A gene family encoding glutathione peroxidase homologues in *Hordeum vulgare* (barley)[1]

Yuri Churin, Silke Schilling, Thomas Börner*

Institute of Biology, Division of Genetics, Humboldt-University, Chausseestr. 117, D-10115 Berlin, Germany

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Abstract We have isolated and characterised three barley cDNAs encoding glutathione peroxidase (GPX) homologues, designated HVGPH1, HVGPH2 and HVGPH3. HVGPH1 may represent a cytosolic form of GPX. The structure of the HVGPH2 N-terminal domain is typical for a plastid transit peptide. A potential peroxisomal targeting sequence occurs near the N-terminus of HVGPH3. Transcript levels of HVGPH1 and HVGPH2 were increased in leaves undergoing stress. In contrast, HVGPH3 mRNA accumulation showed a negative response to stress. Our data indicate that the barley genome bears a small gene family encoding GPX homologues differing in their function and cellular localisation.

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Key words: Glutathione peroxidase; Oxidative stress; Chloroplast; Peroxisome; *Hordeum vulgare*

1. Introduction

Biotic and environmental stresses lead to an increase in antioxidant enzymes such as superoxide dismutases, ascorbate peroxidase, catalases, glutathione reductase and glutathione peroxidase (GPX) [1,2]. GPXs are diverse enzymes that catalyse the reduction of hydrogen peroxide, organic hydroperoxides and lipid hydroperoxides by reduced glutathione [3] and thus help to protect the cells against oxidative damage [4,5]. Several forms of GPX belong to the few proteins known to contain the rare amino acid selenocysteine [6]. In vertebrates various forms of GPX are known to exist: the cellular and cytosolic GPX, the extracellular plasma GPX, the cytosolic gastrointestinal GPX and the phospholipid hydroperoxide GPX (PHGPX). These isoenzymes differ in their structure, substrate specificity and tissue distribution (for review see [3]). In addition, vertebrates also contain epididymal GPXs, that differ from other forms by the replacement of selenocysteine by cysteine at their active site [7,8].

In plants, increased GPX activity was observed after cold treatment of apple callus [9] and after SO_2 fumigation of barley [10]. GPX activity has been detected in cell-free extracts from suspension cultures of several higher plants using both H_2O_2 and organic hydroperoxides as substrates [11]. However, at least part of this activity might have been due to the presence of glutathione *S*-transferase, known to exhibit GPX- in addition to its GSH-transferase activity [12]. Different cDNAs showing similarity to GPXs, in particular to animal PHGPXs, have been isolated from *Nicotiana sylvestris* [13], *Citrus sinensis* [14], *Avena fatua* [15], *Arabidopsis thaliana* [16] and *Lycopersicon esculentum* [17]. Only for two plant GPX homologues the subcellular localisation has been determined: putative PHGPXs from *A. thaliana* and *Pisum sativum* were shown to be targeted to the plastids [18].

Whereas the importance of animal GPXs for the protection of cells against oxygen radicals is well established, the role of GPXs in plants remains to be elucidated. A role in stress response is suggested by several observations. GPX mRNA levels have been shown to increase in tissues of several plant species undergoing stress, including germinating seeds [13,15], mesophyll protoplasts [13], salt-stress in suspension culture cells and plants [14,16,19], infection by viral or bacterial pathogens [20], treatment with heavy metals [13,16], and mechanical stimulation of the plant [17].

Here we report for the first time on a gene family in a monocotyledonous plant showing sequence similarity to animal genes coding for PHGPXs. We cloned and sequenced three cDNAs encoding *Hordeum vulgare* glutathione peroxidase homologues (HVGPHs): HVGPH1, HVGPH2 and HVGPH3. Sequence features of the deduced gene products suggest that they are located in different compartments. Moreover, we have examined the accumulation of HVGPH transcripts in barley leaves subjected to different stress conditions.

2. Materials and methods

2.1. Plant material

All experiments were performed with *H. vulgare* L. cv. Haisa (wild-type) and the mutant line *albostrians* derived from cv. Haisa. This mutant line produces green, white and green-white striped plants [21]. Seedlings were grown in moist vermiculite at 23°C under a 15 h light/9 h dark regime with an illumination of 32 W/m^2 and harvested 7 days after sowing. White *albostrians* seedlings were carefully checked to exclude contamination with small areas of green tissue. Seedlings grown in constant darkness were harvested under green safety light into liquid nitrogen and sorted under normal illumination into aetiolated yellow and white mutant plants. For treatment with Norflurazon (Dr Ehrenstorfer, Augsburg, Germany) seeds were soaked in 50 μM Norflurazon solution for 3 h and subsequently germinated for 7 days under continuous illumination (32 W/m^2). For treatment with NaCl, sorbitol and paraquat, primary leaves of wild-type seedlings were cut into segments of 5 cm length, starting 1 cm below the tip, and were floated in Petri dishes for 24 h on water or other solutions, as indicated in the legends to the figures.

