Expression of a ricin B:F1:V fusion protein in tobacco hairy roots: steps toward a novel pneumonic plague vaccine

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ABSTRACT

Recombinant subunit vaccines consisting of the F1 and V antigens of *Yersinia pestis* have shown remarkable promise for protection of animals and humans against bubonic and pneumonic plague. Previously we demonstrated that intranasal administration of a recombinant protein consisting of the ricin B chain (RTB) fused with an antigen purified from transgenic *Nicotiana tabacum* (tobacco) hairy root cultures was highly effective in stimulating a mucosal antibody response in mice (Medina-Bolivar et al., 2003). In the current study, transgenic tobacco plants and hairy roots were engineered to express a recombinant protein consisting of a hexahistidine-tagged RTB fused to tobacco sequence-optimized F1 and V genes (His:RTB:F1:V). Northern blot analysis of crude leaf extracts revealed that twenty-eight transgenic plant lines expressed a transcript of the predicted mRNA size detectable with the RTB probe. An RTB functional ELISA based on asialofetuin-binding activity was used to screen crude leaf protein extracts from these transgenic plants and select the two lines with the highest levels of fuctional RTB lectin activity. Analysis of the tissue localization of the recombinant mRNA in transformed plants and hairy roots developed from these plants suggested that His:RTB:F1:V expression levels were higher in hairy roots than in any tissue of the original transgenic plants. Asialofetuin-binding ELISA of transgenic hairy root extracts confirmed expression of functional RTB-containing fusion protein. Immunoblotting of lactose affinity-purified protein from hairy root lines using anti-RTB, anti-His-tag, and anti-V antibodies detected a protein of approximately 91 kDa, the predicted size of glycosylated His:RTB:F1:V. Purified His:RTB:F1:V protein will be used for intranasal immunization in mice, in which stimulation of mucosal immunity will be a first step towards an improved pneumonic plague vaccine.

INTRODUCTION

The causative agent of bubonic and pneumonic plague is the bacterium *Yersinia pestis*. While this scourge is infamous for its capacity to cause pandemic and epidemic plague disease, naturally occurring smaller eruptions of both plague forms continue to occur in scattered geographic pockets across the globe (Bertherat et al., 2005; Mudur 1995). *Y. pestis* ranks among the top five potential bioweapon threats due to the rapid contagion of pneumonic plague through spread of aerosolized bacteria from infected patients and near 100% lethality of untreated pneumonic plague cases within 24 hours of infection. Although antibiotics have proven effective in treating most known strains of *Y. pestis*, multidrug resistant plague strains have recently emerged (Galimand et al., 1997; Guiyoule et al., 2001). Moreover, no U.S.-licensed plague vaccine exists. In 1999, usage of the USP whole cell, killed plague vaccine that had been used worldwide for over a hundred years was discontinued in the U.S. due to concerns over significant side effects and questionable vaccine efficacy (Cohen et al., 1967; Meyer 1970; Williamson et al., 1997). These issues underscore the importance of sustained investment in the development of a highly effective vaccine for plague now.

Recombinant subunit vaccines provide a promising avenue toward protection against both bubonic and pneumonic plague and are expected to lack the potential for the undesirable side effects contributed by either killed or attenuated whole organism vaccines. Two *Y. pestis* virulence factor proteins, F1 (Fraction I, encoded by *caf1*) and V (Virulence, encoded by *lcrV*), have dominated recent plague vaccine research and development efforts. F1 is a structural protein of the plague capsular antigen expressed on the bacterial cell surface (Titball et al., 1997) and prevents phagocytosis by host cells (Du et al., 2002). V is a secreted protein that participates with the Type III secretion pathway in introduction of a number of *Y. pestis* outer proteins into host cells (Fields et al., 1999). Subunit vaccines comprised of both the F1 and V proteins have been demonstrated in animal models to confer protection against both the bubonic and pneumonic forms of disease and also have been well-tolerated in humans (Alpar et al., 2001; Anderson et al., 1998; Glynn et al., 2005; Heath et al., 1998; Jones et al., 2000, 2003, 2006; Williamson et al., 1997, 2001, 2005, 2007). Recent reports demonstrate the potential of subunit F1 and V plague vaccines produced in plant model systems to stimulate relevant antibody production in mice (Alvarez et al., 2006) and to protect

against lethal plague challenge in a primate model (Mett et al., 2007).

The premise of this study is that a tobacco-produced F1:V subunit vaccine that includes a fusion partner to target the purified protein to the mucosa of vaccinated individuals may better enhance protection against pneumonic plague compared to non-mucosal formulations, while avoiding the harmful side effects commonly associated with many chemical adjuvants. Previously, we demonstrated that the non-toxic lectin subunit of ricin, ricin B chain (RTB), is useful as a mucosal carrier/adjuvant (Medina-Bolivar et al., 2003). Intranasal administration of recombinant RTB:antigen fusion protein purified from transgenic tobacco hairy root cultures was highly effective in stimulating an antigen-specific mucosal antibody response in mice (Medina-Bolivar et al., 2003). The current report demonstrates successful tobacco-based expression and purification of a novel genetic fusion of the *Y. pestis* F1 and V genes with RTB (His:RTB:F1:V), a novel mucosal adjuvant:antigen strategy.

