Nectar- and stigma-specific expression of a chitinase could partially protect against fire blight in certain apples

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Running title:

Apple flower exudates contain *Erwinia* inhibiting chitinase

Highlight

Certain apple cultivars accumulate to high levels in their nectar and stigma an acidic chitinase III protein that can protect against pathogens including fire blight disease causing *Erwinia amylovora*

Abstract

To attract pollinators many angiosperms secrete stigma exudate and nectar in their flowers. As these nutritious fluids are ideal infection points for pathogens, both secretions contain various antimicrobial compounds. *Erwinia amylovora*, the causing bacterium of the devastating fire blight apple disease, is the model pathogen that multiplies in flower secretions and infects through the nectaries. Although *Erwinia* resistant apples are not available, certain cultivars are tolerant. It was reported that in stigma infection assay, the ‘Freedom’ cultivar was *Erwinia* tolerant while the ‘Jonagold’ was susceptible. We hypothesized that differences in the nectar protein compositions lead to different susceptibility. Indeed we found that an acidic chitinase III protein (Machi3-1) selectively accumulates in the nectar and stigma of the ‘Freedom’ cultivar. We demonstrate that MYB binding site containing repeats of the ‘Freedom’ Machi3-1 promoter are responsible for the strong nectar- and stigma-specific expression. As we found that *in vitro* the Machi3-1 protein impairs growth and biofilm formation of *Erwinia* at physiological concentration, we propose that the Machi3-1 contribute to the tolerance by inhibiting *Erwinia* multiplication in the stigma exudate and in the nectar. We show that the Machi3-1 allele was introgressed from *Malus floribunda* 821 into different apple cultivars including the ‘Freedom’.
Introduction

Plants secrete rewarding, sugar rich fluids such as stigma exudates and nectar to attract pollinators (Tanveer et al., 2014). Based on their stigma Angiosperm can be divided into two groups, plants with dry (E.g.: Arabidopsis thaliana, Arabidopsis) and wet stigma (E.g.: Malus domestica, apple and Nicotiana tabacum, tobacco). While the surface of the dry stigma is covered with a proteinaceous extracuticular layer (pellicle), the wet stigma is covered with stigma exudates (Edlund et al., 2004). This fluid secretion plays a critical role in pollen capture, hydration, growth of pollen tube and serves as a reward for the pollinators. The exudate can also be found at the intercellular spaces of the stigmatic zone and transmitting tissue in mature pistils (Rejón et al., 2014). Floral nectars are secreted by specific glands called nectaries (Heil, 2011; Roy et al., 2017). Nectaries have evolved independently multiple times, and they can differ in their position, morphology, and secretion mechanism (De la Barrera and Nobel, 2004). Despite these differences, nectary development is conserved in most angiosperms (Min et al., 2019). The C-lineage genes regulate the expression of the Crab Claws transcription factor, which is essential for nectary development (Bowman and Smyth, 1999; Lee et al., 2005; Morel et al., 2018). Jasmonic acid (JA) is required for nectar secretion, while auxin mainly controls the volume of the nectar (Radhika et al., 2010; Bender et al., 2013; Roy et al., 2017). JA induces nectary-specific gene expression by stimulating the degradation of JAZ proteins (Kelley and Estelle, 2012), thereby releasing the JAZ repressed transcriptional factor MYB305 or the homologs of MYB305 (Liu et al., 2009; Liu and Thornburg, 2012; Stitz et al., 2014; Schmitt et al., 2018a). MYB305 directly and indirectly promotes the transcription of nectary-specific genes, including nectarins, genes whose proteins products are secreted into the nectar (Liu and Thornburg, 2012). The chemical composition of the stigma exudate and the nectar is relatively different but both secretions are highly nutritious containing high levels of sugars. The stigma exudate is rich in complex sugars and proteins and contains at lower concentration free sugars, amino acids and lipids (Pusey et al., 2008). By contrast, the nectar is rich in sucrose and hexoses and free amino acids. It also contains additional components such as phenolics, secondary metabolites and nectarins (Heil, 2011). The proteome of the floral nectars is relatively simple and frequently contains only a few (sometimes only one or two) dominant proteins (Roy et al., 2017). The protein profile of the stigma exudates is more complex suggesting that it is a physiologically more active extracellular fluid (Rejón et al., 2013). Both secretions are excellent medium for
microbes. Microbial infection of the flower secretions is harmful as microbes can alter the chemical composition of the fluids, and mainly because plant pathogens can infect efficiently by multiplying in these nutritious fluids and then by entering into the plant through the stomata of the nectaries. Thus it is not surprising that plants accumulate antimicrobial components including antimicrobial proteins (such as chitinases and glucanases) in these secretions. Indeed, in the proteome of stigma exudates, the defense and stress response proteins are the dominant GO categories (Sang et al., 2012). Flower nectars frequently contain antimicrobial proteins in very high concentrations (Zha et al., 2016; Ma et al., 2017; Nogueira et al., 2018; Schmitt et al., 2018b) or accumulate nectarins that generate antimicrobial hydrogen peroxide in the nectar (Carter et al., 2007). Furthermore, it was shown that nectar of wild squash is antibiotic and efficiently reduces the symptoms of the bacterial wilt (Sasu et al., 2010). The gram-negative bacterium Erwinia amylovora that is one of the most devastating bacterial pathogens of apple, is the classical example of pathogens that multiply in flower secretions and infects through the nectaries (Farkas et al., 2012; Malnoy et al., 2012). Erwinia first colonizes the stigma and multiplies in the stigma exudates, then the pathogen is washed down by rain or dew into the nectar. The pathogen further multiplies in the nectar and finally enters into the plant through the stomata of the nectaries (Bubán et al., 2003). E. amylovora produces exopolysaccharides that are involved in biofilm formation (Koczan et al., 2009). Pear fruit and apple shoot inoculation assays show that mature biofilm formation is needed for full virulence of Erwinia (Koczan et al., 2011; Piqué et al., 2015). Although Erwinia resistant apple cultivars are not available, certain cultivars are tolerant (Gusberti et al., 2015). These cultivars are infected less frequently and develop reduced symptoms. For instance, after inoculation of the stigmas of the tolerant ‘Freedom’ and the susceptible ‘Jonagold’ cultivars, ‘Freedom’ was less infected, much less bacteria were detected on the surface of the nectaries and tissue coloring symptoms were much weaker on the ‘Freedom’ (Mihalik et al., 2004). It was assumed that the chemical composition of the ‘Freedom’ and ‘Jonagold’ nectars was identical, and proposed that the rough surface of the ‘Jonagold’ nectary was responsible for the more efficient colonization and the stronger symptoms (Mihalik et al., 2004). However, accumulating data indicate that nectarins can play important antimicrobial role (Heil, 2011; Roy et al., 2017). Therefore we wanted to test an alternative (but not mutually exclusive) hypothesis that the nectar protein profiles of the tolerant and susceptible cultivars are different. Indeed, we found that an acidic chitinase III protein (Machi3-1) accumulates to high level in the nectar and the stigma of the tolerant
‘Freedom’ cultivar but not in the susceptible cultivars. We show that different Machi3-1 alleles are present in ‘Freedom’ and ‘Jonagold’ cultivars and that the presence of 5 direct repeats in the promoter of ‘Freedom’ Machi3-1 allele is responsible for the strong nectar- and stigma-specific expression. We demonstrate that the strongly expressing Machi3-1 allele was introgressed from Malus floribunda 821 into different cultivars including ‘Freedom’. Relevantly, we found that Machi3-1 protein can inhibit the growth and biofilm formation of E. amylovora in vitro at physiological concentration. How the stigma- and nectar-specific expression of Machi3-1 could contribute to the Erwinia tolerance and in general to plant defense will be discussed.
Materials and methods

