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Microarray analysis of spring barley cultivars displaying differing sensitivity to physiological leaf spot (PLS)

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Abstract

Physiological leaf spot (PLS) is a disorder of spring barley (*Hordeum vulgare* L.), which has become more pronounced in recent years. The initial symptoms are small chlorotic/brown spots on the upper four leaves, which may develop into necrotic lesions with an irregular shape. As PLS occurs on leaves that are directly exposed to sunlight, it is thought that high light stress could be a trigger for the condition. This study concentrates on two cultivars, Cooper and Crusader, which display differential sensitivity to PLS. Biochemical measurements and enzyme assays revealed substantial difference in levels of ascorbate, type III peroxidases, and superoxide dismutase between the chosen cultivars during the 2003 growing season. A global gene expression study, using these field samples, was performed by microarray analysis. This supported the biochemical findings and highlighted additional sets of genes differentially expressed between the cultivars. Transcripts of particular interest, which appeared, included calcium signalling genes, cold-responsive genes and those involved in the assembly of Photosystem I. We conclude that susceptibility to PLS is related to levels of expression of genes with a role in countering the effects of oxidative stress.

Keywords

abiotic stress • non-parasitic necrosis • physiological leaf spot

Abbreviations: GS, growth stage; PVP, polyvinylpyrrolidone; TCA, trichloroacetic acid; PLS, physiological leaf spot; SOD, superoxide dismutase; APX, ascorbate peroxidase, CAT, catalase, GPX, glutathione peroxidase

Introduction

The disorder termed physiological leaf spot (PLS) has been documented globally since the 1980s (Wu and von Tiedemann, 2002). Since 1997, Irish spring barley (*Hordeum vulgare* L.) growers have encountered this disorder, with severe outbreaks in 1998 and 1999 (Burke, Hackett and O'Sullivan, 2001). Symptom severity varies between cultivars and can fluctuate from year to year, probably as a result of different environmental conditions. PLS is characterised by the appearance of small chlorotic/brown spots, which become larger, darker and more pronounced lesions as the disorder progresses. The initial spots appear on the third leaf at growth stage (GS) 37–39 (Tottman and Broad, 1987) and continue to advance from there to the second leaf and then to the flag leaf and awns at 7-to-10-day intervals until GS 59–65, depending on the sensitivity of the cultivar. This damage to the flag leaf and awns decreases their photosynthetic area and thus affects grain filling (Lu and Lu, 2004) with subsequent yield losses of 10–20% (Wu and von Tiedemann, 2002).

Definitive proof of aetiology in non-parasitic diseases requires an absence of pathogen and reproducibility in healthy plants grown under simulated conditions in a controlled environment. To date, there has never been any biotic agent isolated from affected leaves and PLS can be simulated under growth room conditions (Wu and von Tiedemann, 2004). In addition, a quantitative trait locus (QTL), which affects resistance, has been localised at the *mlo* locus on chromosome 4H (Behn *et al*., 2005). PLS symptom initiation depends on the severity of the stress the plant is subjected to during the main growth period. It has been observed that spot development can occur after a sequence of humid, overcast days followed by sunny, dry days, and is confined to those parts of the plant with direct exposure to sunlight (Wu and von Tiedemann, 2004).

Oxidative stress was determined to be the cause of the lesions as affected leaves contain high concentrations of reactive oxygen species (ROS), particularly superoxide ($O_2^{\bullet-}$) and H_2O_2, surrounding the necrotic area (Wu and von Tiedemann, 2002). An enhanced rate of ROS production because of abiotic stress is coupled with an increase in ROS scavenging enzyme activity in order to detoxify such hazardous accumulation (Rizhsky *et al*., 2002; Apel and Hirt, 2004). The main ROS scavenging enzymes within the plant system are superoxide dismutase (SOD), ascorbate peroxidase (APX) and the Class III peroxidases, catalase (CAT) and glutathione peroxidase (GPX) (Yoshimura *et al*., 2004).

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The aim of the present study was to explore whether differences in PLS susceptibility between spring barley cultivars was related to ROS scavenging capacity or other potential stress defences. To address this, biochemical analysis of field material harvested from trials at Oak Park Research Centre, Carlow, Ireland, was carried out with particular emphasis on ROS scavenging enzyme activity and antioxidant content. A microarray experiment was also conducted to compare the transcriptomes of PLS-susceptible (Cooper) and PLS-tolerant (Crusader) cultivars, in order to determine the extent of differential gene expression on induction of PLS in the field. This report describes numerous subtle changes in expression of genes linked to ROS stress amelioration, which could contribute to cultivar differences in PLS susceptibility.

Materials and Methods

Field material

Field studies of *H. vulgare* L. cvs Cooper and Crusader were conducted at Teagasc, Oak Park Research Centre, Co. Carlow, Ireland, in the summer of 2003. The trials were conducted in twelve 2 m × 20 m plots, six plots per cultivar, in a grid in which the relevant trial plots were separated by blank plots or plots containing cultivars not included in the reported study. All testing was done on the leaves immediately below the flag leaf (F-1), and sampling was carried out on four dates during the month of June, spanning the period when PLS appears on the leaves of the susceptible cultivar, Cooper (see Results). For sampling, a single leaf was removed from each plot, providing six samples for each cultivar. In order to relate the findings to climatic conditions before and during sampling, monthly reports were obtained from the Met Éireann weather station in Co. Kilkenny, Ireland.

Chemicals

All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

Enzyme assays

Five hundred milligram of tissue was frozen with liquid nitrogen and ground to fine powder. Five millilitre of extraction buffer (100 mM potassium phosphate, pH 7 [Sambrook]; 1 mM ethidium bromide (EDTA) [BDH, London, UK]; 1% Triton X-100; 1% polyvinylpyrrolidone (PVP) [soluble, molecular weight (MW) 40,000]) was then added to the ground leaf tissue and agitated until thawed. The resultant mix was then centrifuged at 16,000 ×g for 15 minutes at 4°C. The supernatant was removed and set aside for analysis of Type III peroxidases, SOD and ascorbic acid content. In order to determine APX activity, the extraction buffer above was supplemented with 1 mM ascorbic acid. Soluble protein content was determined using the Bio-Rad Protein Assay Kit, (Bio-Rad Laboratories Inc., UK) according to the Bradford method (Bradford, 1976). Ascorbate peroxidase activity was determined by measuring the decrease in absorbance of the sample at 290 nm (extinction coefficient = 2.8 mM^{-1} cm^{-1}) as described previously (Lee and Lee, 2000). Total SOD activity was determined by the inhibition of nitrite formation from hydroxylamine in the presence of $O_2^{\bullet -}$ generators (Elstner and Heupel, 1976) and by reference to a standard curve. Type III peroxidase activity was measured by monitoring the formation of guaiacol dehydrogenation product at 436 nm (extinction coefficient = 6.39 mM^{-1} cm^{-1}) as described (Lee and Lee, 2000).

