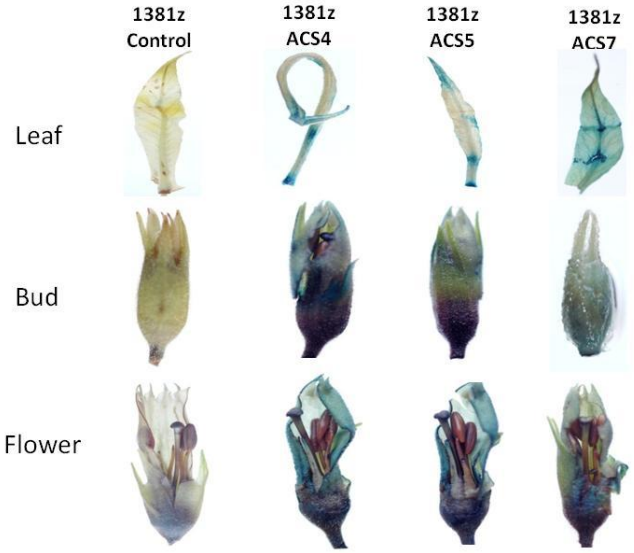
Validation of the developed gene constructs using tobacco as a model system

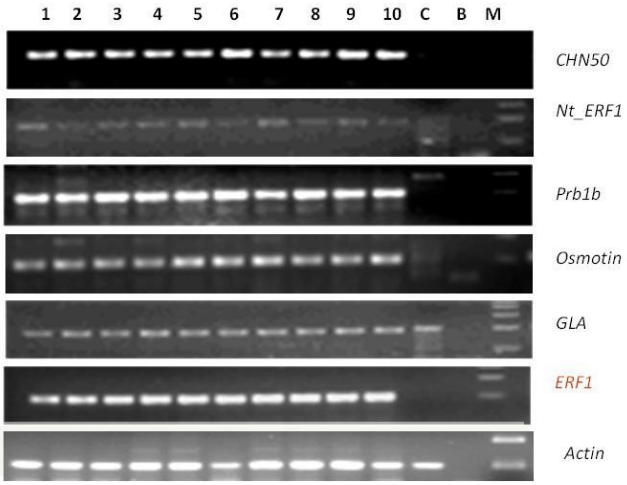
Transgenic tobacco plants have been developed with each of the three cassettes independently and they have been confirmed for the presence of the transgenes. Transgenic plants were analysed for the expression pattern of the promoters using both RT-PCR and by *GUS* staining procedures. RT-PCR indicated that the promoters are expressed in leaves as well as in the inflorescence. Gus staining revealed the elevated expression of the promoters in inflorescence tissue.



Gus staining with leaf, bud and flower from the transgenic plants derived with four different vectors.

The vectors used for developing the transgenic plants are listed on the top. 1381z control: pCAMBIA1381z basal vector; 1381z-ACS4:pCAMBIA1381z with ACS4 promoter cloned upstream of *gus*; 1381z-ACS5: pCAMBIA1381z with ACS5 promoter cloned upstream of *gus*; 1381z-ACS7: pCAMBIA1381z with ACS7 promoter cloned upstream of *gus*

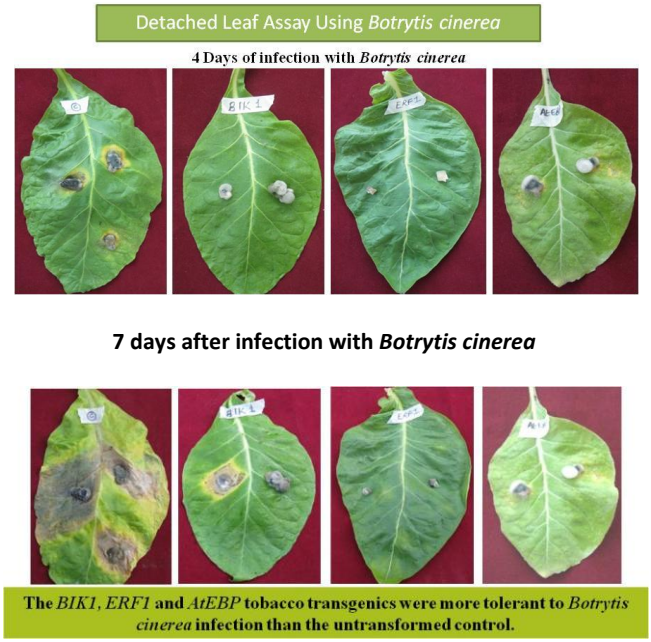
Tobacco transgenic plants carrying single gene cassettes (AtACS4 –*BIK*1, AtACS5 – ERF1 and AtACS7- AtEBP1), were studied for the stable expression of the transgene in the T1 generation. The introduced genes (BIK1, ERF1 and EBP1) are expected to up-regulate many defense related genes. RT-PCR carried out with the T1 plants clearly indicated that many of the pathogenesis related genes were up-regulated compared to control plants. Figure below shows the expression pattern observed in the flower buds of the transgenic plants carrying AtACS5-ERF1 gene cassette.



