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Development of tobacco (*Nicotiana tabacum*) transgenic plants overexpressing ferriitn for oxidative stress tolerance

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Abstract
Transgenic tobacco (*Nicotiana tabacum* cv FCV) special plants expressing a ferritin gene (*pFer/Rok2 and pFlagfer/Rok2*) from alfalfa were obtained through Agrobacterium tumefaciens mediated transformation. Primary transformants over expressing the ferritin *pFer/Rok2* gene products localized into chloroplast and *pFlagFer/Rok2* gene product localized into cytoplasm were confirmed by PCR and Southern analysis. Transgenic plants were evaluated by exposing the leaf discs to high light intensity to induce oxidative stress and then the membrane damage is measured by electrical conductivity test. Ferritin transgenic plants showed less damage as compared to control plants which signifies the relevance of ferritin in protecting the plant under oxidative stress.

Keywords: Ferritin, Oxidative stress, Tobacco, Reactive Oxygen Species, Methyl Viologen

Introduction
Any abiotic stress especially high light intensity under stress, leads to the problem of excess excitation energy in the photosystem. This triggers the Mehler Ascorbate reaction leading to generation of $O_2^{\cdot -}$ and subsequently H_2O_2. If these motifs are not scavenged, it results in the over production of the most toxic hydroxyl $OH^{\cdot -}$ radical. The damage caused by these toxic reactive oxygen intermediates is often referred as the oxidative stress. Iron is toxic to cell when it is free because it acts as catalyst in the production of OH- radicals through Fenton reaction (Arora *et al.,* 2002, Theil *et al*., 2006) [1, 6]. One of the option to regulate the production of $OH^{\cdot -}$ radicals is by minimizing free iron levels. The Ferritin, a class of multimeric proteins, which can sequester Iron, assumes significance. This opens up an option to manage OH- radicals by regulating the ferritin in the plant systems (Udaya Kumar *et al.,* 1999) [7]. A transgenic approach was adopted to study the relevance of the Ferritin and its relevance under stress was assessed by imparting high light stress using methyl vologen and membrane damage was measured using electrical conductivity.

Material and Methods
The *pFer/Rok2* and *pFlagfer/Rok2* (Figure 1A & 1B) constructs coding for Ferritin was obtained as a gift from Dr. Gabor V. Horvath, Institute of plant biology, Biotechnology Institute, Belgium. These constructs were used for *Agrobacterium* mediated gene transformation of wild type tobacco (*Nicotiana tabacum* cv FCV) plants in this study to understand the role of Ferritin in oxidative stress tolerance.

Transformation of FCV special tobacco leaf discs with pFer /Rok2 and pFlagfer/Rok2
The leaf discs were inoculated in plates with MS (Murashige and Skoog) morphogenesis media and then co-cultivated with *Agrobacterium* culture and incubated for 48 hours. The discs after incubation were transferred to fresh plates with selective media (MS media: 2 mg/L BA + 0.2 mg/L NAA + 500 mg/L cefotaxime and 50 mg/L kanamycin) for 20 days and then the regenerated plantlets were later sub cultured and subsequently rooted on MS rooting media containing 0.5 ppm Indole butyric acid (IBA). *In vitro* shoot lets with roots were transferred to the pots containing soilrite and were placed in polythene bags with small holes to avoid build up of humidity. After two weeks the plants were shifted to the mist chamber with 30°C temperature and 85% relative humidity. After two or three weeks the plants were transferred to battery containers in the green house and allowed to establish.

PCR analysis of pfer/Rok2 and pFlagfer/Rok2 putative transgenic plants
DNA from transformed and untransformed Tobacco plants was extracted by C-TAB Method

(Sambrook *et al.*, 1989) [5]. PCR amplification was carried out in thermo cycler (PTC-100™ from MJ RESEARCH, INC.) using *nptII* primers (P1- 5` GAG GCT ATT CGG CTA TGA CTG 3', P2- 5` ATC GGG AGG GGC GAT ACC GTA 3`) with the following thermo cycle profile: 94º C for 4 min, 94º C for 1min (Denaturation), 55.7^0 C for 1 min (Annealing),72º C for 2 min (Extension), GO TO step 2 for 35 more cycles, 72º C for 8 min, 4º C forever. The PCR product (799 bp) of transgenic plants, wild type and the plasmid (*pFer Rok2* and *pFlagfer/Rok2*) was analyzed on the 0.8% agarose gel. PCR products were electrophoresed and transferred to the membrane for southern blotting using ^{32}P labelled random primer labelling kit supplied by Gibco BRL.