RESULTS

Development of the gene construct for tobacco-based expression of a candidate subunit vaccine against pneumonic plague

The transgene construct designed for expression in tobacco of the candidate subunit plague vaccine consisting of RTB in fusion with the *Y. pestis* F1 and V proteins is shown in Figure 1. To direct constitutive expression of the transgene, we utilized the chimeric, synthetic, super-promoter:TEV (Ni et al., 1995; Lee et al., 2007), which consists of activator and promoter sequences from the octopine synthase and mannopine synthase genes of *Agrobacterium tumefaciens* in fusion with the tobacco etch virus translational enhancer. The super-promoter has yielded high-level expression of transgenes in tobacco (Ni et al., 1995; Lee et al., 2007). Because we previously observed that secretion of the expressed transgene product can provide advantages for protein recovery and purification (Medina-Bolivar et al., 2003; Reed et al., 2005), the signal peptide (Pat) from the potato patatin gene was used to direct ER targeting and secretion of the recombinant protein. In addition, targeting into the secretory pathway is needed for glycosylation of RTB. A hexahistidine

tag (His) was included to provide a facile method for detection and protein purification from transgenic plants. Due to the demonstrated ability of the non-toxic subunit of RTB to stimulate mucosal immune responses to a genetically fused model antigen (Medina-Bolivar et al., 2003), RTB was included to act as a mucosal adjuvant for the F1:V plague antigens. To circumvent potential problems with expression of the prokaryotic F1 and V in a plant system, several modifications of the bacterial genes were designed, and the coding region of each gene was commercially synthesized and used in the assembly of the corresponding transgene constructs. The F1 and V gene sequences were optimized for consideration of tobacco codon usage preferences, elimination of cryptic eukaryotic RNA processing signals (including potential premature polyadenylation sites, intron splice sites, and RNA instability elements), and removal of potential N-glycosylation sites. The synthetic F1 and V genes were expected to contribute 15 kDa and 37 kDa to the fusion protein product. The gene construct super-promoter:TEV:Pat:His:RTB:F1:V referred as pP10 was used to generate transgenic tobacco plants and hairy roots for this study.

Generation and screening of transgenic tobacco plants and hairy roots

The binary vector pP10 containing the transgene was mobilized into *Agrobacterium tumefaciens*, which was then used to transform *Nicotiana tabacum* cv. Xanthi according to a petiole-inoculation method previously described (Medina-Bolivar et al., 2003; Medina-Bolivar and Cramer, 2004). The transformed tobacco plants that were regenerated (28 lines) were phenotypically normal. Hairy root lines were developed from all plant lines containing the transgene construct by methods described before (Medina-Bolivar and Cramer, 2004).

As an initial genetic screen of the regenerated transformants, PCR analysis of genomic DNA extracted from the putative transgenic plants was conducted using RTB-specific primers. Figure 2 shows that the predicted 800 bp PCR fragment corresponding to RTB cDNA was observed in all pP10-transformed plant lines and was not generated from non-transformed tobacco cv. Xanthi genomic DNA.
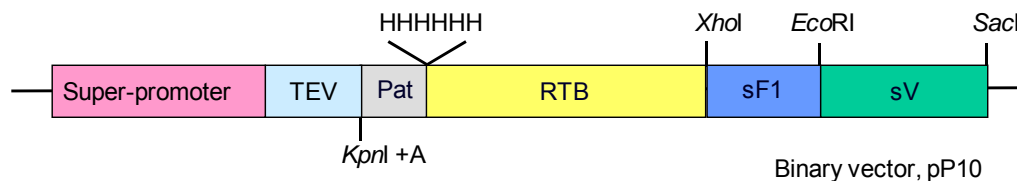

Figure 1. DNA construct utilized for expression of Pat:His:RTB:F1:V in transgenic tobacco plants and hairy roots. Shown are the chimeric super-promoter:TEV (pE1802 backbone, Ni et al., 1995), patatin signal peptide (Pat) used to target the fusion protein for secretion/glycosylation, the hexahistidine tag (His; HHHHHH) included as a purification and detection tag, ricin B chain (RTB), and the synthetic F1 (sF1) and V (sV) genes, as well as the restriction sites used for cloning purposes.
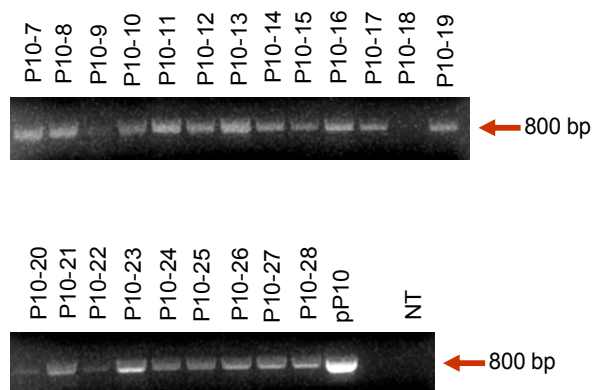
Figure 2. PCR screening of selected putative transgenic plants. RTB-specific primers were used in PCR of genomic DNA isolated from leaves of 22 putative P10 transformants, non-transformed tobacco (NT), and from a positive control plasmid (pP10). The migration position of the expected 800 bp PCR fragment is indicated by the arrows.

To rapidly identify transgenic tobacco plants expressing high levels of RTB:F1:V fusion protein we utilized an asialofetuin-binding ELISA. The lectin function of RTB enables binding to the galactose-terminated glycoprotein asialofetuin with approximately 1000-fold greater affinity compared to RTB's binding affinity for galactose. Previously, we described this ELISA to screen transgenic tobacco plants expressing RTB (Reed et al., 2005). Following this method, all P10 plants were screened for recombinant protein expression. ELISA testing of crude leaf extracts from P10 plant lines revealed in three experiments that lines P10-13 and P10-19 express the highest levels of recombinant protein competent to bind asialofetuin. Figure 3 shows the results of one experiment for 7 representative plant lines, including P10-13 and P10-19, which yielded about 0.025 and 0.015 ng of RTB equivalents per µg total leaf protein, respectively. These values represented 45 and 44 ng RTB equivalents per g FW leaf, respectively. The lines P10-13 and P10-19, including plants and hairy roots, were selected for further studies.