Bacterial strain and plant materials

The bacterial strain *Erwinia amylovora* ref T was grown in TSB (Tryptic Soy Broth) medium at 28°C overnight. The plant materials were collected from the cultivar collection of the Research Institute for Fruitgrowing and Ornamentals (Újfehértó, Hungary) from 2005 to 2015. We used various scab resistant and susceptible cultivars. *Malus domestica* Borkh. cultivars ‘Jonagold’, ‘Sampion’, ‘Golden Delicious’, ‘Gala’ ‘Idared’, ‘Redwinter’, ‘Red Rome’ and the Hungarian landrace ‘Simonffy’ are scab susceptible, while Releika, Resi, Remo, Rewena (Germany) ‘Rajka’, ‘Selena’, ‘Topaz’, ‘Rubin’, ‘Rubinola’ (Czech Republic), ‘Hesztia’ (Hungary) are scab resistant cultivars. F1 hybrids of ‘Freedom’ x ‘Redwinter’ and ‘Freedom’ x ‘Red Rome’ derived from earlier crossbreeding program.

Nectar and stigma collection

Apple nectars were collected from field grown plants. Nectars from transgenic tobaccos, which were grown in the greenhouse, were collected in the morning (9-10 am). Nectars from the same cultivar were usually pooled and stored at -70°C. Stigma samples were pooled from 5 stigmas of the same plant.

Nucleic acid techniques and protein extraction

Genomic DNA was purified with Quick-DNA Plant/Seed Miniprep kit (Zymo Research D6020). RNA extraction was carried out as described (Szittya et al., 2002). To purify plant protein extract, 100 mg plant tissue was homogenized with 400 µl extraction buffer (100 mM NaCl, 100 mM glycine, 10 mM EDTA, 2 % SDS), incubate at 95°C for 5 minutes and centrifuged. Protein concentration was measured at 280 nm.

Stain-free protein profiles and western-blot assays

Nectars and the protein extracts were separated by stain-free 1D SDS-PAGE (Bio-Rad's Mini PROTEAN® TGX Stain-Free™ Gels). For western blot assays, samples were separated by SDS-PAGE, blotted onto Amersham Protran membrane (GE Healthcare, 10600008) and hybridized with rabbit polyclonal antibody serum raised against Machi3-1. ECL Anti-Rabbit IgG Horseradish Peroxidase linked (GE Healthcare, NA934-1ML) secondary antibody was used for detection. Actin antibody (Anti-Actin Plant MerckA0480) was used for control.
Chemiluminescent protein detections were conducted with ECL Western Blotting Substrate (Promega, W1001), according to the manufacturer’s instructions. Western blots were scanned with ChemiDoc MP System and analyzed with ImageLab 5.0 software (Bio-Rad).

**Protein sequencing**

The dominant protein band of ‘Freedom’ nectar was partially sequenced (described in details at Supplementary Materials and Methods S1). Briefly, the excised protein band was in-gel digested as described (Migh et al., 2018). Peptides were analyzed by data-dependent LC-MS using a Waters Q-TOF Premier mass spectrometer online coupled to a nanoAcquity uHPLC system. Raw data was converted into a peaklist using the ProteinLynx PLGS software and the data was searched using the Batchtag Web software of the Protein Prospector search engine. As automated protein identification did not yield high confidence identifications, MS/MS data were inspected manually and high-quality MS/MS spectra were evaluated manually. Protein segments were used for degenerate PCR primer designing.

**PCR cloning of the Machi3-1 gene from Freedom cultivar**

Degenerated oligonucleotides (aldegf and aldegr, respectively) were designed for the predicted N-proximal ADYIWNNF and the C-proximal WNRFYDN peptide segments. cDNA was prepared from total RNA isolated from the nectary rich tissues of ‘Freedom’. PCR product was amplified, subsequently cloned and sequenced. Based on this information specific oligonucleotides were synthesized to clone the genomic region by inverse PCR (invj1 for, invj2 for, invba1 rev, invba2 rev).

**RT-PCR assays**

For quantitative RT-PCR, total RNAs were treated with DNase I (Thermo Fisher Scientific, EN0525), and cDNAs were transcribed using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, K1621). qRT-PCR was carried out with Fast Start Essential DNA Green Master Mix (Roche, 06402712001) in a Light Cycler 96 Real-Time PCR instrument (Roche). For semi-quantitative RT-PCR assays, the same cDNAs were used in conventional PCR reactions using DreamTaq Green PCR Mastermix (Thermo Fisher Scientific, K1081) in a T-Personal thermal cycler (Biometra).

**Machi3-1 genotyping**
The promoter regions of Machi3-1 alleles were amplified with the SaFreeFor and SaFreeRev primers and separated on 1.5 % agarose gel.

Cloning of the transgenic constructs
To generate 5B-Machi3-1 and 2B-Machi3-1 transgenic constructs, the promoter and coding region of 5B-Machi3-1 and 2B-Machi3-1 genes were amplified with the 5B2BproFor and Machi3-1-stopRev primers, and then the PCR products were cloned into HindIII and BamHI cleaved Bin61S vector (Silhavy et al., 2002). To create promoter deletion constructs, promoter segments were amplified using one of the 1.2kbFor, 1kbFor, 0.9kbFor, 0.6kbFor, 0.4kbFor, 0.2kbFor forward primers and the Machi3-1stopRev reverse primer. The fragments were cloned into HindIII and Hpal cleaved 5B-Machi3-1 transformation vector to replace the original promoter regions. The constructs were sequenced. The list of primers are shown as Supplementary Table S1.

