Transgenic Tobacco Expressing a T-Phylloplanin-Fusion Genes Possess Endogenous Resistance to Blue Mold.

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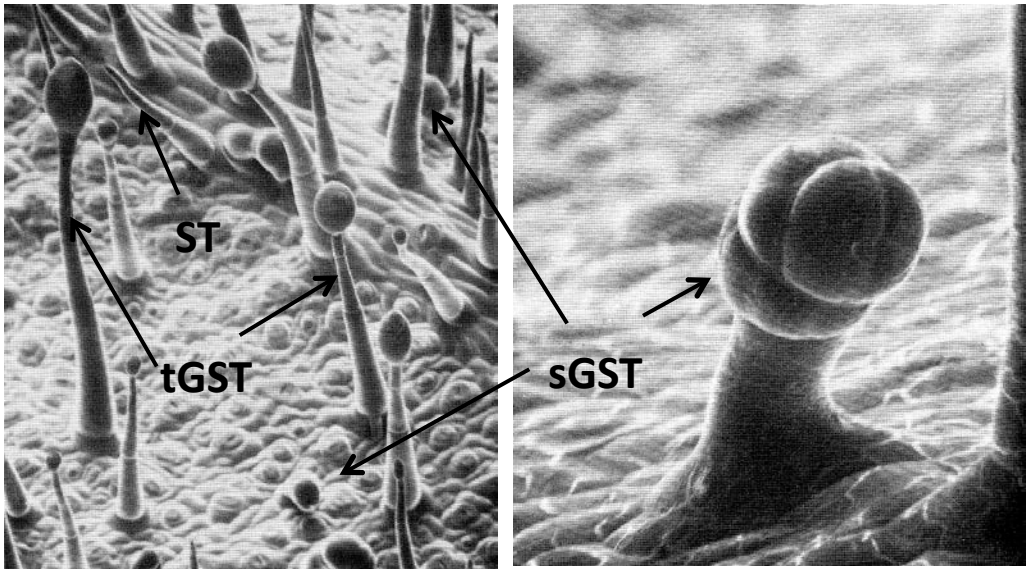


Tall glandular trichomes (tGST)

Short, procumbant glandular trichomes (sGST)

