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

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Summary

The Hallilwell-Asada-Foyer redox cascade (HAF) is viewed as a H_2O_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, dehydroascorbate reductase and ascorbat peroxidase. Necessarily their oxidised counterparts dithioglutathione (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_2O_2 ratios. Thus, the redox states of GSH and ASC can not simply be regarded as indicators for oxidative stress with respect to H_2O_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_2O_2 detoxifying system (HALLIWELL, 1978; HALLIWELL, 1987; ISHIKAWA and SIES, 1989). Detoxification is reached by transfering electrons from nicotineamide adenine nucleoside phosphate (NADPH) to hydrogen peroxide (H_2O_2) *via* the cycle intermediates glutathione and ascorbate. The reactions are catalyzed by the enzymes glutathione reductase (GR), dehyroascorbate 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 pertubations, 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 autput 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_2O_2 which are the input and output ports of the HAF system. Both, the rate parameters and the input (NADPH) to output (H_2O_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_2O_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_2O_2 detoxifying function of HAF can be correlated with the need of residual portions of H_2O_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 (SCHEIBE, 1991; DIETZ, 2005; FOYER and NOCTOR, 2005; STRÖHER 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; JIMENEZ 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_2O_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_2O_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_2O_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 moieity 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 1cm 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 supernatant) the extracts did not contain mitochondria and peroxisomes.

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 dithioglutathione (GSSG) were determined according to GRIFFITH (1980), ascorbate (ASC) and dehydroascorbate (DHA) were determined according to OKAMURA (1980) and the reduced and oxidized coenzymes 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 comparibility 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:

Eqn. 1: $v = k \cdot [A] \cdot [B] \cdot [C]$

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 (LEHNINGER, 1977).

Model calculations occurred with dimensionless contents of the variables glutathione, ascorbate and nicotineamide 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).

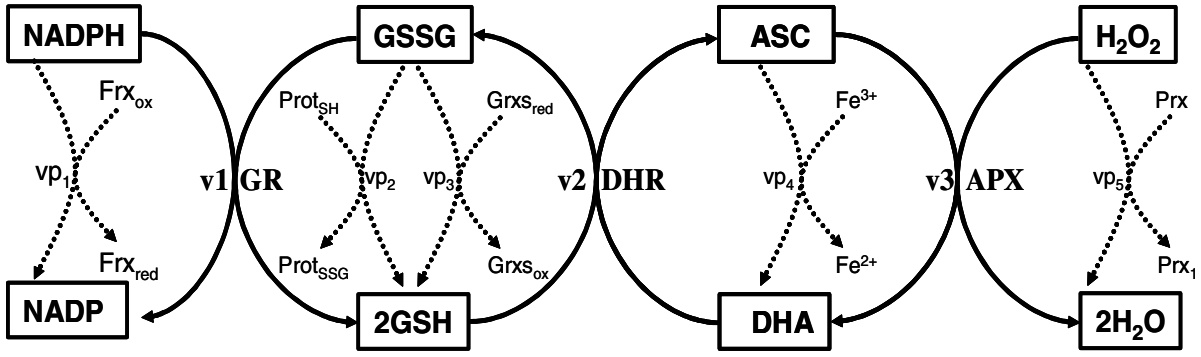


Fig. 1: The redox balancing potential of the antioxidative glutathione-ascorbat cascade (solid lines).
The NADPH pool provides reducing power to the system. Electrons are transferred by the glutathione reductase (GR)(v1) and the dehydroascorbate reductase (DHR)(v2) to the ascorbate moiety (DHA : ASC). Hydrogen peroxide (H_2O_2) finally is reduced *via* the ascorbate peroxidase reaction (APX)(v3). Included into the reaction cascade are interacting reactions (dotted lines). Vp_1, vp_3 and vp_4 symbolize consuming reactions of the reductants, vp_2 and vp_5 are consuming oxidants. Frx_{ox}/Frx_{red} = oxidized and reduced forms of thioredoxins; $Prot_{SH}/Prot_{SSG}$ = thiol protein and glutathionylated protein; Grx_{ox}/Grx_{red} = oxidized and reducued forms of glutaredoxins; Fe^{3+}/Fe^{2+} = oxidized and reduced 2-oxoglutarat (FeII) dioxygenase; Prx_1/Prx oxidized and reduced forms of peroxiredoxins.

