Antioxidant defence and the role of the peroxiredoxins during silique development and seedling establishment in oilseed plants

Dissertation

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Content

Summary

The present work aims at understanding the regulation of the antioxidant defence system during the two most critical stages of development of oilseed plants, namely the transition from lipid based heterotrophic growth to photoautotrophic metabolism in seedlings and the development of siliques during the lipid storage phase of seeds. Special focus is given to peroxiredoxins (Prx), which are involved in detoxification of H_2O_2 and alkyl hydroperoxides.

Arabidopsis thaliana seedlings were analyzed between 1.5 and 5 days after radicle emergence from the seed coat (DARE). They were subjected to either continuous light or light/dark-cycles, in the presence or absence of externally applied sucrose and analyzed for photosynthetic electron transport and for transcript levels of antioxidant enzymes relative to lipid mobilization, superoxide accumulation and the chlorophyll content. Chlorophyll-a fluorescence kinetics showed a transient increase in the electron transport efficiency and transient activation of non-photochemical quenching (NPQ) in seedlings grown in absence of sugar. Sugar application suppressed chlorophyll accumulation after 2.5 DARE and limited activation of NPQ. The genes for antioxidant enzymes were regulated developmentally and in a light-dependent manner. Transient induction of cytosolic Prx and of most of the enzymes of the Halliwell-Asada-cycle was observed in continuous light. In discontinuous light, nuclear genes for chloroplast antioxidant enzymes and for the mitochondrial PrxIIF responded to diurnal fluctuations and showed highest transcript levels in the light. In contrast, cytosolic PrxIID was up-regulated during the nights. Carbohydrates application affected especially APx expression and decreased APx activity, while Prx expression was hardly influenced.

In general, the transcript abundance patterns showed that the chloroplast antioxidant defence system of greening seedlings is dominated by the four peroxiredoxins 2Cys-PrxA, 2Cys-PrxB, PrxIIE and PrxQ. The protein levels were already high at 1.5 DARE under all growth conditions, when APx transcript abundance and activity were low. Between 2.5 and 3 DARE, in parallel to sink-source-transition a change in the control of chloroplast antioxidant protection and metabolism took place. The antioxidant defence system shifted from a

peroxiredoxin-dominated defence to the combined action of the ascorbate-dependent water-water-cycle (Halliwell-Asada-cycle) and the ascorbate-independent peroxiredoxin defence system.

Leaf senescence starts with initiation of flowering. The silique wall then turns to be the main source of photoassimilates supporting seed development (Pechan and Morgan, 1985; Lewis and Thurling, 1994). Growth and photosynthesis generate ROS (Schopfer et al., 2001; Foyer and Noctor, 2000). Activation of antagonizing antioxidants prevents damage in oilseeds at the time of lipid storage. Here, *Arabidopsis thaliana* was used as a model oilseed plant to characterize the antioxidant systems during silique development. The information taken from the study on Arabidopsis was transferred to the oilseed crop *Brassica napus*.

For the characterization of the antioxidant defence systems five stages of siliques development (from an early embryogenic stage to the end of maturation of seeds) were analyzed in *Arabidopsis thaliana*. Chlorophyll-a fluorescence parameters demonstrated high photosynthetic competence in all selected stages. Transcriptional patterns showed significant expression of chloroplastic genes and of the stress-inducible PrxIIC in young siliques compared to mature leaves. The transcript amounts of most of these genes decreased during development. The same pattern was observed for the protein levels of chloroplastic 2Cys-Prx, PrxQ and cytosolic and chloroplastic type II Prxs. The transcript levels of mitochondrial PrxIIF were constitutively high, while the protein amounts increased during silique maturation. In *Brassica napus*, the protein abundance pattern was similar for 2Cys-Prx, PrxQ and type II Prx. Only PrxIIF protein amounts remained constant during development. Analysis on seeds separated from the silique wall revealed expression of the type II Prxs, especially of the mitochondrial PrxIIF, in both organs. In contrast, PrxQ was only detected in silique walls, while 2Cys-Prx was found in silique walls and seeds of the middle stage in *Brassica napus*.