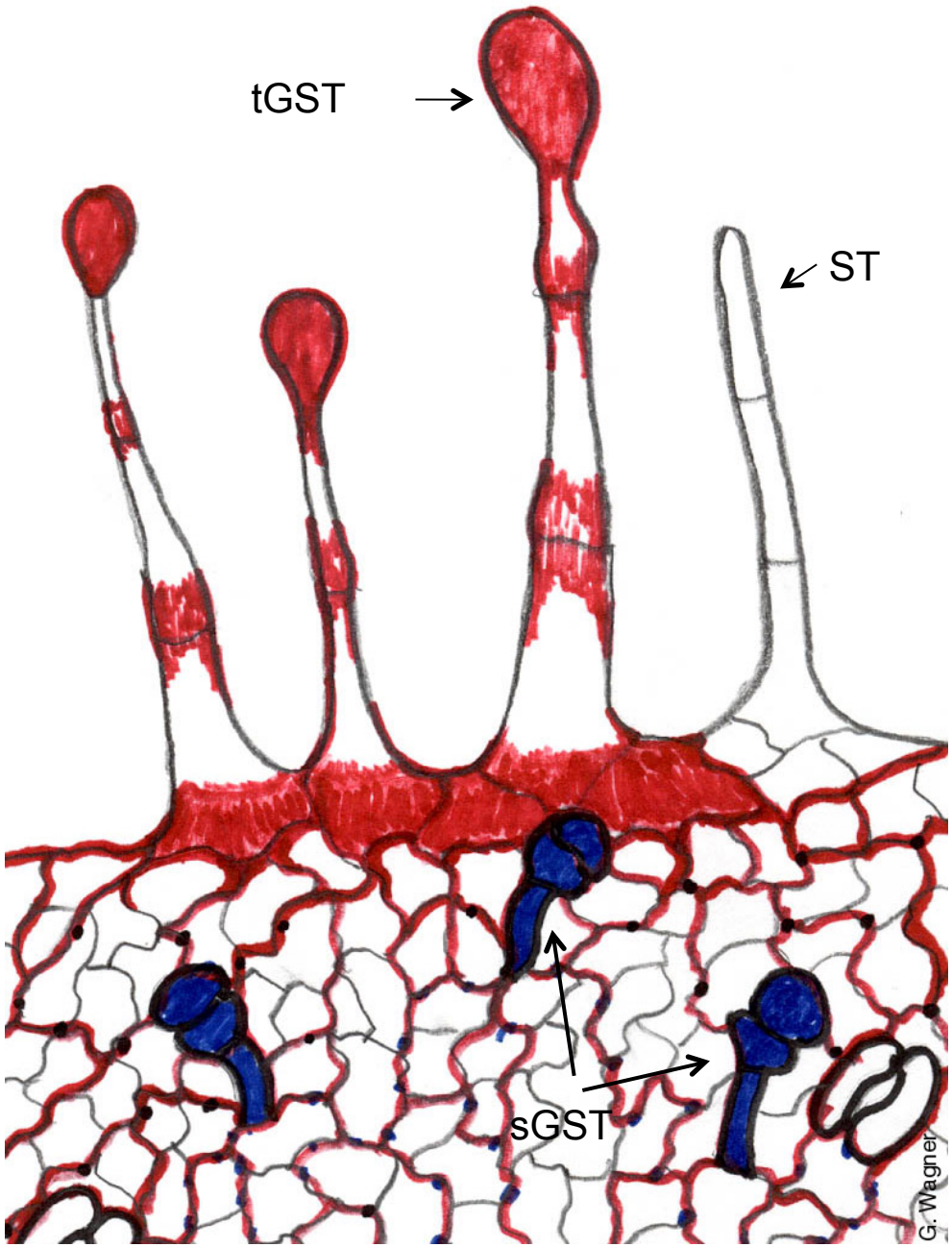
Simple (non-glandular) trichomes (ST)

University of Kentucky

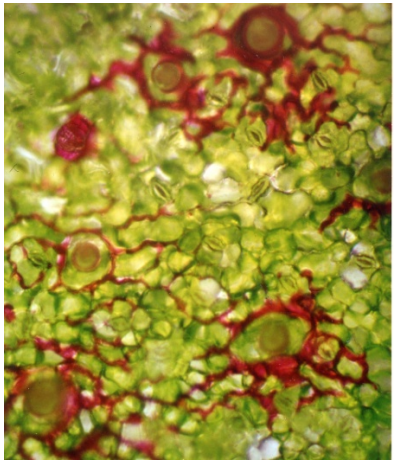
We have isolated water washable Phylloplanin glycoproteins from leaf surfaces of tobaccos, sunflower, and *Datura metel*.

We focused on tobacco and isolated the gene encoding T-phylloplanin from *N. tabacum* cv. T.I. 1068. We have shown that:

1) The **T-phylloplanin gene encodes a family of related, highly glycosylated proteins** with an apparent base size of 127 aa.
2) **T-Phylloplanins inhibit blue mold spore germination and disease** in the leaf spot disease assay.
3) RNAi mediated T-phylloplanin gene knockdown **increases the sensitivity of normally resistant tobacco**,T.I. 1068 to blue mold disease.
4) Leaf water washes containing **T-phylloplanins show antifungal activity against at least one member of all four phyla of fungi and fungi-like pathogens** (Ascomycete, Basidiomycete, Zygomycete, Oomycete). Thus, T-phylloplanins appear to be broad-spectrum fungicides that represent an aerial-surface, first-line-of-defense system in tobacco.
5) The **T-phylloplanin promoter drives expression of GUS and GFP only in sGSTs** (short procumbant trichomes glands), thus T-phylloplanins appear to be produced only in sGSTs.
6) **T-phylloplanins and sGSTs are enriched 4 to 5 fold on the upper leaf surface** where blue mold spore deposition mostly occurs, and are renewed after leaf water washing (simulated rain).
7**) T-phylloplanins are widely dispersed on the tobacco leaf surface**.
8) We have recently shown **protection of turf grasses against *Rhizoctonia solani and Pyricularia oryzae*** in the laboratory (Crop Science 51:2829-2839, 2011) and in the field (3 years- MS in preparation) after spraying with T-phylloplanin containing T.I. 1068 leaf water wash. Protection is lost when the T-phylloplanin is pre-treated with ProteinaseK, or boiled.



