OTKA K 49438
The role of ferritin in enhancing the stress tolerance of grapevine
Closing report

The role of ferritin in protection against conditions evoking oxidative stress was investigated and verified in transgenic grapevine plants expressing *Medicago* ferritin. Main steps of the experimental work were:

1. Creating the transgenic plants
2. Verifying the presence of MsFerr and characterizing the transgenic plants
3. Investigating stress response

Our results show that with appropriate adaptation of experimental techniques, it was possible to introduce MsFerr into grapevine which improved the plants' stress tolerance.

In summary, we found that leaves of MsFerr expressing 'Richter 110' plants retained more photosynthetic activity under – and thus proved more tolerant to – a variety of oxidative stress conditions including lipid peroxidation, free radical attack and salt-stress. The expression of the introduced *Medicago* ferritin gene caused significant decrease of the endogenous genes, possibly due to a feed-back regulation. Relative stress responses of grapevine ferritins were unaltered, consequently protection was due to the introduction of MsFerr, although there was no correlation between the introduced *Medicago* ferritin gene expression level and the extent of stress tolerance.

Results were presented at several conferences, both in Hungary and abroad; were published in 14 journal publications and in one book chapter. An important aspect of the work is that it has served as a basis of a Ph.D. dissertation (by Róbert Oláh, BCU) and was also integrated in an ongoing Ph.D. (of Petra Majer, BRC-Szeged Univ.)

1.

Embryogenic calli were started from anthers of *Vitis vinifera* 'Chardonnay' and *V. berlandieri* x *V. rupestris* cv. 'Richter 110' as previously described (Oláh et al. 2003). The cultures were transformed with *Agrobacterium tumefaciens* strain EHA105 harbouring one of the following gene constructions: the pRok2 vector itself, *pRok2::MsFerr* and *pRok2::FLAG-MsFerr*. Earlier experiments with MsFerr producing tobacco showed that the protein accumulated in the chloroplasts, while the alfalfa ferritin with an amino-terminal FLAG-tag remained in the cytoplasm (Deák et al., 1999). *Agrobacterium*-mediated transformation of embryogenic calli was started with both cutivars. Transgenic plants were selected out on kanamycin containing (20 mg/l) MSEM medium. After one-year selection most of putative transformed 'Richter 110'

embryos showed developmental disorder. Normal shoot formation was highly improved (Fig. 1) by the use of 1 μM BA in the MSEM medium (Oláh et al. 2003) as compared to the previously used protocol allowing us an efficient grapevine transformation and regeneration (Table 1). Germinating embryos were transferred individually to new tubes and exposed to light to induce shoot development. Non-transformed plants were similarly recovered for control experiments on a kanamycin-free medium. Despite of repeated efforts, transformed 'Chardonnay' plants did not grow sufficiently and we were unable to regenerate these for further experiments. A possible explanation is in the general stress sensitivity of this cultivar, which was the reason of selecting 'Chardonnay' for this stress tolerance improvement project. This sensitivity seem to have backfired by hindering transformation.

Successfully regenerated *in vitro* grown 'Richter 110' plants were planted in sterile garden soil:sand:perlite (1:1:1) mixture moistened with sterile tap-water. Plants were kept at 100 % humidity for 10-12 days. Then humidity was gradually reduced to acclimate plants to greenhouse conditions. Plants were then propagated using green softwood cuttings collected from properly developed plants with at least 60-80 cm shoot length and 8-10 internodes. Single-node internodes with the whole leaf were rooted in perlite moistened with acidified tap-water (pH=6.0 adjusted with an 1:1 mixture of 5% nitric acid and 5% phosphoric acid). After three weeks rooted cuttings were transferred into soil mixture (see above) for further growth. Nutrients were supplied by irrigation with 0,5% (w/v) Volldünger (Gartenhilfe GmbH, Linz, Austria). For stress studies plants were grown at least to 4-6 leaved stage.