It is concluded that siliques demand for strong antioxidant protection, especially in the early stages of development. The strong expression of Prx, which scavenge small alkyl hydroperoxides up to complex lipid peroxides, suggest a dominant role of these enzymes in the protection of siliques of oilseed plants.

Abbreviations

ABA	Abscisic acid
APx	Ascorbate Peroxidase
ATP	Adenosine triphosphate
At	*Arabidopsis thaliana*
Bn	*Brassica napus*
BCIP	5-brom-4-chlor-3-indolylphosphate
CAT	Catalase
cDNA	Copy-DNA
Chl	Chlorophyll
DEPC	diethylpyrocarbonate
DHA	Dehydroascorbate
DHAR	Dehydroascorbate Reductase
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylendiamine tetraacetate
EGTA	ethylene glycol-bis [ß-aminoethylether] N,N,N´,N-tetra acetic acid
GSH	Glutathione
GSSG	Oxidized Glutathione
h	Hour
Hv	*Hordeum vulgare* sp *vulgare*
kDa	Kilo Dalton
KD-SOD	Arabidopsis knock down-lines for superoxide dismutase
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
MIPS	Munich Information for Protein Sequences
Min	Minute
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
µL	Microliter
µm	Micrometer
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleoside phosphate
NBT	Nitrotetrazolium Blue
NCBI	National Center for Biotechnology Information
nm	Nanometer
NPQ	Non-photochemical quenche
Prx	Peroxiredoxin
PSI	Photosystem I
PSII	Photosystem II
ΦPSII	Efficiency of photosystem II
P_{680}	Pigment 680 of photosystem II
Q_A	Plastoquinone A
Q_B	Plastoquinone B
qP	photochemical quenche

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
SDS	Sodium dodecyl sulfat
SOD	Superoxide dismutase
UV	Ultra violet

1 Introduction

1.1 *Arabidopsis thaliana* and *Brassica napus* as model plants

Oilseed rape, or canola, (Brassica species) is actually the second most important source of vegetable oil providing 13 % of the world´s supply (Raymer, 2002). The term “canola” is a registered trademark of the Canadian Canola Association and refers to genetically modified cultivars of oilseed rape with less than 2% erucic acid (22:1) and meals with less than 30 mmol of aliphatic glucosinolates per gram (Downey, 1990; Raymer, 2002). Canola is cultivated extensively in Europe, Canada, Asia, Australia, USA and part of South America. The world´s commerce is largely supplied by two species *Brassica napus* L. and *Brassica rapa* L..

Brassica napus is an amphidiploid species resulting from combining chromosome sets of the low chromosome number species *B. olareacea* (2n = 18) and *B. rapa* (2n = 20). For the genome size values of 1105-1235 Mbp have been estimated (Arumuganathan et al., 1991).

Arabidopsis thaliana and *Brassica napus* are close relatives. Both species belong to the Brassicaceae family (Fig. 1). Divergence between the Arabidopsis and Brassica lineages were estimated at 23.1-25.9 and 14.5-20.4 million years ago, respectively (Koch et al., 2000; Yang et al., 1999). Most crop plants other than the Brassica species are only distantly related to *Arabidopsis thaliana*, for example the separation between Brassicaceae and Asteraceae is estimated to have occurred 112-156 million years ago and the monocot-dicot split happened around 170-235 million years ago (Yang et al., 1999).

Arabidopsis thaliana has the smallest genome known for higher plants (130 Mbp) with only 5 chromosomes. This small size facilitated the assembly of comprehensive genetic (http://www.arabidopsis.org) and physical maps (Marra et al., 1999; Mozo et al., 1999). The nucleotide sequence has been determined and annotated for the majority of the genome (Lin et al., 1999; Mayer et al., 1999) and the sequence maps have been extensively cross-referenced with genetic chromosome maps (http://www.arabidopsis.org). In addition, more than 110000 EST sequences are currently available (Höfte et al., 1993; Newmanet al., 1994; Quackenbusch et al., 2000).

