plants lacked visible symptoms (Table 1).

3.2.2. GUS activities of plants stem-inoculated and challenged with P. tabacina

GUS activities (see Section 2.5) from plant tissues collected during the 3 weeks following stem-inoculation and at 1 week following challenge inoculation (see Fig. 1 and Section 2.4) were determined. Since, the goal of this study was to determine which deletion constructs resulted in increases in GUS activity associated with *P. tabacina*-induced SAR, only lines having all *P. tabacina*-inoculated plants that were symptom-free or had mild chlorosis following challenge with *P. tabacina* sporangia, and also having water-inoculated plants that were symptomatic following challenge with *P. tabacina* sporangia, are included in the following analyses. Fig. 3(A) (stem-inoculated with *P. tabacina* sporangia) and Fig. 3(B) (stem inoculated with sterile water) illustrate resulting activities using assay #1 as an example. In general, activities of GUS constructs containing the full-length promoter (1706 bp upstream of the start site of *PR-2d*), and promoter deletion constructs containing 1462, 1047 and 364 bp upstream of the start site of *PR-2d* exhibit greater activity following stem inoculation with *P. tabacina* than with sterile water (Fig. 3(A) and (B)). Plants containing the construct with the full length promoter, stem inoculated with water, also exhibit GUS activity following stem inoculation (Fig. 3(A) and (B)).

One week following challenge inoculation, GUS activity of plants stem-inoculated with water, carrying the construct with the full-length promoter of *PR-2d*, was high (3432 pmol MU/min/mg protein) while the GUS activity of similarly treated plants containing the deletion construct with 364 bp upstream of the start site was only modest (194 pmol MU/min/mg protein; Fig. 3(B)). These results are not unlike those obtained when infecting susceptible (uninduced) plants at 11 weeks (see Fig. 2). In order to more readily compare plants carrying the same construct in the same assay, and to attempt to ameliorate the between-GUS assay differences (Section 3.2.1), the ratio of GUS activities following stem

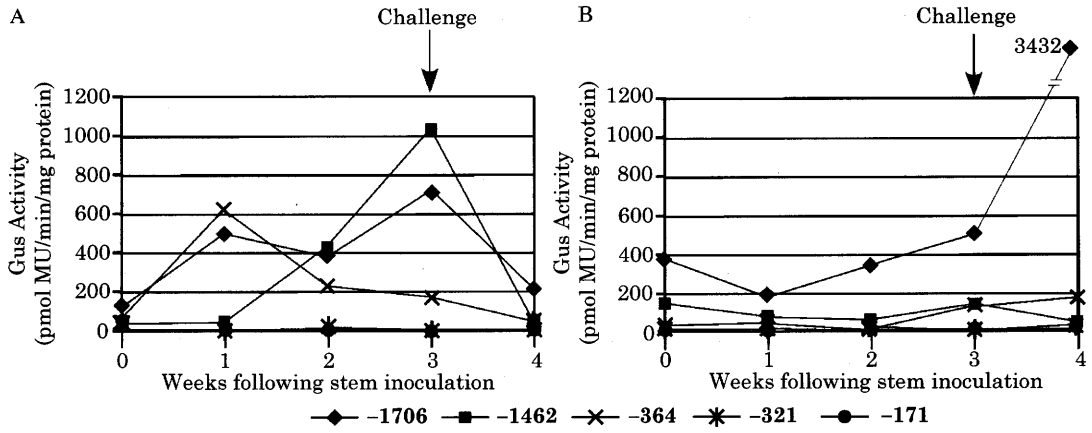
Table 1
Visible symptoms of plants following challenge inoculation

Assay	*P. tabacina*-inoculated				H_2O-inoculated			
	P. t.-challenged		H_2O-challenged		*P. t.*-challenged		H_2O-challenged	
	Total plants	Visible[a] symptoms	Total plants	Visible symptoms	Total plants	Visible[a] symptoms	Total plants	Visible symptoms
#1	11	0	nt[b]	nt	13	9	6	0
#2	10	3	2	0	9	9	6	0
#3	15	7	nt	nt	15	15	4	0
Totals	36	10	2	0	37	33	14	0
%[c]	28		0		89		0	

[a] Number of plants exhibiting any degree of symptoms [mild (chlorosis on one or a few leaves), moderate (chlorosis on most leaves) to severe (chlorosis and necrosis on most or all leaves)] for each set of treatments (stem inoculation and challenge) in each assay.