Following identification of the transgene by PCR, integration of the P10 transgene into the genomic DNA of lines P10-13 and P10-19 was verified by Southern blotting (Figure 4). Genomic DNA was digested with *Sac*I, as this restriction enzyme has only one recognition site within the T-DNA and will help to identify the copy number of inserts in the transgenic plants using an RTB probe. All bands detectable with this probe are likely larger than 8 kb, based on the migration of DNA size markers. Plant lines P10-13 and P10-19 show distinct banding patterns, indicating that they represent two independent transgenic lines. Line P10-13 exhibits two insertion loci, while line P10-19 shows transgene insertion at 4-5 loci. Hairy root lines developed from these cultures also show the same insertion pattern as their parental plant lines (not shown). The RTB-containing positive control plant (line R6-2 developed by Medina-Bolivar et al., 2003) shows the expected pattern of transgene loci (Maureen Dolan, unpublished results).
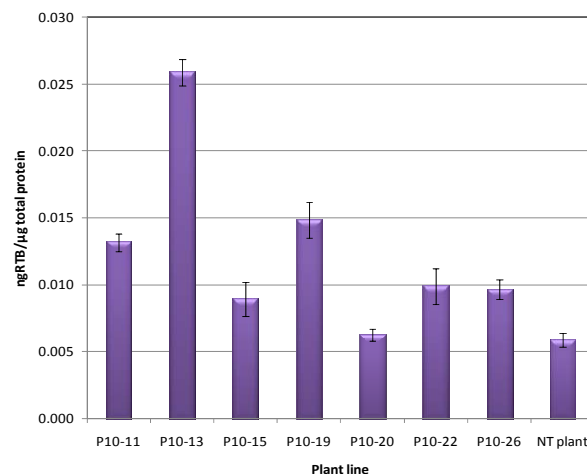

Figure 3. Functional ELISA of P10 protein expression in transgenic plants. An asialofetuin-binding ELISA of crude extracts was conducted with 100 µg of total protein to quantify P10 protein expression. Crude leaf extracts from P10 plant lines (11, 13, 15, 19, 20, 22, and 26) and a non-transformed tobacco sample (NT plant) were assayed for asialofetuin-binding activity. Error bars shows the variation between three repetitions. Statistical analysis showed significant difference in yield of functional the P10 protein in every transgenic plant compared to the control, except for P10-20 (Dunnett's t Test, $\alpha = 0.05$).

Several hairy root lines were developed from plants P10-13 and P10-19. However, hairy roots from plant P10-19 (R1, R2, R4 and R5) showed better growth in liquid culture and these were further analyzed.
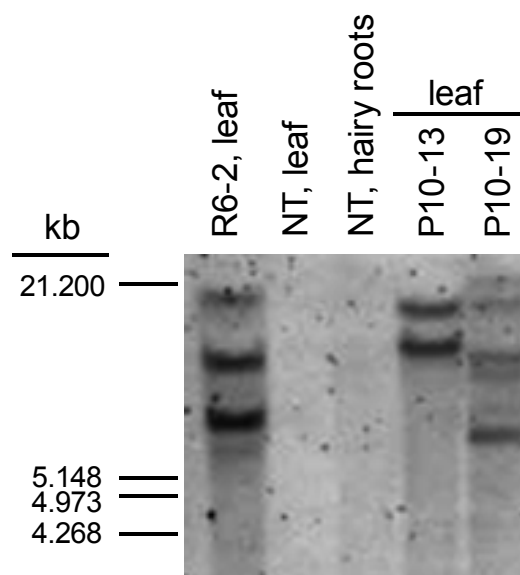

Figure 4. Southern blotting of selected transgenic plant lines. Genomic DNAs isolated from plant lines P10-13 and P10-19, non-transformed tobacco leaf and hairy roots (NT), and a previously characterized positive control line expressing a different RTB-containing transgene (R6-2) were digested with *Sac*I, and processed for Southern blotting with an RTB-specific probe.
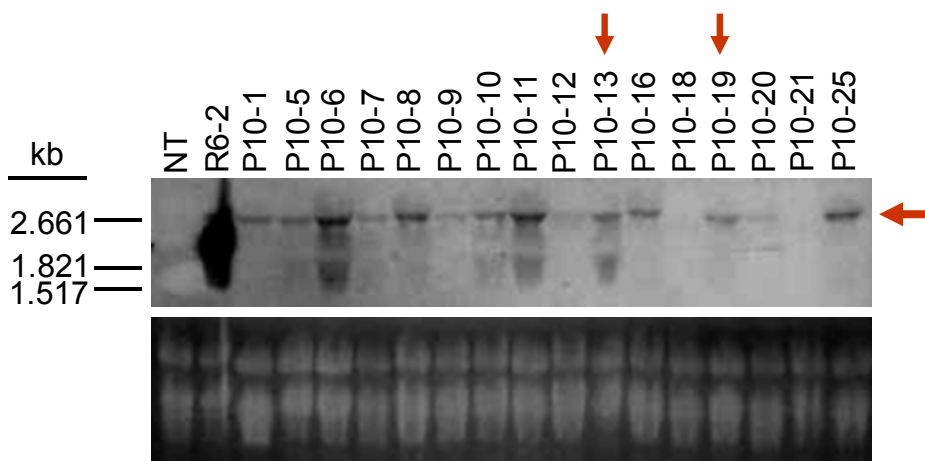
Figure 5. Northern blot of transgenic plant leaf P10 mRNA levels. Total RNA (20 µg) from leaves of P10 plant lines, non-transformed tobacco (NT), and a previously characterized RTB-containing transgenic line (R6-2) was processed for Northern blotting with an RTB-specific probe (top panel). The arrow to the right of the blot indicates the migration position of the 2.9 kb P10 transcript. Arrows above the blot denote two plant lines (P10-13, P10-19) studied in greatest detail herein. Ethidium-bromide staining of the gel prior to RNA transfer demonstrated equal RNA loading per lane (bottom panel).

Basal expression of P10 mRNA in plants and hairy roots

The basal level of P10 mRNA expression in leaves of the transgenic plants was examined by Northern blotting using a probe specific for RTB. The P10 mRNA is predicted to be at least 2.5 kb, and an approximately 2.9 kb transcript was detected with the RTB probe in a total of 22 P10 plant lines. Figure 5 shows a representative RNA blot of 16 P10 lines, two of which did not express detectable levels of P10 mRNA. Relative to the mRNA level observed for the transgene in the positive control R6-2 line, which expresses a different RTB-containing construct, fourteen of the P10 lines shown express very low levels of P10 mRNA. Somewhat surprisingly, the abundance of P10 mRNA in most of the different lines was relatively equivalent, including lines P10-13 and P10-19 which showed the highest levels of asialofetuin-binding ELISA. These results were confirmed by Northern blotting using an F1:V probe (data not shown).