Many of the approaches to analyze biosynthetic pathways in oil crops have been carried out in the model organism *Arabidopsis thaliana* and aimed at elucidating pathways and identifying the involved genes. Currently, the Multinational Brassica Genome Project collects and exchanges information related to Brassica genetics and genomics (http://www.brassica.info). One of the projects coordinated by the AAFC (Agricultural Agri-Food Canada) Brassica/Arabidopsis Genomic Initiative (BAGI; http://brassica.agr.gc.ca) collects information on the molecular and genetic structure and function of Brassica genomes via an effective framework for using Brassica/Arabidopsis comparative genomics to accelerate Brassica research.

Fig. 1: (A) *Arabidopsis thaliana*, (B) *Brassica napus*

1.2 Metabolic changes in germinating seedlings

The most critical stages in the life cycle of higher plants are seed germination and seedling establishment (Bewley and Black, 1994). Germinating and greening seedlings must adapt their metabolic and developmental programmes to the prevailing environmental conditions to achieve photoautrophism before its nutrient reserves become exhausted (Bewley and Black, 1994).

1.2.1 From dormant seed to germination

Like many other plant species, mature Arabidopsis seeds exhibit primary dormancy when freshly released from the mother plant. Dormancy prevents pre-harvest germination (Bentsink and Koorneef, 2002) and is enhanced by the seed coat. The seed coat is a multifunctional organ that plays an important role in embryo nutrition during seed development and in protection against

environmental stressors (Mohamed-Yasseen et al., 1994; Weber et al., 1996). Germination is restricted most of the time by the impermeability of the seed coat to water and/or oxygen or by its mechanical resistance to radicle protrusion (Bewley, 1997). Dormant seeds are unable to germinate under the appropiate environmental conditions without break of dormancy by stratification, after-ripening, or giberellins (Koorneef and Karssen, 1994).

Germination starts with the uptake of water by the quiescent dry seed and terminates with the elongation of the embryonic axis (Bewley and Black, 1994). The visible sign that germination is successfully initiated is usually the emergence of the radicle tip through the seed coat.

1.2.2 The role of light and the photoreceptors during early germination

In addition to the water availability, factors such as light, temperature and storage time, control the onset of germination (Koorneef and Karssen, 1994). Light-dependent germination of *Arabidopsis* seeds is entirely mediated by phytochrome (Casal and Sánchez, 1998). Seedlings covered by soil receive very little light. They show skotomorphogenesis (or etiolation), which is characterized by long hypocotyl elongation and unexpanded cotyledons. This phenotype is achieved via the very low radiance response (VLFR), primarily under the control of the phytochrome PHYA. Once the seedling emerges into the light, PHYA is rapidly degraded, and the effects of PHYB and the cryptochromes start to dominate. Growth in the light induces photomorphogenesis (or de-etiolation). In the natural environment, the switch between etiolated and de-etiolated development allows seedlings to emerge through soil and reach light, where it can follow a developmental pattern optimal for the differentiation of the photosynthetic apparatus (Nemhauser and Chory, 2002; Sullivan and Deng, 2003). The differentiation from proplastids into chloroplasts is associated with the biosynthesis of chlorophyll. For that, light of shorter wavelength than far-red is necessary. Thus, chlorophyll does not accumulate under continuous far-red light, and the seedlings remain yellowish (Barnes et al., 1996).

Above ground on the surface, neighbour detection or shade avoidance programmes are activated. Shade avoidance is mediated by the phytochrome family of photoreceptors by sensing the ratio between red and far-red light (R/FR). As light passes through, or is reflected by, an overhanging leaf there is a selective absorption of red light by the photosynthetic pigments, resulting in light that contains a high proportion of far-red light. Arabidopsis seedlings are capable of sensing very small changes in R/FR, which can act as an early warning system for a potential shade threat (Botto and Smith, 2002). High R/FR ratios severely inhibit hypocotyl elongation, while low R/FR ratios promote it (Casal and Sánchez, 1998).