Plant transformation
Leaf disc transformation was carried out to generate transgenic N. tabaccum plants (Bevan et al., 1985). Transgenic tobaccos were selected on kanamycin containing media and then the regenerants (T0 plants) were grown in the greenhouse.

Expression of recombinant proteins in Pichia pastoris, analysis of protein expression
Machi3-1 protein was expressed with Pichia Expression Kit (Invitrogen K1710-01) using pPICz vector. The protein was expressed according to the manufacturer’s protocol. The signal peptide of Machi3-1 transports the protein to the extracellular space, allowing the purification of the protein from the supernatant. The secreted protein was purified from the supernatant by precipitating with ammonium-sulfate (60% saturation). The precipitate was pelleted (15000 rpm, 10 min., 4°C), resuspended in 5 ml 10 mM Sodium-acetate (pH 5.0) and concentrated with Amicon Ultra-4 Centrifugal Filter Units (Merck Millipore, 10K) ~10 fold, reaching the final protein concentration ~3 mg/ml. Empty pPICz vector transformant P. pastoris was grown and induced as the test strain, and then its supernatant was similarly treated (ammonium-sulfate precipitated, centrifuged, resuspended in 10 mM Sodium-acetate and concentrated to ~3 mg/ml). The purified supernatant of the empty vector transformant P. pastoris was used as negative control in activity assays. SDS-PAGE assay was used to test
that the background of the negative control and the purified Machi3-1 was similar (Fig. S2A and S2B).

**Chitinase and lysozyme activity assay**

Chitinase activity was measured by Schales’ reagent method (Ferrari *et al.*, 2014). Colloid chitin was prepared according to Shen (*et al.*, 2010) with minor modifications. 6 g chitin was suspended in 200 ml 37 % HCl and agitated overnight at 4°C. 1 L of distilled water was added followed by centrifugation at 8000 g for 20 minutes. The pellet was washed with water till the pH reached 5.0.

Colloid chitin (at 3 mg/ml final concentration) was incubated in 200 µl of 50 mM KPO4 (pH 6.0) with increasing amounts (50-400 ng) of Machi3-1 protein. The reactions were rotated at 30°C for 1 hour at 200 RPM. Samples were briefly centrifuged (10 sec) and 100 µl supernatant was transferred to a new tube. 100 µl Schales’ reagent (0.5 M sodium carbonate and 0.5 g/L potassium ferricyanide in water) was added and then boiled at 97°C for 15 minutes. After cooling down to RT, absorbance was measured at 420 nm. Chitinase from *Streptomyces griseus* (Sigma, 9001-06-3) was used for positive control. Purified supernatant of the empty vector transformant *P. pastoris* was used as a negative control.

Lysozyme activity was measured by agar diffusion plate method. *Micrococcus lysodeikticus* (Merck, 4698) was used as the substrate (0.05 mg/ml). 1 % agarose gel containing 1 mg *M. lysodeikticus* in 10 mM sodium-acetate buffer (pH 5.0) was made (20 ml per plate). After solidification, lysozyme (Merck L6876) or Machi3-1 were loaded into the wells (4 mm diameter). Plates were incubated at 30°C for 24 hours.

**In vitro Erwinia growth inhibition assay**

Bacterial *in vitro* growth inhibition assay was carried out with minor modifications as described (Nash *et al.*, 2006). Approximately 10² *E. amylovora* cells were suspended in 200 µl of 10 mM PBS (pH 7.4). The suspension was incubated without shaking for 24 hours at 28°C with different amount of purified Machi3-1 protein, or with purified supernatant of empty vector transformant *P. pastoris* as a negative control. Viable cells were counted by plating.

**Enzymatic detachment of Erwinia biofilm**
In vitro biofilm detachment assay was monitored with crystal violet staining (Koczan et al., 2009; O’Toole, 2011). E. amylovora overnight culture was diluted in LB to 1:100 and 130 µl of the diluted culture was incubated at 30°C in a 96-well TC-treated Tissue culture polystyrene plate (1 x 10^6 cells per well) to allow biofilm formation. After 24 hours the suspension was removed, and then 130 µl of purified Machi3-1 diluted in 50mM KPO4 buffer (pH 6.0) was added to the biofilm covered plates. The reactions were kept at 28°C for 3 hours. Wells were washed 3 times with dH2O, then 150 µl 0.1% crystal violet (CV) was added. After 15 minutes CV was removed, then the plate was washed 3 times with dH2O, and dried for overnight. For quantification 30% acetic acid was added to each well, incubated for 15 minutes and the OD was measured at 550 nm.

Statistics
Bacterial growth inhibition assays were repeated four times in independent experiments. Biofilm detachment experiments were performed in octuplicate and repeated three times. Comparisons between groups were done by ANOVA and Tukey-test to determine P-values. Statistical significance * was set at p<0.05 and *** p<0.001.

Bioinformatical analysis
Sequence analysis was made by BLASTN and BLASTP softwares. Transcription factor binding sites were predicted using PlantTFDB (Plant Transcription Factor Database) (Jin et al., 2017). Protein and DNA sequences were aligned by ClustalW method using the MegAlign program. Structural alignment and homology modelling of Hevamine as the template and the Machi3-1 protein was carried out by the SPDBV (Swiss-PdbViewer) program. Phylogeny tests were made using Bootstrap method (No. of Bootstrap Replications = 1000) and analyzed by UPGMA statistical method using the MEGA6 software.

Results
A class III chitinase-like protein accumulates in the nectar of the Erwinia tolerant ‘Freedom’ apple cultivar

To test our hypothesis that the nectar composition of the fire blight tolerant and susceptible apple cultivars is different, nectar protein profiles of the tolerant ‘Freedom’, the susceptible ‘Jonagold’ and ‘Sampion’ cultivars were studied by 1D SDS-PAGE. None of the nectar
proteins accumulated to high levels in the susceptible cultivars, while the nectar of ‘Freedom’ contained a 29kDa dominant protein (Fig. 1A). Although this protein was present at very high concentration (~50-80 ng/µl) in the ‘Freedom’ nectar, it was not detectable in the nectars of the susceptible cultivars (Fig. 1A). The analysis was repeated in four consecutive years with the same results, therefore the presence of this dominant protein in the ‘Freedom’ nectar was not due to any environmental condition. The 29kDa protein was isolated, partially sequenced, then primers were designed and inverse PCRs were conducted to clone the genomic copy of the gene from the ‘Freedom’ cultivar. The amplified region contained a 894 nucleotide (nt) long intronless coding sequence, a long (1417 nt) upstream and a short (77 nt) downstream regions. Sequence analysis revealed that the predicted ‘Freedom’ nectar protein is a class III chitinase (will be referred to as Machi3-1 for Malus chitinaseIII-1). Class III chitinases belong to the GH18 endochitinase family (Adrangi and Faramarzi, 2013). Machi3-1 is an acidic class III chitinase (calculated isoelectric point is 4.4), which shows strong sequence similarity (66.4%) to the well characterized class III chitinases as PSC (pomegranate seed chitinase) and Hevamine (64.18%) (Terwisscha Van Scheltinga et al., 1996; Lv et al., 2011; Masuda et al., 2015). The critical catalytic amino acids and the cis-peptides (involved in chitin binding) are all conserved. Moreover, homology modelling predicts that the structure of Machi3-1 protein is highly similar to the structure of Hevamine (Fig. S1). Machi3-1 contains an N-terminal signal peptide that destines proteins towards the secretory pathways (Chung and Zeng, 2017). These data suggest that the Machi3-1 protein is a functional, secretable acidic chitinase.