Antioxidant assays

Total foliar ascorbate determination was based on the reduction of ferric ions by ascorbate, coupled with the formation of a red-coloured chelate of ferrous ions and bathphenanthroline (Arakawa *et al.*, 1981). The absorbance of the resulting red-pink solution was read at 534 nm, and the amount of ascorbate in the sample was determined by reference to a standard curve and expressed in mM mg^{-1} protein.

Glutathione content was measured spectrophotometrically using glutathione reductase to quantify reduced glutathione (GSH) according to a published method (Anderson, Chevone and Hess, 1992). The extraction procedure was as follows: 500 mg of tissue was frozen with liquid nitrogen and ground to fine powder. To this, 10 ml of 5% trichloroacetic acid (TCA) was added, mixed thoroughly, poured into a centrifuge tube and centrifuged at 15,000×g for 20 minutes at 4°C. The supernatant was filtered through a 0.45-nm Acrodisc and retained for analysis. The reactions were run in 1.2 ml volumes containing 400 µl reagent 1 [110 mM Na_2HPO_4, 40 mM NaH_2PO_4, 15 mM EDTA (BDH), 0.3 mM Dithiobis-(2-nitobenzoic acid) (DTNB), 0.04% *bovine serum albumin* (BSA)], 320 µl reagent 2 (1 mM EDTA (BDH), 50 mM imidazole, 0.02% BSA, 1.5 U glutathione reductase) and 400 µl of a 1:50 dilution of the acid extract in 5% Na_2HPO_4, pH 7.5. The reaction was started by the addition of 80 µl of 9 mM NADPH. The absorbance was measured after 4 minutes incubation at 412 nm. The sulphhydryl group of GSH reacts with colourless DTNB to produce yellow 5-thio-2-nitrobenzoate (TNB). The rate of TNB production is directly proportional to the GSH concentration; therefore, measurement of TNB absorbance at 412 nm is an accurate method of determining GSH content. The amount of total glutathione present was determined by reference to a standard curve and expressed in mM mg^{-1} protein.

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Pigment analysis

Anthocyanin content was analysed using a modified protocol (Reddy *et al*., 1994). About 0.5 cm^2 of leaf tissue was placed in 5 ml of acidified methanol [1% (v/v) HCl (BDH)]. The samples were then stored in the dark for 24 hours at 4°C. Anthocyanin content was estimated by measuring the absorbance of 200 µl of the extract at 535 nm (extinction coefficient = 31623 mM^{-1} cm^{-1}) and expressed in nM cm^{-2}.

Graphing and analysis

Standard curves were constructed and spectrophotometric data analysed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com (Motulsky and Christopoulos, 2004). The data were analysed with a two-way factorial structure of cultivar × time (2 × 4). As there were repeated measures on the experimental units, a correlation model was included to account for the lack of independence across times. This was implemented using Proc Glimmix from SAS 9.1™ (SAS Institute Inc., Cary, NC, USA) with an unstructured correlation model. Multiplicity of comparisons were taken into account using Tukey-adjusted p-values, and residual checks were made to ensure that the assumptions of the analysis were met.

Total RNA isolation

Total RNA was isolated from three individual leaf samples per cultivar out of the June 11, 2003, sample set using TRI Reagent® (Molecular Research Centre, Inc., Ohio, USA) according to the manufacturer's instructions.

Microarray analysis

Almac Diagnostics, Craigavon, Northern Ireland, conducted the microarray analysis using the commercially available Affymetrix Barley1 GeneChip® (Close *et al.,* 2004) as follows.

One-cycle Target Labelling Assay

Two microgram of total RNA was converted to cDNA via first and second strand synthesis using the GeneChip® Expression 3'-Amplification One-Cycle cDNA Synthesis kit, in conjunction with the GeneChip® Eukaryotic PolyA RNA Control Kit. Cleanup of the double-stranded cDNA was carried out using the GeneChip® Sample Cleanup Module. Biotin-labelled cRNA was synthesised from the double-stranded cDNA using the GeneChip® Expression 3'-Amplification IVT Labelling Kit. To determine an accurate concentration and purity for the newly synthesised biotin-labelled cRNA, a cleanup step was carried out to remove unincorporated nucleoside triphosphates (NTPs) using the GeneChip® Sample Cleanup Module. The cRNA quality was assessed using an Eppendorf Biophotmeter and an Agilent 2100 bioanalyser.

Two-Cycle Target Labelling Assay

Hundred microgram of total RNA was used for cDNA synthesis in the first cycle using the GeneChip® Expression 3'-Amplification Two-Cycle cDNA Synthesis kit, in conjunction with the GeneChip® Eukaryotic PolyA RNA Control Kit. After cDNA generation in the first cycle, the Ambion MEGAscript® T7 Kit was used for the first-cycle in vitro transcription (IVT) amplification to generate unlabelled cRNA. Cleanup of the unlabelled cRNA was carried out using the GeneChip® IVT cRNA Cleanup Module. The unlabelled cRNA was reverse transcribed in the second-cycle first-strand cDNA synthesis using the GeneChip® Expression 3'-Amplification Two-Cycle cDNA Synthesis kit. Cleanup of the double-stranded cDNA was carried out using the GeneChip® Sample Cleanup Module. The resulting double-stranded cDNA was amplified and labelled using the GeneChip® Expression 3'-Amplification IVT Labelling Kit. To determine an accurate concentration and purity for the newly synthesised biotin-labelled cRNA, a cleanup step was carried out to remove unincorporated NTPs using the GeneChip® Sample Cleanup Module. The cRNA quality was assessed using an Eppendorf Biophotmeter and an Agilent 2100 bioanalyser.

