New insights into the dynamics of the glutathione-ascorbate redox system of plants

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Summary
The Halliwell-Asada-Foyer redox cascade (HAF) is viewed as a H$_2$O$_2$ detoxifying system with a great variety of responses against environmental changes. The functional consequences of these responses are interpreted intuitively because a systemic analysis of the inherent dynamic potential of the HAF is lacking. With the help of numerical modelling we show that in wheat roots parameter patterns are established which result in homeostatic states of HAF over a vast range of environmental changes. The reduced fractions glutathione (GSH) and ascorbate (ASC) remain on high levels even during dramatic changes in the enzyme activity ratios of glutathione reductase, dehydro-ascorbate reductase and ascorbat peroxidase. Necessarily their oxidised counterparts diithioglutathione (GSSG) and dehydroascorbate (DHA) stay in these buffered regions on very low concentration levels. Our modelling shows that redox ratios GSH/GSSG and ASC/DHA can be modified additionally via changes in NADPH/H$_2$O$_2$ ratios. Thus, the redox states of GSH and ASC can not simply be regarded as indicators for oxidative stress with respect to H$_2$O$_2$ levels. The involvement of the redox variables in other redox processes than the HAF reaction (redox proteome) and/or their utilisation in metabolism (protein modification, detoxification of xenobiotics) are viewed to cause system relaxations of the redox variables. The re-establishment of their homeostatic ratios follow time courses which are redox moiety specific and are balanced according to the existing parameter patterns. Despite of its detoxification function the HAF balances the glutathione / ascorbate redox state in cells according to the prevailing physiological conditions.

Introduction
The Halliwell-Asada-Foyer cascade (HAF) is a redox cascade which was originally viewed as a H$_2$O$_2$ detoxifying system (HALLIWELL, 1978; HALLIWELL, 1987; ISHIKAWA and SIES, 1989). Detoxification is reached by transferring electrons from nicotineamide adenine nucleo-side phosphate (NADPH) to hydrogen peroxide (H$_2$O$_2$) via the cycle intermediates glutathione and ascorbate. The reactions are catalyzed by the enzymes glutathione reductase (GR), dehydroascorbate reductase (DHR) and the ascorbate peroxidase (APX). The enzyme activity patterns as well as the redox properties of the intermediates are highly responsive to changes of plant growth conditions such as salinity, temperature, ozone, heavy metals, oxygen, pH, and soil fertility and light stress (ARRIGONI, 1995; NOCTOR and FOYER, 1998; DE MARCO and ROUBELAKIS-ANGELAKIS, 1999; PEARCE, 1999; MITTLER, 2002; BLOKHINA et al., 2003; HERNANDEZ et al., 2004; FOYER and NOCTOR, 2005; FOYER et al., 2005; SMIRNOFF, 2005). This responsiveness is viewed as counteraction for fighting oxidative stress, caused by changes of the above mentioned environments.

However, it is widely neglected whether other causes than oxidative stress, resulting from metabolic perturbations, could also lead to similar responses of the HAF cascade. Exploring this problem will be a focus of this paper.

The functioning of any reaction system is primarily dependent on its parameter status (KACSER and BURNS, 1973; REICH and SEL’KOV, 1981) which is given by the values of rate constants of the individual enzymes and their numerical ratios. According to KACSER and BURNS (1973) also co-enzymes and other (signaling) molecules and input to output ratios in reaction cascades can reach parameter status under certain conditions. In the case of the HAF this notion can be applied to stationary concentrations of NADPH and H$_2$O$_2$ which are the input and output ports of the HAF system. Both, the rate parameters and the input (NADPH) to output (H$_2$O$_2$) ratios are regarded as „minimal sets“ of determinants of the dynamic patterns of the HAF system.

A re-evaluation of oxidative stress phenomena in plants which is also based on genetic evidence opens new insights into the potentially damaging effects of reactive oxidative species (ROS) but also directs the attention to their „oxidative signaling“ assignment (FOYER et al., 1997; DAT et al., 2000; FOYER and NOCTOR, 2005; FOYER et al., 2005; HALLIWELL, 2006). According to this extended point of view H$_2$O$_2$ is expected to act as signal molecule, as environmental messenger or enhancer for the regulation of developmental processes (LAGRIMINI et al., 1997; LANDER, 1997; RAO and DAVIS, 2001; PIQUERY et al., 2002; FOREMAN et al., 2003; OVERMYER et al., 2003; FOYER, 2005; FOYER and NOCTOR, 2005). It remains open how the effective H$_2$O$_2$ detoxifying function of HAF can be correlated with the need of residual portions of H$_2$O$_2$ for signaling purposes. Furthermore HAF provides reducing power for the redox proteome and is also supporting glutathione for protein modification reactions as indicated in Fig. 1 (SCHIEBE, 1991; DIETZ, 2005; FOYER and NOCTOR, 2005; STROHER and DIETZ, 2006). In addition glutathione and ascorbate serve particular regulatory and developmental purposes (ISHIKAWA and SIES, 1989; ARRIGONI, 1995; SMIRNOFF, 1996; FOYER et al., 1997; IMENEZ et al., 2002; FOYER et al., 2005; FOYER and NOCTOR, 2005; SMIRNOFF, 2005). The HAF must be regarded as a multifunctional core unit of redox metabolism in cells which serves not only as detoxificant of H$_2$O$_2$, but also provides appropriate reduction power for cellular redox proteome, supports protein modification and balances the redox molecules in accordance with their physiological needs and is involved in the regulation of growth and development of plants via its components H$_2$O$_2$, glutathione and ascorbate.