2.2. South-Western screening

South-Western screening of a cDNA expression library from white seedlings of the barley *albostrians* mutant [22] was performed essen-

*Corresponding author. Fax: (49) (30) 2093-8141.
E-mail: thomas = boerner@rz.hu-berlin.de

[1] The sequences reported in this paper have been deposited in the EMBL data base (accession numbers AJ2338745 for HVGPH1, AJ238697 for HVGPH2 and AJ238744 for HVGPH3).

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tially according to Garabedian et al. [23] with the 108 bp fragment (from −250 to −142) of the wheat *cab1*-promoter [24]. This fragment was amplified by PCR using primers: C11, TTAAGCCAGCGGTCTCTTTCGACTT (from −250 to −226) and C12, CACGCAGAGGCCTTAAATGGATGAG (from −142 to −166) [24] and cloned into the vector pMOSBlue (Amersham). Plasmid with insert was cut with restriction endonucleases *Xba*I and *Bam*HI, labelled by filling in 5′ overhangs with ^{32}P-dCTP and Klenow fragment and purified by electrophoresis on polyacrylamide gel [25].

2.3. RNA isolation and Northern hybridisation

Total RNA was isolated following the protocol of Paulsen and Bogorad [26], separated on 1.4% (w/v) agarose-formaldehyde gels and transferred to Hybond-N+ nylon membrane by capillary blotting. To prepare RNA from the laef sections, 7 day old primary leaves were cut into 1 cm segments. Northern hybridisation was carried out in 0.12 M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, 7% (w/v) SDS and 50% formamide at 42°C. Filters were washed twice in 2×SSC, 0.1% SDS at 65°C for 15 min, then twice in 0.1×SSC, 0.1% SDS at 65°C for 20 min.

2.4. DNA isolation, Southern hybridisation and sequence analysis

Phage DNA was isolated, digested with restriction enzymes, separated by electrophoresis and transferred to nylon membrane by standard methods [25]. Southern hybridisation was carried out in 7% SDS and 250 mM Na[PO_4], pH 7.2, at 65°C (high stringency) or at 55°C

A. HVGPH1

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accctcctccccccaaactcccaactacccgctctgctcctcctgagtcctgactcctagcccgctcgatctccgccgcagccagccatc   90
ccaccgacgccgcctccgtccggccgcacggggaacctctccatggccgccgcgtcctccgccacctccgtccacgacttcaccgtcaag  180
                                        M  A  A  A  S  S  A  T  S  V  H  D  F  T  V  K    16
gatgcaagcggcaaggacGTCGACCTGAGCGTCTACAAGGGGAAGGTTCTTCTCATCGTTAACGTCGCATCCCAGTGTGGCTTAACGAAC  270
 D  A  S  G  K  D  V  D  L  S  V  Y  K  G  K  V  L  L  I  V  N  V  A  S  Q  C  G  L  T  N    46
TCCAACTACACCGAGCTGAGCCAGCTGTACCCCAAGTACAAGGACCAAGGCTTTGAGATCTTGGCTTTCCCATGCAATCAGTTTGGTGGG  360
 S  N  Y  T  E  L  S  Q  L  Y  P  K  Y  K  D  Q  G  F  E  I  L  A  F  P  C  N  Q  F  G  G    76
CAGGAGCCTGGCACCAATGATGAGATCGTTCAGTTTGCCTGCACTCGCTTCAAGGCCGAGTACCCCATTTTTGACAAGGTTGATGTCAAC  450
 Q  E  P  G  T  N  D  E  I  V  Q  F  A  C  T  R  F  K  A  E  Y  P  I  F  D  K  V  D  V  N   106
GGCAACAATGTTTCTCCCCTATACAAGTTCCTGAAGTCTAGCAAAGGCGGCTTTTTCGGCGACAGCATCAAATGGAACTTCTCTAAGTTC  540
 G  N  N  V  S  P  L  Y  K  F  L  K  S  S  K  G  G  F  F  G  D  S  I  K  W  N  F  S  K  F   136
TTGGTTGACAAGGAGGGTCACGTTGTGGACCGCTATGCCCCGACCACTTCCCCCATGAGCATTGAGAAGGATATCAAGAAGCTGCTTTAG  630
 L  V  D  K  E  G  H  V  V  D  R  Y  A  P  T  T  S  P  M  S  I  E  K  D  I  K  K  L  L  -   165
AGCTCTTAGACCTGCTGGCTGGAGCCTGTAGAGAAATCTGTCACTCTGCACTCAAGAAACATGAATAATGTTGTGTCAAAATAAAAATCT  720
GTATGTAGTATTTCAGAGGTTGCATCTACTGTATTTTCTGCATCCTACTCTGTACTTGTTGTATATGTCGTGTTAATGAATTCAATTATT  810
GTAGCTGTTTGATATGTATGCCTTTATTTGGATCATGTTGTTGTCTGGTCAAGTTACTGAAGAACATAATTTATCACATTGGTGCATCAC  900
CTTGATAGCAAAAAA                                                                              915
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B. HVGPH2