Fragmented cRNA Generation

Twenty-five microgram of cRNA generated in the IVT reaction was fragmented using 5× fragmentation buffer and RNase-free water contained within the GeneChip® Sample Cleanup Module. The fragmentation reaction was carried out at 94°C for 35 minutes to generate 35–200 base fragments for hybridisation. The fragmented cRNA quality was assessed using an Agilent 2100 bioanalyser.

Affymetrix Hybridisation Protocol

Before hybridisation, the adjusted cRNA yield in the fragmentation reaction was calculated to account for carryover of total RNA in the IVT reaction. Fifteen microgram of fragmented cRNA was made up into a hybridisation cocktail in accordance with the Affymetrix technical manual corresponding to a 49 format (standard)/64 format array. The hybridisation cocktail was added to the appropriate array and hybridised for 16 hours at 45°C.

Washing, Staining and Scanning Protocol

The array was washed and stained on the GeneChip® fluidics station 450 using the appropriate fluidics script. Once completed, the array was inserted into the Affymetrix autoloader carousel and scanned using the GeneChip® Scanner 3000.

Generation of Stringent Gene List

There were six unscaled data sets used in the experiment, consisting of three Crusader and three Cooper replicants. To

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normalise the data, the values below 0.01 were set to 0.01. Each measurement was divided by the 50th percentile of all measurements in that sample. Each gene was divided by the median of its measurements in all samples. If the median of the raw values was below 10, then each measurement for that gene was divided by 10 if the numerator was above 10, otherwise the measurement was thrown out. The variable was 'cultivars' and distinguished as either 'Crusader' or 'Cooper'. Data interpretation was primarily logged ratio. The axis max was 100 and the axis min was 0.01, and the display was set as continuous. Cross gene error model was active and based on replicates, which were defined by the parameter cultivars. The average base/proportional value was 22.92.

Genes were filtered based on Affymetrix flag calls. The starting gene list was 'All Genes', and the genes were required to display present 'P' or marginal 'M' in six of six samples. Through this process, genes with absent flags were discarded, 12,647 genes passed this filter. The genes were filtered using the expression level filter, using the limits set by the cross gene error model. The starting gene list for this filter was the genes that passed the flag filter (12,647). The data format was control data. The minimum limit was 22.92 and the maximum value was left as default at 12,786. The genes were required to meet these limits in two of two conditions. A total of 12,056 genes passed this filter. The genes were filtered using the fold change filter. The starting gene list for this filter was the genes that passed the cross gene error model filter (12,056). The genes were filtered using the confidence filter via t-test p-value. The minimum p-value was set at 0 and the maximum at 0.05. The multiplicity correction algorithm was set as the Benjamini and Hochberg false discovery rate. About 460 genes passed this filter. These 460 genes are the 'Stringent Gene List'.

Generation of Present/Absent Lists

All six unscaled data files were used for the experimental setup. Again three Crusader cultivar and three Cooper cultivar replicates were used. The data were normalised as before. The variables were set up as before, and the data interpretation was also set up as before. The genes were extracted to MS Excel. In Excel, the Present 'P', Marginal 'M' and Absent 'A' flags were replaced by numerical values and then ratios were extracted, which allowed easy identification of those genes that exhibited consistent present (expression) calls in the Crusader wing and consistent absent (non-expression) calls in the Cooper wing and vice versa. These genes were deemed to be those that follow a possible on-off expression change. The gene list extracted through Excel was copied and pasted back into GeneSpring. The data were then scrutinised for each gene based on normalised data, raw data and p-value. Only those genes that had sound data were kept for the present/absent list and represent those genes that most likely to follow the 'on-off' expression trend. Thirty-one genes were present in this list.

Validation of microarray data

Sequence data for selected contigs from the microarray were obtained from the NetAffx page on the Affymetrix website (http://www.affymetrix.com). Primer pairs specific to these sequences were designed using the EasyPrimer facility on the Cybergene website and were purchased from Sigma-Genosys, as follows: catalase (177 bp); CAT For: 5' AGTTGAATAGTAAAGCCC 3', CAT Rev: 5' AAAAAAGAGGGCAGACAG 3'; Copper/zinc superoxide dismutase (117 bp); CuZn For: 5' CATCGGGCTCCAGGGCTA 3', Cu/Zn Rev: 5' TAGATGGTGTGCTTATTT 3'; heat shock protein 101 (82 bp); HSP For: 5' ATAGAATAGTAAACGGA 3', HSP Rev: 5' TGTCCAACTCGTAGCCAA 3'; cell wall peroxidase 8 (105 bp); Prx8 For: 5' AAGAGATTCCATAGAT 3', Prx8 Rev: 5' GAGCCCCTTGTTCTTG 3'. Validation of the results was performed by reverse transcription-polymerase chain reaction (RT-PCR) using the RETROScript® RT-PCR Kit and the QuantumRNA 18S Universal primers (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions.

Results

SOD, APX and Type III peroxidase

The sampling period was chosen to cover the onset of PLS in the sensitive cultivar, cv. Cooper. On the first sampling date (June 4), leaves of both cultivars were asymptomatic. The first lesions had appeared on cv. Cooper by the second sampling date (June 11), and they became increasingly extensive over the following two sampling dates (June 19 and 26). Only on the final date, there was evidence of the first lesions appearing on the tolerant cultivar (cv. Crusader) and they remained very limited. Figure 1 shows the levels of enzyme activity for ROS scavenging enzymes, and the levels of antioxidants, recorded during the part of the 2003 growing season when the sensitive cultivar (Cooper) began to display symptoms of PLS. Figure 1a shows the level of type III peroxidase. Significant differences in activity between the cultivars occurred on June 4 ($P<0.01$) and June 19 ($P<0.05$). In the first instance, Crusader demonstrated a greater level of peroxidase activity than Cooper. However, by June 19, the activity measured in Cooper displayed a twofold higher level than Crusader. This augmented activity in Cooper corresponded to the advent of PLS symptoms in that cultivar on June 11. Figure 1b details the total SOD