Assessment of stress responses in transgenic plants expressing ferritin genes

In vitro developed ferritin transgenic plants and wild type plants were transferred to pots and maintained under green house conditions. The leaf discs of transformed and control plants were floated on 2µM and 5 µM concentration of methyl viologen and water. One set was kept in dark and other set was kept in light. These two sets were incubated for eight hours in dark. This period of incubation facilitates the entry of the paraquat into the cells. Then one set was exposed to high light intensity of 1600 µ Ein for two hours. The other set was retained in the dark. This served as the control for the one, which has been exposed, to the light. This high light intensity would be sufficient to enhance the generation of the free radicals. The light exposed leaf disc were then transferred to dark and allowed for recovery. During this recovery process the damaged cells leak out the cell content into the solution. After 5 hours the total leachates were collected and the electrical conductivity for inorganic ions was measured (Leopald, 1981, Gonger *et al.*, 2013) [3, 2].

Results and Discussion

Vector confirmation

Genes *pFer/Rok2* and *pFlagFer/Rok2* were mobilized to *Agrobacterium* strains EHA105 by tri-parental mating. The transformed colonies were multiplied and the plasmid DNA was isolated. PCR was performed using *npt II* specific primers. The polymerisation reaction was allowed for extension in presence of dNTPs, *Taq* Polymerase and buffers, which amplifies approximately 796 bp *npt II* coding region (Figure 2).

The putative transformants were selected on the tobacco morphogenetic media containing 100µg /mL kanamycin and were further subcultured on morphogenic MS media and rooted on rooting MS media as mentioned earlier (Plate -1). The rooted plants were initially transferred to 1:1 mixtures of vermiculite and sand and were prehardened under normal room conditions (Plate 2). The prehardened plants were transferred to pots for further observations and seeds were collected by bagging to avoid cross pollination.

PCR analysis of pFer/Rok2 and pFlagfer/Rok2 putative transgenic plants

The genomic DNA isolated from control and transgenic plants were subjected for PCR analysis. It was performed using *npt II* specific primers. The polymerisation reaction was allowed for extension in presence of dNTPs and *Taq* Polymerase, which amplified 800 bp *npt II* coding region. The PCR product was analysed on 1 % agarose gel. The amplified product was obtained in plasmid (lane 10) and transgenic plants (lanes 3, 4, 5, 7, 8 and 9) and was not seen in control (lane 11) plants (Figure 3). These PCR positive plants were used for further physiological and biochemical analysis and they were allowed to attain maturity. The seeds were collected from these plants and stored.

Southern blot analysis

To assess the stable integration of gene of our interest, southern analysis was performed for npt II PCR-product. The genomic DNA was isolated from T_o PCR-positive plants and non transformed plants. PCR analysis was carried out for these plants using *npt II* specific forward and reverse primers. Annealing and the extension of the strands was carried out by *Taq* Polymerase. The PCR product was analyzed on 1.0% agarose gel. The PCR product was transferred on to a nitrocellulose membrane and probed with labeled *npt II*. All the PCR-positives showed a strong hybridization with labeled *npt II* indicating the stable integration of the gene (Figure.4)

Assessment of stress responses in transgenic plants expressing ferritin gene

The relative tolerance to oxidative stress in transgenics expressing ferritin gene was examined by assessing membrane integrity using Methyl viologen test. Ferritin (*FlagFer/Rok2*) transgenics and control plants were selected for the stress studies. Membrane damage is high in light exposed leaf discs as compared to leaf discs kept at dark. The damage is high in 5µM of methyl viologen (MV) than 2 µM and control plants are more sensitive to light stress than ferritin transgenics. The results depicted that ferritin in playing a predominant role in reducing the generation of ROS under stress condition (Figure 5). The results clearly indicates ferritins are not likely to be an essential iron source for plant development, but that they play a significant role in the defence machinery against oxidative stress (Ravet *et al.*, 2009) [9].