Numerical modelling is ideal to unravel the multifaceted functions of the HAF core process. A recent model by POLLE (2001) focused on detoxifying mechanism of the HAF and simulated the functioning of the SOD-ASC-GSH cycle in cells. Here we extend the approach by considering variable system parameter portraits according to REICH and SEL’KOV (1981). These portraits identify the steady-state levels of variables as functions of their parameter sets. They quantify the redox ratios of the variables and allow flux calculations that are characteristic for these steady-states. The focus of this model is addressed to the question how the redox couples GSH / 2 GSSG and ASC / DHA are shifted under changing enzyme activities and how they depend on the support (NADPH) to demand (H$_2$O$_2$) ratio. It will be shown how transitory dynamics of the variables can cause „pseudo steady-states“ (HEINRICH and RAPOPORT, 1977) which can give rise to misinterpretations of experimental data.

Material and methods
In order to deal with a coherent set of data the model calculations were based on a systematic survey of varying growth conditions on
the parameters and variables of HAF in wheat roots (Maldonado, 1998). Only three experiments (4 and 9 days old seedlings grown in the dark, 7 days old seedlings with 3 days under a 14 h photoperiod) from this study are considered in this modelling which show the most significant differences in the responses of the enzyme activities of glutathione reductase (GR), dehydroascorbate reductase (DHR) and ascorbate peroxidase (APX) and of the concomitant redox changes of the GSH / 2GSSG and ASC / DHA moiety ratios. It is intended to elucidate via modelling which effect on the system properties of the HAF arise from these parameter changes.

**Plant material:** Surface sterilized kernels of wheat seedlings (Triticum aestivum L. cv. Bezostaya) were grown in a growth chamber at 20° C in the dark on a 1 cm layer of Vermiculite which was placed on stainless steel sieves. The Vermiculite was watered by wicks which were connected to an aerated water reservoir. The roots were allowed to grow through the sieve into the water. The analytical data considered here stem from roots of dark grown seedlings (4 and 9 days old, respectively) and from roots of seven days old seedlings which had been grown under a 14 h photoperiod for three days before harvest. The harvested root samples were frozen in liquid nitrogen and used for subsequent enzyme activity determinations. The moieties of nicotinamide adenine dinucleoside phosphate (NADP), glutathione (GSH) and ascorbate (ASC) were analyzed from freshly prepared plant extracts. Roots of seedlings were analyzed because they are lacking the chloroplast antioxidative system. According to the applied method (10 000 * g

**Enzyme activities and metabolites:** The enzyme activities of the glutathione reductase (E.C. 1.6.4.2) (GR), the dehydro-ascorbate reductase (E.C. 1.8.5.1) (DHR) and the ascorbate peroxidase (E.C. 1.11.1.11) (APX) were determined from 10 000 g supernatants according to procedures by Smith et al. (1988), Gogorcena et al. (1995) and Kato et al. (1997), respectively. From these determinations the rate parameters Vmax / Km were calculated and used for modelling. The concentrations of variables glutathione GSH and of dithio-glutathione (GSSG) were determined according to Griffith (1980), ascorbate (ASC) and dehydroascorbate (DHA) were determined according to Okamura (1980) and the reduced and oxidized co-enzymes NADPH and NADP were measured as described by Matsumura and Miyachi (1980). These primary data of parameters and variables (NADPH, glutathione and ascorbate) provide unique starting sets of data from one plant species and from directly comparable experiments for modelling.

**Modelling of the antioxidative system:** The rate equations for modelling the HAF (Fig. 1, solid lines) were formulated according to their scaled expressions (Fig. 2). This formalism depicts the core processes of the HAF (Fig. 2) in form of its dimensionless variables and its scaled enzyme parameters (Reich and Sel’kov, 1981). Scaled data facilitate compatibility of the system properties of the HAF under changed sets of parameters at varying growth conditions. Scaling converts experimentally measured results of highly differing magnitudes to comparable ranges between 0 and unity. Scaled data of reactions of different orders are dimensionless which is a necessity for explicit mathematical modelling.

Interferences of the HAF (Fig. 1) with components of additional cellular redox processes like the reactions of the thiol redox proteins (dotted lines) are considered but are viewed as kinetically subordinate in comparison to the core process reactions (see later). In our model calculations was used a coherent set of experimental data from one plant organ. This is different to the modelling approach by Polle (2001) who used a mix of data from different sources.

The formalism for modelling HAF is given by the following approach. The rate of reactions can be related to the concentrations of reactants by an equation of the type:

$$v = k \cdot [A] \cdot [B] \cdot [C]$$

where $v$ is the reaction rate; $k$ is the rate constant with a complex overall order and $[A]$, $[B]$ and $[C]$ are substrate concentrations. The order of a reaction is a purely experimental quantity (Laidler, 1978) and is from no concern in calculations with scaled and dimensionless entities. In our example each of the three reactions (Fig. 1, solid lines) represents combinations of high and low substrate concentrations. Kinetically we are dealing with pseudo monomolecular pseudo first order reactions (Llehninger, 1977).

Model calculations occurred with dimensionless contents of the variables glutathione, ascorbate and nicotinamide adenine dinucleoside phosphate (Fig. 2). Scaling of the variables is justified by assuming „moiety conservation“ of the redox couples which can be expected for „snap shot conditions“ of a current physiological state in the moment of harvest. The scaling formalism is given in equations 2a-c, including moiety conservation expressions of the variables (Eqns. 3a-c).