[b] 'nt' indicates 'not tested.'

[c] Percentage of plants exhibiting visible symptoms following each set of indicated treatments (stem inoculation and challenge) over three assays. The total number of *P. tabacina*-stem-inoculated plants challenged with *P. tabacina* sporangia exhibiting symptoms was significantly less than water-stem-inoculated plants challenged with *P. tabacina* sporangia and exhibiting symptoms (at $P\leq 0.05$).



C

Challenge

Line	Weeks following stem inoculation 0	1	2	3	4
Assay #1					
−1706	0.29	2.55	1.19	1.46	0.06
−1462	0.36	0.65	8.85	8.43	1.39
−364	5.58	16.76	8.33	1.6	0.16
−321	0.80	1.0	2.33	1.33	0.60
−171	1.25	1.25	0.8	6.0	0.20
Assay #2					
−1706	5.67	3.20	3.94	5.30	0.13
−1047	15.08	49.38	6.29	161.62	0.33
−364	0.25	6.63	17.3	12.13	0.14
−321	1.00	0.76	0.43	1.00	0.36
−171	6.00	7/0*	0/0*	0/0*	1.5
Assay #3					
−1706	1.29	63.0	2.86	0.06	0.37
−1462	1.5	1.5	0.43	7.0	0.41
−1047	2.76	0.88	5.42	16.7	3.11
−321	1.67	0/0*	0/0*	0/0*	0.20
−171	3.00	2.00	1.00	0/2*	0.50

Fig. 3. GUS activity of plants during development of *P. tabacina*-induced SAR and following challenge inoculation. GUS activity (pmol MU/min/mg protein) detected in tissue collected from plants carrying different constructs at 0, 1, 2 and 3 weeks following stem inoculation, with *P. tabacina* sporangia or with water, then at week 4, following challenge with *P. tabacina* sporangia, indicated with a vertical arrow. Only lines having all symptomless or mildly chlorotic *P. tabacina*-stem-inoculated plants and also having symptomatic water-stem-inoculated plants in a given assay are shown. (A) GUS activities (pmol MU/min/mg protein) obtained from tissues of plants stem-inoculated with *P. tabacina* sporangia and subsequently challenged with sporangia: −1706 (one plant), −1462 (two plants), −364 (two plants), −321 (two plants) and −171 (two plants). GUS activities were assessed in two groups (all treatments for the same line done within the same group) and normalized utilizing a commercially-prepared β-glucuronidase enzyme assessed at the same time in each group (Section 2.5). (B) GUS activities (pmol MU/min/mg protein) obtained from tissues of plants stem-inoculated with *P. tabacina* sporangia and, subsequently challenged with sporangia: −1706 (four plants), −1462 (three plants), −364 (three plants) −321 (three plants) and −171 (three plants). GUS activities were assessed in two groups (all treatments for the same line done within the same group) and normalized utilizing a commercially-prepared β-glucuronidase enzyme assessed at the same time in each group (Section 2.5). (C) Table showing ratio of GUS activity (pmol MU/min/mg protein) of *P. tabacina*-inoculated plants divided by GUS activity (pmol MU/min/mg protein) of water-inoculated plants from tissues obtained at indicated time points following stem inoculation and 1 week after challenge inoculation (week 4). Asterisk indicates that activities were low and actual GUS activity of sporangia-inoculated plants (pmol MU/min/mg protein)/water-inoculated plants (pmol MU/min/mg protein) is shown. Three time points from the water controls of the line containing the −1046 construct in assay #3 needed to be repeated and the GUS activities resulting were normalized with the GUS control for those of the other time points and the *P. tabacina*-induced plants from the same line. Plants excluded from analysis are: from assay #1, those carrying the −607 construct because plants stem-inoculated with water lacked symptoms following challenge with *P. tabacina* sporangia; and those carrying −1492 and −607 constructs from assay #2, and carrying −607 and −364 from assay #3 because *P. tabacina* stem-inoculated plants exhibited symptoms following challenge inoculation with *P. tabacina*.