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CGAATAAAAGGAAATAAAAAAGCCGAAGCAGATATCGTTCTCCTCCCCGCGCACCCACGATTTCCGCTATAAAGCCCGGTCTCCGATCCC   90
CGATGCCTTCTCGCCCCGCCCCGTTCATCCACCGCCTCCGCCTCCTGAGAAGCGCCGTCGTCTCTCCCTCCTCCGCGCCGCTGCCGTGCA  180
   M  P  S  R  P  A  P  F  I  H  R  L  R  L  L  R  S  A  V  V  S  P  S  S  A  P  L  P  C    29
GCCGCCCGCGCCTCGCCGTCCCGTTCGCGCAGCCGCCGATCCGCGCCCCCGCCTTCGCCGCCGCCGGCTCGCCGCTGCTCCGCCCGGTCG  270
S  R  P  R  L  A  V  P  F  A  Q  P  P  I  R  A  P  A  F  A  A  A  G  S  P  L  L  R  P  V    59
GCGCCCGATTCTCGCTGTCCAGCAACATGGCCGCCGCCGCCTCCTCCGCCTCCTCCGTCCACGACTTCACCGTCGAGGATGCAAGTGGAA  360
G  A  R  F  S  L  S  S  N  M  A  A  A  A  S  S  A  S  S  V  H  D  F  T  V  E  D  A  S  G    89
AAGATGTCGATCTGAGCACCTACAAGGGGAAGGTTCTCCTCATTGTCAATGTTGCATCCCAGTGTGGATTGACCAATTCCAACTATACGG  450
K  D  V  D  L  S  T  Y  K  G  K  V  L  L  I  V  N  V  A  S  Q  C  G  L  T  N  S  N  Y  T   119
AACTCGCTCAGTTGTATGAGAAGTACAAGGACCAGGGTTTTGAGATCCTTGCTTTCCCATGCAACCAGTTTGGTGGGCAGGAACCTGGCA  540
E  L  A  Q  L  Y  E  K  Y  K  D  Q  G  F  E  I  L  A  F  P  C  N  Q  F  G  G  Q  E  P  G   149
CTAATGAGGAAATTGTTCAGTTTGCTTGCACTCGCTTCAAGGCCGAGTATCCAATTTTTGACAAGGTTGATGTCAATGGTGACAATGTTG  630
T  N  E  E  I  V  Q  F  A  C  T  R  F  K  A  E  Y  P  I  F  D  K  V  D  V  N  G  D  N  V   179
CACCTGTCTACAAGTTTCTGAAGTCAAGCAAAGGCAGTCTCTTCGGGGACAACATCAAATGGAACTTCTCCAAGTTCTTGGTTGACAAGG  720
A  P  V  Y  K  F  L  K  S  S  K  G  S  L  F  G  D  N  I  K  W  N  F  S  K  F  L  V  D  K   209
ATGGGAATGTTGTGGATCGCTACGCGCCGACCACCTCCCCCCTCAGCATCGAGAAGGACATCAAGAAGCTGCTCGCGAGTTCTTAAATCT  810
D  G  N  V  V  D  R  Y  A  P  T  T  S  P  L  S  I  E  K  D  I  K  K  L  L  A  S  S  -      237
TTATGCTGGATCAACTCGACGCATCGGTACACCTGAAGCTTCAATAATGTTGTAATAAGGGTCGTGCTCGGAACTGGCTATGTTATGCGT  900
GCTCAGCAGCCTCCGATACCTTGCGCATTCCAATCTCGTATTGCCCGTCTCGTAGTATATATGACATGTAATGGATTGGGTGGAATGCAT  990
TACTTCTGTTGCAAAAAAAAAAAAAAAAAAAAAAA                                                         1026
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C. HVGPH3