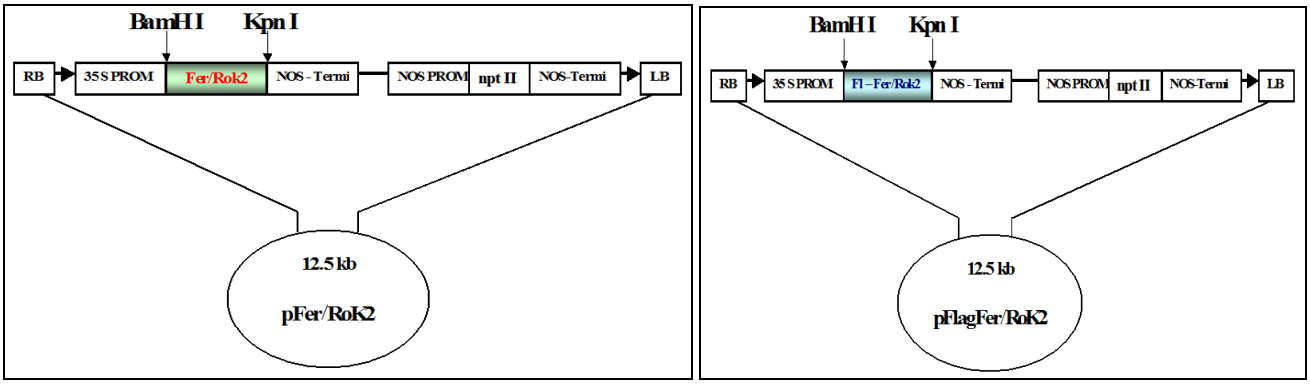

Fig 1A &1B: Plasmid map showing pFer/Rok2 and pFlagFer/Rok2 in which Ferritin complete cDNA cloned into BamH I-Kpn I site of Rok2 plant expression vector

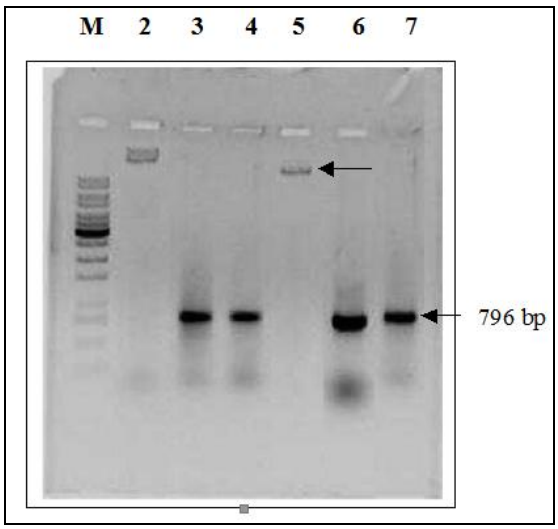

Fig 2: PCR analysis showing the successful mobilization of pFer/Rok2 and pFlagFer/Rok2 constructs into Agrobacterium strains EHA 105.

M: Gene Ruler 1 kb marker. Lane 2: Undigested plasmid DNA of pFer/Rok2 constructs. Lane 3and 4: PCR amplification of *pFer/Rok2* constructs isolated form Agrobacterium Strain EHA105 after mobilizing the constructs into Agrobacterium, Lane 5: Undigested plasmid DNA of *pFlagFer/Rok2* constructs., Lane 6 and 7: PCR amplification of *pFlagFer/Rok2* constructs isolated form Agrobacterium Strain EHA105 after mobilizing the constructs into Agrobacterium.

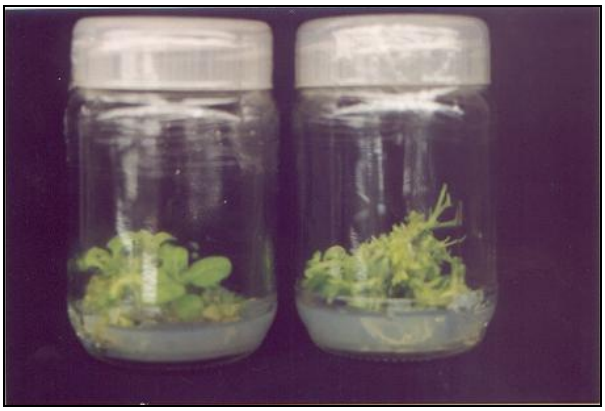

Plate 1: Subcultured transgenic plants overexpressing Ferritin on tobacco morphogenic media

The putative tobacco transgenic plants overexpressing *Fer/Rok2* and *Flagfer/Rok2* gene constructs were selected on kanamycin media. Later the plantlets were subcultured on tobacco morphogenic media for further multip lication.

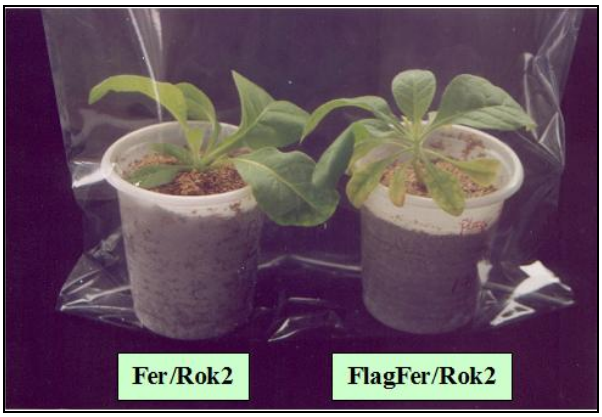

Plate 2: Photograph of a well rooted transgenic plants overexpressing *Fer/Rok2* and *Flagfer/Rok2* construct

The putative transgenic plants over expressing *Fer/Rok2* and *Flagfer/Rok2* gene constructs were rooted on tobacco rooting media (½ MS +0.5mg/L IBA).