1.2.3 Plant hormones involved in germination

Plant hormones are important regulator of the developmental program of seedlings. They interact with light signals promoting or inhibiting photomorphogenic growth (Nemhauser and Chory, 2002). Auxin promotes hypocotyl elongation. The response is regulated at four levels: biosynthesis, metabolism, transport and response. Light affects auxin transport as well as auxin-signaling (Jensen et al., 1998). Auxin is produced primarily in the apical tip of the growing shoot and transported towards the roots in the differentiated vasculature. An asymetric redistribution of auxin transport or altered auxin responsiveness, may mediate seedling phototropism by PHOT1 (Friml et al., 2002; Stowe-Evans et al., 1998).

Auxin is antagonized by cytokinin, which also plays a role in photomorphogenesis by regulating the de-etiolation response (Hwang and Sheen, 2001). Cytokinins are also negative regulators of ethylene signalling (Vogel et al., 1998). Ethylene has been shown to regulate cell expansion in a light- and tissue-dependent manner. In the dark, ethylene inhibits cell elongation, while in the light, it promotes the opening of the apical hook, a process involving cell expansion, as well as elongation of the hypocotyl (Raz and Ecker, 1999; Smalle et al., 1997).

In addition, brassinosteroids reverse ABA-induced dormancy and stimulate germination (Steber and McCourt, 2001). They are also linked to the de-etiolation

process and have been implicated in repressing PHYA-mediated VLFR (Li et al., 1996; Luccioni et al., 2002) and a role for brassinosteroid response in hypocotyl elongation in both light and dark has been proposed (Nemhauser and Chory, 2002).

Gibberellins play an important role in promoting seed germination. Two mechanism of action were proposed: first, induction of genes encoding enzymes that reduce the mechanical resistance to radicle protrusion; second, a direct effect on the growth potential of the embryo (Karssen and Lacka, 1986). The first mechanism has not been proven in Arabidopsis. The second is assumed to be restricted by abscisic acid (ABA). Gibberelin is required to overcome the ABA-induced dormant state and may also act in de-etiolation process (Sun, 2000).

The hormone ABA is required during seed development, keeping the seed in an embryonic program and to prevent precocious germination (Nambara et al., 1992; Ooms et al., 1993). ABA is involved in the acquisition of desiccation tolerance during late maturation, supposedly through the induction of the late embryogenesis-abundant (LEA) proteins (Ooms et al., 1993). ABA levels decrease at the end of seed maturation. It was found that for the maintenance of dormancy in imbibed seeds *de novo* ABA synthesis is required (Debeaujon and Koorneef, 2000; Grappin et al., 2000). Consistently, the decrease of endogenous ABA levels is necessary for the onset of germination (Price et al., 2003). Pritchard et al. (2002) demontrated that although ABA treatment inhibits expression of genes encoding enzymes of β-oxidation and glyoxilate cycle, significant levels of expression still occur. This observation indicates that ABA in Arabidopsis seeds prevents germination but not lipid mobilization.

1.2.4 Lipid breakdown

With onset of germination an extensive conversion of storage lipids to soluble carbohydrates takes place (Canvin and Beevers, 1961). The main pathways involved are β-oxidation, the glyoxylate and tricarboxylic acid (TCA) cycles and gluconeogenesis. The subcellular compartments involved in these pathways have to coordinate the exchange of metabolites (Trelease and Doman, 1984). The release of fatty acids from triacylglycerol (TGA) stored within oil bodies is

catalysed by lipases. Free fatty acids are activated to acyl-CoA esters and enter the glyoxysome, where they are broken down to acetyl-CoA via the β-oxidation spiral (Kindl, 1987). Acetyl-CoA serves as a substrate for the glyoxylate cycle forming succinate, which passes from glyoxysomes into mitochondria and enters the TCA cycle. It is converted to malate, which is exported to the cytosol and oxidized to oxaloacetate. This is converted to PEP, which ultimately fuels the synthesis of soluble carbohydrates in gluconeogenesis (Beevers, 1980). Several studies have shown that, when the glyoxylate cycle is operative in germinating oilseeds, the activity of the decarboxylation steps of the TCA cycle are suppressed, favouring the synthesis of carbohydrate over respiration (Millhouse et al., 1983; Falk et al., 1998). The transition from heterotrophic to photoautotrophic metabolism is then accompanied by a rapid increase in the TCA cycle activities. It starts with activation of gluconeogenesis metabolism and switches then to respiration (Falk et al. 1998).