RT-PCR analysis confirming the up-regulation of many defense related genes in the flower buds of the transgenic plants carrying AtACS5-ERF1 gene cassette

Defense related genes analysed for the expression pattern are mentioned on the right hand side of the figure. House keeping gene Actin was used as the control to study the relative expression of other genes. Lanes 1-10: 10 T1 pants carrying ERF1 cassette; Lane C: untransformed control plant; B: Blank (no template) – PCR control M: molecular size marker

The obtained T1 generation plants were subjected to *Botrytis cinerea* infection studies using detached leaf assay and the transgenic plants showed decreased rate of infection compared to the untransformed control plants.



Detached leaf assay with transgenic tobacco plants carrying individual gene cassettes

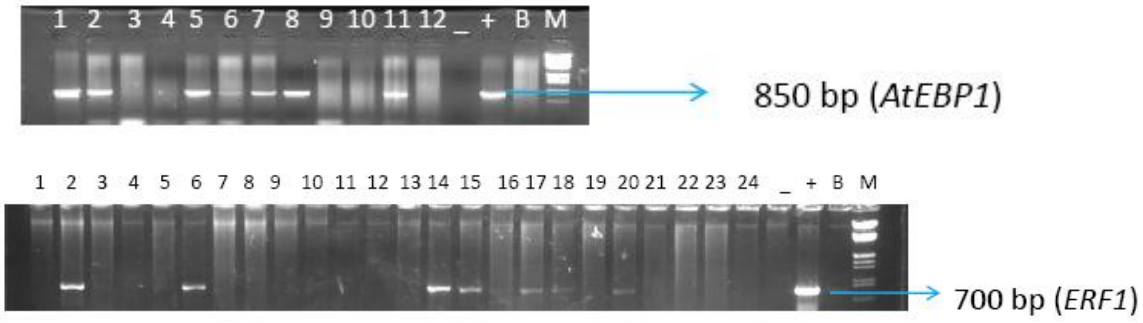
Leaves of the transgenic plants carrying individual gene cassettes (AtACS4 –*BIK*1, AtACS5 – ERF1 and AtACS7-AtEBP1) were subjected to B. cinerea infection and the leaves were observed for 7 days for the appearance of the disease symptoms. Leaf from the control (untransformed) plant was used as the control.

As empirically observed, tobacco transgenic plants had been realized with the three single gene cassettes (ACS4-BIK1, ACS5-ERF1 and ACS7-AtEBP1) independently and had been confirmed for the presence and expression of the introduced gene cassettes. To assess the cumulative effect of expressing more than one gene for imparting resistance against fungi, the gene cassettes were pyramided by crossing the plants carrying single gene cassettes. At least 24 progeny plants of each of the crosses (ERF X BIK1, BIK1 X At EBP and AtEBP X ERF1), in the transgenic greenhouse and presence and expression of the gene cassettes was confirmed using PCRs and RT-PCRs. Number of plants positive for the presence of the gene cassettes is indicated in Table below.

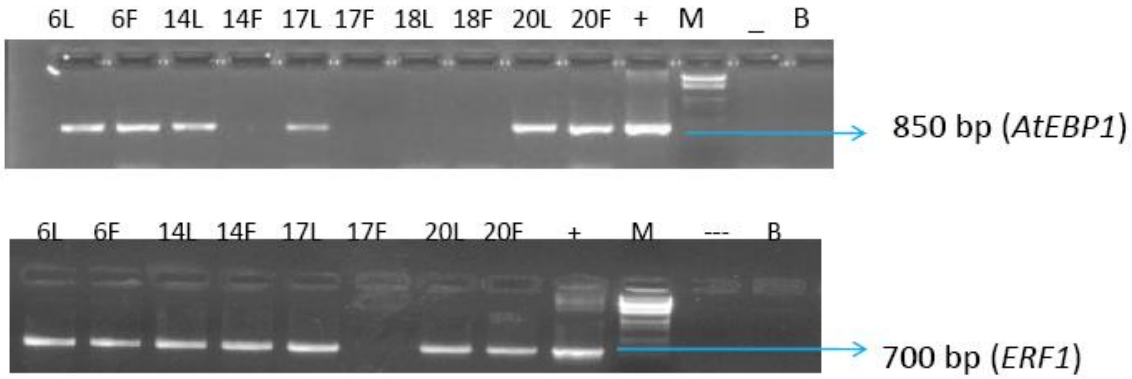
Table. Number of PCR positive plants obtained in the segregating population of cross between plants carrying single gene cassettes

AtEBP-19 X ERF1 16-13		***At EBP-2X BIK1 5-2***		***BIK5-5 X E16-14-***	
Only AtEBP (A)	**2**	**Only AtEBP (A)**	**8**	**Only BIK1 (B)**	**2**
Only ERF1(E)	**3**	**Only BIK1(B)**	**2**	**Only ERF1 (E)**	**16**
Both A&E	**7**	**Both A&B**	**4**	**Both B&E**	**6**

Plants that showed presence of the two cassettes were confirmed for the expression of the gene cassettes. PCR analysis of the cross AtEBP1 X ERF1 and the expression of both cassettes is represented in the figures below.