Eqn. 2a: $\alpha = ASC/A_0 \; ; \; 1-\alpha = DHA/A_0$

Eqn. 2b: $\beta = GSH/G_0 \; ; \; 1/2 \cdot (1-\beta) = GSSG/G_0$

Eqn 2c: $\eta = NADPH/N_0 \; ; \; 1-\eta = NADP/N_0$

with,

Eqn. 3a: $A_0 = ASC + DHA$

Eqn. 3b: $G_0 = GSH + 2GSSG$

Eqn. 3c: $N_0 = NADPH + NADP^+$

The dimensionless concentration of H_2O_2 is defined as

Eqn. 4: $r = H_2O_2 / Km_{H_2O_2}$

with

$Km_{H_2O_2}$, the half saturation constant of ascorbate peroxidase (TAKEDA et al., 1998).

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

Eqn. 5: $v_1 = k_1 \cdot 1/2 \cdot (1-\beta) \cdot \eta(GR)$

Eqn. 6: $v_2 = k_2 \cdot (1-\alpha) \cdot \beta^2 (DHR)$

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

where,

GR, *DHR* and *APX* represent the scaled reactions of the gluthatione 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 α and β:

with

Eqn. 8: $\alpha := k2 \cdot \dfrac{\beta^2}{\left(k2 \cdot \beta^2 + k3 \cdot r\right)}$

Eqn. 9: $\beta := a - \dfrac{b}{a} + c$

with

a, b and *c* as written in equation 10.

Eqn. 10:

$$a := \left[\frac{-1}{6} \cdot k3 \cdot \frac{r}{k2^2} \cdot \frac{(k2 \cdot Ka - 2 \cdot k3 \cdot r \cdot k2)}{Ka} + \frac{1}{2} \cdot k3 \cdot \frac{r}{k2} \ldots \right.$$
$$+ \frac{1}{27} \cdot \frac{(k2 \cdot Ka - 2 \cdot k3 \cdot r \cdot k2)^3}{\left(k2^3 \cdot Ka^3\right)} + \frac{1}{9} \cdot \sqrt{k3} \cdot \sqrt{\left[\begin{array}{l} \left(-k3^3 \cdot r^4 \cdot Ka \cdot k2 - 8 \cdot k3^3 \cdot k2^2 \cdot r^4 + 12 \cdot k3^2 \cdot k2^2 \cdot r^3 \cdot Ka \ldots \right) \ldots \\ + 10 \cdot k3^2 \cdot r^3 \cdot Ka^2 \cdot k2 \\ + k3^2 \cdot r^3 \cdot Ka^3 \end{array} \right] - 6 \cdot k3 \cdot k2^2 \cdot Ka^2 \cdot r^2 \ldots}$$
$$\left. + 2 \cdot k3 \cdot r^2 \cdot Ka^3 \cdot k2 + \left(r \cdot k2^2 \cdot Ka^3\right) \cdot \frac{\sqrt{3}}{\left[k2^{\left(\frac{3}{2}\right)} \cdot Ka^{\left(\frac{3}{2}\right)} \right]} \right]^{\left(\frac{1}{3}\right)}$$

$$b := \frac{1}{3} \cdot k3 \cdot \frac{r}{k2} - \frac{1}{9} \cdot \frac{(k2 \cdot Ka - 2 \cdot k3 \cdot r \cdot k2)^2}{\left(k2^2 \cdot Ka^2\right)}$$

$$c := \frac{1}{3} \cdot \frac{(k2 \cdot Ka - 2 \cdot k3 \cdot r \cdot k2)}{(k2 \cdot Ka)}$$

The rate constants *k1*, *k2* and *k3* in equations 8 and 10 represent the scaled rate parameters. *Ka* is the product of the scaled values of *k1* and η. Combining both constants saves computer capacity for calculations.