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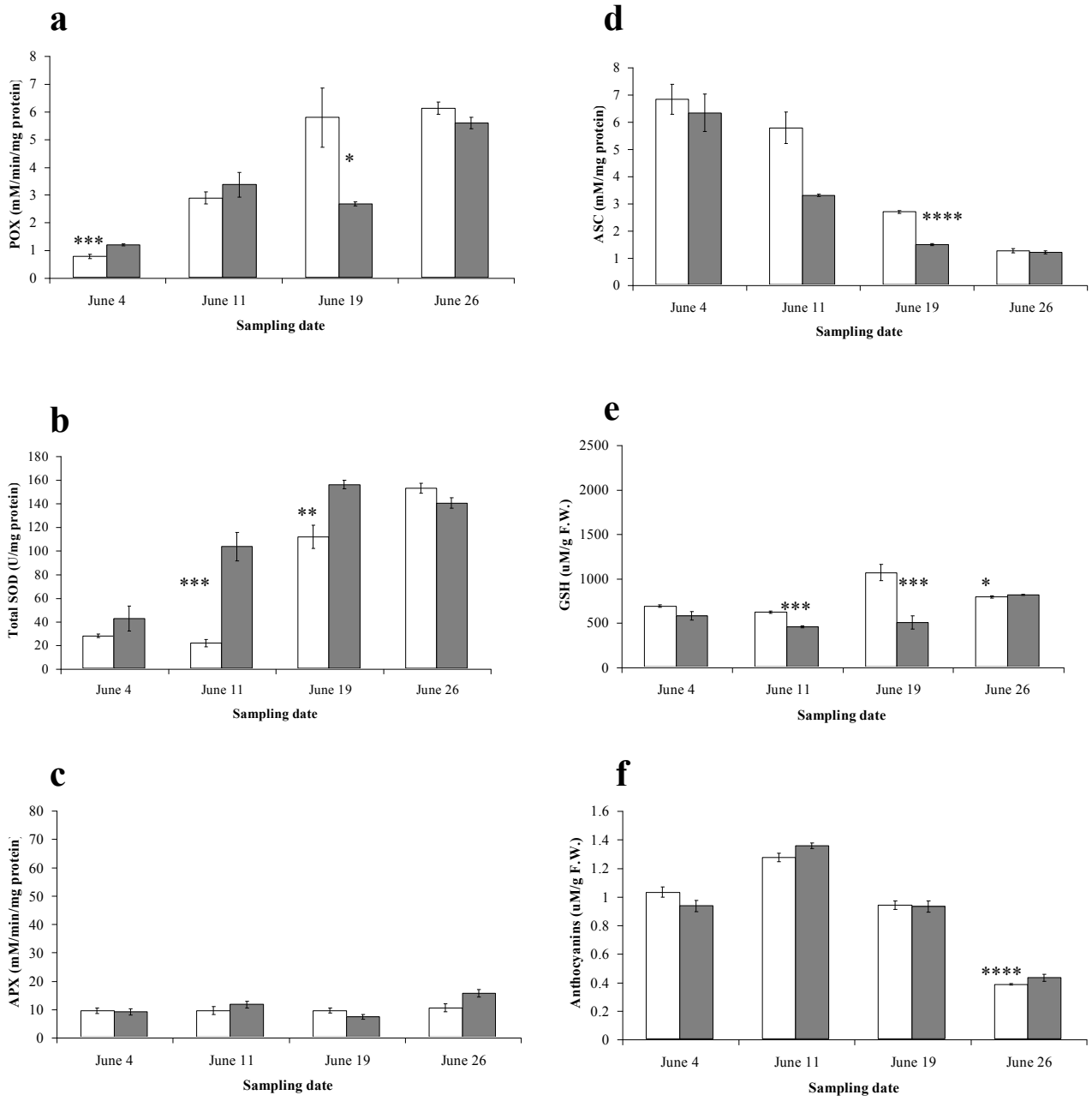

Figure 1. ***Activity of ROS scavenging enzymes (*****a, b, c*****) and levels of antioxidants (*****d, e, f*****) in F-1 leaves of*** *H. vulgare* ***cvs. Cooper (white bars) and Crusader (grey bars), during June 2003. (a) Type III peroxidase (POX), (b) total superoxide dismutase (SOD), (c) ascorbate peroxidase (APX), (d) reduced ascorbate (ASC), (e) reduced glutathione (GSH) and (f) anthocyanin content. Values are means ± SE (n = 6).***

activity. Significant differences were recorded for the sampling dates, June 11 ($P<0.01$) and June 19 ($P<0.01$). The greatest fold-change occurred on June 11 where Crusader contained 4.8 times the amount of SOD activity compared to Cooper. Figure 1c reflects the APX activity. There was little change throughout the sampling period in either cultivar and no significant differences were recorded.

Ascorbic acid, glutathione and anthocyanins

During the sampling period in June 2003, the total ascorbate content declined steadily as revealed in Figure 1d. The only significant difference occurred on June 19 ($P<0.001$). On this date, Cooper leaves possessed 1.8 times the amount of ascorbate than those of Crusader plants. Figure 1e details total glutathione, measured as GSH content, during the sampling

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period. Significant differences between the cultivars were found on June 11 (P<0.001), June 19 (P<0.001) and June 26 P<0.001). The anthocyanin content of leaves is shown in Figure 1f. A single significant difference was observed on June 26 (P<0.001).

Weather data

In order to examine climatic conditions before and during sampling, Monthly Reports were obtained from the Met Éireann station in Kilkenny, Ireland. These observances are displayed graphically in Figure 2. It is clear that the amount of sunshine fluctuates daily from high to low between June 4 and June 11, before PLS initiation. The mean air temperatures were slightly cooler during early spot development than in the rest of June 2003.