PCR analysis of the progeny plants of AtEBP1 X ERF1 cross for the presence of EBP1 and ERF1 cassettes



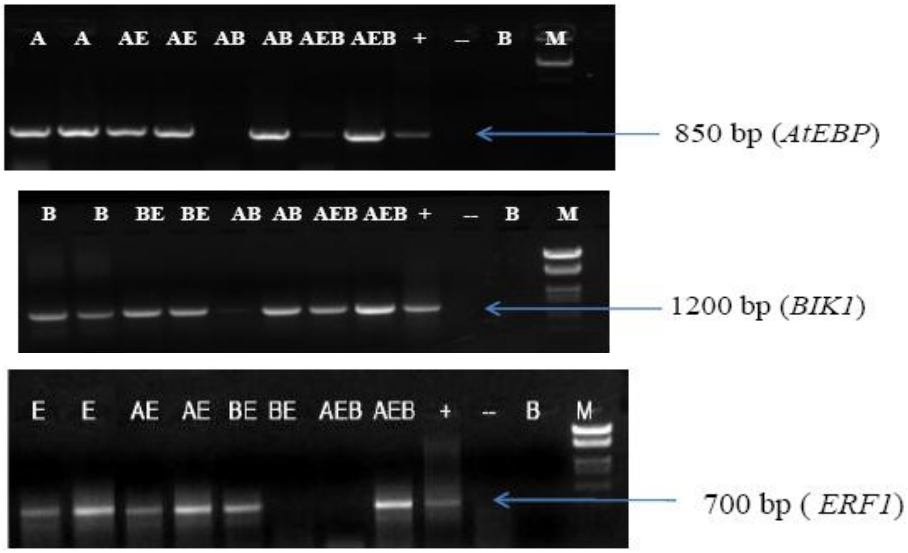
RT-PCR analysis of progeny plants of AtEBP1 X ERF1 cross that were PCR positive for both the cassettes

Plants that expressed two genes (e.g. AtEBP & ERF1) were crossed with tobacco plants carrying the corresponding third gene cassette (e.g. BIK1) to stack all the three gene cassettes. Thus three cross combinations were made to realize plants carrying all the three cassettes. Progeny of two of the crosses were analysed to identify plants carrying all possible combinations of the three cassettes. Table below indicates the results obtained with one of the crosses (AtEBP1 & ERF1 X BIK1) after PCR analysis and the results indicated Mendelian segregation of the three gene cassettes.

Table. Number of PCR positive plants obtained in the segregating population of the cross AtEBP & ERF1 (AE20) X BIK1(5-5)

Only AtEBP (A)	**10**
Only BIK1 (B)	**10**
Only ERF1 (E)	**8**
Both A& E	**8**
Both B & E	**6**
Both A & B	**9**
All three (A,E & B)	**8**
NULL	**18**

Representative plants carrying different combinations of gene cassettes were subjected to RT-PCR to confirm the expression patterns of the transgenes (Figure below). With these analyses, plants expressing either single or two or three gene cassettes have been identified and these plants will be subjected to disease bioassay.



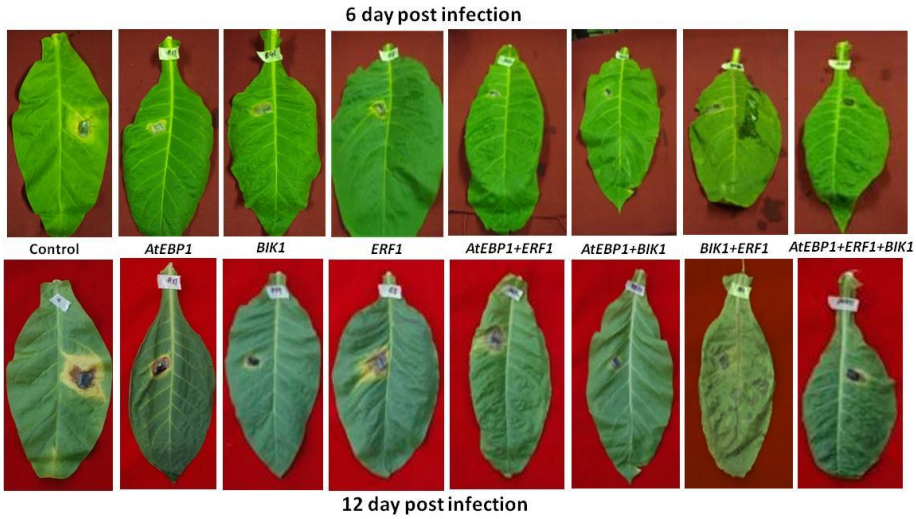
RT-PCR analysis of the representative PCR positive plants of the cross AtEBP & ERF1 (AE20) X BIK1(5-5)