Additional redox rates (vp_s) in Fig. 1 represent low leveled time hierachies 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 x 10^9 $M^{-1}min^{-1}$ (POLLE, 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 RAPOPORT (1977). Finally, MDHA most likely fulfils 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, Massachsetts, 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).

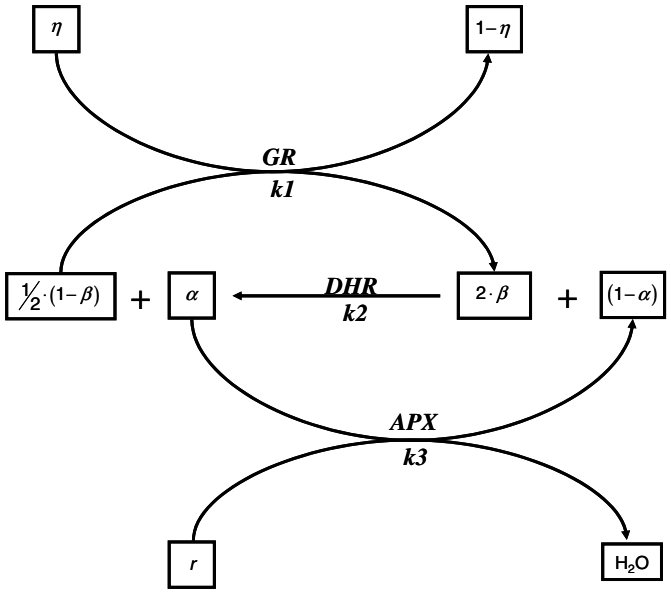


Fig. 2: Symbolism for modelling the antioxidative cascade.
The antioxidative cascade as depicted in Fig. 1 is presented with the symbols used for model simulations. *k1*, *k2* and *k3* are the rate parameters of the reactions of the glutathione reductase (GR), the dehydroascorbate reductase (DHR) and the ascorbate peroxidase (APX) respectively (see methods for details). The greek letters symbolize the scaled fractions of the variables: η is NADPH/N_0, (1-η) is NADP / N_0; α is ASC / A_0, (1-α) is DHA / A_0, β is GSH / G_0 and ½ · (1-β) is GSSG / G_0. r is the scaled concentration of H_2O_2 and is defined as [H_2O_2] / Km_{H2O2}, where Km_{H2O2} is the half-saturation concentration of H_2O_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 systemic 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_2O_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 ($NADP^+$+NADPH), G_0 (GSH+ 2 GSSG) and ASC_0 (ASC+DHA) in root extracts of wheat seedlings.
The differences of the dimensionless values (Tab. 1) of the variables reflect the effect of the three growth conditions considered. NADP changed between 0.42 and 0.64. The fraction of 2GSSG in the seedlings grown in the dark ranged between 0.1 and 0.15 but reached a value of 0.48 under a 14 h photoperiod. DHA varied between 0.36 and 0.49. The fractions of the reduced forms of the variables are the differences to unity of the presented values. It can be seen (Tab. 1) that the HAF under the considered growth conditions is kept on a highly reduced state with respect to glutathione while ascorbate tends to less reduced fractions. The reduced nictotinadenine dinucleoside phosphate fraction shifts to approximately 0.5. In the latter case it should be considered that major portions of the reduced co-enzyme are assumed to be firmly bound to redox enzymes. NADPH is therefore most likely represented by a higher fraction in roots as detected in measured soluble extracts of this organ.

Tab. 1: The scaled oxidized fractions of the variables nicotinamide adenine dinucleoside phosphate (NADP), dithioglutathione (GSSG) and dehydro ascorbate (DHA) (lanes 1, 2 and 3) and their total moieties: N_o is NADP + NADPH, G_o is 2 GSSG + GSH and ASC_o is DHA + ASC (lanes 4, 5 and 6) from root extracts of three different growth experiments with wheat seedlings. The wheat seedlings were grown according to the conditions highlighted in the headlines.