Microarray data and validations

The stringent list generated 460 differently expressed probe sets, and the degree of differential expression of each probe set was determined using the gene expression ratio of Cooper to Crusader. Positive expression ratios indicated a higher transcript level in Cooper compared to Crusader, whilst negative ratios indicated the reverse situation. Gene identification was achieved by using the Affymetrix probe sequences available from the NetAffx facility on the company website (http://www.affymetrix.com) in a BLASTX search of the NCBI non-redundant database. Hereafter the term probe set will be replaced by gene for simplicity. Out of the 460 genes represented on the stringent list, 138 were classified as unknown because their most significant hit was either unclassified or no match could be found. The remaining genes were assigned to functional groups and are displayed graphically in Figure 3. The groups were also divided on the basis of their expression ratios, a total of 175 genes possessed a positive ratio and 147 were negative. Figure 4 shows the positive to negative breakdown of genes in the functional groups represented by Figure 3. Because stress related and defence genes are particularly likely to be linked to differential sensitivity to PLS, these are listed in Table 1, together with a selection of other relevant genes. A total of 31 genes were designated to the present/absent list and are listed in Table 2.

The validation method of choice for this experiment was relative quantitative RT-PCR. Candidate genes were chosen for validation on two counts: first, the difference between the cultivars could be easily assessed and, second, the possible relationship between the candidate and the PLS model. The final selections were catalase 1 (*Triticum aestivum*), cytosolic Cu/Zn SOD (*Zea mays*) and the cell wall peroxidase Prx-8 (*H. vulgare*) from the stringent list. A fourth validation candidate was chosen from the present/absent list: heat shock protein 101 (HSP101, *T. aestivum*) shown to be present in Crusader only. The RT-PCR products generated were separated by agarose gel electrophoresis and normalised to the 312 bp 18S ribosomal RNA band as shown in Figure 5.

Discussion

The aim of this research was to analyse the disparate susceptibilities of two spring barley cultivars (*H. vulgare* L. cvs Cooper and Crusader) to the PLS phenomenon. The experimental approach was carried out on two fronts. Initially, a biochemical analysis of ROS scavenging enzyme activity and the antioxidant capacity of field grown barley was implemented.

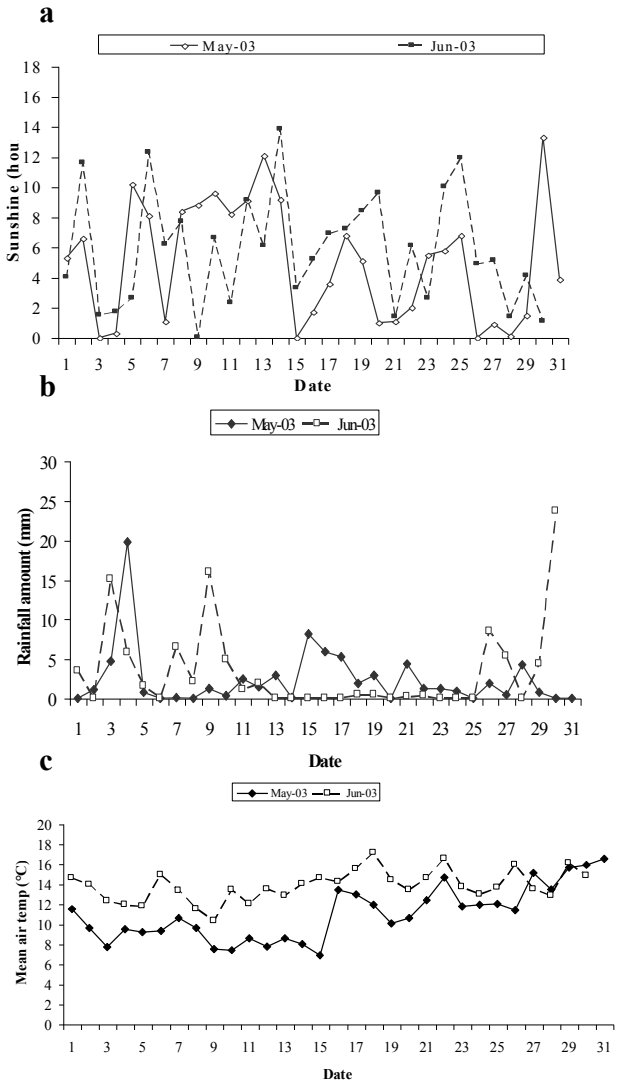

Figure 2. ***Weather data recorded before [May 2003] and during [June 2003] sampling. (a) Hours of sunshine, (b) total rainfall and (c) daily mean air temperature.***

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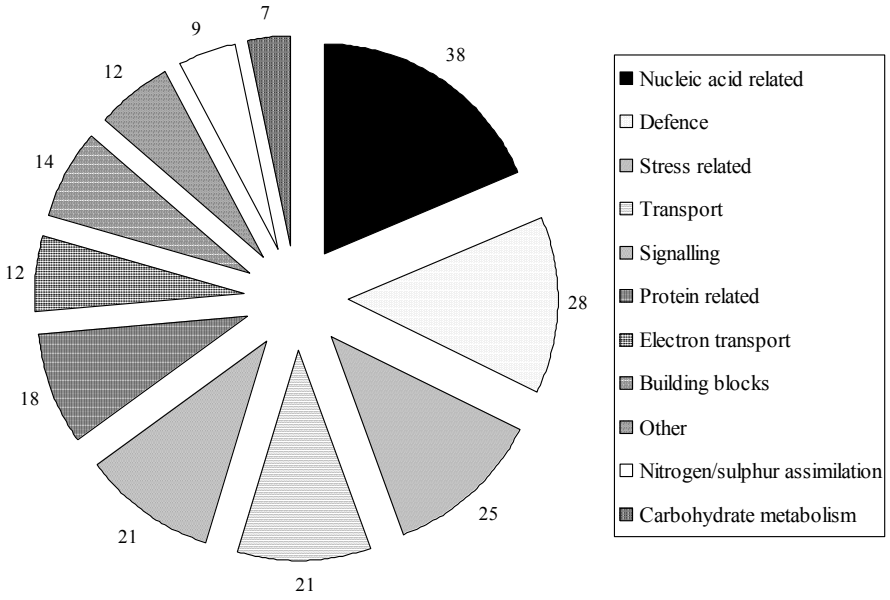

Figure 3. Pie chart showing the major functional groups appearing on the stringent list generated from the microarray data, n = 205.