In Arabidopsis, lipids constitute between 30 % and 40 % of the seed dry weight (Eastmond et al., 2000). Lipid breakdown is not essential for seedling germination but for seedling establishment (Hayashi et al., 1998). It is consistent with the suggestion that soluble carbohydrates present in many seeds are sufficient to support germination and that major storage reserves are used primarily during post-germinative growth (Bewley and Black, 1994). Lipids are not generally considered to be quantitatively important respiratory substrates in plants (ap Rees, 1980). In castor bean (*Ricinus communis*), for example, oil is converted almost stoichiometrically to sugars and then exported (Beevers, 1980). In this plant the major storage organ is the endosperm, which is completely consumed during seedling development. In other oilseed plants as Arabidopsis or Brassica storage reserves are deposited in the cotyledons of the embryo (Eastmond and Graham, 2001). It was shown that lipids can provide a major source of carbon for respiration in these species (Eastmond et al., 2000). After germination, seedling development is epigeal, and the cotyledons are progressively transformed into photosynthetic organs. The glyoxylate cycle may be less predominant over respiration in Arabidopsis because there is a reduced need for the intracellular transport of carbon skeletons (Eastmond et al., 2000). However, this alternative

pathway cannot provide an anaplerotic supplement for the TCA cycle (Eastmond and Graham, 2001). The glyoxylate cycle has been suggested to have an anaplerotic function in replenishing intermediates of the TCA cycle (Graham et al., 1994). Thus, in the abscence of the glyoxylate cycle another carbon source is required for post-germinative growth under non optimal light conditions (Eastmond et al., 2000).

1.2.5 Sugar effects on germination

Soluble sugars play an important role in the regulation of many genes involved in physiological and developmental processes including photosynthesis, nitrate assimilation, assimilate storage, and the mobilization of starch and lipids (Graham, 1996; Koch, 1996; Jang et al. 1997; Smeekens and Rook, 1997). Endogenous sugars as sucrose, glucose and fructose remain low during etiolated seedling development (Dijkwel et al., 1996). Addition of exogenous sugars showed delay of germination and lipid mobilization (To et al., 2002; Graham et al., 1994; Dekkers et al., 2004) and repression of the activation of genes coding for enzymes involved in photosynthesis during the establishment of the photosynthetic machinery (Krapp et al., 1993; Dijkwel 1996, 1997). These results indicate the importance of sugar signalling in the early activation of photosynthesis.

1.3 Metabolic changes during seed and silique development

Setting seeds is the last phase in the plant life cycle. In *Arabidopsis thaliana* and *Brassica napus*, as in other angiosperms, after double fertilisation in the embryo sac, embryo and endosperm develop separately. The triploid endosperm develops in two steps: a coenocytic stage then a cellularised and differentiated stage (Oelsen, 2001) before being almost totally resorbed during maturation (Mansfield and Briarty, 1993). Embryo development can be divided into three stages (Fig. 2): (1) early morphogenesis, in which the embryo develops from two cells to the early torpedo stage; (2) maturation, in which cell expansion and differentiation concomitant with accumulation of storage products occurs; (3) and the late

maturation stage, in which the embryo becomes metabolically quiescent and tolerant to desiccation (Mansfield and Briarty, 1992).