Tissue localization of transgene mRNA by Northern blotting of young leaves, mature leaves, stem, and roots from plant line P10-13 revealed that the P10 transcript is expressed ubiquitously but is not highly abundant in any organ. The highest levels were observed in roots, whereas the transcript was expressed at very low levels in stem, and was barely detectable in either young or mature leaves (data not shown). This experiment confirms that leaf P10 mRNA levels are generally low, as noted during the initial leaf RNA blot (Figure 5). This observation that leaf P10 mRNA levels are low and basal root levels are moderately higher suggested that the hairy root culture expression system may be better suited than whole plants for production of P10 using the super-promoter.

To assess the levels of P10 transcript in hairy root lines derived from plant line P10-19 total RNA from lines P10-19 R1, R3, R4, and R5 was analyzed by Northern blotting. The P10 transcript was expressed at very similar levels in each of the P10-19 hairy root lines tested (Figure 6), while those

levels were significantly higher than those observed in the leaf samples (data not shown).

Asialofetuin-binding ELISA of hairy root protein extracts

In our previous asialofetuin-binding ELISA of leaf protein extracts from transgenic plants, two lines P10-13 and P10-19 were selected as the highest expressors of functional P10 protein. In addition, the tissue localization of P10 mRNA showed that roots had higher basal levels of RNA than leaves, suggesting that hairy roots could also yield higher levels of the recombinant protein P10 protein. To assess for functional P10 protein, hairy roots lines P10-19 R1, R3, R4 and R5, were analyzed using this ELISA.
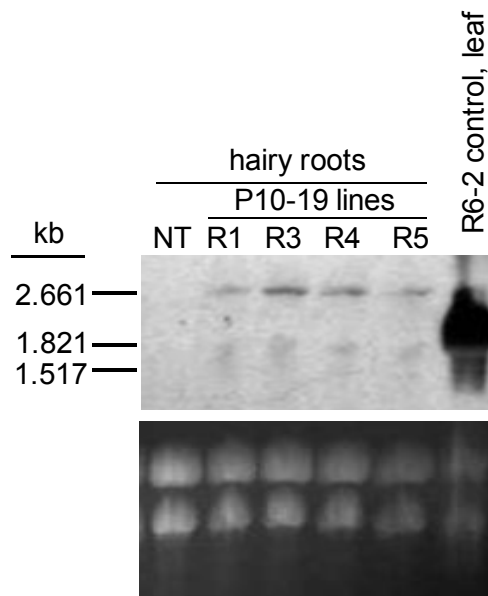

Figure 6. Transgene mRNA expression in hairy root lines derived from P10-19 plant. Total RNA extracted from hairy root lines generated from plant

line P10-19 (R1, R3, R4, R5), from leaf and hairy roots of non-transformed tobacco (NT), and from leaf of a positive control line (R6-2) was processed for Northern blotting using an RTB-specific probe (top panel). The bottom panel shows the ethidium-bromide stained gel prior to RNA transfer, indicating equal RNA loading per lane.

Figure 7 shows a representative of two independent experiments in which the levels of functional recombinant protein observed in hairy root tissue (lines P10-19 R1, R3, R4 and R5) were at least 10-fold greater than the levels observed in leaf tissue of the P10-19 plant line (Figure 3). The R1 and R5 hairy root lines produced 299 and 244 ng RTB equivalents/g FW root, respectively. No functional P10 protein was detected in the medium of any of the root lines therefore only extracts from hairy root tissue were further analyzed by Western blotting.
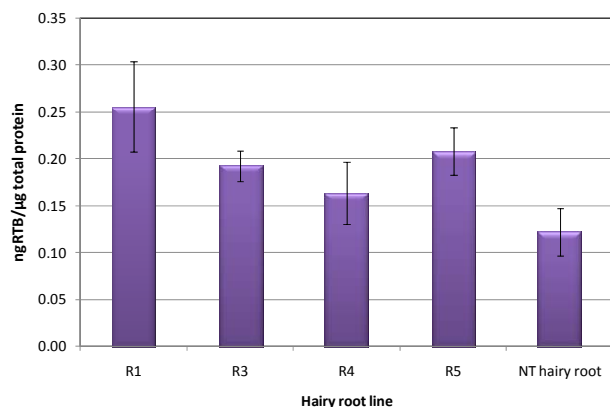

Figure 7. Functional ELISA of P10 protein expression in transgenic hairy root lines. An asialofetuin-binding ELISA of crude extracts was conducted with 100 µg of total protein to quantify P10 protein expression in hairy roots derived from plant P10-19 (R1, R3, R4, and R5); a non-transformed tobacco hairy root sample was also considered (NT hairy root). Error bars shows the variation between three repetitions. Statistical analysis showed significant difference in the yield functional P10 protein for lines R1 and R5 compared to the control; the other lines didn't shown this difference (Dunnett's t Test, α = 0.05).

Purification of P10 protein from hairy roots and detection by Western blotting

Crude extracts of P10-19 hairy root lines R1, R4, and R5 were subjected to lactose affinity chromatography to purify P10 protein based on the carbohydrate-binding ability of RTB. Western blotting of the affinity-purified samples revealed the presence of an approximately 91 kDa protein detectable with antibodies specific for RTB, the His-tag, and V antigen (Figures 8A, 8B and 8C, respectively) in all P10 hairy root lines tested. This product is consistent with the predicted size of fully intact recombinant P10 protein, glycosylated at the two potential N-glycosylation sites within RTB. An approximately 90 kDa doublet was observed in P10 hairy root lines R1, R4, and R5 using the His-tag-specific antibody (Figure 8B). The bottom band of the doublet is predicted to be P10 protein, whereas the upper band co-purifying in much lower abundance is also detected in the non-transgenic control. Immunoblotting with the anti-RTB antibody detected four likely P10 degradation products (three in the 35 kDa range, and one of nearly 50 kDa) in varying quantities in the purified samples from the different P10 lines but not in the non-transformed control (Figure 8A). Two to three probable P10

degradation products were observed from P10 hairy roots in the His-specific blot (Figure 8B), which also revealed four additional non P10-derived bands that are also evident in the non-transformed control. Detection of the 91 kDa P10 protein by the RTB and His-tag antibodies has been replicated several times, while the profile of observed degradation products has varied slightly between experiments. The V-specific antibody detected a doublet of approximately 91 kDa and one in the 35 kDa range in the purified sample from the P10-19, R5 hairy root line. Co-purifying proteins in the purified sample of the non-transformed line were detected. However, a unique protein of 91 kDa was only observed in samples from the transgenic P10-19, R5 hairy root (Figure 8C).