4d Dark (reference)					
Scaled values			Total concentrations (per g DW)		
NADP	2GSSG	DHA	N_o (µM)	G_o (µM)	ASC_o (µM)
0.42	0.12	0.49	0.382	1.51	33.8
9d Dark					
Scaled values			Total concentrations (per g DW)		
NADP	2GSSG	DHA	N_o (µM)	G_o (µM)	ASC_o (µM)
0.64	0.15	0.36	0.118	1.0	31.1
4d Dark + 3d 14 / 10 photoperiod					
Scaled values			Total concentrations (per g DW)		
NADP	2GSSG	DHA	N_o (µM)	G_o (µM)	ASC_o (µM)
0.43	0.48	0.49	0.388	0.92	100.0

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) µmole 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 extend the GSH pool) has the potential to detoxify bursts of H_2O_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 (FUKUOKA and ENOMOTO, 2001) or the isocitrate dehydrogenase contradicts the view of its function as a primary reductant to detoxify oxidants like H_2O_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 (MALDONADO 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 arisis 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_2O_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_2O_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_2O_2 survive on low levels if the reaction fades out at its final course of H_2O_2 destruction. It remains open wether these basic differences in the detoxification rates of H_2O_2 are involved in the regulation of H_2O_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. *k1, k2* and *k3* are presented in Tab. 2. The rate parameters are formulated as Vmax / 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 = Vmax * S / [Km + S] to the linear form v = [Vmax / Km] * S).

The measured Vmax 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 *k2* 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_2O_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_2O_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 SEL'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.