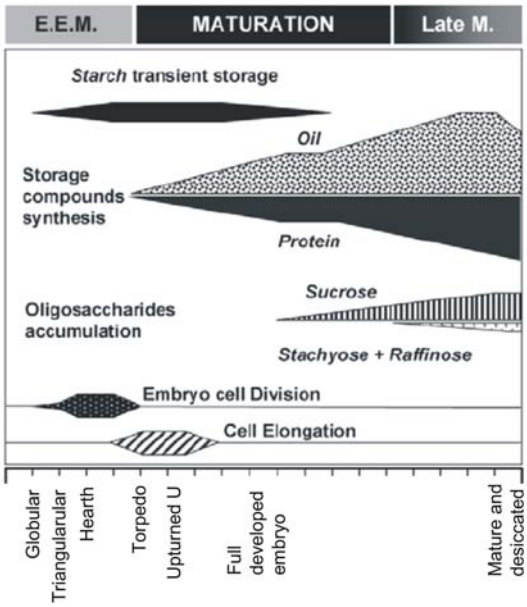

Fig. 2: Summary of the major physiological and metabolic events affecting *Arabidopsis thaliana* seeds. (Baud et al., 2002)

1.3.1 The silique wall as the principal source of photoassimilates during seed development

Following the rapid increase in flowering branches from the shoot apical meristem, leaf senescence starts (Pechan and Morgan, 1985). With the rapid decline of functional leaf area, one major source of photoassimilate is lost. Consequently, silique wall photosynthesis turns to be the main source of assimilates (Lewis and Thurling, 1994). Its photosynthetic capacity is equivalent to that of the leaf on a chlorophyll basis (King et al., 1998). Studies in canola reveal that, unlike many starch-storing leaves of C_3 dicotyledoneous species, the silique walls use newly fixed carbon to produce preferentially sucrose. At early stages, sugars are stored temporally as hexoses in the silique wall (King et al., 1997). At the onset of rapid seed growth, hexose content drops in the silique wall, suggesting mobilisation to the seed. Sucrose levels in carpels remain stable during development, supporting seed growth and silique metabolism (King et al., 1997).

1.3.2 Photosynthetic capacity of Arabidopsis embryos

Embryos within oilseeds from cruciferous plants are green during development and photosynthetically active (Eastmond et al., 1996, Asokanthan et al., 1997; King et al., 1998; Ruuska et al., 2004). Works on canola showed that the rate of photosynthesis in developing embryos increases until onset of dessication and declines afterwards. By maturity, embryos degrade chlorophyll and loose their photosynthetic activity (Eastmond et al, 1996). Considering that seeds are predominantly sink tissues, Asonkathan et al. (1997) classified their plastids as heterotrophic chloroplasts, which are formed from pregranal plastids in green embryos and dedifferentiate into protoplast in mature, quiescent seeds. The pigment composition of these embryonic plastids is similar to that from shade-adapted plants, with increased Chl-b to Chl-a ratios and significant carotenoids contents for an appropriate utilization of the weak, green light that is transmitted through the silique wall (Ruuska et al., 2004). Three main roles of embryo photosynthesis have been proposed: (1) production of ATP and reducing power to provide the energy for fatty acid synthesis (Eastmond et al., 1996, Asokanthan et al., 1997, Ruuska et al., 2004); (2) O_2 evolution to prevent anoxia inside the seed (Rolletschek et al., 2002); (3) and refixation of respiratory CO_2 and the CO_2 released by fatty acid biosynthesis during the conversion of pyruvate to acetyl-CoA (King et al., 1998, Ruuska et al., 2004).

1.3.3 Metabolic changes during seed development

The three developmental stages in seed growth, namely early embryo morphogenesis, maturation and desiccation, are characterized by evolution of the carbohydrate content, fatty acid synthesis and lipid storage, amino acid and protein storage and acquisition of desiccation tolerance.

Baud et al. (2002) gave an overview considering these metabolic events in seeds of *Arabidopsis thaliana* ecotype Wassilewskija (Fig. 2). The early morphogenesis stage is characterized by high accumulation of hexoses and starch, that tends to decrease along with development. Hexoses might support cell division and then the transition to cell expansion and storage product synthesis (King et al., 1997), while starch, which accumulates transiently in the embryo and in the two cell

layers of the outer integument, might contribute to the production of mucilage in the outer cell layer and the formation of the small pyramidal domes (Western et al., 2000; Beeckman et al., 2000).