"Models are to be used, and not believed!"



Our working model suggests that diterpenes and sugar esters (red color) are secreted by tGST and escape gland containment then flow down to the epidermal surface where they river-out onto the surface in channels between anticlinal walls of epidermal cells. T-Phylloplanins (blue) are produced by sGST, escape the glands through pores, and flow down to the epidermal surface where, due to their amphipathic nature they "mix" with diterpene/sugar ester and distribute on the surface. When a fungal spore arrives, it's germination is inhibited by phylloplanin and disease is suppressed.

ENGINEERING BLUE MOLD RESISTANCE

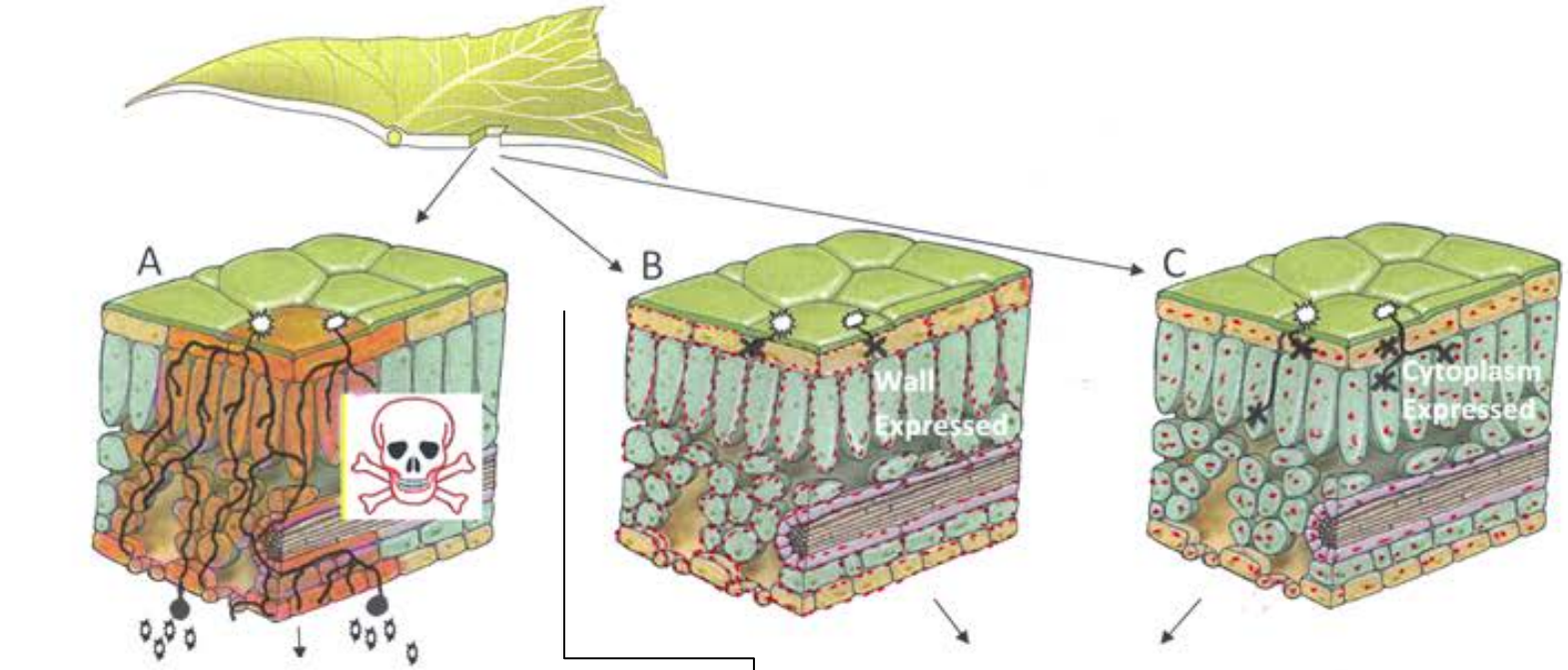
We have shown that spraying T.I. 1068 leaf water wash containing T-phylloplanins can prevent blue mold disease on leaves of sensitive KY14 tobacco that were subsequently spotted with blue mold spores, and we also observed disease resistance on turf grasses treated with spores or hyphae in the laboratory, or exposed naturally to *Rhizoctonia solani* and *Pyricularia oryzae* diseases in the field. These observations begged the question, could endogenous protection of plants against fungal diseases be achieved by engineering endogenous expression of the T-phylloplanin gene in sensitive plants? To test this we have expressed T-phylloplanin-fusion genes in KY14, a blue mold sensitive tobacco.