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GTCGACCCACGCGTCCGGTTGTTCAGGGTGCTGCCGCTTAACTCTTCTCCAATCCTTCCTCTAGTTATTGCTGAAAGCAAAGATGGGCGC   90
                                                                                  M  G  A     3
AGCAGAGTCGTCTTCCAAGCTTGGTGGTTCCGTCCATGACTTCGTCGTTAAGGATGTGAGAGGAAATGATGTGGAGCTCAGCAGATACAA  180
  A  E  S  S  S  K  L  G  G  S  V  H  D  F  V  V  K  D  V  R  G  N  D  V  E  L  S  R  Y  K   33
GGGGAAAGTCCTGCTTATTGTCAATGTCGCATCTCGATGCGGTCTGGCCAATTCCAACTACACGGAAATGGGCCAGCTCTATGAGAAATA  270
  G  K  V  L  L  I  V  N  V  A  S  R  C  G  L  A  N  S  N  Y  T  E  M  G  Q  L  Y  E  K  Y   63
CCGGGAGAAAGGTTTGGAGATATTGGCGTTCCCCTGCAATCAATTTGCCGGGCAGGAACCAGATAGCGATGAGAAGATTGTGGAGTTTGC  360
  R  E  K  G  L  E  I  L  A  F  P  C  N  Q  F  A  G  Q  E  P  D  S  D  E  K  I  V  E  F  A   93
TTGCGACCGCTTCCAAGCACAGTTTCCTATTTTTCGCAAGGTGGACGTGAATGGCAACAATGCTGCCCCGCTGTACAAGTTCTTGAAGTC  450
  C  D  R  F  Q  A  Q  F  P  I  F  R  K  V  D  V  N  G  N  N  A  A  P  L  Y  K  F  L  K  S  123
AGAGAGGGGCGGTCTATTCGGAGAGCGTATCAAATGGAACTTCACCAAGTTTCTAGTTGACAAAGAGGGGCATGTCATGAATCGATATGC  540
  E  R  G  G  L  F  G  E  R  I  K  W  N  F  T  K  F  L  V  D  K  E  G  H  V  M  N  R  Y  A  153
ACCGACCTGGTCCCCACTCGGCATTGAGAATGATATCAAGAAGCTGTTGGAGGTTTGAGGTATCTCAAGGGAAGATCATGCGTCGTCTGT  630
  P  T  W  S  P  L  G  I  E  N  D  I  K  K  L  L  E  V  -                                   171
GTCATGTTTTCGCTACTATATGTGTATGTACACTTGCTCTATCATGAGTAACCCCAGCCTGTTGTATGCTACTCTGTTTTCTACACTGTG  720
AAAGGAAAGCAACATTCCGCTGAAATAAAAAAAAA                                                          755
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Fig. 1. Nucleotide and deduced amino acid sequences of the barley cDNAs encoding HVGPH1 (A), HVGPH2 (B) and HVGPH3 (C). Numbers at the right margin refer to the base pair and the amino acid positions. Codons for cysteine in GPX active sites are in bold and underlined. A: The 5′ end of the w541 insert is in lower-case characters. B: Two in-frame methionine residues are in bold and underlined.

Table 1
Amino acid sequence similarity of putative GPXs from barley (HVGPH1, HVGPH2, HVGPH3), *A. thaliana* (ATPG1, GPX1), *C. sinensis* (GPXCs), *N. sylvestris* (GPXNs), *L. esculentum* (GPXLe), *S. oleracea* (GPXSo) and *P. sativum* (GPXPs)

	HVGPH1	HVGPH2[a]	HVGPH3	ATGP1	GPXCs	GPXNs	GPXLe	GPXSo	GPX1[a]	GPXPs[a]
HVGPH1	100	95.2	82.4	90.3	89.1	89.7	89.7	89.7	80.6	79.4
HVGPH2		100	78.7	89.9	89.2	86.4	85.8	87.6	79.2	78.0
HVGPH3			100	79.9	80.2	76.9	76.3	82.5	71.4	72.0
ATGP1				100	90.4	89.9	88.8	92.3	78.0	79.8
GPXCs					100	89.2	87.4	89.8	76.1	78.4
GPXNs						100	97.0	89.3	76.2	78.6
GPXLe							100	88.2	76.2	78.0
GPXSo								100	77.4	76.8
GPX1									100	89.9
GPXPs										100

[a]Amino acid sequences starting with region of high homology were used for comparison

(low stringency). Probes for hybridisation were labelled to high specific activity by random priming (using an Amersham *redi*prime kit) according to the procedure recommended by the manufacturer (Amersham, Braunschweig, Germany). Filters were washed twice in 2×SSC, 0.1% SDS at temperature of hybridisation for 15 min, then twice in 0.1×SSC, 0.1% SDS at 65°C for 20 min, if specific probe was used.

DNA sequences were analysed using a thermal cycle amplification system (Bio-Rad, Hercules, CA, USA) and an ABI 373 automatic DNA sequencer (Applied Biosystems, Perkin Elmer). Database searches and general sequence comparisons relied on the BLAST world wide web server at the National Center for Biotechnology Information [27]. Amino acid alignments were performed using the ClustalW program [28].