Sucrose, which is the most abundant disaccharide in dry seeds of *Arabidopsis thaliana*, and oligosaccharides, such as stachyose and raffinose, are synthesised during maturation and late maturation (Baud et al., 2002). Sucrose stock is suggested to be an energy resource (Bentsink et al., 2000), which is easily and rapidly available during seed germination. According to the water "replacement hypothesis", sucrose also serves as a protective agent (Leprince et al., 1990; Vertucci and Farrant, 1997). It is postulated that the hydroxyl groups of sugars substitute water and provide hydrophylic interactions for membrane and protein stabilisation. Other oligosaccharides might assist in the protection of membranes from desiccation damage by restricting or preventing crystallisation of sucrose (Caffrey et al., 1988).

At maturation, rapid fatty acid synthesis overlaps with increasing photosynthetical activity in the embryo (Eastmond and Rawsthorne, 2000). The incoming sucrose is broken down via glycolysis. Intermediates are imported into the plastids for the synthesis of fatty acids, which are later exported to the cytosol for synthesis of triacyl glycerol (TAG) at the endoplasmatic reticulum (White et al., 2000) and storage in oilbodies (Murphy et al., 1989). At the desiccation stage, a decrease in the lipid content by at least 10 % in *Brassica napus* (Chia et al., 2005) and 28 % in *Arabidopsis thaliana* (Baud et al., 2002) takes place. It was proposed that the lipid breakdown supports the synthesis of storage proteins, late embryogenesis abundant proteins (Cuming, 1999) and other metabolites associated with seed maturation, whose biosynthesis continues into the phase of desiccation (Baud et al., 2002). Low activity of lipid-breakdown enzymes was observed during the main period of oil accumulation in canola seeds, indicating a "housekeeping" function in the turnover of membrane lipids or in the regulation of cellular acyl-CoA levels (Chia et al., 2005).

Protein storage occurs mainly in the maturation stage and continued at a lower rate during late maturation (Baud et al., 2002). The water content decreases almost linearly during seed maturation to 45 % due to a gradual accumulation of

dry matter (Baud et al., 2002). This decline in seed moisture is not considered dehydration, because the cellular water potential remains constant up to maturation drying. During the late maturation stage, desiccation takes place to a water content of 7 % (Baud et al., 2002). Desiccation tolerance mechanisms are activated by dehydration and by the plant hormone abscisic acid (Ingram and Bartels, 1996), which also inhibits premature germination (Bewley, 1997). Accumulation of anhydrobiotic molecules, late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs) are part of the desiccation tolerance program (Hoekstra et al, 2001).

1.4 ROS production and implication in plant metabolism

1.4.1 ROS sources

Aerobic metabolism in animals and plants leads to generation of ROS. In plant cells, the production of ROS such as superoxide anion radical ($O_2^{\cdot -}$), the hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) takes place in chloroplasts, mitochondria, peroxisomes, the plasma membrane and the apoplastic space (Bolwell, 1999).

In chloroplasts, the main generation site of superoxide radicals ($O_2^{\cdot -}$) is photosystem I at the level of ferredoxin-$NADP^+$ reductase and by the Mehler reaction (Asada, 1999). Photosystem II contributes with smaller amounts of H_2O_2, which is generated at the Q_B site, if the plastoquinone pool is highly reduced (Navari-Izzo et al., 1999). Furthermore, production of $O_2^{\cdot -}$ and hydroxyl radicals at photosystem II has been reported, and P_{680}, pheophytin and protein Q_A have been proposed to generate superoxide (Navari-Izzo et al., 1999).

In plant mitochondria an alternative respiratory pathway leads to reduction of oxygen to water under conditions of low energy demand (Turrens, 1997). This consists of a non-proton-pumping NAD(P)H dehydrogenase that bypasses complex I and an alternative oxidase that accepts electrons directly from the ubiquinone pool without participation of the cytochrome c oxidase pathway through complexes III and IV (Turrens, 1997; Halliwell and Gutteridge, 2000).

NADH dehydrogenase produces superoxide radicals that are dismutated to H_2O_2 which induce the alternative oxidase. Through this mechanisms, the uncoupling of electron transfer from ATP production takes place, which prevents overreduction of the respiratory electron chain (Møller, 2001; Smith et al., 2004).