Transformed plants were tested by PCR analysis. Plant DNA was isolated from young leaves by Quiagen Plant DNA minikit according to the supplier's instructions. A 700 bp region of the *nptII* gene was amplified by the *nptII*F (5'-ATCGGGAGCGGCGATACCGTA-3') and *nptII*R (5'-AGGCGAGGCGGCTATGACTG-3') primers (Hoffmann et al. 1997). A 513 bp region of the alfalfa ferritin gene was amplified by the FerFw1 (5'-GTCACGGTGTGTGGCACTTTGA-3') and FerRev2 (5'-AGACAGAGCCAATTCCATGGCA-3') primers. To verify the absence of *Agrobacterium* vector in putatively transformed plants, DNA samples were also tested with the VCF (5'-ATCATTTGTAGCGACT-3') and VCR (5'-AGCTCAAACCTGCTTC-3') primers (Sawada et al. 1995), which amplifies a 730 bp virulence region located on the separate helper Ti plasmid.
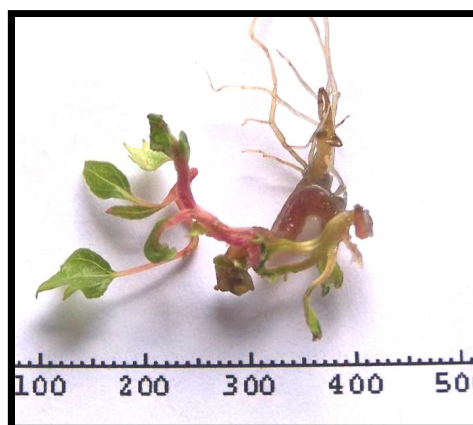
Figure 1.
The induction of normal shoot formation using BA ('Richter 110')

2.

We have obtained several independently regenerated plants after co-cultivation with *A. tumefaciens* EHA105 harbouring the three different gene constructions. From among these, 16 transformed lines were selected on the basis of PCR and Western blot analysis. The PCR analysis of the putative transformants showed that both *nptII* (Fig. 2) and *MsFer* genes (Fig. 3) were present in the tested lines but *virC* gene-specific amplicons were missing (data not shown).

Table 1.
The result of induction of normal shoot formation using 1 µM BA in the case of *Agrobacterium tumefaciens* EHA105 (pRok2Ferr) transformed 'Richter 110'

No. of experiment	MSEMkm*	MSEMkm+BA*
1	0/38 (0%)	5/26 (19,2%)
2	0/30 (0%)	2/23 (8,7%)
3	3/33 (9%)	9/25 (36%)
4	0/32 (0%)	2/15 (13,3%)
5	1/17 (5,8%)	6/17 (35,3%)
6	0/28 (0%)	7/22 (31,8%)
Average (s.d.)	2,46% (3,95)	24,05% (11,87)

*MSEM medium (Oláh et al. 2003) with km= kanamycin (20 mg/l) and/or BA = benzyl-adenine (1 µM). One data represents the number of developed shoots from somatic embryos / total number of embryogenic calli during a 3-month period.
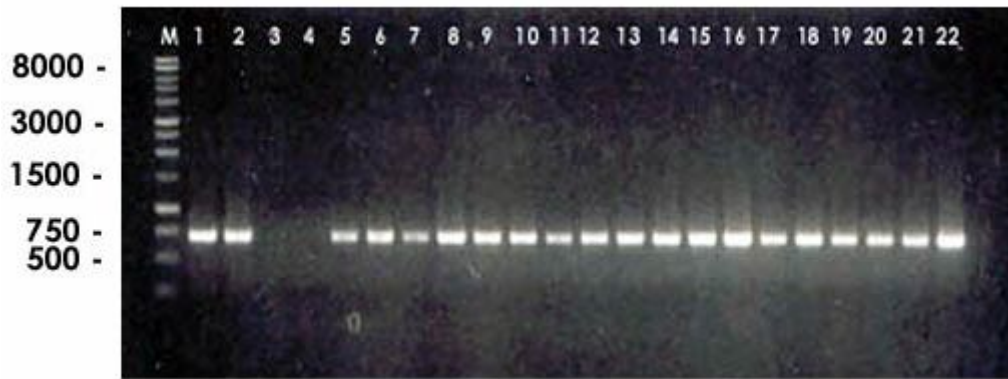
Figure 2.
PCR detection of the transferred DNA in grapevine plants using *nptII* specific primers. Lanes 1-2: *A. tumefaciens,* lane 3: 0 control (without template DNA), lane 4: control 'Richter 110', lanes 5-22: 18 independent transformed 'Richter 110' lines.
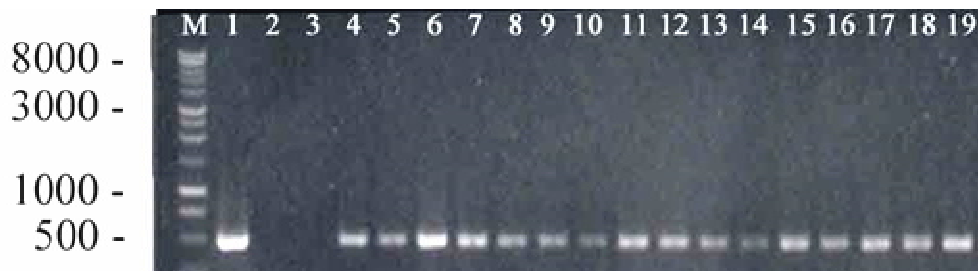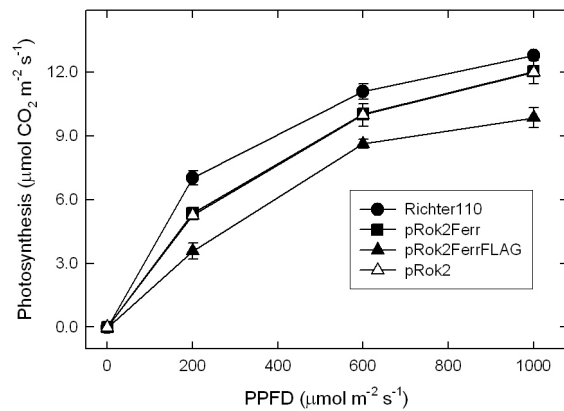