Machi3-1 is an active chitinase

Basic class III chitinases frequently have dual chitinase and lysozyme activities, while the acidic class III chitinases have strong chitinase but only weak or no lysozyme activity (Ma et al., 2017). To characterize the Machi3-1 protein, it was expressed in P. pastoris and the chitinase and lysozyme activities of the purified protein were tested in vitro.

To measure the chitinase activity of the purified Machi3-1 protein, Schales’ procedure using colloidal chitin for a substrate was carried out (Ferrari et al., 2014). S. griseus chitinase and the supernatant of empty vector transformed P. pastoris were used as positive and negative controls, respectively. Machi3-1 proved to be a relatively efficient chitinase; its activity was ~25% of the S. griseus chitinase (Fig. 2A). The Machi3-1 had a barely detectable lysozyme
activity in *Micrococcus* lysis assays (Fig. S2C). Thus we concluded that Machi3-1, like most acidic chitinase III proteins, has strong chitinase and very weak lysozyme activity.

*Machi3-1 inhibits growth and biofilm formation of E. amylovora in vitro*

Next we tested if Machi3-1 had an antibacterial effect against *E. amylovora*. Bacterial cultures were incubated with Machi3-1 protein purified from *P. pastoris* supernatant or with supernatant of empty vector transformant *P. pastoris* for a negative control. We found that at high concentration (40-80 ng/µl) Machi3-1 significantly reduced the growth of *E. amylovora* (Fig. 2B). Relevantly, the Machi3-1 protein is present in the nectar of Freedom cultivar in similar ~50-80 ng/µl concentration (Fig. S2B).

Biofilm formation is required for successful *Erwinia* infection (Koczan et al., 2011). As chitinases can impair biofilm formation (Chung et al., 2014), we wanted to study if Machi3-1 modifies biofilm formation of *E. amylovora*. Pre-formed *E. amylovora* biofilm was treated with purified Machi3-1 protein, and then biofilm detachment was quantified by crystal violet staining assay (O’Toole, 2011). As Fig. 2C shows, the Machi3-1 efficiently detached the preformed *E. amylovora* biofilm at physiological concentration. Taken together, the Machi3-1 acidic chitinase III protein efficiently impairs the growth and biofilm formation of *E. amylovora in vitro* at physiological concentration.

*Expression of Machi3-1 gene in ‘Freedom’ and ‘Jonagold’ cultivars*

To analyze the expression pattern of Machi3-1 gene, polyclonal antibody was produced, and accumulation of the Machi3-1 protein was studied in different tissues of the ‘Freedom’ and ‘Jonagold’ cultivars (Fig. 3A and Fig. S3). Confirming our earlier data, the Machi3-1 protein accumulated to high levels in the Freedom nectar but was barely detectable in the Jonagold nectar (Fig. S3). Moreover, in the ‘Freedom’ cultivar the Machi3-1 protein was also abundant in the stigma tissue but accumulated to low levels in the nectary, leaf, petal, stamen and ovary samples. In the ‘Jonagold’ cultivar, the Machi3-1 protein accumulated to low levels in all samples (Fig. 3A). Next we studied the expression of Machi3-1 at mRNA level in the nectary, stigma and leaf samples of the two cultivars (Fig. 3B). In the ‘Freedom’ cultivar, Machi3-1 transcript expressed to very high levels in both the nectary and the stigma but it was barely detectable in the leaves. By contrast, Machi3-1 mRNA accumulated to low levels in all ‘Jonagold’ samples (Fig. 3B). Taken into consideration that (i) 5B-Machi3-1 mRNA
expressed in the ‘Freedom’ nectary, while the protein accumulated in the nectar, and that (ii) the Machi3-1 protein has an export signal, we conclude that the 5B-Machi3-1 is a nectarin gene, which expresses in the nectary cells and then its protein product is secreted into the nectar.

*The promoter regions of the ‘Freedom’ and ‘Jonagold’ Machi3-1 alleles are different*

We postulated that variations in the Machi3-1 promoters are responsible for the strikingly different mRNA expression between ‘Freedom’ and ‘Jonagold’ cultivars. Therefore, the coding and the promoter regions of the Machi3-1 gene were also cloned from the ‘Jonagold’ cultivar, and then the ‘Freedom’ and ‘Jonagold’ Machi3-1 genes were compared. The nucleotide sequences of the coding regions and the predicted protein sequences are almost identical (only 4/894 nt and 2/298 amino acids are different) indicating that the coding region of Machi3-1 is well conserved in different apple cultivars (Fig. S4). However, while the promoter regions show strong overall similarity, significant differences were found in the middle region of the promoter (Fig. 3C and Fig. S5-7). The ‘Freedom’ contains a 38 nt long insertion and a 15 nt long deletion relative to the ‘Jonagold’ promoter (Fig. 3C). More interestingly, both promoters contain 59-64 nt long direct repeat segments (referred to as boxes). However, the ‘Jonagold’ promoter contains only 2 boxes, while 5 boxes are present in the promoter of the ‘Freedom’ Machi3-1 gene (Fig. 3C). Thus the ‘Freedom’ Machi3-1 promoter is longer than the Jonagold promoter (1417 nt and 1202 nt, respectively). The box1 and box5 of the ‘Freedom’ promoter resemble to the box1 and box2 of the ‘Jonagold’ promoter respectively, while the ‘Freedom’ box2, 3 and 4 are more similar to each other (Fig. S6). Further studies revealed that Machi3-1 was present in heterozygous form in both ‘Freedom’ and ‘Jonagold’ cultivars (Fig. S8), the second allele in both cultivars was a putative pseudogene. The promoter of the pseudogene (ps promoter for pseudogene promoter) contained 2 boxes. The three alleles will be referred to as 5B-Machi3-1, 2B-Machi3-1 and ps-Machi3-1, respectively (Fig. 3C and Fig. S8).