inoculation with *P. tabacina* sporangia and of GUS activities following stem-inoculation with water at each time point, for each transformed line, and within each assay, was calculated (Fig. 3(C)). *P. tabacina*-stem inoculated plants containing the GUS construct with the full-length *PR-2d* promoter or the constructs containing 1462, 1047 and 364 bp upstream of the start site of *PR-2d* exhibited GUS activities greater than plants with the same construct stem-inoculated with water during

Table 2
Genes having regions that are putatively homologous in the region upstream of base pair 364 in the *PR-2d* promoter

Gene	Base pairs in:		Per cent identity	Putative function	Accession no.	Source
	PR-2d[a]	Homolog				
GRP	−1237 to −1184	−1487 to −1404	92	*N. tabacum* glycine-rich protein precursor	M37152	43
GGL4	−1302 to −1112	−1370 to −726	80	*N. tabacum* β-1,3-glucanase	AF141654	–[b]
EPSPS	−1335 to −1046	−731 to −401	62	*Petunia hybrida* shikimate pathway enzyme	M37029	3
G261	−1302 to −980	−602 to −270	61	*Petunia inflata* nitrate induced protein	AF530594, AF530595	47

[a] *GRP* and *GGL4* were indicated by Blast. *GGL4*, *EPSPS* and *G261* were indicated by FASTA.
[b] Unpublished sequence submitted to GenBank by Chen, Hou and Lin.

the 3 week period following stem inoculation (Fig. 3(C)). Differences between *P. tabacina*-inoculated plants and water-inoculated plants are particularly noticeable in plants carrying the GUS promoter constructs with 1047 and 364 bp upstream of the start site (Fig. 3(C)). Activities in plants carrying GUS constructs with promoter deletions 321 or 171 bp upstream of the start site were consistently low and activity could be undetectable (Fig. 3). These data suggest that one or more elements upstream of 321 bp before the start site of *PR-2d* are involved in the regulation of transcription following stem inoculation with *P. tabacina* sporangia.

GUS activity of plants inoculated with water and subsequently mock-challenged ranged from 3 pmol MU/min/mg protein for plants carrying the −171 and the −321 constructs in assays 1 and 3, respectively, to 121 pmol MU/min/mg protein for a plant carrying the −1706 construct in assay #1. Two plants stem-inoculated with *P. tabacina* sporangia and subsequently mock challenged in assay #2 had GUS activities of 118 pmol MU/min/mg protein (carrying the −1706 construct) and 181 pmol MU/min/mg protein (carrying the −364 construct).

3.3. Response of NahG plants to P. tabacina stem inoculation

Wild type plants and plants carrying the bacterial salicylate hydroxylase gene *(NahG)* [10] were stem inoculated with either *P. tabacina* or water at 9 weeks following planting. Stem inoculation was delayed by 1 week, to prevent inoculation of the meristem in short plants [5]. At the time of stem inoculation, the mean height of wild type Xanthi plants was 66.8 ± 5.1 cm and that of *NahG* plants was 37.1 ± 9.0 cm. One week following challenge inoculation, the only plants that lacked visible symptoms were wild-type Xanthi plants stem-inoculated with *P. tabacina* (2 of 3) and mock-challenged plants (one each water-inoculated plant of the wild type line and of that carrying *NahG*). All other plants exhibited symptoms of chlorosis and one *P. tabacina*-stem inoculated *NahG* plant exhibited necrosis. Sporulation was attempted from leaf tissue of all plants (see Section 2.) but was not detectable on any wild type Xanthi plants or on the *NahG* mock-challenged plant. Mean sporulation on *NahG* plants stem-inoculated and challenged with *P. tabacina* was 2.0×10^3 sporangia/cm^2 and on those stem-inoculated with water and challenged with *P. tabacina* was 1.8×10^4 sporangia/cm^2.