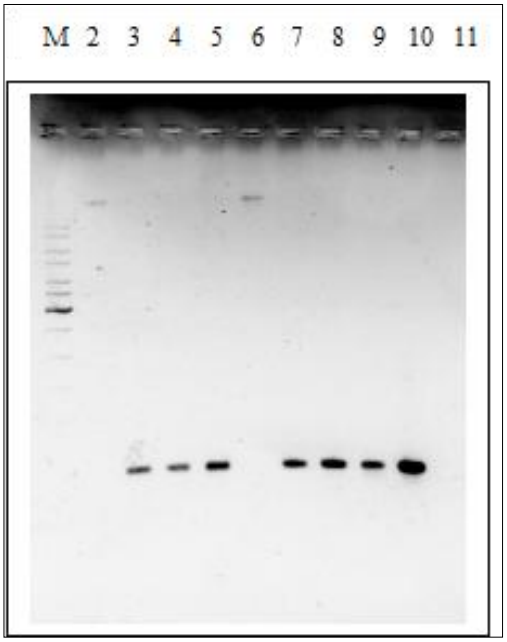

Fig 3: Confirmation of putative transformants by PCR analysis for genomic DNA carrying the *pFer/Rok2* and *pFlagFer /Rok2* constructs over expressing Ferritin genes.

M: Gene ruler 1 kb ladder. Lane 2&6: Unamplified genomic DNA of *pFer/Rok2* and *pFlagFer /Rok2* over expressing tobacco plants, Lane 3, 4 and 5: DNA from transformed plants expressing *pFer/Rok2*. Lane 7, 8 and 9: DNA from transformed plants expressing *pFlagFer/Rok2*. Lane 10: Plasmid DNA of *pFer/Rok2* constructs. Lane 11: DNA form untransformed plants

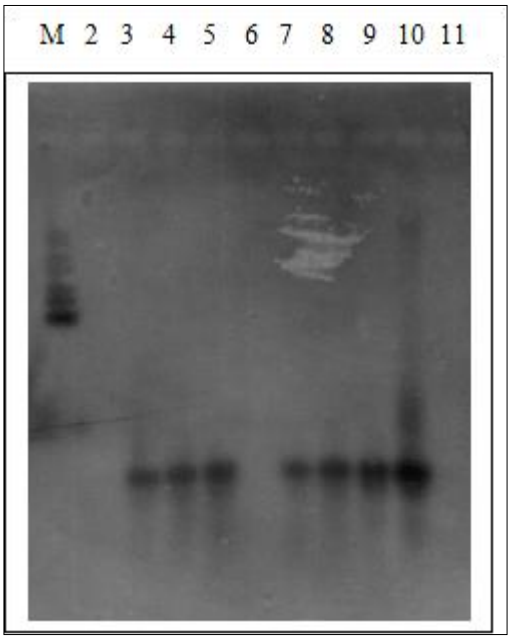

Fig 4: Southern analysis of *npt II* PCR product.

Lane1: Gene ruler 1 kb ladder, Lane 2 & 6: Genomic DNA from tobacco transgenic plants overexpressing ferritin, Lane 3, 4, 5, 7, 8, 9: PCR product amplified from tobacco transgenic plants overexpressing ferritin, Lane 10: PCR product amplified from plasmid.

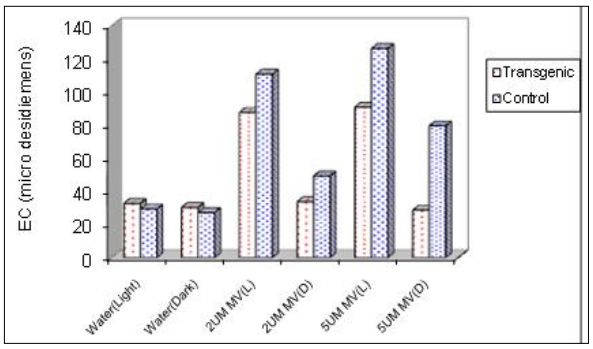

Fig 5: Effect of oxidative stress on membrane integrity in transgenic and control plants

Conclusion

The transgenic plants over expressing Ferritin accumulated relatively higher levels of ferritin. These transgenic plants also showed increased tolerance under methyl viologen induced ROS generation at hight light stress. Further experiments are in progress to analyze the other transformed plants for oxidative stress tolerance and for the relative expression of transcripts and protein.

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