5B-Machi3-1 allele co-segregate with high Machi3-1 protein level in the nectar of hybrid apples

Multiplication of a repeat region in a promoter can dramatically increase transcriptional activity (Espley et al., 2009). We assumed that 5 box containing 5B-Machi3-1 promoter is responsible for the strong nectary-, and stigma-specific expression of Machi3-1 mRNA and
indirectly for the nectar- and stigma-specific accumulation of Machi3-1 protein in the ‘Freedom’ cultivar. To confirm that the 5B-Machi3-1 promoter is essential for the specific expression, the F1 hybrids from ‘Freedom’ × ‘Red Rome’ and ‘Freedom’ × ‘Red Winter’ (Free. × R.R. and Free. × R.W.) crosses were studied. The Machi3-1 protein is not detectable in the nectars of the ‘Red Rome’ and ‘Red Winter’ cultivars and both are homozygous for the ps-Machi3-1 alleles (Fig. 4 and Fig. S9). The ‘Freedom’ harbors one 5B-Machi3-1 and one ps-Machi3-1 allele and contains Machi3-1 protein in the nectar. The F1 progenies segregated close to the 1:1 for 5B-Machi3-1/ps-Machi3-1 heterozygous and for ps-Machi3-1/ps-Machi3-1 homozygous plants (Free. × R.R. F1 segregated for 7:7, while Free. × R.W. F1 hybrids segregated for 11:13). Only 6 Free. × R. R. and 8 Free. × R. W. F1 plants developed flower in the year of the study. We found that Machi3-1 protein accumulated to easily detectable levels in the nectar of all F1 progenies that inherited the Freedom 5B-Machi3-1 allele (Fig. 4 and Fig. S9), while the progenies that inherited the ‘Freedom’ ps-Machi3-1 allele did not accumulate the protein in their nectar (stigma samples were not collected). These results indicate that the Machi3-1 gene is present in a single copy in ‘Freedom’, and that the 5B-Machi3-1 allele is required and sufficient for the intense nectar-specific (and likely stigma-specific) protein expression.

The 5B-Machi3-1 promoter can confer nectary- and stigma-specific expression in tobacco

If the trans factors that are responsible for the nectar- and stigma-specific expression of the 5B-Machi3-1 allele in apple are also present in other dicot plants, the 5B-Machi3-1 promoter can be used as an efficient biotechnology tool for nectary- and stigma-specific expression. To test this assumption transgenic tobacco lines were generated with constructs containing the promoter and the coding region of the 5B-Machi3-1 or the 2B-Machi3-1 alleles (Fig. 5A). We found that the Machi3-1 protein was easily detectable in the nectar of 4 out of 16 5B-Machi3-1 transgenic tobacco lines (Fig. 5A). By contrast, the transgenic protein could not be detected in any of the 2B-Machi3-1 transgenic tobacco nectars (0/17 plants). We have also studied the accumulation of the Machi3-1 protein in the stigma of two 5B-Machi3-1 plants that expressed the Machi3-1 protein in their nectars and in two 2B-Machi3-1 plants, which did not accumulate the protein (Fig. 5C and 5D). We found that the expression in the nectar and the stigma correlated. In 5B-Machi3-1 transgenic plants, the Machi3-1 protein accumulated to high levels both in the nectar and the stigma. By contrast, in the 2B-Machi3-1 transgenic plants the Machi3-1 protein could not be detected in either the nectar or the stigma samples.
These results show that all trans factors that are required for the specific expression are also present in tobacco. Thus, the 5B-Machi3-1 promoter can be used for efficient nectary- and stigma-specific expression in various dicot plants.

The 5 box region of the Machi3-1 promoter is required for efficient expression

To directly prove that the boxes of the 5B-Machi3-1 promoter are required for the specific expression, plants were transformed with deletion constructs generated from the 5B-Machi3-1 plasmid (Fig. 5B), and then we studied the accumulation of the Machi3-1 protein in the nectars of the transformants (stigma samples were not collected in this experiment). The results suggest that the 5 boxes are essential for the efficient expression, only 1 out of 52 plants accumulated Machi3-1 protein in the nectar at detectable levels when constructs lacking the 5 boxes were used (we combined the results of 0.6, 0.4 and 0.2 constructs, see Fig. 5B). By contrast, 15/48 plants expressed Machi3-1 protein in the nectar (Fig. 5B) when the promoter contained the 5 box region (combining the results of 1.2, 1.0 and 0.9 constructs).

MYB305 could play an important role in the regulation of 5B-Machi3-1

As the 5 box promoter region is required for the efficient and specific expression of the 5B-Machi3-1 gene, we assumed that transcription factors that selectively bind to this region play important role in the regulation. To identify 5 box region specific transcription factor binding sites, we compared the 5 box and 2 box promoter regions of the 5B-Machi3-1 and 2B-Machi3-1 alleles. Interestingly, we identified 4 potential MYB binding sites in the 5 box and only 1 in the 2 box promoter region (Fig. S7). In tobacco, the MYB305 transcription factor directs the nectary-specific expression of many genes including nectarins (Liu et al., 2009). We hypothesized that MYB305 and the apple homolog of MYB305 (referred to as MdMYB305, gene: MDP0000344978) directs the expression of 5B-Machi3-1 in the nectary as well as in the stigma cells. If these assumptions are correct, MYB305 and 5B-Machi3-1 (but not 2B-Machi3-1) mRNAs are co-expressed. To test it, qRT-PCR and semi qRT-PCR assays were conducted to monitor the expression of MdMYB305 in ‘Freedom’ and ‘Jonagold’ cultivars (Fig. 6A and Fig. S10A). The MdMYB305 mRNA expressed similarly in both cultivars, it was abundant in the nectary and stigma samples but was barely detectable in leaves (Fig. 6A and Fig. S10A). As the ‘Freedom’ 5B-Machi3-1 (but not the ‘Jonagold’ 2B-Machi3-1) mRNAs expressed similarly (Fig. 3B and Fig. S10A), we concluded that in apple the MdMYB305 transcript is co-expressed with the 5B-Machi3-1 but not with the 2B-Machi3-
mRNA. In line with our results, a recent RNA-seq experiment showed that in ‘Golden Delicious’ cultivar (SRA-NCBI: SRP125281 study), which contains one copy of each of the 2B-Machi3-1 and the ps-Machi3-1 alleles, the MdMYB305 mRNA expressed to high levels in the stigma and style and that, 2B-Machi3-1 transcript accumulated to low levels in these tissues (Fig. S10B.).