3.4. Database search of region upstream of −364 bp in the PR-2d promoter

To determine whether regions containing known regulatory elements, besides SARE, may be present in the region 1706–321 bp from the start site of *PR-2d*, database searches were conducted with known public databases. Four genes resulted using two different search strategies (Section 2.7) and similar sequences all fall in a region from 1335 to 980 bp upstream of the start site of *PR-2d* (Table 2). The genes are from tobacco or petunia (Table 2). Only *GGL4*, a β-1,3-glucanase gene from tobacco (Chen, Hou and Lin, direct submission), resulted in both types of searches and is the only other β-1,3-glucanase gene, besides *PR-2d*, that resulted in the searches. Interestingly, *GRP* from tobacco, which encodes a glycine-rich repeat precursor protein, has a promoter region with the greatest percentage similarity to a region in the promoter of *PR-2d* and is TMV and SA-inducible [43]. The two other genes are both from petunia. *EPSPS* encodes for an enzyme that catalyzes an essential step in the shikimate pathway giving rise to products necessary for the synthesis of aromatic secondary metabolites such as lignins, flavanols and anthocyanins [3]. The other petunia gene is induced by nitrates and is involved in self-incompatibility [47].

4. Discussion

This work provides further evidence for the regulation of *PR-2* genes after infection with the virulent pathogen *P. tabacina* and following induction of SAR by *P. tabacina* sporangia. Previous work had shown that inoculation underneath the epidermis of susceptible tobacco with 10^6 *P. tabacina* sporangia resulted in a necrotic lesion (1–10 mm in length) developing in the cambium and

external phloem but leaving the pith, cortex and xylem apparently free of infection [5]. Such plants have increased resistance to a subsequent challenge and this resistance may be proportional to the length of the necrotic stem region [5]. It is not clear whether SAR associated with the development of the *P. tabacina* stem lesion is similar to the SAR following HR and necrotic lesions formed when *N* gene tobacco is inoculated by TMV [32]. No gene-for-gene type R gene is known to be involved in the interaction between *P. tabacina* and commercially cultivated tobacco [14], yet regulation of *PR-2* genes to *P. tabacina*-induced SAR appears to be, at least in part, similar to SAR induced by TMV in *N*-gene tobacco or by exogenous application of SA to leaf disks ([15,38]; reviewed in [22]).

It had previously been shown that at least three PR-2 proteins, PR-2a, PR-2b and PR-2c, accumulated following infection of susceptible *N. tabacum* by *P. tabacina* [41]. We demonstrated that a fourth PR-2 also responds to *P. tabacina* infection, by assessing the GUS activity of plants transformed with *PR-2d* promoter/GUS construct. Plants containing the full-length construct (−1706) responded at levels significantly above those containing a construct with only 321 bp upstream of the start site of *PR-2d* (Fig. 2). The results of this assay suggest that there exists at least one element located upstream of 321 bp in the promoter of *PR-2d* that is involved in regulation of *PR-2d* following infection with *P. tabacina*. In the region from 364 to 321 bp upstream of the start site has previously been identified a regulatory element (SARE), characterized to respond to TMV infection and to exogenous application of SA [15,38]. Additionally, although not statistically significant, presumably due to high plant-to-plant variability, there appears to be greater GUS activity in plants carrying the full-length promoter than those carrying constructs having 607 or 364 bp upstream of the start site of *PR-2d* (Fig. 2). This suggests that a second element may be in the region from 1706 to 607 bp upstream of the start site of *PR-2d* that also responds to infection by *P. tabacina*. These results are consistent with what has been previously observed in plants infected by TMV or leaf disks treated exogenously with SA [15].

Consistent with previous studies [5,51], SAR was induced by injection of *P. tabacina* sporangia into the stem of 8-week old tobacco plants. In this study, plants apparently protected from infection, as assessed visibly, were noted rather than a reduced level of infection in all plants as has been done in previous studies [5,51]. Nonetheless, from 53 to 100% of plants lacked visible symptoms in individual assays (Table 1). This stringent method of scoring infection was done in order to identify *P. tabacina*-injected plants apparently resistant to challenge with *P. tabacina* sporangia, as these plants were of value for the analyses that followed.