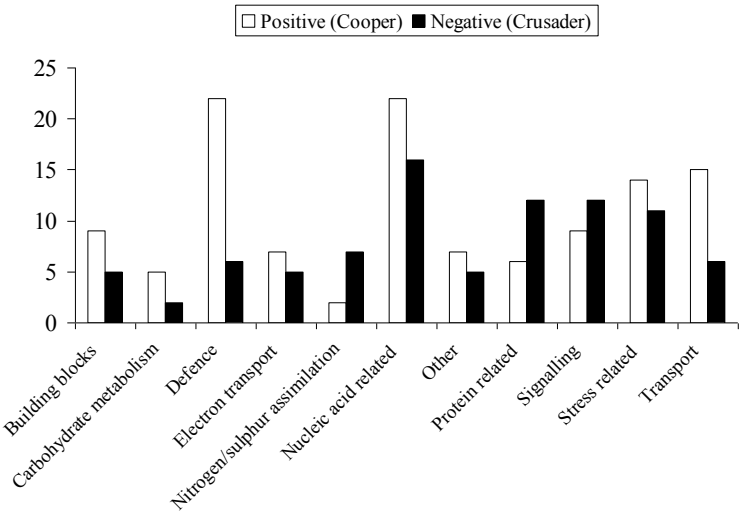

Figure 4. Transcript amount per functional group, separated on the basis of their relative expression ratios, n = 205. Positive genes accumulate in Cooper, negative in Crusader.

In an effort to gain in-depth molecular information, a global gene expression study of both cultivars was also conducted, using the Affymetrix Barley1 GeneChip®. The total RNA used for the microarray study was isolated from leaves gathered when Cooper displayed initial PLS symptoms, whilst Crusader did not. Amongst numerous differences in the transcript profiles of the two cultivars, several merit comment in relation to known or postulated features of the PLS disorder.

Firstly, there were pronounced differences in the foliar levels of enzymes associated with redox changes in stressed cells and other pathways connected with abiotic stress responses. Transcripts for Class III peroxidases were elevated in the sensitive cultivar, Cooper. These enzymes have a broad range of functions within plant cells (Passardi *et al.*, 2005) including the stimulation of ROS production associated with the hypersensitive response (HR), a process mimicked by the lesions found during PLS. Cooper leaves also exhibited high levels of phenylalanine ammonia-lyase (PAL) and cinnamate-4-hydroxylase (C4H) transcripts. These are the first two enzymes of the general phenylpropanoid pathway, which synthesises many compounds also associated with defence, for example, lignins, anthocyanins and phytoalexins. This finding, in association with the heightened peroxidase presence and ROS generation, is consistent with oxidative cross-linking of lignin precursors in Cooper leaves, a

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Table 1. Members of the stringent list of differentially regulated genes between Cooper (symptomatic PLS sensitive cultivar) and Crusader (asymptomatic PLS-tolerant cultivar). All genes designated as stress related are included, along with a selection of other relevant genes. The values (expression ratios) indicate the relative levels of transcript, with a positive value indicating higher expression in Cooper, and a negative value higher expression in Crusader.

Function	Accession	Expn	Ratios
Stress related			
Abiotic			
Betaine aldehyde dehydrogenase	BAB62846	−6.7	
Nuclease I	BAA82696		2.5
putative acid phosphatases	CAB71336	−4.8	
putative subtilase	BAB89065	−4.1	
Putative universal stress protein USP1	AAM09541		2.3
r40g2	CAA70174	−4.1	
Ribosomal protein L37, cytosolic	P49212		4.4
Temperature stress-induced lipocalin	AAL75812		2.1
trehalose-6-phosphate phosphatase	NP_851171	−4.0	
Cold acclimation protein WCOR413	AAB18207		2.2
Cold acclimation protein WCOR615	T06812	−5.8	
Phosphoglucan water dikinase	NP_198009	−7.9	
ROS scavenging			
Ascorbate peroxidase, thylakoid bound	T12282	−6.7	
Catalase 1	BAA13068		15.7
Cu/Zn superoxide dismutase, cytosolic	P11428		4.0
Peroxidase [*Triticum aestivum*]	AAM76682		2.9
Peroxidase 2 precursor [*Triticum aestivum*]	CAA59485		5.9
Peroxidase 8 (Prx-8) [*Hordeum vulgare*]	CAB99487		4.0
peroxidase 8 [*Triticum monococcum*]	AAW52722		4.8
Putative ascorbate peroxidase, thylakoid bound	Q9THX6	−5.4	
Putative peroxidase [*Oryza sativa*]	AAM08517		2.3
Putative peroxidase [*Oryza sativa* (japonica)]	XP_469867		2.9
Thiols			
Glutaredoxin I	T12219	−4.3	
Putative thioredoxin m2	CAC69854	−5.4	
Thioredoxin h	AAD33596		2.7
Thioredoxin x	AAF15952	−4.4	
Defence (partial list)			
Disease resistance response protein-like	NP_914706		3.2
Glucan endo-1,3-beta-glucosidase	AAA32960		2.3
Harpin-induced gene 1 homologue	AAB97367		2.0
Hv-1a, pathogenesis related	CAA52893		3.5
Hypersensitive-induced reaction protein	AAN17462		3.7
Jasmonate-induced protein	CAA58110		7.2
PR protein	P16273	−10.0	
PR protein 4	CAA71773		5.3
PR 10a	AAF85972		5.4
Oxalate oxidase	AAC25777		3.0

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continued ***Table 1. Members of the stringent list of differentially regulated genes between Cooper (symptomatic PLS sensitive cultivar) and Crusader (asymptomatic PLS-tolerant cultivar). All genes designated as stress related are included, along with a selection of other relevant genes. The values (expression ratios) indicate the relative levels of transcript, with a positive value indicating higher expression in Cooper, and a negative value higher expression in Crusader.***

Function	Accession	Expn	Ratios
Phenylpropanoid metabolism (from the 'building blocks' list)			
Cinnamic acid 4-hydroxylase	BAB71717		2.1
Inducible phenylalanine ammonia-lyase	AAG02280		2.3
Phenylalanine ammonia-lyase [*Triticum aestivum*]	CAA68064		2.0
Phenylalanine ammonia-lyase [*Hordeum vulgare*]	CAA89005		6.0
Putative dihydroflavonal-4-reductase	AAK92621	−7.0	
Electron transport and chloroplast function (partial list)			
Chlorophyll a/b binding protein	NP_173349	−4.7	
Chlorophyll a/b binding protein 25	P04782	−6.3	
Chlorophyll a/b binding protein type 1b	PQ0764	−5.3	
NifU-like protein	BAB97140		2.7
Chloroplast RNA processing 1 (CRP1)	T01685	−4.1	
Other genes			
Phosphatidylinositol-4-phosphate 5-kinase	AAB65487	−-5.2	