![Fig. 1: The redox balancing potential of the antioxidative glutathione-ascorbate cascade (solid lines).](image-url)

The NADPH pool provides reducing power to the system. Electrons are transferred by the glutathione reductase (GR) (v1) and the dehydroascorbate reductase (DHR) (v2) to the ascorbate moiety (DHA : ASC). Hydrogen peroxide (H₂O₂) finally is reduced via the ascorbate peroxidase reaction (APX) (v3). Included into the reaction cascade are interacting reactions (dotted lines). Vp₁, Vp₂ and Vp₃ symbolize consuming reactions of the reactants, Vp₁ and Vp₃ are consuming oxidants. Frx_o/Frx_red = oxidized and reduced forms of thioredoxins; Prot_o/Prot_red = thiol protein and glutathionylated protein; Grx_o/Grx_red = oxidized and reduced forms of glutaredoxins; Fe³⁺/Fe²⁺ = oxidized and reduced 2-oxoglutarat (FeII) dioxygenase; Prx_o/Prx_red = oxidized and reduced forms of peroxiredoxins.
Eqn. 2a: \[ \alpha = \frac{ASC}{A_0} ; \ 1 - \alpha = \frac{DHA}{A_0} \]

Eqn. 2b: \[ \beta = \frac{GSH}{G} ; \ \frac{1}{2} (1 - \beta) = \frac{GSSG}{G} \]

Eqn. 2c: \[ \eta = \frac{NADPH}{N_0} ; \ 1 - \eta = \frac{NADP^*}{N_0} \]

with,

Eqn. 3a: \[ A_0 = ASC + DHA \]

Eqn. 3b: \[ G_0 = GSH + 2GSSG \]

Eqn. 3c: \[ N_0 = NADPH + NADP^* \]

The dimensionless concentration of H₂O₂ is defined as

Eqn. 4: \[ r = \frac{H₂O₂}{K_m H₂O₂} \]

with \(K_m_{H₂O₂}\), the half saturation constant of ascorbate peroxidase (TAKEDA et al., 1998).

In analogy to equation 1 the scaled and dimensionless forms of the rate equations derived from Fig. 2 are:

Eqn. 5: \[ v_1 = k_1 \cdot \frac{1}{2} (1 - \beta) \cdot \eta(GR) \]

Eqn. 6: \[ v_2 = k_2 \cdot (1 - \alpha) \cdot \beta^3(DHR) \]

Eqn. 7: \[ v_3 = k_3 \cdot \alpha \cdot r(APX) \]

where,

GR, DHR and APX represent the scaled reactions of the glutathione reductase (Eqn 5), the dehydroascorbate reductase (Eqn 6) and the ascorbate peroxidase (Eqn 7) respectively. Scaling of the rate parameters is achieved as depicted in Tab. 2.

Assuming that the antioxidative system is in steady-state, rate equations can be equated \(v_1 = v_2 = v_3\) and solved symbolically for the variables \(\alpha\) and \(\beta\), with

Eqn. 8: \[ \alpha = k_2 \cdot \frac{\beta^3}{(k_2 \beta^2 + k_3 r)} \]

Eqn. 9: \[ \beta = \alpha - \frac{b}{a} + \epsilon \]

with \(a\), \(b\) and \(c\) as written in equation 10.

Eqn. 10:

\[
a = \left[ \frac{-1}{6} k_3 \cdot \frac{r}{K_a} \left( k_2 - 2k_3 \cdot r \cdot K_a \right) + \frac{1}{2} k_3 \cdot \frac{r}{K_a} \right] + \frac{1}{27} \frac{\left( k_2 - 2k_3 \cdot r \cdot K_a \right)^2}{k_2^3 + K_a^3} \times \left[ \left( k_3 \cdot r \cdot K_a \cdot k_2 - 8k_3^3 \cdot k_2^2 \cdot r \cdot K_a \right) + 12k_3^3 \cdot k_2^2 \cdot r^2 \cdot K_a \right] \ldots \right] - 6k_3^2 \cdot k_2^2 \cdot K_a^2 \cdot r^3 \ldots
\]

\[
b = \frac{1}{3} \left( \frac{k_2 \cdot K_a - 2k_3 \cdot r \cdot K_a}{k_2} \right)^2 - \frac{1}{9} \left( \frac{k_2 \cdot K_a}{k_2} \right)^2 \]

\[
c = \frac{1}{3} \left( \frac{k_2 \cdot K_a - 2k_3 \cdot r \cdot K_a}{k_2} \right)
\]

The rate constants \(k_1, k_2\) and \(k_3\) in equations 8 and 10 represent the scaled rate parameters. \(K_a\) is the product of the scaled values of \(k_1\) and \(\eta\). Combining both constants saves computer capacity for calculations.

Additional redox rates \((v_p)\) in Fig. 1 represent low leveled time hierarchies as compared to the fast reaction rates \(v_1, v_2\) and \(v_3\). They are omitted from the model calculations. The latter reactions are considered to adjust the redox ratios of the glutathione and ascorbate moieties.

The assumptions for the model calculations are:

1) The redox system establishes steady-states.
2) The enzymes and their substrates / products are freely diffusible and not separated by compartmentalization.
3) The fraction of NADPH of the total coenzyme moiety (NADPH + NADP) was taken to be 60% in accordance with the experimental results.
4) The electron transfer through the core system is unidirectional because of the irreversibility of the reaction step of APX.

The mono-dehydro ascorbate reductase (MDHR) has not been included into the model because it was practically not measurable in the root supernatant extracts. According to the experimental procedure we deal most likely with the cytosolic fraction of roots (isocitrate dehydrogenase was not measurable). Secondly, mono-dihydroascorbate (MDHA) disproportionates with a rate constant of \(2 \times 10^9 \text{M}^{-1}\text{min}^{-1}\) (POLE et al., 2001; BLOKHINA et al., 2003) which assigns the reaction to a time hierarchy which is more efficient than the enzyme catalyzed reactions of the HAF. Only reactions with comparable time scales can form steady-state aggregates as defined by HEINRICH and RAPPOPORT (1977). Finally, MDHA most likely fulfills particular physiological functions in the context of trans-membrane redox catalysis (MITTLER, 2002). This view is underlined by the fact that the cytosolic isoform of this catalyst is located in the plasma membrane (ASADA, 1999).

Transmembrane separation and transport of DHA and ASC as well as disproportionation of MDHA may influence the respective pools of our model but will be equilibrated by relaxations which are processes considered in our approach. Omitting MDHR does not violate the general concept of our model.