3. Results

3.1. Isolation and sequence analysis of cDNAs encoding putative glutathione peroxidases from barley

The first barley cDNA encoding a putative PHGPX was fortuitously identified during screening of a cDNA expression library from white seedlings of the barley *albostrians* mutant for DNA binding proteins using a 108 bp fragment of the wheat *cab1*-gene promoter: one clone, designated w54, was found to bind the probe through two rounds of screening and plaque purification. Further analysis revealed an unspe-

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HVGPH3  ------------------------------------------------------------MGAAESSSKLGGSV
GPXSo   ------------------------------------------------------------MASDSSAQPKSV
HVGPH1  ------------------------------------------------------------MAAASSATSV
HVGPH2  MPSRPAPFIHRLRLLRSAVVSPSSAPLPCSRPRLAVPFAQPPIRAPAFAAAGSPLL-RPVGARFSLSSNMAAAASSASSV
ATGP1   ------------------------------------------------------------MAASSEPKSL
GPXNs   ------------------------------------------------------------MASQSSKPQSI
GPXLe   ------------------------------------------------------------MATQTSNPQSV
GPXCs   ------------------------------------------------------------MASQSKTSV
GPX1    MVSMTTSSSSYGTFSTVVNSSRPNSSATFLVPSLKFSTGISNFANLSNGFSLKSPI-NPGFLFKSRPFTVQARAAAEKTV
GPXPs   -MASMAFSTTFFTPLRDFNQPRTNSTPSTSLPFTKSSIASSKSPFFQLGFSQQASSNFPIVPSKTRSFSVNAKAIKDKTI
                                                                          :    ::

HVGPH3  HDFVVKDVRGNDVELSRYKGKVLLIVNVASRCGLANSNYTEMGQLYEKYREKGLEILAFPCNQFAGQEPDSDEKIVEFAC
GPXSo   HEFVVRDARGNDVDLSIYKGKVLLIVNVASQCGLTNSNYTEMTELYEKYRELGLEILAFPCNQFGNQEPGSNEEVLEFAC
HVGPH1  HDFTVKDASGKDVDLSVYKGKVLLIVNVASQCGLTNSNYTELSQLYPKYKDQGFEILAFPCNQFGGQEPGTNDEIVQFAC
HVGPH2  HDFTVEDASGKDVDLSTYKGKVLLIVNVASQCGLTNSNYTELAQLYEKYKDQGFEILAFPCNQFGGQEPGTNEEIVQFAC
ATGP1   YDFTVKDAKGNDVDLSIYKGKVLLIVNVASQCGLTNSNYTELAQLYEKYKGHGFEILAFPCNQFGNQEPGTNEEIVQFAC
GPXNs   YDFTVKDAKGNDVDLSIYKGKVLIIVNVASQCGLTNSNYTDLTEIYKKYKDQGLEILAFPCNQFGGQEPGSIEEIQNMVC
GPXLe   YDFTVKDAKGKDVDLSIYKGKVLIIVNVASQCGLTNSNYTDMTELYKKYKDQGLEILAFPCNQFGGQEPGNIEDIQQMVC
GPXCs   HDFTVKDAKGQDVDLSIYKGKLLLIVNVASQCGLTNSNYTELSQLYDKYKNQGLEILAFPCNQFGAQEPGDNEQIQEFAC
GPX1    HDFTVKDIDGKDVALNKFKGKVMLIVNVASRCGLTSSNYSELSHLYEKYKTQGFEILAFPCNQFGFQEPGSNSEIKQFAC
GPXPs   YDFTVKDIDKKDVSLSKFKGKVLLIVNVASRCGLTSSNYTELSHLYENFKNKGLEVLAFPCNQFGMQEPGSNEEIKQFAC
        ::*.*.*   :** *. :***:::******:***:.***::: .:* :::  *:*:********. ***.  ..: ::.*