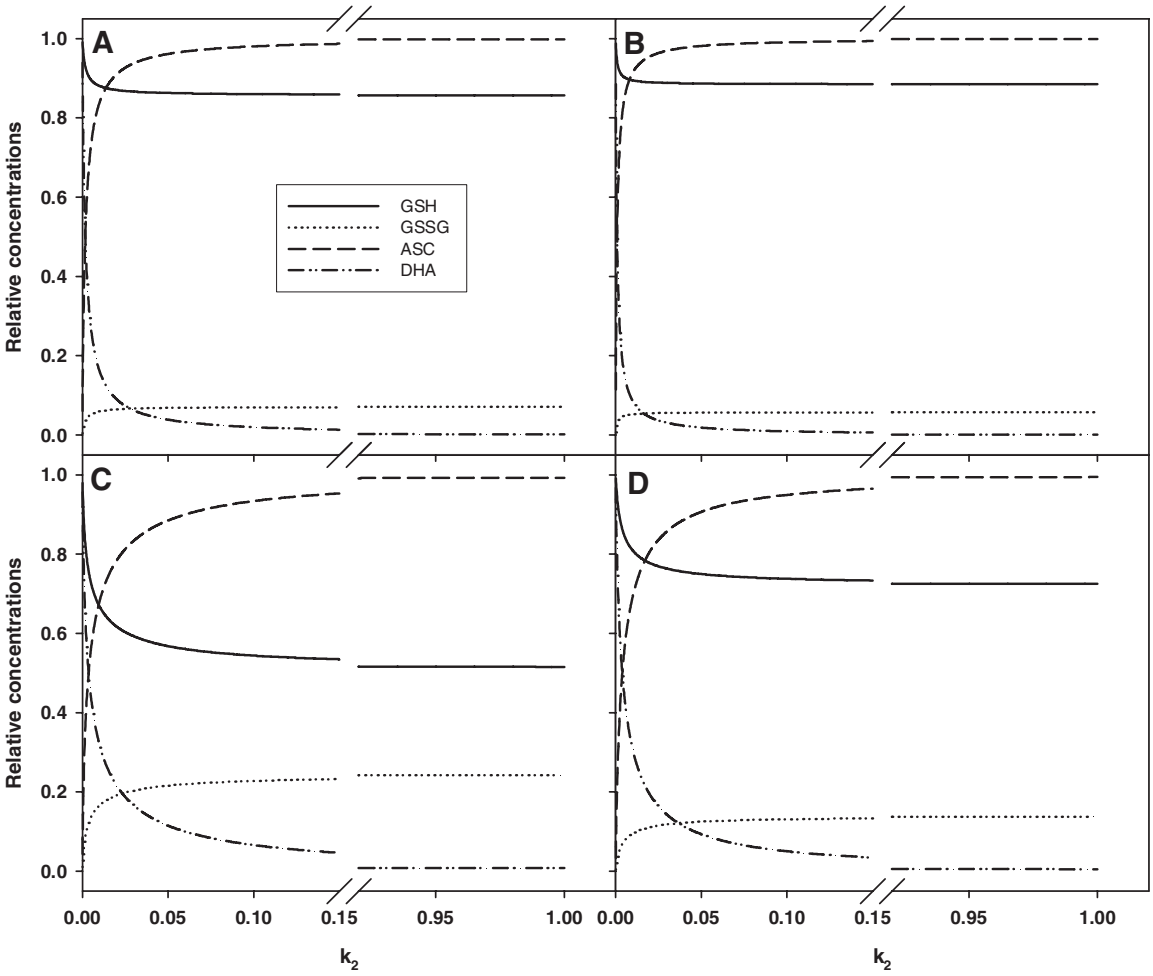
Under the growth conditions tested (Tab. 2) the DHR activity shows the most variable responses. Therefore parameter *k2* (x-axis in Fig. 3) was allowed to change between unity and zero in the parameter portrait while parameters *k1* and *k3* were kept constant. The calculated steady values of the moieties of glutathione and ascorbate are found on the y-axis of Fig. 3. Following the x-axis downward to *k2* = 0.1 continuously changes the experimentally determined parameter ratio *k1* : *k2* : *k3* = 0.036 : 1 : 0.01 to *k1* : *k2* : *k3* = 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 – visualizes homeostasis and is a system immanent property. This property primarily arises from the ratio of the rate parameters *k1* : *k2* : *k3* under a given ratio of NADPH : H_2O_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 background and not on intuitive reasons.

However, the homeostatic property of the system vanishes if the *k2*-values are approaching zero. The differences of the parameter ratios *k1* : *k2* : *k3* 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 exsists a robust and stable branch (*k1* = 1→0.1) with highly unequal parameter values and a sensitive branch (*k1* < 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 parameter 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

Tab. 2: Vmax values of the glutathione reductase (GR), the dehydroascorbate reductase (DHR) and the ascorbate peroxidase (APX) of dark grown seedlings (4 d; 9 d) and of seedlings with a 14/10 photoperiod during the last 3 d (lanes 2, 3 and 4) and the Km values of their oxidized substrates (lane 5) respectively. On the right hand side of the table are arranged the rate parameters of the three enzymes (Vmax / Km, upper row in lanes 5, 6 and 7) and their scaled values *k1*, *k2* and *k3* (lower row in lanes 5, 6 and 7). Scaling was achieved by dividing k1 and k3 by k2.

Growth conditions	4 d dark (reference)	4 d dark plus 3 d 14/10 photoperiod	9 d dark		4 d dark (reference)	4 d dark plus 3 d 14/10 photoperiod	9 d dark
	Vmax [µM * min^{-1}*g^{-1}DW]			Km [µM]	Vmax / Km [min^{-1}*g^{-1}DW] (upper row) *k* = scaled rate constants (lower rows)		
Glutathione reductase	4	7	2	GSSG 1.4	2.86 *k1* = 0.036	5.0 *k1* = 0.015	1.43 *k1* = 0.036
Dehydroascorbate reductase	20	82	10	DHA 0.25	80 *k2* = 1	328 *k2* = 1	40 *k2* = 1
Ascorbate peroxidase	2	6	1.5	H_2O_2 2.5	0.8 *k3* = 0.01	2.4 *k3* = 0.007	0.6 *k3* = 0.015