Model calculations have been performed with MATHCAD 6 (Mathsoft Inc. Cambridge, Massachusetts, USA) according to the reaction mechanism depicted in Fig. 2. The time courses of relaxation events were calculated with ModelMaker (Version 3, Cherwell Scientific, Oxford, UK) which integrates numerically the differential equations 5, 6 and 7 using the Runge-Kutta algorithm. The Figs. have been created in SigmaPlot (Version 8.02, SPSS, Inc. USA).
The antioxidative cascade as depicted in Fig. 1 is presented with the symbols used for model simulations. $k_1, k_2$ and $k_3$ are the rate parameters of the reactions of the glutathione reductase (GR), the dehydroascorbate reductase (DHR) and the ascorbate peroxidase (APX) respectively (see methods for details). The Greek letters symbolize the scaled fractions of the variables: $\eta$ is NADPH/N$_0$, $(1-\eta)$ is NADP/N$_0$; $\alpha$ is ASC/A$_0$, $(1-\alpha)$ is DHA/A$_0$; $\beta$ is GSH/G$_0$ and $\gamma$ · $(1-\beta)$ is GSSG/G$_0$. $r$ is the scaled concentration of H$_2$O$_2$ and is defined as $[\text{H}_2\text{O}_2]/K_{\text{m}2}\text{H}_2\text{O}_2$, where $K_{\text{m}2}\text{H}_2\text{O}_2$ is the half-saturation concentration of H$_2$O$_2$ of the APX.

### Results and discussion

**Steady state solutions of HAF with scaled variables and parameters:** The goal of this investigation was to elucidate species properties of the Halliwell-Asada-Foyer detoxification system by means of numerical modelling. It was analyzed which steady-state redox ratios of glutathione and ascorbate can be established under varying enzyme parameter conditions, how the steady fluxes of electrons (the detoxifying potential) change during parameter alterations and how shifted input to output ratios (NADPH / H$_2$O$_2$) effect the redox patterns of the variables. Finally it was studied how relaxation processes equilibrate the variables after system perturbations. Tab. 1 summarizes the scaled values of the oxidized fractions of the variables NADP, GSSG and DHA as well as their total moieties N$_0$, G$_0$ and ASC$_0$ in root extracts of wheat seedlings. The wheat seedlings were grown according to the conditions highlighted in the headlines.

<table>
<thead>
<tr>
<th>4d Dark (reference)</th>
<th>9d Dark</th>
<th>4d Dark + 3d 14 / 10 photoperiod</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaled values</td>
<td>Total concentrations (per g DW)</td>
<td>Scaled values</td>
</tr>
<tr>
<td>NADP 2GSSG DHA N$_0$ G$_0$ ASC$_0$</td>
<td>NADP 2GSSG DHA N$_0$ G$_0$ ASC$_0$</td>
<td>NADP 2GSSG DHA N$_0$ G$_0$ ASC$_0$</td>
</tr>
<tr>
<td>0.42 0.12 0.49</td>
<td>0.382 1.51 33.8</td>
<td>0.64 0.15 0.36 0.118 1.0 31.1</td>
</tr>
<tr>
<td>0.43 0.48 0.49</td>
<td>0.388 0.92 100.0</td>
<td>0.43 0.48 0.49 0.388 0.92 100.0</td>
</tr>
</tbody>
</table>

The total moieties of the co-enzyme (N$_0$), glutathione (G$_0$) and ascorbate (ASC$_0$) in wheat root extracts follow the ranking N$_0$ < G$_0$ << ASC$_0$ (Tab. 1). The absolute values of the total moieties under the chosen experimental conditions of N$_0$, G$_0$, and ASC$_0$ varied between 0.12 to 0.39 µmol g$^{-1}$ dry weight (DW), 1 to 1.5 µmol g$^{-1}$ DW and 30 to 100 (or more) µmol g$^{-1}$ DW respectively. This ranking is most likely also valid for respective extracts of other plants and might even be generalized (FOYER and NOCTOR, 2005).

From the ranking of the pools of variables it is concluded that only the ASC pool (and to a lesser extent the GSH pool) has the potential to detoxify bursts of H$_2$O$_2$ which are caused by environmental cues (OKUDA et al., 1991). This effect will hardly be reached with the co-substrate NADPH which typically appears in low free concentrations in cells. Its low rate of generation in compartments like the cytoplasm by metabolic reactions like the pentose phosphate shunt (FUJII et al., 1991) or the isocitrate dehydrogenase contradicts the view of its function as a primary reductant to detoxify oxidants like H$_2$O$_2$. With NADPH as primary donor of reducing power it must be considered that the reservoirs of GSH and ASC can only be filled up under low oxidative demand. This point of view is supported by the fact that the glucose-6-phosphate dehydrogenase is regulated by a NADPH-dependent negative feedback loop. Increased NADPH pools would inhibit its regeneration by a highly redox sensitive regulatory mechanism of the pentose phosphate shunt (MASON and PAHLICH, 1997). Most likely the antioxidative cascade functions as a pull-regulated system (OLIVER, 2002) the NADPH support of which could become the limiting process for fueling glutathione and ascorbate pools under severe oxidative stress in non-chloroplast compartments.

An advantage of a high ASC pool for detoxification arisises from its participation in two-substrate mechanisms of catalysis as in the ascorbate peroxidase (APX) reaction. Based on ordinary enzyme kinetics it can be shown that a high substrate pool of ASC in combination with a low pool of H$_2$O$_2$ results in very effective and complete destruction of the oxidant. This is in contrast to the reactions of catalase or the GSH or NADPH dependent H$_2$O$_2$ reductases. The latter enzymes catalyse also two substrate reactions but with low substrate concentrations of the reductants and the oxidant. The consequence is that residual fractions of H$_2$O$_2$ survive on low levels if the reaction fades out at its final course of H$_2$O$_2$ destruction. It remains open wether these basic differences in the detoxification rates of H$_2$O$_2$ are involved in the regulation of H$_2$O$_2$ concentrations which are needed for signaling.
Parameter patterns determine the redox ratios of the variables:
The scaled rate parameters of GR, DHR, and APX i.e. $k_1$, $k_2$ and $k_3$
are presented in Tab. 2. The rate parameters are formulated as $V_{\text{max}} / \text{Km}$
ratios according to the generally accepted view that enzymes in
cells are reacting at substrate concentrations in the range or even the
lower range of their Km values (which changes the simple Michaelis
and Menten equation from $v = V_{\text{max}} * S / [\text{Km} + S]$ to the linear form
$v = [V_{\text{max}} / \text{Km}] * S$).