Figure 3.
PCR detection of the transferred DNA in grapevine plants using FerrFw1 and FerrRev2 primers. Lane 1: *A. tumefaciens* EHA105 (pRok2Ferr), lane 2: 0 control (without template DNA), lane 3: non-transformed 'Richter 110' DNA, lanes 4-19: 16 independent transformed 'Richter 110' lines.

Thus the tested regenerated plants were free of contaminating *Agrobacterium* cells and they can be considered as true transgenic lines. Next, the *in vitro* grown plants were acclimated to greenhouse conditions. Grapevines transformed with EHA105(pRok2Ferr) showed normal morphology and photosynthesis (Figs. 4 and 5). While pRok2Ferr transformed plants were indistinguishable from non-transformed ones, plants transformed with the FLAG construct (*pRok2::FLAG-MsFerr*) showed dwarf phenotype (data not shown) and early leaf senescence, although their photosynthesis was close to that of non-transformed and pRok2Ferr plants before the onset of senescence (Fig. 5). In this experiment, rates of CO_2 assimilation and transpiration were determined using a portable infrared gas analyser (LiCor, USA). Incident photosynthestic photon flux density (PPDF) was increased stepwise, registering the plants' responses to 200, 600, and 1000 μmol m^{-2} s^{-1} PPFD after 10 min. Gas exchange measurements were carried out with reference air from the greenhouse environment (380-420 μmol mol^{-1}, corresponding to the growth conditions).

Figure 4.
In vitro grown non-transformed (first from left) and three transformed 'Richter 110' plants acclimated to greenhouse conditions

Figure 5.
Photosynthesis measured as CO_2 assimilation in leaves of non-transformed controls and MsFerr transformed 'Richter 110' plants.

Expression levels of both endogenous grapevine and introduced *Medicago* ferritin genes were determined before and followed during stress experiments in comparison to internal control genes. To this end, we had to overcome two major initial obstacles.

One of these was the processing of grape leaves which are different from most plant material routinely used in our laboratory (such as tobacco or *Arabidopsis*). Harder texture, high polysaccharide and polyphenol contents made conventional RNA-isolation methods inefficient. The most robust and widely used Trisol method has failed to provide grapevine RNA in acceptable quantity and quality. We then tested several RNA isolation kits from leading molecular biology companies (Sigma, Roche, Macherei-Nagel, Qiagen, Gentra, Omega) with moderate success, then concluded in developing a new isolation method specially optimized for this plant material.

Second, neither genomic sequence data nor sequences of ferritin genes were available for *Vitis sp.* at the beginning of the project. Homology searches of publicly available EST databases with consensus sequences of plant ferritin genes resulted in finding two genes with high homology. Primer pairs were designed and obtained which amplify these two sequences with high specificity and no cross-reactivity with each other or with the *Medicago* ferritin gene.