Table 2. Gene transcripts that registered present or absent in Cooper and Crusader. P, present; A, absent. Entries which are not followed by parentheses are *Hordeum vulgare* ***Genbank accessions.***

Genbank	Cooper	Crusader	Name
AAD44033	P	A	ARK protein (Ta)
XP_550628	P	A	Unknown protein (Os)
NP_188924	P	A	Protein carrier (At)
-	P	A	-
AY095303	P	A	Cytochrome c synthase 1 (CCS1) (Cr)
ABA95304	P	A	Disease resistance gene (Os)
NP_567420	P	A	Glycosyl transferase (At)
AJ001341	P	A	putative acyl-CoA oxidase
XP_474191	P	A	Putative ATPase (Os)
AAG32473	P	A	putative glutathione-S-transferase (Os)
NP_195093	P	A	Aldo-keto reductase/oxidoreductase (At)
ABA91051	A	P	Hypothetical protein from R-gene-rich regions of chromosome 11 and 12 (Os)
AAF01280	A	P	heat shock protein 101 (Ta)
AY266444	A	P	MLA7
NP_922442	P	A	putative glutathione-S-transferase (Os)
NP_188993	A	P	Cyclopropane-fatty-acyl-phospholipid synthase/oxidoreductase (At)
AAQ5541	A	P	MLA10
AAM22751	A	P	Clone mth2-7a7 (Mt)
NP_567642	A	P	APG8A – GABA receptor associated (At)
NP_174556	A	P	C1D11 – RNA binding (At)
AF523679	A	P	MLA13

Abbreviations: ***Ta:*** *Triticum aestivum****; Os:*** *Oryza sativa****; At:*** *Arabidopsis thaliana****; Cr:*** *Chlamydomonas reinhardtii****; Mt:*** *Medicago truncatuta.*

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feature of HR and the similar generation of lesions on the leaf surface during PLS.

Another enzyme associated with ROS scavenging, ascorbate peroxidase, exhibited higher activity in Crusader than that in Cooper, a feature that correlated with transcript accumulation of the thylakoid-membrane-bound isoform in Crusader leaves. Ascorbate peroxidase is demonstrably associated with tolerance to oxidative stress (Davletova *et al.* 2005).

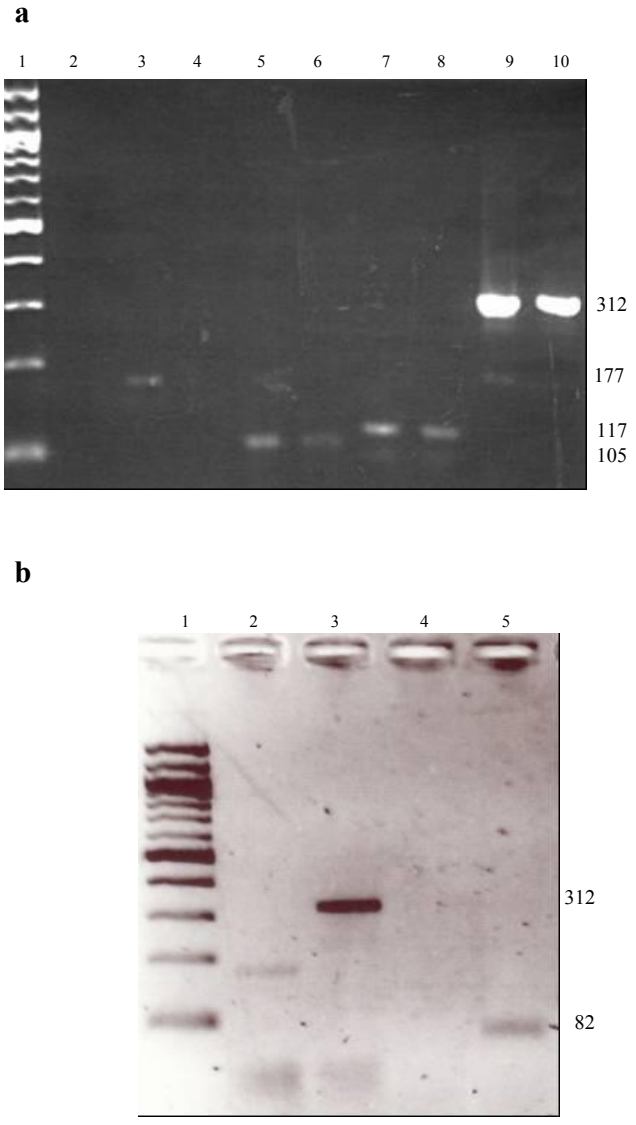

Figure 5. ***Microarray validation using a 30-cycle RT-PCR, visualised on a 2% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. (a) Lane 1: 100 bp DNA ladder (NEB); Lane 2: negative control; Lane 3: CAT1 (Cooper); Lane 4: CAT1 (Crusader); Lane 5: Prx-8 (Cooper); Lane 6: Prx-8 (Crusader); Lane 7: Cu/Zn SOD (Cooper); Lane 8: Cu/Zn SOD (Crusader); Lane 9: 18S rRNA (Cooper) and Lane 10: 18S rRNA (Crusader). (b) Lane 1: 100 bp ladder; Lane 2: Cooper CAT1 + 18S; Lane 3: Crusader CAT1 + 18S; Lane 4: Cooper HSP101 and Lane 5: Crusader HSP101. The image was inverted to emphasise the absence of a band in the Cooper lane.***

An established feature of PLS, supported by the array data, is its dependence on high light intensity. Shading of PLS-sensitive plants in the field prevents incidence of the disorder, indicative of a light-induced response (Wu and von Tiedemann, 2004). The array data reveal cultivar differences in a number of transcripts associated with the maintenance of functional photoapparatus. Examples include three transcripts for chlorophyll *a/b* binding (CAB) proteins, and *crp*1 (chloroplast RNA processing 1) transcript, in Crusader. Furthermore, an nifU-like protein uncovered in the Cooper subset may be necessary for maintaining photosynthetic electron transport (Yabe *et al*., 2004).