HVGPH3  DRFQAQFPIFRKVDVNGNNAAPLYKFLKSERGGLFGERIKWNFTKFLVDKEGHVMNRYAPTWSPLGIENDIKKLLEV--
GPXSo   TRFKAEYPIFDKVDVNGSNAAPIYKFLKSSKGGLFGDGLKWNFTKFLVDKDGNVVDRYAPTTSPKSIEKDVKKLLGIQK
HVGPH1  TRFKAEYPIFDKVDVNGNNVSPLYKFLKSSKGGFFGDSIKWNFSKFLVDKEGHVVDRYAPTTSPMSIEKDIKKLL----
HVGPH2  TRFKAEYPIFDKVDVNGDNVAPVYKFLKSSKGSLFGDNIKWNFSKFLVDKDGNVVDRYAPTTSPLSIEKDIKKLLASS-
ATGP1   TRFKAEYPIFDKVDVNGDKAAPVYKFLKSSKGGLFGDGIKWNFAKFLVDKDGNVVDRFAPTTSPLSIEKDVKKLLGVTA
GPXNs   TRFKAEYPIFDKVDVNGDNAAPLYKFLKSSKGGFFGDSIKWNFSKFLVDKEGNVVDRYSPTTTPASMEKDIKKLLGVA-
GPXLe   TRFKAEYPIFDKVDVNGDNAAPLYRFLKSSKGGFFGDGIKWNFSKFLIDKEGHVVDRYSPTTSPASMEKDIKKLLGVA-
GPXCs   TRFKAEFPIFDKVDVNGDNAAPLYKHLKSSKGGLFGDSIKWNFSKFLVDKEGNVVERYAPTTSPLSIEKDIKKLLETA-
GPX1    TRFKAEFPIFDKVDVNGPSTAPIYEFLKSNAGGFLGGLIKWNFEKFLIDKKGKVVERYPPTTSPFQIEKDIQKLLAA--
GPXPs   TKFKAEFPIFDKVDVNGPFTAPVYQFLKSSSGGFFGDIVKWNFEKFLVDKNGKVVERYPPTTSPFQIEKDIQKLLAA--
         :*:*::*** ******  .:*:*..***. *.::*  :**** ***:**.*:*::*:.** :*  :*:*::***
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Fig. 2. Comparison of the amino acid sequences of putative plant GPXs. Alignment of barley HVGPH1, HVGPH2 and HVGPH3 with putative GPXs from: *Spinacia oleracea* (GPXSo) [30], *A. thaliana* (ATGP1) [16], *A. thaliana* (GPX1) [18], *N. sylvestris* (GPXNs) [13], *L. esculentum* (GPXLe) [17], *C. sinensis* (GPXCs) [14], and *P. sativum* (GPXPs) [18]. Asterisks and dots indicate identical and similar amino acid positions, respectively. Amino acid sequences corresponding to conserved regions are marked as follows: the GPX selenocysteine active site (or GPX signature 1) is in bold; the GPX signature 2 is in bold and underlined; domain WNF(S/T)KF found in most plant and mammalian GPXs is in bold and italics.

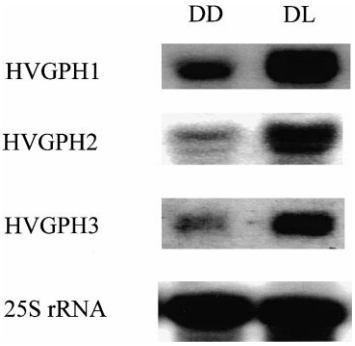

Fig. 3. The effect of light on the accumulation of HVGPH transcripts in barley seedlings. Total RNA was isolated from aetiolated (DD) or 6 h illuminated (DL) primary leaves of wild-type seedlings.

cific binding to DNA (data not shown). The respective phage was isolated and its insert was subcloned into the vector pBluescript KS+ (Stratagene) for further analysis. Sequence analysis of the 717 bp w54 insert showed similarity to PHGPX genes. Unspecific DNA binding of a GPX is not a surprising finding because several enzymes were shown to bind to nucleic acids even though they lack known binding motifs [29]. Clone w54 did not represent a full-length cDNA. To obtain a complete cDNA clone, the w54 insert was used as a probe to rescreen the cDNA library at high stringency. Sequence analysis of the longest recombinant clone identified, designated w541, demonstrated that it represented an extended version of w54 containing additional 198 bp at the 5′ end (Fig. 1A). The longest open reading frame within clone w541 encodes a protein of 165 amino acids with a predicted molecular mass of 18.3 kDa (Fig. 1A). Comparison of the w541 deduced amino acid sequence with protein sequences available in NCBI data bases revealed that this protein is most closely related to putative PHGPXs from plants. We designated this protein as HVGPH1. We have then used the w54 insert to screen the cDNA library at low stringency. Three positive clones were recovered from approximately 50 000 recombinant plaques. Inserts from isolated phages were subcloned into pBluescript KS+ and sequenced. Sequence analysis revealed open reading frames with high similarity to GPX genes in all three clones. Two of the three cDNA clones were obviously derived from identical mRNAs. The longer one of the two cDNAs, designated w12, was selected for further analysis. The third cDNA was designated w15. The cDNA insert of 1026 bp from clone w12 was found to contain two ATG codons that are in frame: a first ATG at position 93, a second one at position 297 (Fig. 1B). The longest open reading frame encodes a protein of 237 amino acids with a predicted molecular mass of 25.7 kDa (HVGPH2). The shorter open reading frame encodes a protein of 169 amino acids with a predicted molecular mass of 18.6 kDa (Fig. 1B). Clone w15 contained a cDNA insert of 755 bp. The longest open reading frame encodes a protein of 171 amino acids with a predicted molecular mass of 19.3 kDa (HVGPH3) (Fig. 1C).

A comparison of the three HVGPHs from barley and putative PHGPXs from other plants revealed a high sequence similarity ranging between 71 and 96% (Table 1, Fig. 2). Amino acid sequences of all analysed GPXs contain three conserved domains: GPX signature 1, GPX signature 2 and domain WNF(S/T)KF found in most plant and mammalian GPXs (Fig. 2) [31–34]. These three domains contain amino acid residues which form part of the proposed catalytic site of GPX, namely C and G in GPX signature 1, Q in GPX signature 2 and WNF in the third conserved domain (Fig. 2) [35].