To further support that 5B-Machi3-1 and MYB305 transcripts are co-expressed, Machi3-1 and MYB305 mRNA levels were studied in the stigma tissues of 5B-Machi3-1 and 2B-Machi3-1 transgenic tobaccos (Fig. 6B and Fig. S10C). The MYB305 mRNA expressed to high levels in the stigma of both transgenic lines. Relevantly, the 5B-Machi3-1 transgenic mRNA was also abundant in the stigma, whereas the 2B-Machi3-1 mRNA expressed to low levels (Fig. 6B and Fig. S10C). The fact that the MdMYB305 and MYB305 mRNAs co-express only with the 5B-Machi3-1 transcript, and that it is transcribed from the 4 MYB binding site containing 5B-Machi3-1 gene suggests that MYB305 homologs play an important role in the regulation of 5B-Machi3-1 expression.

The 5B-Machi3-1 might be introgressed from the Malus floribunda 821 to different cultivars

We found that the ‘Freedom’ cultivar contains the 5B-Machi3-1 allele in heterozygous form, while ‘Red Rome’, ‘Red Winter’ and ‘Jonagold’ cultivars harbored the 2B-Machi3-1 and/or the ps-Machi3-1 alleles. To clarify how widespread is the 5B-Machi3-1 allele, we PCR genotyped several more apple cultivars (Fig. 7A and Fig. S11). We found that the 5B-Machi3-1 allele is present in heterozygous or homozygous form in the genome of many (but not all) apple cultivars that contain the M. floribunda 821 derived Vf scab resistance gene, while it was not found in the cultivars that did not harbor the Vf gene (Gessler and Pertot, 2012). The Machi3-1 protein was present in the nectar of all the 5B-Machi3-1 allele containing cultivars (Fig. 7A) but it was not detectable in the nectar of cultivars lacking the 5B-Machi3-1 allele. These data confirm that the 5B-Machi3-1 allele is sufficient for the accumulation of Machi3-1 protein in the nectar.

Various Vf scab resistant apple cultivars including ‘Freedom’, ‘Releika’ and ‘Topaz’ contain the 5B-Machi3-1 allele (Fig. S11). Although these cultivars were generated in different breeding programs in the USA (‘Freedom’), Germany (‘Releika’) and Czech Republic (‘Topaz’), progenies from the M. floribunda 821 clone and ‘Rome Beauty’ (F26829-2-2) crossing were used in each programs (Gessler and Pertot, 2012). Therefore, we postulated that
the 5B-Machi3-1 allele was introgressed from the M. floribunda 821 ancestor. Indeed, we found that the M. floribunda 821 contains a 5B-Machi3-1 allele (Fig. 7B), which is almost identical (295/298 amino acids of the predicted proteins are identical) to the Freedom 5B-Machi3-1 allele (Fig. S12). The promoter of M. floribunda 821 5B-Machi3-1 is also highly similar to the promoter of the ‘Freedom’ 5B-Machi3-1 gene, it contains the 5 boxes and all 4 MYB binding sites are present (Fig. S12 and S13). We hypothesize that Machi3-1 protein is also abundant in the nectar and the stigma of M. floribunda 821 (flowers were not available to test this prediction). Machi3-1 is present in heterozygous form in M. floribunda 821, the second Machi3-1 allele has a specific promoter with 3 boxes (3B-Machi3-1) (Fig. 7B). Taken together, these data indicate that the 5B-Machi3-1 allele was introgressed from the M. floribunda 821 clone into various apple cultivars.

Discussion

Here we show that the 5B-Machi3-1 allele, which was introgressed from the M. floribunda 821 ancestor into certain apple cultivars, encodes an acidic chitinase III protein that accumulates to very high levels in the stigma and the nectar, the primary niches of the Erwinia infection. As its protein product inhibits in vitro the growth and biofilm formation of Erwinia at physiological concentration, we hypothesize that the Machi3-1 protein could partially protect apple against Erwinia infection and defend nutritious flower secretions from microbial infections.

Regulation of the expression of the 5B-Machi3-1 allele

We found that the Machi3-1 acidic chitinase III gene is present in at least three different forms in various apple cultivars, one is a pseudogene, while the two other alleles (5B-Machi3-1 and 2B-Machi3-1) encode very similar proteins (Fig. S4). However, the regulation of the two active alleles is markedly different. The 2B-Machi3-1 allele expresses to low levels in all studied tissues, while the 5B-Machi3-1 mRNAs shows very intense expression in the nectary and stigma (Fig. 3). Our segregation, association and transgenic assays demonstrate that the 5 boxes of the 5B-Machi3-1 promoter is required for the nectary- and stigma-specific transcript and for the nectar- and stigma-specific protein expressions (Fig. 4, 5 and 7). Previously it was shown that MYB305 (or its homologs as
MYB21 and MYB24) transcription factor plays a critical role in the expression of nectar proteins in tobacco, snapdragon and Arabidopsis (Roy et al., 2017). MYB305 is activated by JA (and probably by auxin), then it binds to the promoters of many nectary-specific genes (including certain nectarins) and promotes their transcription (MYB305 might also stimulate indirectly the accumulation of other nectar proteins). We identified four potential MYB binding sites on the 5 box region of the 5B-Machi3-1 allele but only one on the 2B promoter (Fig. S7). We demonstrated that in addition to the nectary (Liu et al., 2009), tobacco MYB305 is also expressed to high levels in the stigma (Fig. 6). Moreover, we showed that the apple homolog MdMYB305 transcripts are also abundant in the nectary and stigma tissues, while it accumulates to low levels in the leaf (Fig. 6). We propose that the 5B-Machi3-1 gene is similarly regulated in apple and transgenic tobacco plants (Fig. 7C). During late phase of flower development, JA increases the MYB305 level in the nectary and probably in the stigma, and then MYB305 binds to the 5 box region and promotes the transcription of 5B-Machi3-1 gene. As Machi3-1 protein contains a signal peptide, it can be secreted into the nectar explaining why 5B-Machi3-1 mRNA is abundant in the nectary, while the Machi3-1 protein accumulates in the nectar. We hypothesize that Machi3-1 protein is also secreted from the stigma cells. As both apple and tobacco have wet stigma (Sang et al., 2012; Losada and Herrero, 2012), we assume that in both plants Machi3-1 protein is secreted into the stigma exudate (Fig. 7C) (also see below).

Interestingly, the strong expression of MYB305 in nectary and stigma is not restricted to the plants having wet stigma such as tobacco or apple. The Arabidopsis homolog MYB21 is also expressed to high levels in both nectary and in the papilla cells of the dry stigma (Osaka et al., 2013).