The measured $V_{\text{max}}$ values in Tab. 2 support the impression that the
respective antioxidative cascades of plants grown under the three
conditions have developed fairly different „antioxidative properties“. However, the scaled values elucidate that despite of this impression
the parametric system design is very similar in the two dark grown
batches but seems to be different in the light / dark grown seedlings
(Tab. 2).

The agreement between the measured and modelled redox moieties
of glutathione and ascorbate is shown in Fig. 3. The dimensionless
glutathione and ascorbate values are found at the y-axis when $k_2$ is at
unity. In 4d and in the 9d old seedlings (Fig. 3 A and B) the modelled
dimensionless GSH values were 0.86 and 0.88 respectively, while in
the dark-light treatment a value of 0.52 was found (Fig. 3C). The redox
states of glutathione predicted by the model are in good agreement
with the measured values at the given set of parameters and at the
H$_2$O$_2$ values indicated. In contrast, the simulated and observed ASC
values under the same parametric conditions are in disagreement. ASC
was predicted to be close to unity in all three cases. In reality the ASC
was present in far more oxidized states in all three batches (Tab. 1).
Most likely the high fraction of DHA is a preparation artefact as far as
it results from mixing highly oxidised apoplastic and vascular portions
(BURKEY et al., 1995; FOYER et al., 2001) during homogenization with
the intracellular fluid. The model predicts also, that ASC under steady
conditions is practically insensitive to changes of H$_2$O$_2$ under the
measured parameter patterns.

The robustness of the antioxidative system as judged from the
parameter portraits: The parameter portrait of a system describes the „qualitative behaviour in the space of permissible parameter values“
(REICH and SEI’KOV, 1981). With respect to HAF this means that
systematic changes of the set of the system parameters in a parameter
portrait can visualize and predict the possible stationary values of
glutathione and ascorbate.

Under the growth conditions tested (Tab. 2) the DHR activity shows
the most variable responses. Therefore parameter $k_2$ (x-axis in Fig. 3)
was allowed to change between unity and zero in the parameter portrait
while parameters $k_1$ and $k_3$ were kept constant. The calculated steady
values of the moieties of glutathione and ascorbate are found on the
y-axis of Fig. 3. Following the x-axis downward to $k_2 = 0.1$ conti-
nuously changes the experimentally determined parameter ratio $k_1 : $ $k_2 : k_3 = 0.036 : 1 : 0.01$ to $k_1 : k_2 : k_3 = 0.036 : 0.1 : 0.01$. This is a
dramatic parameter perturbation which might be caused in situ by
environmental cues or developmental traits. Surprisingly this dramatic
parameter change is rather inefficient with respect to changes of the
redox moieties of glutathione and ascorbate which stay at their initial
redox patterns irrespective of the assumed enzyme parameter changes.
The enzyme parameter patterns as measured in wheat roots reflect
obviously a construct which has the potential to buffer the redox ratios
of the variables. Despite of major parameter shifts this behaviour –
which provides stability to the redox ratios of the variables – visual-
izes homeostasis and is a system immanent property. This property
primarily arises from the ratio of the rate parameters $k_1 : k_2 : k_3$ under
a given ratio of NADPH : H$_2$O$_2$. Stable pools of the reductants are the
result despite of dramatic changes of the enzyme activity patterns.
This is to our knowledge the first report of this kind which explains
homeostasis of redox components of HAF on a mechanistic back-
ground and not on intuitive reasons.

However, the homeostatic property of the system vanishes if the $k_2$-
values are approaching zero. The differences of the parameter ratios
$k_1 : k_2 : k_3$ are approaching the approximate range of 1 : 1 : 1. Under
such a regime of parameters the ratios of GSH : 2GSSG and ASC :
DHA are highly responsive to further parametric changes. A new
reaction (and model) situation would arise with DHR approaching
very low values. Thus, the parameter portrait of the antioxidative
system uncovers a bi-phasic character. There exists a robust and stable
branch ($k_1 = 1\rightarrow 0.1$) with highly unequal parameter values and a
sensitive branch ($k_1 < 0.1$) which arises from a set of parameter ratios
which approach equal values. This structural difference within the
reaction cascade elucidates that experimentally observed enzyme
activity changes do not necessarily indicate functional changes with
respect to the redox ratios of the variables glutathione and ascorbate.
However, it is important for functioning whether an environmental
cue hits the system at its homeostatic or its sensitive branch. The
responses must be expected to be quite different. Therefore the param-
eter portrait visualizes the „functional phenotype“ of the system in
cells / tissues. The portrait also reflects system changes which might
occur during plant development or under the influence of varying
growth conditions. In order to better understand „stress induced“
metabolic responses it is necessary to consider the actual state of the
HAF system at the moment of perturbation.