DISCUSSION

In this study, a DNA construct including the F1 and V antigens of *Yersinia pestis* fused to the innovative, potential mucosal adjuvant protein, RTB, was successfully expressed in tobacco with the goal of developing a novel, recombinant pneumonic plague vaccine (P10 protein = His:RTB:F1:V). We predict that the ability of RTB to bind galactose present at mucosal surfaces may be crucial to its function as a mucosal adjuvant. Therefore, we initially used a functional ELISA that tests the ability of binding to the galactose terminated glycoprotein, asialofetuin, to screen for transgenic tobacco lines with high levels of P10 protein. Initial results from more than 20 P10 plant lines assayed using this ELISA identified two lines P10-13 and P10-19 as the highest expressors of P10 protein in leaves. Interestingly, the levels of functional P10 protein in line P10-19 were at least 10-fold greater in hairy roots than in leaves. These results are consistent with the RNA blots which showed that transgenic plant roots (not shown) and hairy roots express higher P10 transcript levels than leaf. Though plant line P10-13 outperformed the others in three independent ELISA experiments using leaf extracts, P10-13 hairy root lines were discarded due to an undesirable growth phenotype, and subsequent analyses focused on hairy roots derived from the P10-19 plant. It is formally possible that plant lines other than P10-13 and P10-19 expressed a higher overall level of recombinant protein that was not competent to bind asialofetuin under the conditions of this assay, and also possible that overall expression levels may emerge as more important than carbohydrate-binding ability. Nevertheless, we selected several P10-19 hairy root lines for further analysis. Four P10-19 hairy roots lines (R1, R3, R4, R5) yielded fairly comparable RTB equivalents in the asialofetuin-binding ELISA, and lines R1, R4, and R5 were subsequently analyzed for P10 protein expression by Western blotting.

Surprisingly, RNA blot analysis of leaf tissue harvested from 28 potentially transgenic P10 plant lines identified by PCR did not sufficiently distinguish the plants to allow down-selection of the lines for subsequent investigation. Moreover, though Southern blotting showed that plant line P10-19 possesses at least twice the transgene copy number of line P10-13, the two lines expressed comparable P10 transcript levels in leaf. Moderately higher leaf transcript levels detected in a few lines did not correlate with higher levels of P10 protein in those lines in later experiments. According to observed wound inducibility of

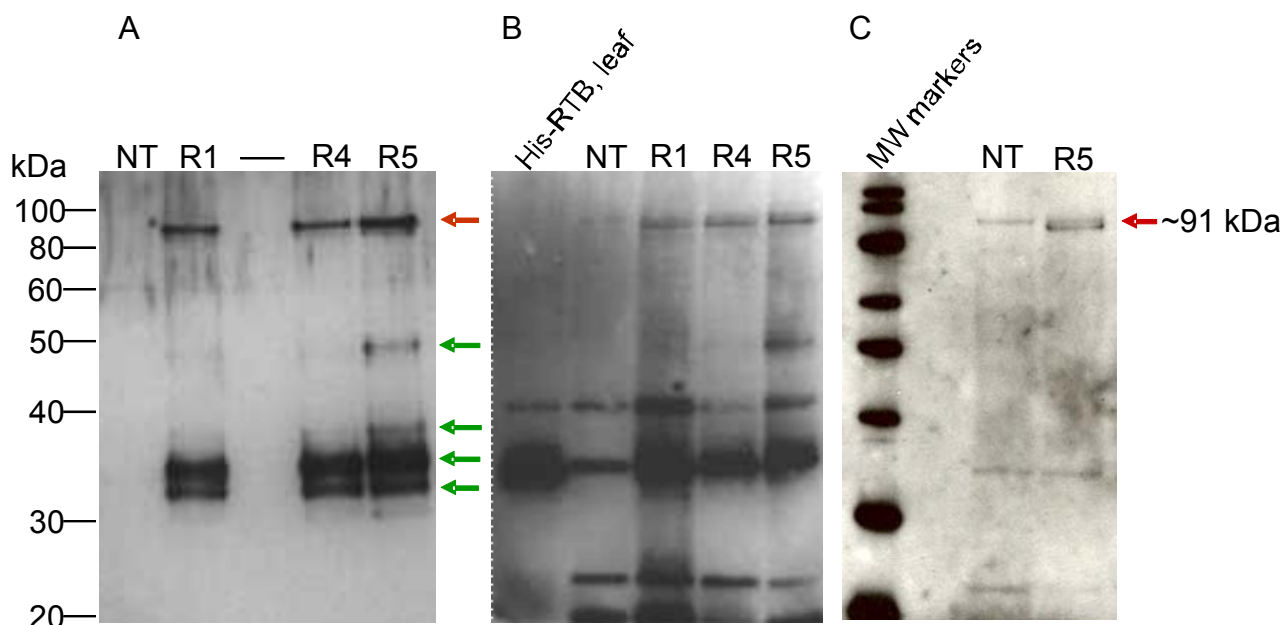the super-promoter in a separate study, conducted in our