When apparently resistant plants were analyzed for GUS activity, a transcriptional response of *PR-2d* during *P. tabacina*-induced SAR was indicated. Plants containing the full length *PR-2d* promoter fused with the GUS reporter gene, and promoter deletion constructs containing 1462, 1047 or 364 upstream of the start site (plants containing the −607 GUS construct were not included in these analyses) consistently demonstrated an increase in activity during the 3 weeks following stem inoculation with *P. tabacina* sporangia as compared with plants treated with water (Fig. 3; Table 2). There is no consistent increase in activity in plants carrying GUS constructs with either 321 or 171 bp upstream of the start site of *PR-2d* and undetectable GUS activity levels could result. This suggests that one or more elements exist upstream of 321 bp of the start site of *PR-2d*. Again, this supports a role for the previously characterized SARE [15,38], which would be included in the −1706, −1462, −1047 and −364 constructs, during response of *PR-2d* to inoculation with *P. tabacina* sporangia, and suggests that, besides TMV infection and exogenous SA application, *P. tabacina* induction of SAR may also act to upregulate *PR-2d*, through SARE, perhaps via SA. *P. tabacina* stem-inoculated plants with no visible symptoms or few chlorotic spots following challenge, had a reduced level of GUS activity conferred by the *PR-2d* constructs. When Northern analysis was conducted, using a clone from another *PR-2* gene (*PR-2c*) as a probe, RNA extracted from plants stem-inoculated with *P. tabacina* and having no visible symptoms following challenge, exhibited reduced hybridization as compared with RNA extracted from susceptible water-inoculated plants (data not shown). Pan and colleagues reported an approximately two times increase in β-1,3-glucanase activity 1 week following challenge of *P. tabacina*-inoculated plants and an approximately nine times increase in activity in water-inoculated plants [33]. This increase was associated with increased activities of PR-2b and PR-2c, and, to a lesser extent, PR-2a and two other β-1,3-glucanase isoforms, as compared with water-inoculated controls [33]. Northern analysis conducted for the present work, utilizing *PR-2c* as a probe, suggested that there may be an increase in *PR-2c*-hybridizing transcripts 1 week following challenge in plants stem inoculated with *P. tabacina* and subsequently challenged with *P. tabacina* sporangia, as compared with mock-challenged *P. tabacina*-induced plants (data not shown), which is not in conflict with previous work [33]. In the present work, *P. tabacina*-inoculated plants that were apparently unprotected, and exhibited moderate to severe symptoms following challenge, had GUS activities comparable to infected water-inoculated plants carrying the same construct (−607) 1 week following challenge. A ratio comparison of GUS activities (MU/min/mg protein) from *P. tabacina*-inoculated plants exhibiting moderate to severe chlorosis and/or necrosis 1 week following challenge, to GUS activities of water inoculated plants exhibiting similar symptoms were 0.87 and 1.27 in assays #2 and #3, respectively (data not shown; see Fig. 3), supporting the contention that the reduced GUS activity in plants protected against *P. tabacina* challenge may be due to limited or no

infection in these plants. The present work suggests that the level of activity of PR-2d may decline between 3 and 4 weeks following SAR induction with *P. tabacina* sporangia (Fig. 3).

That transcription of *PR-2d* may be developmentally regulated [15] is further supported by evidence in this work. For example, plants having high levels of GUS activity prior to stem inoculation are evident (e.g. Fig. 3(C), assay #2). This is consistent with previous reports of the activities of β-1,3-glucanase genes [6]. Plants carrying the full-length promoter construct and the deletion construct containing 1462 bp upstream of the start site of *PR-2d* exhibited reduced and/or less consistent increases, over water-inoculated controls, during the 3 weeks following stem inoculation with *P. tabacina*, suggesting that if there exists an element that is involved in upregulation of *PR-2d*, it may be in the region of the promoter from 1706 to 1047 base pairs upstream of the start site. Additionally, due to the extended time in the greenhouse (11 weeks), the *PR-2d* promoter/GUS constructs may have responded to environmental conditions such as fluctuations in temperatures, differing light intensities, insects, application of pesticides, or other disturbances; it has been documented that genes for PR proteins can be upregulated in plants in response to temperature extremes, drought [1,9,50,52], salt [50], light levels [53], UV light [4] or insects [8]. Needless to say, the well-tended plants utilized in this study were not exposed to such extremes. But it can be speculated that exposures to even subtle environmental changes could conceivably affect the expression of responsive genes.