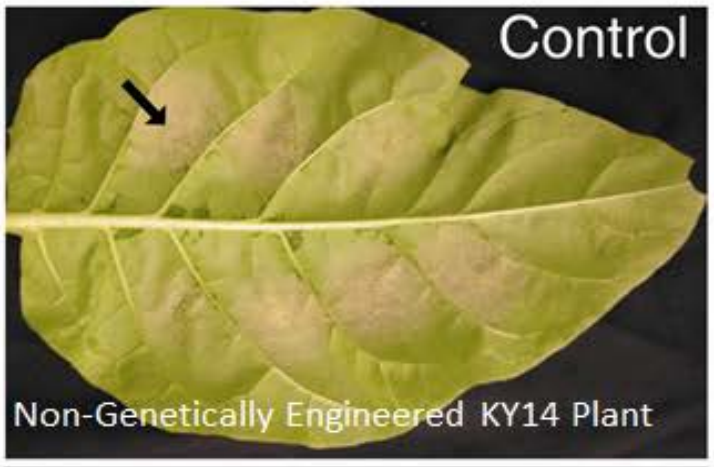
THE STRATEGY:

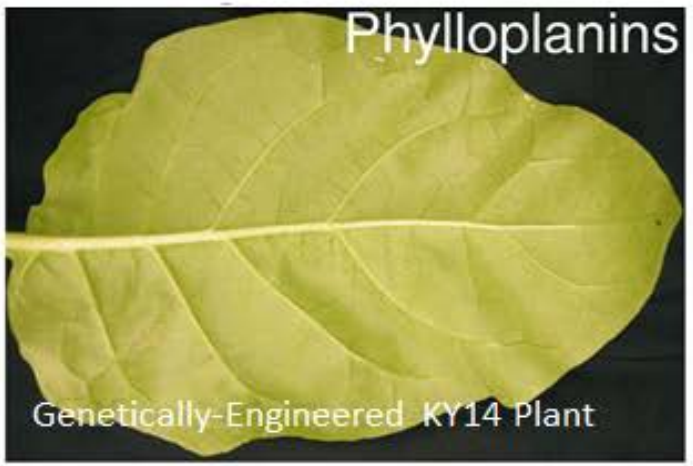
Introduce a fusion gene encoding *N. tabacum* cv. T.I. 1068 T-phylloplanin fused to GUS or GFP into the blue mold sensitive *N. tabacum,* KY14 to determine if these fusion genes:

1) Are expressed.
2) If co-suppression is avoided using a fusion gene.
3) If cytoplasmic or cell wall targeting is more effective in reducing disease assessed using the leaf spot assay for blue mold.