Figure 8. Western blot analyses of lactose affinity-purified protein from P10 hairy root lines. Extracts were prepared from 20 g FW of 12-14 day-old, P10-19 hairy root lines (R1, R4, R5), 10 g FW of hairy roots of non-transformed tobacco (NT), and 5 g FW of leaves of the positive control transgenic line, His:RTB (Reed et al., 2005) and subjected to lactose affinity purification. For the P10 and non-transformed hairy root samples, about 3 g FW equivalent of hairy root samples were loaded per gel lane, while 0.5 g FW equivalent of His:RTB leaf was loaded for Western blots using: **(A)** rabbit polyclonal anti-RTB primary antibody at 1:5000 dilution, secondary goat anti-rabbit-HRP (Sigma, St. Louis, MO) at 1:3000, with nitrocellulose membrane; **(B)** monoclonal anti-His tag primary antibody at 1:1000 (Novagen, Madison, WI), secondary goat anti-mouse-HRP (Sigma, St. Louis, MO) at 1:5000, with nitrocellulose membrane; and **(C)** monoclonal anti-V primary antibody at 1:100 dilution (BioDesign International, Saco, ME), goat anti-mouse-HRP (Sigma, St. Louis, MO) at 1:5000, with PVDF membrane. The red arrows denote the expected migration position for full-length P10 protein, while the green arrows denote likely P10 degradation products.

lab (Ñopo et al., manuscript submitted) we also attempted to elevate P10 transcript expression levels by tissue wounding. However, significantly or reproducibly higher levels of P10 mRNA in leaf tissue of wounded plants compared to the non-wounded controls were not obtained (data not shown). We expect from the current study and other work in our lab, as well as a recent publication from the developer of the super-promoter (Lee et al., 2007) that this promoter is developmentally regulated with leaf age, such that small age differences in the leaf samples harvested may partially explain the variable P10 transcript levels across the three leaf Northern blot experiments reported herein. Perhaps the failure of the transcript to accumulate to higher levels in P10 plants reflects gene silencing of the construct or inherent instability of the mRNA, despite our previous success in expressing recombinant RTB constructs in tobacco (Medina-Bolivar et al., 2003; Reed et al., 2005) and our current efforts to eliminate RNA instability elements when designing the synthetic F1 and V genes.

Tissue localization of P10 mRNA showed relatively low levels of expression in all plant tissues, with expression in roots showing the most promise for potential harvest of useful quantities of P10 protein. Therefore, we immediately initiated generation of hairy roots from all available P10 plant lines with the expectation that hairy roots may express the vaccine candidate at levels conducive to protein purification and downstream assessments.

Immunoblot analysis showed that the predicted 91 kDa P10 protein was purified from P10-19 R1, R4 and R5 hairy root lines. We speculate that the cloning junction between RTB and F1 may have generated a protease recognition site within the recombinant protein, resulting in reduced recovery of full-length P10 protein coupled with recovery during purification of the observed His-RTB-containing products in the 35 kDa range. Similarly, our previous work with RTB-GFP expressed in tobacco leaves and hairy roots showed that this recombinant fusion protein was subject to degradation (Medina-Bolivar et al., 2003). The banding patterns observed in the 35 kDa range in both the anti-RTB and anti-His blots recapitulate our recently published data for the expression and lactose affinity purification of His-RTB in tobacco (Reed et al., 2005). Cursory attempts to stabilize P10 during extraction and purification by the inclusion of protease inhibitors and chelating agents did not increase the yield of recombinant protein.

Our recovery of the fully-glycosylated 91 kDa form of P10 is significant because we expect that RTB glycosylation

may be important for its function at the mucosal epithelium at the time of immune stimulation by the antigen. Our successes with affinity purification and ELISA demonstrate functionality of P10 as a lectin, including both galactose- and lactose-binding capabilities known for RTB. Additionally, our Western blot data show that three distinct domains of the 91 kDa P10 molecule (V, RTB, and His portions) are probably largely intact. This is an important consideration for accurate presentation of our intended F1:V antigen.

We anticipate that further optimization of the gene sequence may be required to increase levels of P10 RNA. In addition, we expect increased yield of purified P10 protein from hairy roots to be realized through optimization of the extraction and purification processes. An immunization trial utilizing purified P10 protein is being designed to test the ability of the recombinant antigen to stimulate both systemic and mucosal antibody responses in nasally immunized mice, which will be considered the initial indicators of a "good" response. As we aim for increased P10 yield from hairy roots, the potential instability of the P10 mRNA and the potential inducibility of the super-promoter system by various stimuli are some of the targets to be addressed.

CONCLUSION

This report demonstrates successful expression and purification of a functional, full-length, glycosylated, His:RTB:F1:V protein fusion product from tobacco hairy roots. This study also suggests that the hairy root system may be especially well suited for expression of transgenes driven by the super-promoter system, as supported in depth by another report from our laboratory (Ñopo et al., manuscript submitted). Assessment of the purified His:RTB:F1:V recombinant protein's immunomodulatory potential *in vivo* will further address whether this novel antigen:adjuvant combination may prove effective for induction of mucosal immunity against pneumonic plague.

MATERIALS AND METHODS

Construction of the binary vector (pP10) used for expression of recombinant 6His:Pat:RTB:F1:V vaccine in tobacco plants and hairy roots.

DNA oligomers encoding the tobacco sequence-optimized F1 gene flanked by *Xho*I and *EcoR*I restriction sites, and the sequence-optimized V gene flanked by *EcoR*I and *Sac*I restriction sites were custom synthesized commercially (Blue Heron Biotechnology; Bothell, WA). The F1 fragment was subcloned into pUC119 as an *Xho*I/*EcoR*I fragment to create plasmid pUC119-F1. The V gene was subcloned into pUC119 as a *EcoR*I/*Sac*I fragment to create plasmid pUC119-V. Plasmid pBC-RTB-F1-V was created by ligating the RTB *Xba*I-*Xho*I fragment from pBC-R6-2 (Medina-Bolivar, personal communication), the F1-containing *Xho*I/*EcoR*I fragment from pUC119-F1, and the V-containing *EcoR*I/*Sac*I fragment from pUC119-V into the *Xba*I/*Sac*I fragment of pBC in a single reaction (Stratagene; La Jolla, CA). The *Kpn*I/*Sac*I fragment of pBC- RTB-F1-V that contains the carboxyl-terminal portion of the RTB gene plus F1:V was subcloned into pE1802 containing the super-promoter:TEV module (Ni et al., 1995) to create the plasmid

pP9. Plasmid pP9 was digested with *Kpn*I and treated with calf intestinal alkaline phosphatase prior to ligation with the *Kpn*I fragment from pBC-R9-His (Reed et al., 2005) containing Pat (signal peptide from patatin), a 6XHis purification tag, and the amino-terminal portion of RTB to create pP10. Immediately downstream to the *Kpn*I site and upstream of the DNA sequence encoding the initial methionine of the patatin signal peptide, an adenine was added (Figure 1). The ligation junction within pP10 at the *Kpn*I site internal to the RTB coding sequence was sequenced to verify the predicted DNA sequence. Plasmid pP10 was used to generate transgenic tobacco plants and hairy roots in the current work.