Evidence from the microarray suggests that the plants underwent additional abiotic challenges. Both cultivars had an increased amount of cold-responsive transcripts, which is not unexpected when the relevant weather data is examined. In the week prior to the sampling date, the air temperature did not rise above 15°C and the mean grass temperature for the period was 3.4°C. The array data from Crusader revealed several transcripts associated with cold tolerance, including those for a phosphoglucan water dikinase, phosphatidylinositol-4-phosphate 5-kinase, betaine aldehyde dehydrogenase (BADH) and trehalose-6-phosphate phosphatase (T6PP), implying that Crusader may have a superior cold-acclimation responses. Furthermore, the Crusader subset included two heat shock proteins: a DnaJ homologue (heat shock protein binding) and HSP101, the latter of which was absent in Cooper. These findings all indicate a superior capacity of Crusader to endure environmental stress, in comparison with Cooper.

In conclusion, microarray analysis reveals a number of marked differences in transcript profiles and abundance between PLS-sensitive and PLS-tolerant cultivars of *H. vulgare* when symptoms become apparent on the former. A number of these relate to enzymes associated with ROS scavenging and the maintenance of a functional photoapparatus. These differences are consistent with a differential response to abiotic stress, as is also reflected in biochemical analysis of ROS scavenging enzymes (APX, SOD and type III peroxidase) and antioxidants (ascorbate, glutathione) in these two cultivars. The interaction of these factors is complex and should not be over-interpreted. However, given the likelihood that PLS is likely to remain a persistent and growing problem for Irish crop land, these findings can provide useful clues to possible strategies for addressing this condition, both by altered agricultural practises and breeding using both traditional and contemporary approaches.

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References

Anderson, J.V., Chevone, B.I and Hess, J.L. 1992. Seasonal variation in the antioxidant system of Eastern White pine needles - Evidence for thermal dependence. *Plant Physiology* **98:** 501-508.

Apel, K. and Hirt, H. 2004. Reactive oxygen species, Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* **55:** 373-99.

Arakawa, N., Tsutsumi, K., Sanceda, N.G., Kurata, T. and Inagaki, C. 1981. A rapid and sensitive method for the determination of ascorbic acid using 4,7-diphenyl-1,10-phenanthroline. *Agricultural and Biological Chemistry* **45:** 1289-1290.

Behn, A., Hartl, L., Schweizer, G. and Baumer, M. 2005. Molecular mapping of QTLs for non-parasitic leaf spot resistance and comparison of half-sib DH populations in spring barley. *Euphytica* **141:** 291-299.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72:** 248-254.

Burke, J.I., Hackett, R. and O'Sullivan, E. 2001. The barley leaf spot problem - causes and control. Paper presented at the National Tillage Conference, January 2001. http://www.teagasc.ie/publications/2001/tillageconference/paper03.htm

Close, T.J., Wanamaker, S.I., Caldo, Turner, S.M., Ashlock, D.A., Dickerson, J.A., Wing, R.A., Muehlbauer, G.J., Kleinhofs, A. and Wise, R.P. 2004. A new resource for cereal genomics: 22K barley GeneChip comes of age. *Plant Physiology* **134:** 960-968.

Davletova, S., Rizhsky, L., Liang, H., Shengquiang, Z., Oliver, D.J., Shulaev, V., Schlauch, K. And Mittler R. 2005. Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. *Plant Cell* **17,** 268-281.

Elstner, E.F. and Heupel, A.1976. Inhibition of nitrite formation from hydroxylammonium chloride: A simple assay for superoxide dismutase. *Analytical Biochemistry* **70:** 616-620.

Lee, D.H. and Lee, C. H. 2000. Chilling stress-induced changes in antioxidant enzymes in the leaves of cucumber, in gel enzyme activity assays. *Plant Science* **159:** 75-85.

Lu, Q. and Lu, C. 2004. Photosynthetic pigment composition and Photosystem II photochemistry of wheat ears. *Plant Physiology and Biochemistry* **42:** 395-402.

Motulsky, H. and Christopoulos, A. 2004. Fitting Models to Biological Data using Linear and Nonlinear Regression. A Practical Guide to Curve Fitting. Oxford University Press, New York.

Passardi, F., Cosio, C., Penel, C.and Dunand, C. 2005. Peroxidases have more functions than a Swiss army knife. *Plant Cell Reports* **24:**255-265.

Reddy, V.S., Goud, K.V., Sharma, R. and Reddy, A.R. 1994. Ultraviolet-B-responsive anthocyanin production in a rice cultivar is associated with a specific phase of phenylalanine ammonia lyase biosynthesis. *Plant Physiology* **105:** 1059-1066.

Rizhsky, L., Hallak-Herr, E., Van Breusegem, F., Rachmilevitch, S., Barr, J.E., Rodermel, S., Inzé, D. and Mittler R. 2002 Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. *Plant Journal* **32:** 329-342.

Tottman, D.R. and Broad, H. 1987. The decimal code for the growth stages of cereals, with illustrations. *Annals of Applied Biology* **110:** 441-454.

Wu, Y-X. and von Tiedemann, A. 2002. Evidence for oxidative stress involved in physiological leaf spot formation in winter and spring barley. *Phytopathology* **92:** 145-155.

Wu Y-X. and von Tiedemann, A. 2004. Light-dependent oxidative stress determines physiological leaf spot formation in barley. *Phytopathology* **94:** 584-592.

Yabe, T., Morimoto, K., Kikuchi, S., Nishiob. K., Terashimac, I. and Nakai, M. 2004. The Arabidopsis chloroplastic NifU-like protein CnfU, which can act as an iron-sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and Photosystem I. *Plant Cell* **16:** 993-1007.

Yoshimura, K., Miyao, K., Gaber, A., Takeda, T., Kanaboshi, H., Miyasaka, H. and Shigeoka, S. 2004. Enhancement of stress tolerance in transgenic tobacco plants over expressing Chlamydomonas glutathione peroxidase in chloroplasts or cytoplasm. *Plant Journal* **37:** 21-33.

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