ENGINEERING BLUE MOLD RESISTANCE in KY14



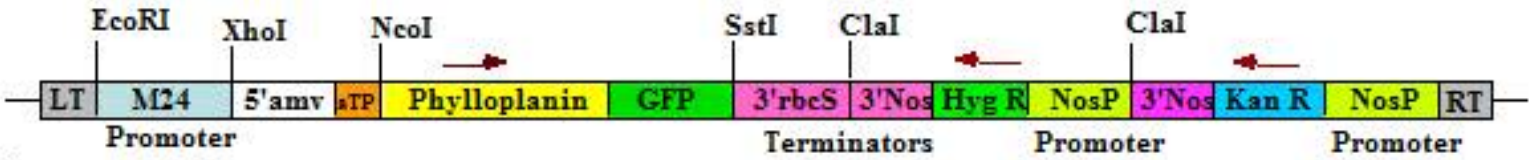




Gene Constructs:

4) pKM24H – Vector control.

5) pKM24H-aTP-T-Phy-GFP (for extracellular expression).

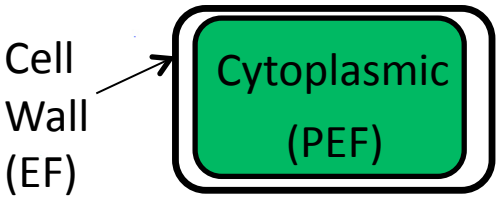


6) pKM24H-T-phyllo-GFP – Identical to the above but without aTP (for cytosol expression).

Schematic maps of the plant expression constructs (pKM24-*T-phyllo*, vector control and related *T-phyllo-GFP* constructs) for chimeric *T-Phyllo* gene expression (GenBank accession no. AY705384, etc.) are showns. The modified full-length transcript promoter (M24) of the *Mirabilis mosaic virus* (Maiti et al., 2002; Dey and Maiti, 1999; Dey and Maiti 1999) directs the coding sequence of *T-phyllo-GFP* genes. A translational enhancer sequence (5'amv) 35-nt long 5'-untranslated region of AlMV RNA 4 was fused with the gene. The apoplast targeting sequence (aTP) of *Arabidopsis* 2S2 protein gene (Krebbers et.al.,1988) was fused with the coding sequence of *T-phyllo-GFP* in construct 5. LT, left T-DNA border; RT, right T-DNA border; *HygR*, hygromycin selectable marker gene directed by nopaline synthase promoter (NosP); the 3'-terminator sequences (Terminators) of ribulose bisphosphate carboxylase small subunits (3'RbcS) and nopaline synthase (3' Nos) genes are also shown. The KanR is also present.The EcoRI, XhoI, SstI, NcoI and ClaI restriction sites used to assemble these expression vectors are shown.

Results for GFP: GFP4 – cell wall (*)

GFP5 – cytoplasmic (*)



		Segregation	RT PCR	qRT-PCR (Comparative C_T method)	Disease results	GFP Concentration		
Line	Genr^n	KanR:KanS	GFP+ Phylloplanin	Phylloplanin		µg GFP /mg TP protein	µg GFP /mg EF protein	µg GFP /mg PEF protein
Control KY14				1 ± 0.12	15(16), 7(16, 9(16) 12(16), 15(16)	0	0	0
aTP-GFP-9	T2	8.9:1		0.78 ± 0.17	8(16), 10(16),3(16), 5(16)	0.383 ± 0.04	7.962 ± 0.81	0.258 ± 0.03
* GFP4-23-2	T2	Homozygous	+	586.12 ± 40.3	0(24),0(24), 0(24)	0.781 ± 0.08	6.433 ± 0.65	0.228 ± 0.03
* GFP4-35	T2	1.7:1	+	169.79 ± 17.2	0(16),0(16), 0(8),0(24), 0(16),6(16)	0.659 ± 0.07	5.994 ± 0.61	0.361 ± 0.04
GFP4-14	T2	Homozygous	+	6267.34 ± 617.3		0.168 ± 0.02	0	0.009 ± 0.0003
GFP4-21	T2	32:8	+	3923.5 ± 380.5		0.373 ± 0.006	1.32 ± 0.178	0.609 ± 0.047
GFP4-135	T2	Homozygous	+	4891.82 ± 480.8		0.545 ± 0.076	0.826 ± 0.142	0.00 ± 0.0
GFP4-223	T2	Homozygous	+	2335.52 ± 227.3		0.303 ± 0.049	0.721 ± 0.12	0.00 ± 0.0
* GFP5-8	T2	30.6:1	+	3.51 ± 0.32	8(16),7(16), 3(16),11(16), 10(16)	0.52 ±0.05	0.16 ± 0.02	0.62 ± 0.05