Machi3-1 protein might interfere with Erwinia infection at two different steps

We found that the Machi3-1 acidic chitinase III protein is present at very high concentration in the nectar and the stigma of the 5B-Machi3-1 allele containing cultivars (Fig. 3 and 7). During apple infection, Erwinia propagates first in the stigma exudate, then in the nectar and finally enters into the plant through the stomata of the nectary (Bubán et al., 2003; Farkas et al., 2012). As biofilm formation is critical for the fruit and shoot infection of Erwinia (Koczan et al., 2011), it is likely that biofilm formation is also required for efficient flower infection. In vitro, the Machi3-1 protein inhibits growth and
biofilm formation of *Erwinia* at a concentration it is present in the nectar of 5B-Machi3-1 allele containing apple cultivars (Fig. 2). These findings suggest that the Machi3-1 protein can interfere with the propagation and infection of *Erwinia* in the nectar of the 5B-Machi3-1 allele containing cultivars. Moreover, the Machi3-1 protein might also inhibit *Erwinia* infection at the stigma. Machi3-1 was one of the most abundant protein in the ‘Freedom’ stigma sample (Fig. 5D, see the + control sample and Fig. S3) that contains both the stigma tissue and exudate. Machi3-1 has a signal peptide, therefore we assume that it is secreted from the stigma cells and accumulates in the exudate. The Machi3-1 protein might be so abundant in the stigma exudate that it can interfere with the propagation of *Erwinia*. Thus we propose that accumulation of Machi3-1 protein could protect apples from *Erwinia* infection by forming two barriers, it interferes with the replication and biofilm formation of the *Erwinia* in the stigma exudate as well as in the nectar, thereby protecting the plants. Our data shows that the 5B-Machi3-1 allele was introduced into different cultivars from *M. floribunda* 821 clone. We propose that the inhibitory Machi3-1 protein level in the stigma exudate and the nectar could contribute to the partial *Erwinia* resistance of these *M. floribunda* 821 derived cultivars. Importantly, if 5B-Machi3-1 contributes to the *Erwinia* resistance, this effect is not detectable in the frequently used shoot inoculation assays. Finally, as chitinases have wide spectrum antimicrobial effect (Cletus *et al.*, 2013), the high concentration of Machi3-1 protein could effectively protect the nectar and the stigma exudate from various microbial infection. Thus it can keep the optimal composition of these important fluid secretions, thereby enhancing the efficiency of pollination and fertilization.

**Supplementary Data**

**Supplementary Table S1.** List of primers.

**Supplementary Materials and methods S1.** Protein sequencing.

**Fig. S1.** Homology modelling of Machi3-1 and Hevamine proteins by Swiss-PdbViewer.

**Fig. S2.** Machi3-1 has a very weak lysozyme activity.

**Fig. S3.** Machi3-1 expresses to high levels in the stigma and the nectar of ‘Freedom’ apple cultivar.
Fig. S4. Alignment of the coding regions of 5B-Machi3-1 and 2B-Machi3-1 genes.

Fig. S5. Similarity of the promoter regions of 5B-Machi3-1 and 2B-Machi3-1 genes.

Fig. S6. Comparison of the boxes from the 5B-Machi3-1 and 2B-Machi3-1 promoters.

Fig. S7. Predicted transcription factor binding sites in the 5 box and 2 box regions.

Fig. S8. The structure of the three Machi3-I alleles.

Fig. S9. Machi3-1 protein expression co-segregate with the 5B-Machi3-1 ‘Freedom’ allele.

Fig. S10. The MYB305 and 5B-Machi3-1 transcripts are co-expressed.

Fig. S11. Genotyping of different apple cultivars for Machi3-1 alleles.

Fig. S12. The ‘Freedom’ and M. floribunda 821 5B-Machi3-1 genes are highly similar.

Fig. S13. Predicted transcription factor binding sites in the 5 box region of the promoter of M. floribunda 821 5B-Machi3-1 gene.

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Figure Legends

Fig. 1. Machi3-1 acidic chitinase III protein accumulates to high levels in the nectar of ‘Freedom’ apple cultivar. (A) The nectar protein profile of ‘Jonagold’ (Jon.), ‘Sampion’ (Sam.) and ‘Freedom’ (Free.) cultivars were studied by SDS-PAGE. Note that Machi3-1 protein accumulates only in the ‘Freedom’ nectar. M. shows size marker. (B) Multiple sequence alignment of Machi3-1 with PSC (Pomegranate seed chitinase) and Hevamine. The N-terminal signal peptide regions were omitted from the alignment. Substrate binding cleft are shown as ●, while the active site is marked with ○. The three magnesium binding sites of PSC (3 to 5 amino acid/binding site) are shown by empty, light green and dark grey columns.

Fig. 2. Machi3-1 has an antibacterial effect against E. amylovora. (A) In vitro chitinase assay was conducted with purified Machi3-1 protein and with S. griseus chitinase as positive and with supernatant from empty vector transformed strain as negative control. Note that the positive control is ~ 4 times more effective than the Machi3-1. (B) Machi3-1
inhibits the growth of *E. amylovora*. (C) Biofilm detachment assay shows that Machi3-1 impairs biofilm formation. Significancy levels: * p-value <0.05, *** p-value <0.001.

**Fig. 3.** Expression of Machi3-1 in ‘Freedom’ and ‘Jonagold’ apple cultivars. (A) Machi3-1 protein is abundant in the stigma of ‘Freedom’ cultivar. Western-blot assay was conducted to monitor the expression of Machi3-1 protein in leaf, stigma (Sti.), nectary (Nect.), ovary (Ova.), stamen (Sta.) and sepal (Sep.) samples in ‘Freedom’ and ‘Jonagold’ cultivars. Actin probe was used as loading control. ‘Freedom’ nectar was used as positive control (+con.) for the Machi3-1 blot. Note that Machi3-1 accumulates to low levels in the ‘Freedom’ nectary but it is very abundant in the nectar and that actin, which lacks signal peptide, does not accumulate in the nectar. (B) Expression of Machi3-1 mRNA. Quantitative RT-PCR assay was conducted to study the expression Machi3-1 mRNA in different organs of ‘Freedom’ and ‘Jonagold’ cultivars. (C) Non-proportional schematic representation of the different Machi3-1 alleles. White box shows the coding region. Black triangles indicate the allele specific insertions. The differently colored boxes represent the direct repeats. Note that *ps-Machi3-1* is a pseudogene.

**Fig. 4.** Machi3-1 nectar expression co-segregate with the 5B-Machi3-1 ‘Freedom’ allele. F1 progenies from ‘Freedom’ X ‘Red Rome’ crossing were genotyped for the Machi3-1 alleles (upper panel). ‘Freedom’ and ‘Red Rome’ genotypes (Free. and R.R., respectively) are also shown. M. indicates DNA size marker (1.5, 1.0, 0.7 and 0.5 kb bands are shown). The accumulation of the Machi3-1 protein in the nectars of the flowering F1 plants (others did not flower) was studied by stain-free gel visualization (Sta.) and by western-blot assay (Wes.).