Observations of PASTORI et al., (2000) in maize leaves resulted in a
parameter pattern of the HAF of GR : DHR : APX of 0.9 : 1 : 10

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>4 d dark (reference)</th>
<th>4 d dark plus 3 d 14/10 photoperiod</th>
<th>9 d dark (reference)</th>
<th>4 d dark plus 3 d 14/10 photoperiod</th>
<th>9 d dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ [µM * min$^{-1}$g$^{-1}$DW]</td>
<td>4</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Km [µM]</td>
<td>2.86</td>
<td>$k_1 = 0.036$</td>
<td>5.0</td>
<td>$k_1 = 0.015$</td>
<td>1.43</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>GSSG 1.4</td>
<td>DHA 0.25</td>
<td>H$_2$O$_2$ 2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydroascorbate reductase</td>
<td>20</td>
<td>82</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbate peroxidase</td>
<td>2</td>
<td>6</td>
<td>1.5</td>
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</tbody>
</table>

$k = \text{scaled rate constants (lower rows)}$
Modelling the glutathione-ascorbate redox system of plants

These findings identify a completely different situation in maize leaves as is described for wheat roots. The data point to a sensitive pattern of the HAF rather than a homeostatic situation. The concomitant redox ratios $\text{GSH : GSSG}$ that are close to unity in leave extracts and about two in mesophyll extracts support this point of view. The ascorbate redox ratios ($\text{ASC : DHA}$) are about 1.3 and 0.14 and mirror an entirely different situation than observed in wheat roots. Comparison of the model predictions with literature data is hard to achieve mainly because of incomplete sets of comparable system parameters and variables.

**Input to output parameters:** Kacsar and Burns (1973) have shown that under certain steady conditions co-enzymes and other (signaling) molecules can receive the state of parameters. According to the formulation of our reaction model (Fig. 2), which symbolized a snapshot condition at the moment of harvest of the plants, the reductant NADPH and the oxidant $\text{H}_2\text{O}_2$ are treated as parameters. It will be shown that in combination with the enzyme parameters they must also be viewed as determinants of the redox state of glutathione and ascorbate.

Under physiological conditions it can be assumed that the rates of generation of NADPH and of $\text{H}_2\text{O}_2$ and hence their levels are not necessarily interdependent. Therefore the concentration ratios of NADPH and of $\text{H}_2\text{O}_2$ will vary individually according to the physiological condition of cells. The effect of this variation has been modelled for two scenarios. In these calculations the enzyme rate parameters have been kept constant:

1) NADPH to $\text{H}_2\text{O}_2$ ratio is 4 to 1 (Fig. 3A: $\eta = 0.58, r = 0.15$)
2) NADPH to $\text{H}_2\text{O}_2$ ratio is 2 to 1 (Fig. 3D: $\eta = 0.3, r = 0.15$)

The homeostatic level of GSH at high values of NADPH relative to $\text{H}_2\text{O}_2$ is 0.86 (Fig. 3A) while it decreases to about 0.73 at drastically decreased values of NADPH relative to $\text{H}_2\text{O}_2$ (Fig. 3D). At the same time the homeostatic fractions of ASC remains nearly unaffected in both cases. If $\text{H}_2\text{O}_2$ is increased relative to a fixed NADPH value a similar effect on GSH (and ASC) is obtained (not shown). The effect on the GSH level from decreasing NADPH levels relative to $\text{H}_2\text{O}_2$ or from increasing $\text{H}_2\text{O}_2$ levels relative to NADPH is vice versa. As has been shown for the enzyme rate parameters it is the ratio of these additional sets of parameters and not their absolute values which in-

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**Tab. 3:** The parameter portrait of the antioxidative cascade.

The normalized redox values of GSH, GSSG, ASC and DHA ($y$-axis) are presented as functions of varying scaled values of $k_2$ ($x$-axis) at constant values of $k_1$ and $k_3$ respectively. Relative levels of GSH and GSSG are depicted as solid and dotted lines and those of ASC and DHA are presented as dashed and dashdotted lines. The calculations were performed with the following parameters (taken from Tab. 2): A: 4 days old dark grown seedlings, $k_1 = 0.036, k_3 = 0.01, \text{NADPH} = 0.6, \eta = 0.58, r = 0.15$. The calculated values of GSH and ASC (at $x = \text{unity}$) are 0.86 and 0.997 respectively. B: 9 days old dark grown seedlings, $k_1 = 0.036, k_3 = 0.015, \eta = 0.36, r = 0.05$. The calculated values of GSH and ASC (at $x = \text{unity}$) are 0.88 and 0.998 respectively. C: 7 days old seedlings which are grown in a 14 / 12 h photoperiod during the last 3 days. $k_1 = 0.015, k_3 = 0.007, \eta = 0.57, r = 0.3$. GSH and ASC (at $x = \text{unity}$) are calculated to be 0.52 and 0.99 respectively. D: GSH and ASC under decreased NADPH ($\eta = 0.3$), the rest of parameters and variables as in Fig. 3A. The calculated values of GSH and ASC (at $x = \text{unity}$) are 0.73 and 0.99 respectively.
fluence the redox ratios of the glutathione and ascorbate moieties. It is evident from these calculations that decreased / increased stationary concentrations of GSH can be caused by changing the enzyme rate parameter patterns, or the ratios of NADPH levels relative to H$_2$O$_2$. Under the parameter patterns considered the level of ASC remains always on a highly reduced state. The redox ratios of the variables glutathione and ascorbate and their changes can not simply be interpreted as indicators of increased or decreased oxidative stress, i.e. changed levels of H$_2$O$_2$.

**Electron fluxes and detoxifying efficiencies:** The detoxifying efficiency of the HAF is best characterized by the electron fluxes through the system under the prevalent parametric conditions. Therefore fluxes have been calculated with the measured parameters (Tab. 1) and the redox levels of glutathione and ascorbate at k2 values of 1, 0.1, 0.05, 0.005 and 0.001 respectively (Fig. 4). The relative fluxes derived from scaled parameters and variables show also bi-phasic patterns of different intensities and are bending downward at very low k2 values (Fig. 4). This effect is most pronounced in plants that are grown under light. Their parameter conditions promote highest electron fluxes despite that GR and APX and also GSH obtain lowest values. In comparison to this the fluxes in 4d and 9d old dark grown seedlings range on lower rates. All three fluxes approach similar values when k2 is becoming a limiting reaction step. Under these conditions the regeneration of ASC is fading out and the system does not agree any more to the concept formulated in Fig. 2. It is surprising how far the rate parameter ratios can be changed without being mirrored by respective flux changes in the plateau ranges of the profiles. The example illustrates that electron flux (as metabolic fluxes in general) reflect sytem properties and can not be deduced intuitively from pool sizes of the redox variables or from enzyme activity changes of single reaction steps. It can be concluded that moderate enzyme activity alterations of the HAF, which are reported in the literature, are from minor consequences for changes of its detoxifying potential under respective parameter patterns.