Normalization of gene expression data to internal controls such as housekeeping genes is a widely used method for decreasing experimental errors. We tested six of the most frequently used such genes for their expression in the studied grapevine species (Fig. 6). We found that elongation factor 1α, malate dehydrogenase and S-adenosylmethionine decarboxylase had somewhat different expression patterns in the different species, while Actine, Ubiquitin, and

Glyceraldehyde 3-phosphate dehydrogenase were more similar and were thus chosen as internal controls.
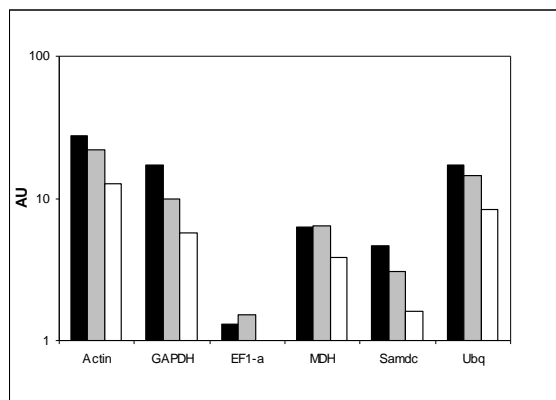
Figure 6. Expression of housekeeping genes in grapevine species
Expression levels of the indicated genes were assessed using Q-PCR in 'Richter 110' (black bars), 'Chardonnay' (grey bars) and transgenic 'Richter-110' (white bars) harbouring *Medicago* ferritin gene.

3.

Stress conditions in leaves were achieved by floating leaf diskettes on water solutions of one of the following elicitors of oxidative damage: paraquat, NaCl or *tert*-butyl-hydroperoxide (*t*-BHP). All treatments were carried out in light (50 μmol m^{-2} s^{-1} PPFD). Photochemical yield of the electron transport was calculated from parameters measured with a pulse amplitude modulated chlorophyll fluorimeter (Imaging PAM or Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany), according to Schreiber et al. (1997).

Salt stress by 250 mM NaCl resulted in more than 65% loss of photosynthesis in 'Richter 110' while this loss was only around 50% in all three pRok2Ferr lines (Fig. 7A).

Oxidative damage to lipids can lead to a variety of alkyl hydroperoxides, which are particularly detrimental due to their ability to initiate and propagate free radical chain reactions. *t*-BHP is a small molecule mimicking the effect of oxidative membrane damage. It is specially effective in the presence of free iron since it can generate very reactive ferryl (IV)-centered radicals (Schackleford et al. 2003). Responses of individual lines to 4 mM *t*-BHP varied, but two out of the three studied pRok2Fer lines, Fer9 and Fer11 showed significantly less stress-induced loss of photosynthesis than 'Richter 110' plants (Fig.7B).

Next, leaf disks were floated on 400 nM paraquat for 6 h in darkness, then for 2 h under 50 μmol m^{-2} s^{-1} PPFD. The dark period was used to ensure the osmotic uptake of the herbicide, which in the light is univalently reduced by the photosynthetic electron transport of chloroplasts to its cation radical, rapidly donating electrons to oxygen, and thus producing superoxide radicals *in*

6

situ (Babbs et al. 1989). Such artificial, high flux superoxide production exceeds the antioxidant ability of the plant and the excess superoxide and other ROS propagate oxidative damage in the membrane. Fig. 7C shows that leaves from two pRok2Fer plants lost less photosynthetic activity during this stress than non-transformed 'Richter 110' leaves.
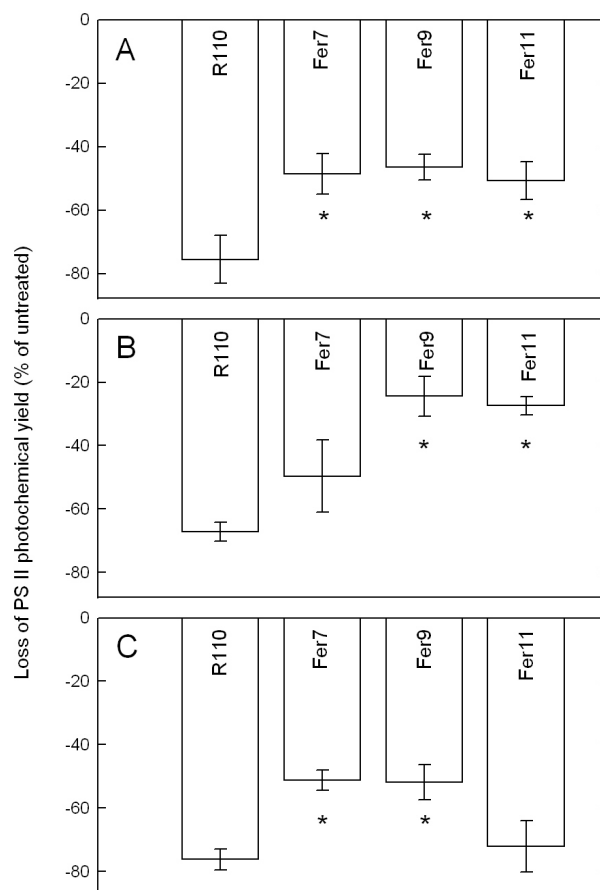
Figure 7.
Stress responses of non-transformed control (R-110) and transgenic MsFerr expressing (Fer7, Fer9, Fer11) 'Richter 110' to NaCl (A), tert-butyl-hydroperoxide (t-BHP) (B) and paraquat (C).