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 *k2* (x-axis) at constant values of *k1* and *k3* 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, *k1* = 0.036, *k3* = 0.01, NADPH = 0.6, η = 0.58, r = 0.15. The calculated values of GSH and ASC (at x = unity) are 0.86 and 0.997 respectively. B: 9 days old dark grown seedlings, *k1* = 0.036, *k3* = 0.015, h = 0.36, r = 0.05. The calculated values of GSH and ASC (at x = 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. *k1* = 0.015, *k3* = 0.007, η = 0.57, r = 0.3. GSH and ASC (at x = unity) are calculated to be 0.52 and 0.99 respectively. D: GSH and ASC under decreased NADPH (η = 0.3), the rest of parameters and variables as in Fig. 3A. The calculated values of GSH and ASC (at x = unity) are 0.73 and 0.99 respectively.

(20°C) and 2.7 : 1 : 0.18 (15°C). 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 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 (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: KACSER 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 snap shot condition at the moment of harvest of the plants, the reductant NADPH and the oxidant H_2O_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 H_2O_2 and hence their levels are not necessarily interdependent. Therefore the concentration ratios of NADPH and of H_2O_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 H_2O_2 ratio is 4 to 1 (Fig. 3A: η = 0.58, r = 0.15)
2) NADPH to H_2O_2 ratio is 2 to 1 (Fig. 3D: η = 0.3, r = 0.15)

The homeostatic level of GSH at high values of NADPH relative to H_2O_2 is 0.86 (Fig. 3A) while it decreases to about 0.73 at drastically decreased values of NADPH relative to H_2O_2 (Fig. 3 D). At the same time the homeostatic fractions of ASC remains nearly unaffected in both cases. If H_2O_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 H_2O_2 or from increasing H_2O_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-

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_2O_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_2O_2.

Electron fluxes and detoxifying efficiencies: The detoxifying efficience 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 biphasic 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 only from minor consequences for changes of its detoxifying potential under respective parameter patterns.
Changing the input to output ratios NADPH / H_2O_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 k_3 (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.

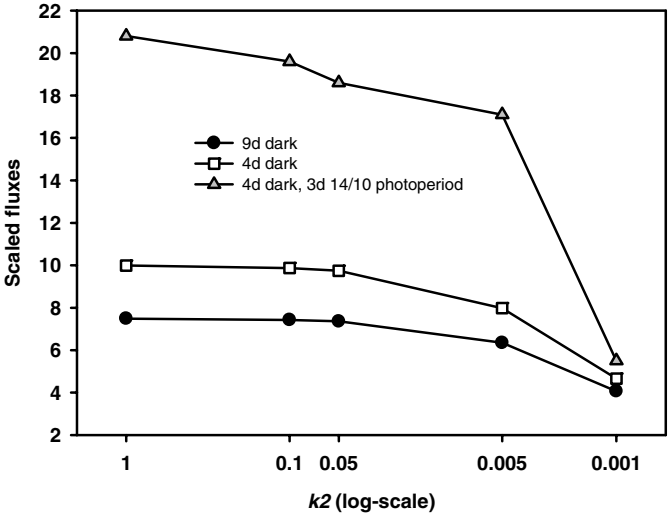


Fig. 4: Electron fluxes calculated with the parameters and variables of Figs. 3 A to C at decreasing values of *k2*. The fluxes exert well established homeostatic ranges down to a nearly 100 fold decrease of *k2* relative to constant values of *k1* and *k3*.