The "+" Sign indicating lines +ve for the assay.

*qRT-PCR results were analyzed using the comparative C_T method and presented as fold changes compared with the reference (control Ky14 wild plants were taken as reference). Gene specific primers for phylloplanin were used to evaluate phylloplanin transcript level. Tobacco *α-tubulin* was taken as an internal control to normalize the expression of phylloplanin during qRT-PCR. The specificity of each primer pair was verified by determining the melting curve of PCR products at the end of each run. Data presented as the mean ± s.d. of three independent experiments.

TP: Total protein; EF: Extracellular Fluid; PEF: Post-extracellular Fluid

Results: GUS4 – cell wall (*)

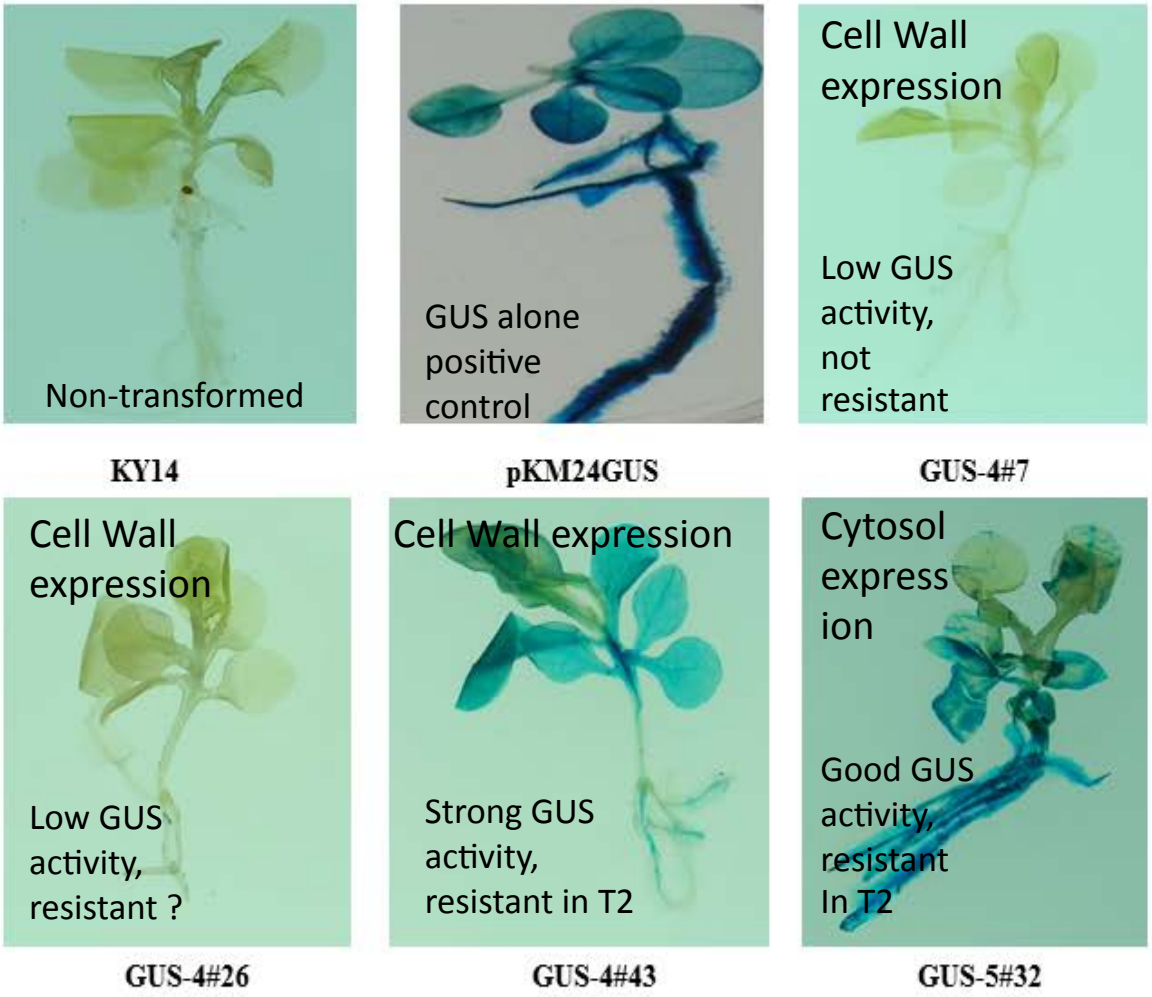
GUS5 – cytoplasmic (*)