Regarding the role of SA during induction of SAR with *P. tabacina*, it has been shown that free SA increases following stem inoculation with *P. tabacina* sporangia, over water-inoculated plants, which was associated with an increase in resistance [39]. Additionally, GUS analysis of leaf disks removed from plants containing construct −364, carrying the smallest promoter deletion with the SARE [38], and treated with 1 mM SA, suggests that SA is involved in the upregulation of *PR-2d* during *P. tabacina*-induced SAR. Further support was provided when *NahG* plants, and plants of the recipient *N. tabacum* line, were stem inoculated with sporangia or water. All *NahG* plants, despite the treatment, were susceptible to challenge with *P. tabacina.* Additionally, these plants supported sporulation in this assay while wild-type plants subjected to the same treatments did not. This suggests that not only were *NahG* plants unable to accomplish SAR following inoculation with *P. tabacina* sporangia, but they may have increased susceptibility to a *P. tabacina* challenge, due to suppression of age-related resistance to *P. tabacina*, which has been previously documented to occur in tobacco [35,49]. *NahG* plants have been used to demonstrate that SA is involved in age-related resistance in *Arabidopsis* and tobacco [16,17,20]. In the present study, stem inoculation needed to be delayed by 1 week, to prevent inoculation of the meristem of *NahG* plants [5], since they were smaller than plants of the untransformed cultivar, a phenomenon not known to be reported. However, *NahG* overexpression effects on cell growth in *Arabidopsis* defense mutants, has been shown [36,46].

Previous work [15] had suggested that there may be at least one additional region, upstream of −364 bp of *PR-2d*, involved in response to infection with TMV in whole plants and to endogenous application of SA to leaf disks. The present work may suggest, although not statistically significant, that there may exist an element in the region between −1706 and −607 in the promoter of *PR-2d* that responds to infection by *P. tabacina* (Fig. 2). When database searches using the region from −1706 to −321 bp as the query were conducted, the only four chromosomal plant genes with defined function that shared homology in the promoter regions were from solanaceous plants They all shared similarities in the region from −1237 to −1184 in the *PR-2d* promoter. This suggests that this region may contain a conserved element involved in regulation of all five genes. One tobacco gene, *GRP*, containing a promoter region similar to a region in the *PR-2d* promoter, is known to be induced following infection by TMV in *N* gene tobacco, or by exogenous application of salicylic acid. Other genes containing homologies in the same region are a tobacco putative β-1,3-glucanase gene (*GGL4*; Chen, Hou and Lin, direct submission) and two petunia genes, one with homologies to a nitrate-induced gene (*G261*; [47]), and a gene essential to the shikimate metabolic pathway (*EPSPS*; [3]). Further experimentation would need to be done in order to assess whether elements in this region are involved in response to pathogens or in regulation during development.

This work provided evidence that the accumulation of PR-2 proteins following stem inoculation with *P. tabacina* sporangia previously documented [33] may be regulated, at least in part, at the transcriptional level. It provided evidence that the *PR-2d* gene responds to infection by *P. tabacina* in susceptible plants. Additionally, evidence was provided that SAR induced by stem inoculation with *P. tabacina* is associated with upregulation of the *PR-2d* gene. This work also supports a role for SA in *P. tabacina*-induced SAR thus suggesting that regulation of *PR-2* genes during *P. tabacina*-induced SAR may be similar to regulation of these genes during TMV inoculation of *N*-gene tobacco or regulation following exogenous application of SA.

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