Generation of transgenic tobacco plants and hairy roots

Plasmid pP10 was mobilized into *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method (Holsters et al., 1978). *A. tumefaciens* containing binary vector pP10 (Figure 1) was used to transform *N. tabacum* cv. Xanthi according to a petiole-inoculation method described previously (Medina-Bolivar and Cramer 2004). Transgenic T_0 plantlets were micropropagated in Phytatray™ II vessels (Sigma, St. Louis, MO) containing mMS medium (Medina-Bolivar et al., 2003), supplemented with 100 mg/L kanamycin. Cultures were maintained under a 16 h photoperiod, at 24°C with average light intensity of 98 μmol m^{-2} s^{-1}. Hairy roots were developed from the designated transgenic plant lines and maintained as described before (Medina-Bolivar and Cramer, 2004).

Control plant material

Control plant materials included plant line R6-2 (de35S:TEV:Pat:RTB:GFP, Medina-Bolivar et al., 2003) and R9-His (de35S:TEV:Pat:RTB:His, Reed et al., 2005).

Genetic screening of putative transgenic tobacco plants and hairy roots by PCR

Detection of RTB DNA in putative transgenic plants was performed by PCR as follows. Genomic DNA was isolated using DNeasy mini kit (Qiagen, Valencia, CA) and subjected to PCR using RTB primers RB5 (5'-CGCTCTAGAGCTGATGTTTGTATGGAT-3') and RB3a (5'-GAGCTCCTCGAGAAATAATGGTAACCATAT-3') with cycling parameters: 94°C for 3 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min 15 s; 72°C for 10 min. The expected size of the PCR product is 800 bp.

Isolation of Genomic DNA and Southern Blotting

Genomic DNA was isolated from tobacco leaf and hairy root tissue using DNeasy Plant Maxi kit (Qiagen, Valencia, CA). *Sac*I-digested genomic DNA (10 μg each sample) was resolved on a 0.7% agarose gel with DIG-labeled DNA molecular weight markers (Roche, Boulder, CO). After electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination to document equal DNA loading per well. The DNA was partially depurinated by incubation of the gel in 250 mM HCl for 20 min with shaking, followed by a brief rinse in sterile water. DNA was denatured by incubation of the gel in 0.5 M NaOH, 1.5 M NaCl with shaking, twice for 15 min each, followed by a brief

rinse in sterile water. The gel was neutralized by incubation in 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5 with shaking, twice for 15 min each. The gel was equilibrated in 20X saline sodium citrate (SSC) buffer for 10 min and the DNA was transferred to positively-charged nylon membrane (Roche, Boulder, CO) by capillary transfer overnight in 20X SSC. Following transfer, the membrane was rinsed briefly in 2X SSC, and the DNA was cross-linked to the damp membrane by UV illumination. Probe preparation and Southern blot hybridization and detection are described below.

RNA Extraction and Northern Blot

Total RNA was extracted by the phenol/SDS method (Ausubel et al., 2002) with modifications. Leaf or hairy root tissue (1 g) was ground in liquid nitrogen and transferred to a tube containing 10 mL RNA extraction buffer (0.18 M Tris, 0.09 M LiCl, 4.5 mM EDTA, 1% SDS, pH 8.2) and 0.3 aqueous volume of TE-equilibrated phenol, pH 4.3. Samples were vortexed vigorously for 1 min. Chloroform was added (0.3 aqueous volume), followed by vortexing for 30 seconds. Samples were centrifuged at 17,700 x g, 10 min, at 4°C to separate the aqueous and organic phases. Phenol/chloroform extraction of the aqueous layer was repeated twice, followed by two extractions with chloroform alone. The aqueous layer was transferred to a fresh tube on ice, and precipitated by addition of 0.1 volume 3 M NaOAc, pH 5.2, and 2.5 volumes 100% ethanol and incubation at –70°C for 30 minutes. Samples were centrifuged at 17,700 x g, 15 min at 4°C, and pellets were resuspended in 1 mL DEPC-treated water. RNA was selectively precipitated by addition of LiCl to a final concentration of 2 M and incubation at 4°C overnight, followed by centrifugation at 17,700 x g, 20 min, at 4°C. The RNA pellet was washed with 1 mL of ice cold 2 M LiCl and resuspended in 100 µL DEPC-treated water. For northern blots, 20 µg of RNA per sample was processed as described before (Reed et al., 2005) except that a digoxigenin (DIG)-labeled RTB probe was used, as described below.

Preparation of DIG-labeled RTB probe

The RTB probe was prepared by PCR from plasmid p10 as follows. The reaction contained 1 ng of plasmid template, 0.5 µM each RTB primer (RB5 and RB3a, described above), 0.2 mM each dATP, dCTP, dGTP, 0.167 mM dTTP, 0.033 mM DIG-dUTP (Roche, Boulder, CO), 1.5 mM MgCl$_2$, and 2.5 units Taq polymerase in a final reaction volume of 100 µL. PCR parameters were: denaturation at 94°C for 3 min; 35 cycles of 94°C for 30s, 50 °C for 30s, 72°C for 1 min 15 sec; 72°C for 10 min.