		Segregation	RT PCR	qRT-PCR (Comparative Ct method)	Disease results	GUS Activity			GUS Staining
Line	Genr^n	KanR:KanS	GUS+ Phylloplanin	Phylloplanin		nmol 4-MU produced/ min/mg TP protein	nmol 4-MU produced/ min/mg EF protein	nmol 4-MU produced/ min/mg PEF protein	
Control KY14				1 ± 0.14	15(16), 7(16, 9(16) 12(16), 15(16)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-
pKM24-GUS	T2	2.1:1	-	0.88 ± 0.12	not done	47.34 ± 4.5	39.11 ± 4.0	52.36 ± 5.1	+
GUS4-7	T2	2.92:1	+	60.67 ± 7.0	4(16),0(16), 1(8),0(8), 9(24)	0.019 ± 0.002	0.21 ± 0.0034	0.00 ± 0.00	-
GUS4-26	T2	2.83:1	-	1476.25 ± 138.0	0(16),0(16)	0.005 ± 0.0003	0.335 ± 0.038	0.00 ± 0.00	-
* GUS4-43	T2	Homozygous	+	804.28 ± 78.0	0(16),0(16), 0(16),3(16), 3(24)	1.114 ± 0.13	4.64 ± 0.49	2.58 ± 0.27	+
* GUS5-32	T2	2.45:1	+	892.46 ± 85.0	0(16),0(16), 0(16),1(16), 4(24)	11.294 ± 1.2	3.59 ± 0.4	14.24 ± 1.3	+

The "+" Sign indicating lines +ve for the assay.

*qRT-PCR results were analyzed using the comparative Ct method and presented as fold changes compared with the reference (control Ky14 wild plants were taken as reference). Gene specific primers for phylloplanin were used to evaluate phylloplanin transcript level. Tobacco *α-tubulin* was taken as an internal control to normalize the expression of phylloplanin during qRT-PCR. The specificity of each primer pair was verified by determining the melting curve of PCR products at the end of each run. Data presented as the mean ± s.d. of three independent experiments.

GUS staining of T2 plants expressing T-phylloplanin in cytoplasm and cell wall.



GFP- To assure results of extracellular fluid analysis, experiments were repeated and malate dehydrogenase (a cytoplasmic enzyme) was assayed in EF and PEF.