3.2. Accumulation of HVGPH mRNAs under various stress conditions

To examine whether HVGPHs respond to light, Northern blot analysis of total RNA was carried out. RNA was isolated from aetiolated (DD) and from first dark-grown and than illuminated (DL) wild-type leaves. Transcript levels for all three HVGPHs were very low in aetiolated leaves and found to be drastically elevated after 6 h of illumination (Fig. 3).

Stress-related genes were recently reported to show an enhanced expression in white compared with green leaves of the barley *albostrians* mutant [22]. To determine if the expression of HVGPH mRNAs is also affected in white leaves of the *albostrians* mutant, we examined steady-state levels of HVGPH transcripts in green and white mutant leaves. For comparison, total RNA from wild-type leaves treated with the bleaching herbicide Norflurazon was used. Norflurazon inhibits the synthesis of carotenoids in plant leaves. The lack of these protective pigments results in the destruction of pigment-protein complexes by photo-oxidation and blocks chloroplast development [36]. HVGPH1 and HVGPH2 exhibited nearly the same transcript accumulation pattern: a very low steady-state level in green leaves that was drastically increased in white leaves of the mutant and in wild-type leaves treated with Norflurazon (Fig. 4). In contrast, the level of HVGPH3 mRNA was highest in green, untreated leaves whereas its accumulation in white leaves and in wild-type Norflurazon-treated leaves was barely detectable (Fig. 4).

To examine the effect of different stress factors on HVGPH mRNAs, we subjected primary leaves of 7 days old wild-type barley seedlings to salt/osmotic stress (400 mM NaCl and 1 M sorbitol, respectively) and oxidative stress produced by the herbicide paraquat. Northern blot analysis of total RNA isolated from treated leaves revealed that HVGPH1 and HVGPH2 transcript abundance markedly increased in leaves treated with any of those stress factors (Fig. 5). Interestingly, HVGPH2 mRNA accumulation increased stronger under salt stress than by paraquat treatment whereas HVGPH1 transcript level was higher after paraquat treatment. In contrast, the HVGPH3 mRNA accumulation was drastically decreased under all stress conditions (Fig. 5).

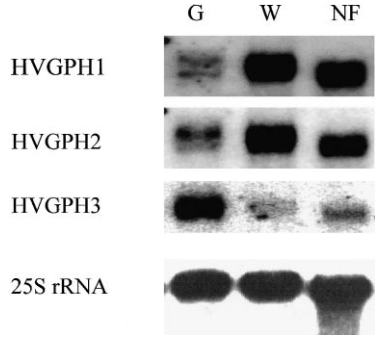

Fig. 4. Steady-state level of HVGPH transcripts in green (G) and white (W) seedlings of the *albostrians* mutant and in wild-type, Norflurazon-treated (NF) seedlings.

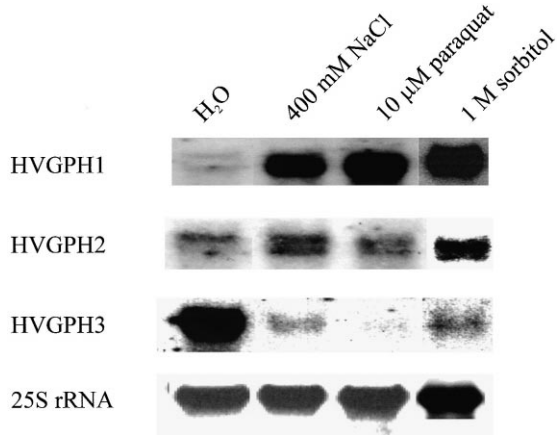

Fig. 5. Accumulation of HVGPH mRNAs in barley leaves undergoing stress. Total RNA was isolated from segments (5 cm in length) of wild-type primary leaves floated on water (H_2O), 400 mM NaCl, 10 μM paraquat, or 1 M sorbitol solutions for 24 h.