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

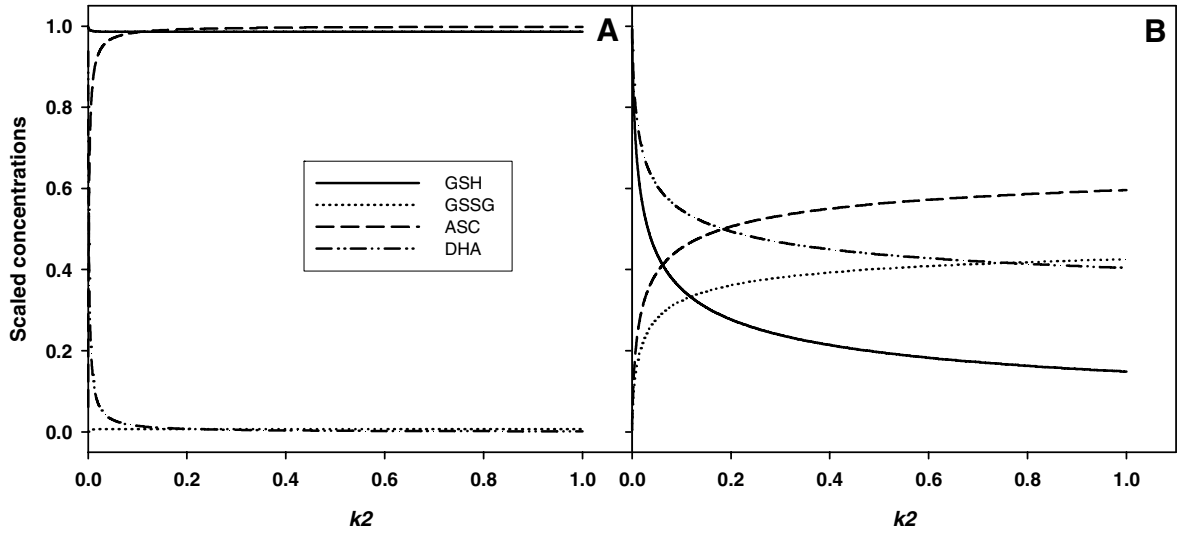


Fig. 5: The effect on glutathione and ascorbate moieties of increased *k1* and *k3*.
The effect of a 10 fold increase of *k1* (Fig. 5A) or of *k3* (Fig. 5B) on the amount of GSH and ASC. Other conditions as described in Fig. 3A. GSH and ASC remain reduced under increased *k1* (Fig. 5A) but drop dramatically under increased *k3* (Fig. 5B).

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 *k1* remains low and moves on a plateau over a wide range of *k2* 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 (*k3*) activities are expected to increase the rate of detoxification of H_2O_2. Assuming a 10-fold increase of *k3* in 4 d old seedlings changes the ratio of *k1 : k2 : k3* 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. 5 B). The predicted steady flux profile over changing *k2* is very different (Fig. 6, *k3*) to the control (Fig. 4, 4d). The system has lost its homeostatic plateau and is changed into a *k2*-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 H_2O_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 overexpression of tAPX increases the resistance against O_2^- under given growth conditions but does not reinforce defense under changed light or heavy metal environments (MURGIA et al., 2004). Even so we are convinced that modelling provides useful insights into the responsiveness and functioning of complex metabolic units like HAF which can not be gained by guessings. But modelling „physiological reality" (POLLE, 2001) is a much more complex task than modelling a defined „core unit" of metabolism.