Changing the input to output ratios NADPH / H$_2$O$_2$ from 4:1 to 2:1 result in high fluxes if k2 is unity (Fig. 6, in-out 2:1). But the entire system undergoes a shift from homeostatic to sensitive patterns in respect to decreasing k2. It should be noted that a very similar flux profile is reached under 10 fold increased k3. Flux responses at decreased NADPH input or at a 10-fold increase of k3 (Fig. 6, in-out 2:1 and k3) are nearly indentical. Thus, two entirely different parameter changes result in nearly identical system responses which again elucidates that system responses are hardly to foresee intuitively.

**Modelling assumed genetic transformations of the HAF:** Tailoring of plants for improving their (oxidative) stress performance via transformation of particular reaction steps is a widely followed concept (BROADBENT et al., 1995; NOCTOR et al., 1997; XIANG et al., 2001; STROHM et al., 2002; RIZHSKY et al., 2002b; MURGIA et al., 2004; MITTLER and POULOS, 2004; YAMAMOTO et al., 2005). From the discussion in the previous section it can be seen which results might be received by over-expressing enzymes of the HAF. If in 4 d dark grown seedlings the k1 would be over-expressed 10-fold then the measured parameter ratio k1 : k2 : k3 would change from the observed ratio 0.036 : 1 : 0.01 to the assumed ratio 0.36 : 1 : 0.01. The model predicts for this tailored parameter constellation that concentrations of ASC and GSH approach their maximal levels, which is nearly unity for

![Fig. 4: Electron fluxes calculated with the parameters and variables of Figs. 3 A to C at decreasing values of k2. The fluxes exert well established homeostatic ranges down to a nearly 100 fold decrease of k2 relative to constant values of k1 and k3.](image)

![Fig. 5: The effect on glutathione and ascorbate moieties of increased k1 and k3. The effect of a 10 fold increase of k1 (Fig. 5A) or of k3 (Fig. 5B) on the amount of GSH and ASC. Other conditions as described in Fig. 3A. GSH and ASC remain reduced under increased k1 (Fig. 5A) but drop dramatically under increased k3 (Fig. 5B).](image)
ASC (Fig. 5A). Due to the equilibrium of the GR reaction GSH can not exceed 0.9 on the relative scale. In contrast to expectations (increasing support of the cascade with GSH), the electron flux at increased $k_1$ remains low and moves on a plateau over a wide range of $k_2$ changes (Fig. 6). This is easy to understand since the substrate DHA is on its lowest level under these conditions (Fig. 3) and necessarily opposes the parameter increase.

Checking these predictions experimentally is difficult. Overexpression of a particular enzyme like GR might enhance also other enzymes like APX and DHAR (STROHM et al., 2002) and therefore change the entire rate parameter patterns. Furthermore the effects arising from overexpression of a particular gene might be limited to a given developmental state and is different under other developmental conditions (STROHM et al., 2002). Another question is whether changes of single gene expressions can be reached in vivo as practiced by modelling.

Increased APX ($k_3$) activities are expected to increase the rate of detoxification of $\text{H}_2\text{O}_2$. Assuming a 10-fold increase of $k_3$ in 4 d old seedlings changes the ratio of $k_1 : k_2 : k_3$ to 0.036: 1 : 0.1. The stationary GSH pool under these conditions is predicted to drop to a level of < 0.2 and the ASC pool decreases to 0.6 (Fig. 5B). The predicted steady flux profile over changing $k_2$ is very different (Fig. 6, k3) to the control (Fig. 4, k4). The system has lost its homeostatic plateau and is changed into a $k_2$-sensitive slope (Fig. 6, k3). This parametric alteration causes a system change and switches it from a stable to a sensitive functional form. If electron flux is taken as a measure for antioxidative capacity then the system should become less effective with respect to $\text{H}_2\text{O}_2$ detoxification under this parameter constellation.

Overexpression of particular enzymes might show the expected response under certain growth conditions. But even slightly different growth conditions might perturb the system and evoke „flexible” and unpredictable responses. It has been shown for instance that overgrowth conditions might perturb the system and evoke „flexible” and unpredictable responses. It has been shown for instance that overgrowth conditions might perturb the system and evoke „flexible” and unpredictable responses.

Glutathione and ascorbate consumption cause relaxations: Glutathione and / or ascorbate can be withdrawn from the HAF system for instance by intercellular transport (FOYER et al., 2001) or by ASC dependent hydroxylation of extracellular proteins (DE TULLIO et al., 1999). Export and re-import of GSH and / or GSSG and ASC and / or DHA among cellular compartments such as the apoplast or the vascular system are also reported (SMIRNOFF et al., 1996; HOREMANS et al., 2000; HEBER et al., 2003; YAMAMOTO, 2005). In any of these cases the redox balance within the HAF will be disturbed and a re-adjustment according to the given parameter patterns by a relaxation process will be initiated. The time scales of the relaxations are different for any reaction because of individual relaxation times $\tau$ which are approximated by $K_m / V_{max}$ values in enzyme catalyzed reactions (BUCHER and RUSSMANN, 1963; HIGGINS, 1963). The smaller the relaxation time the more effective is the transition process.

In 4 d old seedlings (Tab. 1) are observed ASC levels (Fig. 3) which are out of redox balance and far too low according to model calculations. Under the given set of parameters a relaxation would be unavoidable under these conditions (Fig. 7). The pool of ASC which is about 0.5 at time zero increases instantaneously and approaches its stationary value close to unity in an extremely short period of time. This reduction occurs on the cost of the GSH pool which at the same time drops from 0.86 to a value of less than 0.5. This drop is caused by the high $k_2$ value or its respective relaxation time $\tau_2$ which is 0.013.