4. Discussion

The cDNAs analysed in this study are closely related to cDNAs isolated from several plants and encoding putative PHGPX [13–18]. Only for one of these gene products, the activity of a PHGPX has been experimentally proven [37]. The HVGPH2 cDNA contains two ATG codons that are in-frame (Fig. 1B). The amino acid sequence between first and second in-frame methionines is high in serine, contains no acidic residue, and has a net positive charge (+7), features reminiscent of plastid targeting sequences [38,39]. Hence, HVGPH2 is likely to be plastid localised as recently demonstrated for an Arabidopsis and a pea GPX ([18]; Fig. 2). This conclusion is further substantiated by a computer-aided analysis of the putative targeting sequence using the program PSORT (data not shown). HVGPH1 and HVGPH3 have no apparent plastidial or mitochondrial presequences. Moreover, a stop codon not far upstream of the start codon for HVGPH1 rules out the possibility that an additional targeting sequence is encoded. HVGPH1 represents most probably a cytosolic form of the enzyme. HVGPH3, however, contains near the N-terminus an SSS sequence (position 6–8) (Fig. 1C), which has been shown to be a peroxisomal targeting signal in cotton catalase [40]. The peroxisomal catalases CAT-2 from barley, CAT-A from rice and CAT-3 from maize also contain this tripeptide near the N-terminus (position 9–11) [41]. Thus, HVGPH3 may be a peroxisomal glutathione peroxidase. While animal PHGPX genes were found to include the codon TGA for selenocysteine, the codon for the corresponding amino acid residue in homologous plant genes, including HVGPH1 and HVGPH2 cDNAs (Fig. 1A,B), is TGT [17,18,42], which encodes a cysteine residue [43]. The corresponding codon in HVGPH3 is TGC (Fig. 1C). Thus, the three barley enzymes, as the other known putative plant GPX, should contain cysteine instead of selenocysteine in their catalytic centre.

It has been observed that the expression of some antioxidant genes is regulated by light [44–46]. The transcript accumulation of all three HVGPHs was increased after illumination of aetiolated leaves.

It has been shown that the steady-state level of putative GPX mRNA and/or GPX protein increases in plant tissues under stress conditions like high osmolarity, salt, H_2O_2 and pathogen infection [13–15,20,47] suggesting a function of these enzymes in the stress response of plants. The data obtained from our studies on HVGPH transcript levels suggest that the putative chloroplast HVGPH2 and the putative cytosolic HVGPH1 may play a role in the response of barley plants to stress, whereas the putative peroxisomal HVGPH3 should have another, hitherto unknown function. Treatment of barley leaf segments with NaCl or sorbitol induced the accumulation of both HVGPH1 and HVGPH2 transcripts (Fig. 5). Levels of HVGPH1 and HVGPH2 mRNA were also much higher in white *albostrians* leaves and Norflurazon-treated wild-type leaves than in green, untreated leaves (Fig. 3). Enzymes with suggested or proven function in stress response show much higher levels of their transcripts in white than in green leaves of *albostrians* mutant. White *albostrians* leaves are deficient in chlorophyll and carotenoids [21,22]. Norflurazon acts as a non-competitive inhibitor of phytoene desaturase [48] and blocks the synthesis of all photo-protective carotenoids. If carotenoid-free chloroplasts are exposed to bright light they suffer extensive oxidative damage [49]. Therefore, it is probable that the increase of mRNA accumulation for HVGPH1 and HVGPH2 is a response to oxidative stress. This view is supported by our results obtained with paraquat-treated leaves. Bipyridyl herbicides such as paraquat are redox-active compounds that become reduced within the cell and subsequently transfer their electrons to oxygen, forming the superoxide anion [50,51]. Their main activity is exhibited in the light, where photosystem I is responsible for their reduction [52]. Significant increases in mRNA steady-state levels of some antioxidant enzymes in response to paraquat stress were observed [46,53]. Moreover, it has been recently shown that *Escherichia coli* cells expressing the cDNA for a putative *Citrus* GPX, were more tolerant to paraquat [54]. The treatment of barley leaf segments with paraquat increased the transcript level for HVGPH1 and HVGPH2 drastically (Fig. 5). The cytosolic compartment is most likely also involved in antioxidant responses to paraquat [1]. Thus, HVGPH1 and HVGPH2 may contribute to antioxidant defences in cytosol and chloroplast, respectively.

In contrast, analysis of mRNA levels for HVGPH3, which, as we suppose, is localised to peroxisomes, showed a distinctly different pattern. The HVGPH3 transcript level was reduced after treatment of barley leaf segments with NaCl, sorbitol and paraquat as well as in white seedlings of the *albostrians* mutant and in Norflurazon-treated wild-type leaves Interestingly, similar observations have been reported for other peroxisomal enzymes. The maize *Cat2* mRNA level was very low in the leaves of mutants *lw3* and *w3*, that suffer from photooxidation due to the lack of protective carotenoids [55,56]. Moreover, it has been shown that activities of peroxisomal enzymes such as glycolate oxidase, hydroxypyruvate reductase and catalase were very low after treatment of plants with Norflurazon [57,58]. In white *albostrians* leaves not only activities but also mRNA accumulation for glycolate oxidase and catalase were drastically reduced compared to green leaves [59]. Thus, HVGPH3 may be involved in peroxisomal metabolism rather than in the defence of plants against oxidative stress.

Although the role of plant GPX homologues is still obscure, the data presented here support the view that the plant GPX homologues may play a role in the protection of cells

against oxidative damage. For the first time we could demonstrate the existence of three GPX homologues in a plant which probably are localised to different compartments and could show that their transcript accumulation responses in different ways to various stress conditions.

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