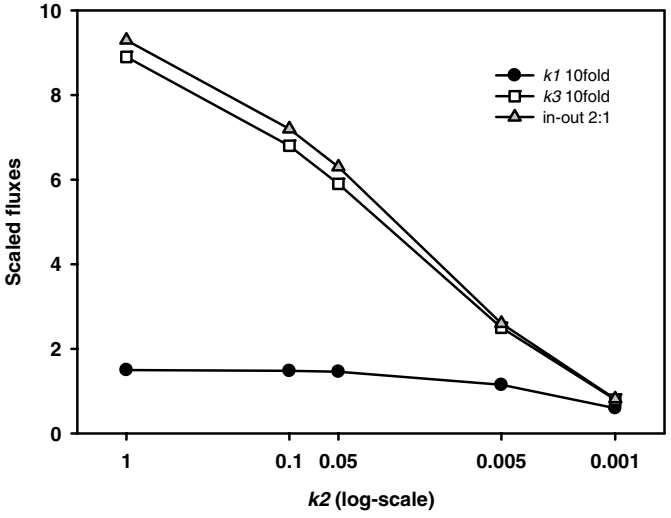


Fig. 6: Electron fluxes under increased rate parameters *k1* or *k3* (other conditions as defined in Fig. 5A) and under decreased amounts of NADPH relative to H_2O_2 (η : r is 0.3 : 0.15). Increasing the rate parameter *k1* is ineffective in respect to the homoeostatic patterns of the system. Increasing amounts of *k3* or decreasing levels of NADPH relative to H_2O_2 make the system sensitive to changes of the parameter *k2*.

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 τ which are approximated by Km / Vmax values in enzyme catalyzed reactions (BÜCHER and RÜSSMANN, 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 *k2* value or its respective relaxation time τ_2 which is 0.013. In contrast to ASC the regeneration of the stationary GSH level is slow because of the lower relaxation time τ_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

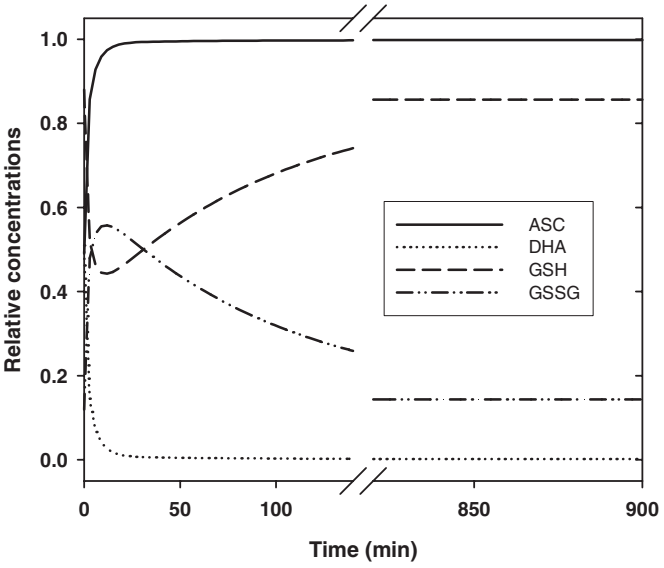


Fig. 7: Adjustment of the unbalanced DHA : ASC moiety over time according to the parameter patterns of 4d old seedlings. According to the system modelling (Fig. 3A) the measured ASC fraction (Tab. 1) of seedlings is too low. The balance of the variables in dependence of the measured rate parameters is achieved in a two step relaxation process. DHA is reduced immediately after the onset of the relaxation process on the cost of GSH which drops from 0.86 to about 0.45. In a second step GSH is approaching its steady value at a much lower rate than ASC. The GSH : GSSG values up to about 250 time units might be regarded as „pseudo steady-state" values.

values after experimental perturbations it must be assumed that they are part of a so called „pseudo steady-state“ (HEINRICH 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 H_2O_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 H_2O_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 H_2O_2 while the support with NADPH remains undisturbed. However, increased burdens of H_2O_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 emphazise 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 minimal and unique pathway needs still be analyzed and characterized by a classical kinetic approach.

Based on these results it is doubtful whether system properties like homeostasis or efficiency of functioning of thiol linked redox processes can be deduced from genome expression data (FOYER and NOCTOR, 2005) or from proteome based insights (DIETZ, 2005). These techniques are best suited to identify system components. Insights in functionality however, have to be based on kinetic data which have to be analysed via system modelling. System characteristics like homeostasis, emerge from the „supramolecular organization of the enzymes“ (SRERE, 1993; SRERE, 1994). This is a functional level beyond gene expression or proteom patterns.

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 remaine 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 H_2O_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 H_2O_2 ratio. Decreasing concentrations of NADPH relative to H_2O_2 cause identical redox properties of the redox variables with increasing H_2O_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 H_2O_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 H_2O_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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