	Protein Concentration (mg/g tissue)			MDH activity (Units/mg protein)		
Lines	TP	EF	PEF	TP	EF	PEF
Control KY14	15.771 ± 1.23	0.0529 ± 0.006	15.312 ± 1.32	1.32 ± 0.123	0.0016 ± 0.0001	1.356 ± 0.132
aTP-GFP-9	15.586 ± 1.06	0.055 ± 0.006	15.78 ± 1.25	2.089 ± 0.185	0.003 ± 0.0002	1.85 ± 0.186
* GFP4-23-2	16.71 ± 1.35	0.0516± 0.004	16.178 ± 1.22	1.581 ± 0.156	0.002 ± 0.001	1.384 ± 0.129
GFP4-35	17.645 ± 1.68	0.0568 ± 0.005	17.269 ± 1.19	1.411 ± 0.148	0.002 ± 0.001	1.329 ± 0.128
GFP4-14	17.465 ± 1.38	0.0648 ± 0.007	17.281 ± 1.39	1.694 ± 0.153	0.0017 ± 0.0018	1.916 ± 0.169
GFP4-21	14.941 ± 1.45	0.0593 ± 0.004	13.39 ± 1.41	1.439 ± 0.13	0	1.479 ± 0.15
GFP4-135	16.297 ± 1.64	0.068 ± 0.006	15.85 ± 1.56	1.869 ± 0.191	0	1.672 ± 0.163
GFP4-223	15.621 ± 1.48	0.0565 ± 0.005	14.93 ± 1.38	1.477 ± 0.134	0	1.866 ± 0.153
* GFP5-8	15.414 ± 1.568	0.0608 ± 0.004	14.86 ± 1.7	1.27 ± 0.128	0	1.624 ± 0.136

Data presented as the mean ± s.d. of 7 plants from each line. One unit of MDH is the conversion of 1mM NADH per min at 22^{0}C
TP: Total protein; EF: Extracellular Fluid; PEF: Post-extracellular Fluid

GUS- To assure results of extracellular fluid analysis, Experiments were repeated and malate dehydrogenase (a cytoplasmic enzyme) was assayed in EF and PEF.

	Protein Concentration (mg/g tissue)			MDH activity (Units/mg protein)		
Line	TP	EF	PEF	TP	EF	PEF
Control KY14	15.298 ± 1.63	0.07 ± 0.007	14.173 ± 1.34	1.684 ± 0.153	0.002 ± 0.001	1.18 ± 0.12
pKM24-GUS	14.98 ± 1.03	0.05 ± 0.006	12.837 ± 1.33	1.48 ± 0.162	0	1.272 ± 0.113
GUS4-7	15.809 ± 1.026	0.0493± 0.005	13.488 ± 1.28	1.512 ± 0.153	0	1.689 ± 0.159
GUS4-26	17.866 ± 1.59	0.057 ± 0.003	15.84 ± 1.34	1.613 ± 0.153	0	1.342 ± 0.141
GUS4-43	17.263 ± 1.82	0.0726 ± 0.006	16.162 ± 1.28	1.34 ± 0.132	0	1.56 ± 0.143
GUS5-32	16.173 ± 1.43	0.0538 ± 0.005	15.81 ± 1.11	1.37 ± 0.126	0.0028 ± 0.001	1.23 ± 0.141

Data presented as the mean ± s.d. of 7 plants from each line. One unit of MDH is the conversion of 1mM NADH per min at 22^0C
TP: Total protein; EF: Extracellular Fluid; PEF: Post-extracellular Fluid

Conclusions:

1) We have achieved expression of T-phylloplanin-GFP and T-phylloplanin-GUS fusion genes in the blue mold sensitive tobacco, KY14.

2) Plants that produced substantial fusion proteins (measured as GFP or GUS) showed substantial resistance to blue mold using the leaf spot assay.

3) Targeting to the cytoplasm or cell wall were both successful in T2 plants, however best results were obtained with the GFP fusion.

4) Best results were obtained with T-phylloplanin-GFP.

4) Recent results show that cell wall GUS fusions may be lost after the T2 generation while cell wall GFP fusions are retained.

5) We will apply to APHIS to test promising lines in the field in 2012.