Effect of flooding (hypoxia) was studied on green cuttings: 3-weeks old plants were transferred from the soil-sand-perlite growth mixture to aerated 1/10 Hoagland solution and grown further for 3 weeks. A test solution was supplemented with 50 mM sodium bicarbonate (pH=8.3) and was flushed with N_2 gas before the plants were transposed into it for a 2-weeks treatment. Untreated plants were left in aerated Hoagland without bicarbonate. After 2 weeks, plant performance was evaluated by measuring photosynthetic gas exchange and photochemical yields using the techniques detailed above. In this experiment, transgenic plants did no show significantly higher tolerance to hypoxia/bicarbonate than non-transferred ones (data not shown).

Expression levels of endogenous grapevine ferritin genes and of the *Medicago* ferritin gene were also followed under various stress conditions. As illustrated by the example of paraquat stress in Fig. 8., this stress (as well as others, data not shown) resulted in an increased, nearly two orders of magnitude higher expression of one of the *Vitis* ferritin genes. This result is in good agreement with our original working hypothesis that ferritin plays an important role in mitigation of various stress conditions in grapevine. On the other hand, the expression level of the *Medicago* ferritin gene in the transgenic grapevine did not change significantly upon stress treatments due to the fact that it was expressed under the control of the 35S promoter of constitutive expression (Fig. 8B.).
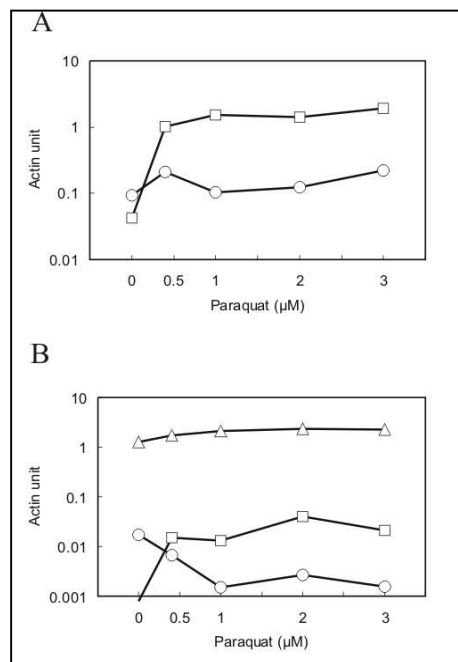
Figure 8. Expression levels of ferritin genes in grapevine leaves
The expression of the two *Vitis* ferritin genes (circles and squares, respectively) and the *Medicago* ferritin gene (triangles) was assessed in paraquat treated leaves. These levels are shown relative to actin gene levels in non-transformed (A) and transgenic (B) 'Richter 110' leaves.

It is important to note that the expression of *Medicago* ferritin resulted in significantly reduced expression levels of the endogenous ferritin genes (compare Figs. 8A and B), which may be explained as a feed-back regulation or some pleiotropic effect of the introduced gene. The level of the introduced *Medicago* ferritin gene was similar to that of its *Vitis* homolog under stress. In the absence of stress (0 μM paraquat data in Fig. 8.) the intracellular level of total ferritin mRNA was more than an order of magnitude higher in the transgenic plants than in the non-transformed ones. In this way, MsFerr expressing plants are already in a stress-responsive, high iron

scavenging capacity condition, unlike non-transformed plants, for which some oxidative damage is needed as a signal to achieve this state. The observed differences in the physiological stress responses of transformed and non-transformed grapevine plants may be explained by this advantage of the former ones. On the other hand, the relatively small extent of stress tolerance achieved by MsFerr expression may be due to similarities of total ferritin mRNA levels under extensive stress conditions.

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