Hybridization and detection of Northern and Southern blots

The membrane was pre-hybridized in DIG Easy Hyb solution (Roche, Boulder, CO), containing DIG Blocker (Roche, Boulder, CO) at 65°C for 1 h. The probe was denatured by heating at 95°C for 10 min and the blot was hybridized overnight at 65°C in 2 µL probe/mL DIG Easy Hyb solution containing DIG Blocker preheated to 65°C. Following hybridization, the blot was washed twice for 20 min each in 2X SSC, 0.1% SDS, and then twice for 20 min each in 0.5X SSC, 0.1% SDS, preheated to 65°C. For chemiluminescent detection, the blot was processed according to the manufacturer's recommendations using the DIG Wash and Block Buffer Set (Roche,Boulder, CO), and developed using anti-DIG-alkaline phosphatase conjugate (Roche, Boulder, CO) and CDP-Star substrate (Roche, Boulder, CO).

Protein Extraction

Tobacco hairy root tissue was harvested from culture, drained on paper towels, and stored at -70°C until use. Protein extracts were prepared as follows: hairy root tissue was ground under liquid nitrogen with mortar and pestle and transferred to a centrifuge tube containing Tris-ascorbate buffer [100 mM Tris base, 100 mM ascorbic acid, 150 mM NaCl, 2.5% (w/v) PVP-40, 0.1% Tween 20; pH 7.0] at 3:1 buffer to tissue ratio and vortexed vigorously for 1 min. Extracts were centrifuged at ~ 13,000 x g for 10 min at 4°C and supernatants were filtered through four layers of Miracloth^{TM} (Calbiochem-EMD Biosciences, San Diego, CA). The total protein concentration of crude protein extracts was measured using the Advanced Protein Assay (Cytoskeleton, Denver, CO) in a Bio-Tek EL808 Ultra Microplate Reader. Total protein determinations were made in reference to bovine albumin serum (BSA) standards dissolved in Tris-ascorbate buffer.

Asialofetuin-binding ELISA

The functionality of recombinant protein in the protein extracts of transgenic lines was determined via binding to asialofetuin, the glycoprotein fetuin treated with sialydase to expose galactose-terminated glycans as we described before (Reed et al., 2005). Two hundred microliters of asialofetuin (Sigma; St. Louis, MO) at a concentration of 300 µg/mL in modified PBS (mPBS) buffer (100 mM Na-phosphate, 150 mM NaCl, pH 7.0) was bound per well of an Immulon 4HBX (Fisher, Pittsburg, PA) microtiter plate for 1 h at room temperature (RT). The coating solution was discarded, and the wells were blocked with 200 µL of 3% BSA, 0.1% Tween 20 in PBS for 1 h at RT. The blocking solution was discarded and 100 µL each of RTB standards (described below) and sample protein extracts (prepared as above) was applied in triplicate wells and incubated for 1 h at RT. The blocking solution was discarded, and wells were washed three times with PBS, 0.1% Tween 20. Rabbit anti-*R. communis* lectin (RCA60) polyclonal antibody (Sigma, St. Louis, MO) diluted at 1:4000 in blocking buffer (as above) was applied and incubated for 1 h at RT, then discarded. The wells were then washed as before. Alkaline phosphatase-conjugated goat-anti-rabbit IgG (Bio-Rad; Hercules, CA) was applied at a 1:3000 dilution in blocking buffer and incubated for 1 h at RT, then discarded. The wells were washed three times as before, and 100 µL pNPP (p-nitrophenyl phosphate disodium salt) substrate (Pierce, Rockford, IL) was applied per well. The reaction was stopped after 15 min by the addition of 50 µL

of 2 N NaOH. Absorbance (A_{405nm}) was read in a Bio-Tek EL808 Ultra Microplate Reader. One hundred μg of total protein extract from leaves or hairy root tissue or 100 μL of media from hairy root cultures were assessed compared to a standard curve consisting of serially diluted castor bean-derived RTB (Vector Labs, Burlingame, CA) in Tris-acorbate buffer (above), in concentrations ranging from 2.5 ng to 500 ng RTB per well, and were plotted as $1/A_{405nm}$ vs. 1/ng RTB per well.

Lactose affinity chromatography

Recombinant protein was purified from hairy root tissue by carbohydrate-affinity chromatography using lactose-conjugated agarose resin (EY Labs, San Mateo, CA). A 500-μL aliquot of resin was added to clarified crude protein extracts prepared as above from 10 g FW hairy root tissue in 50-mL conical tubes and incubated with continuous immersion on a rotary Labquake shaker (Barnstead, IA) for 1-2 h at 4°C and washed three times for 5 min in 5 mL ice-cold PBS buffer (Sambrook et al., 1989), pH 7.4. A final wash was conducted in 0.5 mL volume, during which the resin was transferred to a 1.5 mL microfuge tube for elution. Bound protein was eluted with 0.5 ml ice-cold elution buffer (0.5 M galactose in PBS, pH 7.4). Eluted fractions were concentrated approximately 10-fold in Centricon-10 units (Millipore, Bedford, MA) at 4°C according to the manufacturer's instructions and stored at -20°C for subsequent analyses.

Western blotting

Protein samples were resolved by SDS-PAGE NuPAGE[TM] 10% Bis-Tris acrylamide gels in an X-Cell SureLockTM Mini-Cell apparatus (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations using MOPs running buffer. After electrophoresis for 60 min at 200 volts, protein was transferred to 0.2 μm Trans-Blot® nitrocellulose membrane (Bio-Rad, Hercules, CA) or PVDF as indicated using an X-Cell II[TM] blot module and NuPAGE transfer buffer containing 20% MeOH for 1.5 h at 30 V. Blots were blocked overnight shaking at 4°C in blocking solution (PBS containing 0.1% Tween-20, 3% BSA) followed by incubation with the designated primary antibody at the designated concentration in blocking solution for 1 h. Membranes were washed three times for 15 min each in PBS, 0.1% Tween-20 and incubated with secondary antibody as indicated for 45 min to 1 h and washed 3 times as above. Chemiluminescent detection was performed using Immun-Star[TM] AP substrate (Bio-Rad; Hercules, CA) and Nitro-Block Enhancer II[TM] (Tropix, Bedford, MA).

Statistical analysis

All statistics were determined by ANOVA (Dunnett's t Test to compare P10 protein expression against a control, with α = 0.05), with the aid of the Statistical Analysis Systems statistical software, release 8.00.

Acknowledgements

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