**Fig. 5.** 5B-Machi3-1 transgenic tobacco plants express the Machi3-1 protein in the nectar and the stigma. (A) Non-proportional schematic representation of the 5B-Machi3-1 and 2B-Machi3-1 transgenic constructs. The promoter and the coding regions from the 5B-Machi3-1 and 2B-Machi3-1 alleles with the 35S terminator segment were used to generate transgenic tobacco lines. The ratio of transgenic plants expressing/non-expressing Machi3-1 protein to detectable levels in the nectar is shown at the right side (nectar Machi3-1 +/-). (B) The 5 box promoter region is required for efficient expression in the nectar. Transgenic tobacco plants were generated with 5B-Machi3-1 promoter deletion constructs, and then
the Machi3-1 protein expression in the nectar was tested. (C-D) Machi3-1 protein accumulates in the nectar and the stigma tissues of certain 5B-Machi3-1 transgenic tobacco plants. The accumulation of the Machi3-1 protein was studied by Stain-free gel visualization (Sta.) and by western-blot assay (Wes.). (C) The nectar profile of 2-2 independent T0 2B-Machi3-1 (T0 plants 5 and 7) and 5B-Machi3-1 (T0 plants 6 and 8) transgenic plants. We selected 5B-Machi3-1 plants, which accumulate the transgenic protein in the nectar. Cleav. shows putative cleavage fragment of the Machi3-1 protein. (D) The protein profile of stigma samples of the same transgenic lines. ‘Freedom’ apple stigma extract was run as positive control (+cont.). * marks Machi3-1 band in the stigma samples. Note that Machi3-1 protein is one of the most abundant protein in ‘Freedom’ stigma sample. Actin probe was used as loading control for the western-blot. Note that although stigma sample of 5B-Machi3-1 plant 6 is underloaded, the Machi3-1 protein is still easily detectable.

Fig. 6. 5B-Machi3-1 is co-expressed with MYB305. (A) In apple, the MdMYB305 is expressed in the nectary and the stigma but not in the leaf. qRT-PCR was conducted to monitor the expression of MdMYB305 in the nectary, stigma and leaf samples of ‘Freedom’ and ‘Jonagold’ cultivars. Note that MdMYB305 expressed to comparable level in both cultivars. (B) In 5B-Machi3-1 transgenic tobacco, Machi3-1 mRNA is co-expressed with MYB305 transcript in the stigma. qRT-PCR analysis of the expression of MYB305 and Machi3-1 mRNAs in the stigma of 5B-Machi3-1 (5B) and 2B-Machi3-1 (2B) transgenic tobaccos.

Fig. 7. The 5B-Machi3-1 allele containing apple cultivars express the Machi3-1 protein in the nectar. (A) The Machi3-1 protein is abundant in the nectar of 5B-Machi3-1 allele containing apple cultivars. Different apple cultivars were genotyped for Machi3-1 and their nectar samples were stain-free visualized (Sta.) (Fre.-‘Freedom’, Pri.-‘Prima’, Raj.-‘Rajka’, Rel.-‘Releika’, Sel.-‘Selena’, Top.-‘Topaz’, Sam.-‘Sampion’, R.W.-‘Red Winter’). (B) M. floribunda 821 contains a 5B-Machi3-1 allele. (C) Model of regulation of Machi3-1 expression.
Fig. 2.

A. Chitinase assay

B. Growth inhibition assay

C. Biofilm detachment assay
Fig. 3.

A. Western blot analysis showing the expression of Machi3-1 in various tissues and organ systems. 

B. Bar graph representing Machi3-1 mRNA expression levels in Stigma, Nectar, and Leaf tissues for Freedom and Jonagold varieties.

C. Diagram illustrating the genomic structure of Machi3-1 in different varieties, indicating the presence of BOX elements and nt lengths.
Fig. 4.

|   | M.  | 1   | 2   | 3   | 4   | 5   | 6   | Free. R.R. |
|---|-----|-----|-----|-----|-----|-----|-----|------------|
| F1 Freedom X Red Rome | 5B-Machi3-1 ps-Machi3-1 |
| Sta. | Machi3-1 |
| Wes. | Machi3-1 |
Fig. 5.

A) Transgenic constructs

- 2 BOX 15 nt
- Machi3-1

2B-Machi3-1

- 38 nt
- 5 BOX

5B-Machi3-1

Nectar Machi3-1 +/−

- 2B-Machi3-1
- 5B-Machi3-1

2 BOX 15 nt

Machi3-1

- 35S

0/17

4/16

B) Constructs containing 5 Box

- 1,2 kb

Machi3-1

- 35S

8/17

15/48

- 1,0 kb

Machi3-1

- 35S

5/16

- 0,9 kb

Machi3-1

- 35S

2/15

C) T₀ plants: nectar samples

| T₀ | 2B-Machi3-1 | 5B-Machi3-1 |
|----|-------------|-------------|
| 5  | Sta.        | Machi3-1    |
| 7  |             | Cleav.?     |
| 6  |             | Machi3-1    |
| 8  |             |             |

D) T₀ plants: stigma samples

| T₀ | 2B-Machi3-1 | 5B-Machi3-1 |
|----|-------------|-------------|
| 5  | Sta.        | Machi3-1    |
| 7  |             |             |
| 6  |             |             |
| 8  |             |             |

 Constructs lacking 5 Box

- 0,6 kb

Machi3-1

- 35S

0/13

1/52

- 0,4 kb

Machi3-1

- 35S

0/19

- 0,2 kb

Machi3-1

- 35S

1/20

Wes.

Actin
Fig. 6.

A

MdMYB305 expression in Freedom and Jonagold cultivars

1.25  2.87  2.42  3.07  0.01  0.03

[Bar chart showing expression levels for Stigma, Nectary, and Leaf for Freedom and Jonagold.]

B

Machi3-1 and MYB305 expression in the stigma of transgenic tobaccos

0.00  0.50  1.00  1.50  2.00  2.50

[Bar chart showing expression levels for Machi3-1 and MYB305 for 5B, 2B, and T₀.]

[Legend indicates Freedom and Jonagold for MdMYB305 expression.]
Fig. 7.

A

Fre. Pri. Raj. Rel. Sel. Top. Sam. R. W.

5B-Machi1-3
ps-Machi3-1
2B-Machi3-1

Sta.

Machi3-1

B

Jon. Free. Flo.821

5B-Machi3-1

3B-Machi3-1

C

Late flowering → Stigma, nectary JA  Myb305  5B-Machi3-1 transcription  5B-Machi3-1 export into nectar and stigma exudate