In contrast to ASC the regeneration of the stationary GSH level is slow because of the lower relaxation time $\tau_1$ of the GR which is 0.34.

The consequence of these differences in the relaxation times of the two enzymes is that the GSH level reaches its original stationary value of 0.86 long after the adjustment of the ASC pool. These remarkable differences in the time courses of the adjustments of the stationary values of perturbed systems can also be observed in physiological responses of even higher complexity as the HAF like the the prolin acclimation in water stressed plants (PAHLICH, 1996).

As long as it is open whether variables have reached their stationary...
values after experimental perturbations it must be assumed that they are part of a so called „pseudo steady-state“ (HENRICH and RAPOPORT, 1977). The variables are not yet balanced according to the parameter patterns and are still moving on a transitory trajectory. The level of the variables can be far away from their final and balanced states while accompanying metabolites have reached their balanced state already. In the presented case the decreasing levels of GSH at high ASC contents in the initial phase of the relaxation will hardly provide a safe base for intuitive interpretations of the physiological significance of the state of HAF.

**Thiol-disulfide proteome, a subsystem of HAF?** It is assumed that NADPH, GSH and also ASC are electron supporters of the thiol-disulfide proteome which therefore are competing reactions to the detoxification of $\text{H}_2\text{O}_2$. The genetic multiplicity of the glutathione ascorbate cycle genes (MITTLER and POULOS, 2005) and those of the thiol-disulfide proteome (DIETZ, 2005) in plants can cause highly diverse and tissue specific combinations of the proteom’s components with the antioxidative cascade. This multiplicity can not be treated with a simplifying dynamic model. But some aspects of the supposed interactions can be deduced from our findings. It can be assumed that the redox cycles of the thiol-disulfide proteins operate at lower concentration and rate scales than those of the antioxidative cascade. Consumption of GSH or ASC or direct changes of their redox ratios will necessarily cause relaxations of the HAF. In any case the redox variables glutathione and ascorbate will be balanced according to the parameter structure of the antioxidative cascade. These suggestions might be taken as evidence that the antioxidative cascade functions as a superior balancing system which mirrors the cellular redox state according to the parameter ratios of the HAF.

**Does the antioxidative system operate at a steady-state?** The described properties of the antioxidative system are strictly valid only if the antioxidative system operates at steady-state. This assumption includes steady values of NADPH and $\text{H}_2\text{O}_2$. A steady-state of the HAF can most likely be established only under conditions when a moderate and continuous electron demand is needed for the detoxification of continuously produced metabolic $\text{H}_2\text{O}_2$ while the support with NADPH remains undisturbed. However, increased burdens of $\text{H}_2\text{O}_2$ (e.g. caused by ozone spikes), changed availabilities of NADPH, environmentally induced sudden enzyme activity changes will perturb the system due to drastically changed input to output ratios and enzyme rate parameter patterns. Thus, the system is forced into relaxations with fluctuating concentrations of the variables according to individual relaxation times of the particular reactions (Fig. 7). The system is removed from its initial steady state and will stay in motion until its self-organized search for a new possible steady-states is reached (von BERTALANFFY et al., 1977; PEACOCK, 1983).

We emphasize the core process of the antioxidative system as a non-decomposable set of enzymes (PAPIN et al., 2003) which looses its particular system properties if parametric components are included or deleted. This undissectable reaction unit might be part of a broader metabolic network with superimposed regulatory properties. Advanced analytical techniques (SCHWENDER et al., 2004) and mathematical network approaches, which are based on whole genome responses (PAPIN et al., 2003), might help to uncover missing components of an extended antioxidative network. However the basic functioning of a particular system properties if parametric components are included or deleted. The HAF as indicated in Fig. 1 can, most likely function as a redox balancing mechanism which organizes the redox ratios of glutathione and ascorbate according to the prevailing condition of cells. The observed insights into the HAF system were only possible via numerical modelling which we recommend as a tool in stress physiology to support intuitive interpretations.

**Conclusions**

The modelling of the antioxidative system has uncovered properties which hitherto have been overlooked. The particular enzyme activity ratios of the antioxidative Halliwell-Asada-Foyer (HAF) cascade of wheat roots give rise to the formation of homeostasis. In the homeostatic range the redox ratios of the glutathione and ascorbate moieties remain on a stable level as opposed to rather dramatic enzyme activity changes. The ratios of the homeostatic redox moieties 2GSSG : GSH and DHA : ASC depend on the ratios of the respective enzyme parameter patterns of the system when the pools of NADPH and of $\text{H}_2\text{O}_2$ are in steady-state. Under the parameter conditions considered the redox moieties of glutathione and ascorbate were kept on highly reduced states. The measured level of DHA most likely represents a fraction which is located in a non-regenerating compartment, probably the apoplast or the vascular system. Major changes of the redox ratios of the glutathione and ascorbate moieties can also result from changes of the NADPH to $\text{H}_2\text{O}_2$ ratio. Decreasing concentrations of NADPH relative to $\text{H}_2\text{O}_2$ cause identical redox properties of the redox variables with increasing $\text{H}_2\text{O}_2$ burdens relative to an unchanged NADPH pool. The redox state of the glutathione and ascorbate moieties can not be regarded as indicators for oxidative stress in terms of increased $\text{H}_2\text{O}_2$ levels. The electron fluxes through the system exert also homeostatic branches. Neither increased enzyme activities nor increased levels of the reduced states of the variables are indicators for increased fluxes or increased antioxidative capacities. High electron fluxes can occur under low fractions of GSH and ASC and the system might be highly efficient with respect to $\text{H}_2\text{O}_2$ detoxification under these conditions. The HAF as indicated in Fig. 1 can, most likely function as a redox balancing mechanism which organizes the redox ratios of glutathione and ascorbate according to the prevailing condition of cells. The observed insights into the HAF system were only possible via numerical modelling which we recommend as a tool in stress physiology to support intuitive interpretations.

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