To bring three gene cassettes together, transgenic plant (BE8) that expressed two genes, *BIK1* and *ERF1*, was crossed with transgenic tobacco plant (A17) carrying *AtEBP1* and the progeny plants were analyzed for the presence of different gene cassettes using PCRs with different gene specific primers. This analysis identified the number of plants carrying combinations of gene cassettes as indicated in Table below. PCR positive plants were further analysed for the expression of the gene cassettes using RT-PCR and this confirmed expression of respective cassettes in leaf and flowers.

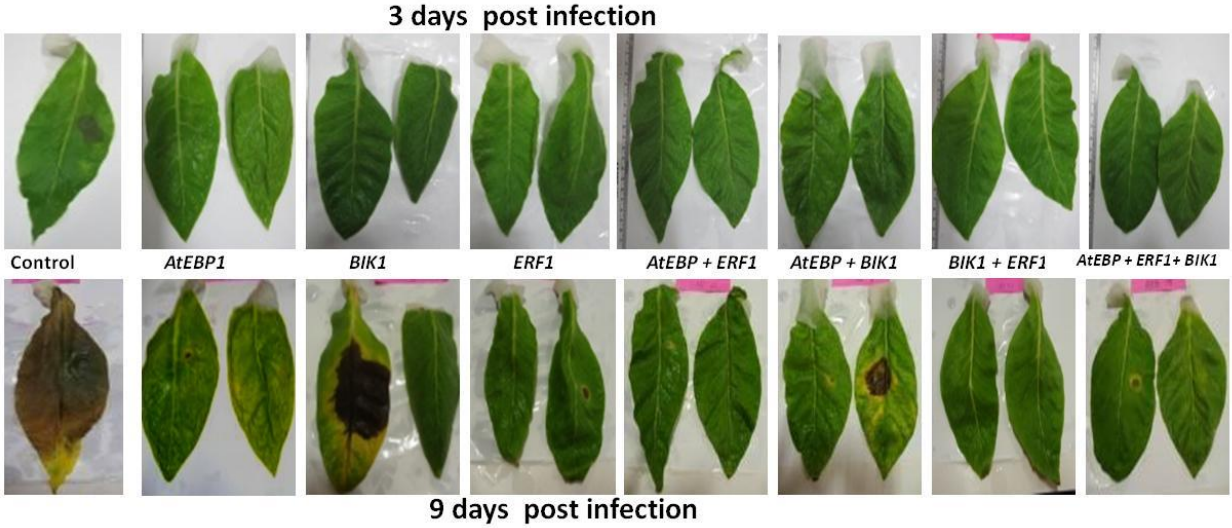
Table. PCR positive plants obtained in the segregating population of the cross between BE8 (carrying both *BIK1* and *ERF1* cassettes) and A-17 (carrying *AtEBP1* cassette)

Gene cassettes carried	No of plants
Only AtEBP (A)	7
Only BIK1 (B)	2
Only ERF1 (E)	12
Both A& E	5
Both B & E	4
Both A & B	4
All three (A,E & B)	3
NULL	22
Total	59

With these analyses, plants expressing either single or two or three gene cassettes were identified and these plants were subjected to disease bioassay with necrotrophic fungi *Alternaria alternata* and *Phytophthora parasitica pv. nicotianae.* Repeated experiments have indicated that transgenic plants, irrespective of the number of transgene cassette carried, showed better tolerance than null plant or untransformed control plants. Differential tolerance to the pathogens is represented in Figures below. For Alternaria, transgenics with stacked gene cassettes showed better tolerance than those with single gene cassettes with *AtEBP1+BIK1* combination showing the best response. However, response to Phytophthora disease infection showed a different pattern with *BIK1+ERF1* and *AtEBP1+ERF1* combinations showing highest level of tolerance. Transgenic plants carrying gene cassettes in different combinations will further be evaluated for tolerance to diseases using inflorescence.



Detached leaf bioassay with *Alternaria alternata* of transgenic plants carrying different gene cassettes (Gene cassettes carried by the transgenics are indicated)



Detached leaf bioassay of transgenic plants carrying different gene cassettes with *Phytophthora parasitica var. nicotianeae* pathogen (Gene cassettes carried by the transgenics are indicated)