Cell wall-bound peroxidases generate H_2O_2 in the apoplastic space (Bolwell, 1999). In addition, in the plasma membrane, NADPH oxidases analogous to that of the mammalian system produce superoxide (Bolwell, 1999). Evidence of NADPH oxidase dependent superoxide production was reported in rice (putative plasma membrane NADPH oxidase; Sagi and Fluhr, 2001). Direct genetic evidence that two components of a plant NADPH-oxidase are required for ROS production during plant defense responses is based on studies with knock-out plants of two Arabidopsis *rboh* genes, *AtrbohD* and *AtrbohF*, which largely eliminate ROS during disease resistance reactions in response to avirulent pathogens (Torres et al., 2002).

Peroxisomes are organelles with an essentially oxidative metabolism. Different peroxisome types have specialized functions in plant cells. The main metabolic processes leading to H_2O_2 generation are the photorespiration-associated reactions in leaf peroxisomes (Reumann, 2000); the fatty acid β-oxidation and the glyoxylate cycle in glyoxysomes of storage tissues in oilseeds and in senescing tissues (del Río et al., 1998) and the enzymatic reaction of flavine oxidases and the disproportionation of superoxide radicals (del Río et al., 1996; Reumann, 2000). Superoxide generation takes place at least at two sites: in the peroxisomal matrix by the action of xanthine oxidase and in the peroxisomal membrane dependent on NAD(P)H (López-Huertas et al., 1999; del Río et al., 1998, 2002).

Nitric oxide ($NO^{\cdot}$) is generated in the peroxisomes by the enzyme nitric oxide synthase (NOS; Barroso et al., 1999). H_2O_2, nitric oxide and $O_2^{\cdot -}$ can be released to the cytosol. Cytosolic production of $O_2^{\cdot -}$ takes place at an NAD(P)H-dependent $O_2^{\cdot -}$ production site in the peroxisomal membrane, which is apparently formed by a small electron transport chain using O_2 as electron acceptor (del Río et al., 1998; López-Huertas et al., 1999). When $NO^{\cdot}$ permeates the peroxisomal membrane, in the presence of superoxide, the oxidant peroxinitrite ($OONO^-$) can be generated ($NO^{\cdot} + O_2^{\cdot -} \rightarrow OONO^-$). (Bolwell, 1999; Clark et al., 2000).

1.4.2 Damage risks by ROS accumulation

Within the chloroplast, formation of hydroxyl radicals from superoxide ion via a Cu(II)-catalyzed Haber-Weiss mechanism causes rapid loss of oxygen evolution activity (Yruela et al., 1996). H_2O_2 causes DNA breakage and inactivates thiol-containing enzymes such as the thioredoxin-modulated enzymes of the chloroplast stroma (Charles and Halliwell, 1981). Hydroxyl radicals are highly reactive and can be formed non-enzymatically in the presence of iron ions (Fenton reaction). They can modify proteins and make them more susceptible to proteolytic attack (Casano et al., 1994) they also cause the loss of membrane integrity by lipid peroxide formation. Peroxinitrite is a powerful oxidant, which inhibits the activity of catalase and ascorbate peroxidase (APx; Clark et al., 2000) and regulates the conversion of xanthine dehydrogenase into the superoxide-generating xanthine oxidase (Distefano et al., 1999). Catalase is inactivated by light (Schäfer and Feierabend, 2000), which leads to accumulation of H_2O_2 and superoxide radicals, causing further inhibition of catalase and APx, CuZn-superoxide dismutase (CuZnSOD) and Fe-superoxide dismutase (FeSOD; Pan and Yau, 1992), which are important scavenging systems in plant cells.

Evidence for singlet oxygen ($^1O_2$) formation, which results from the interaction of triplet chlorophyll with molecular oxygen, was demonstrated in leaves upon photoinhibition of the PSII reaction centres (Hideg et al., 2001; Fryer et al., 2002). Singlet oxygen reacts rapidly with organic molecules generating hydroperoxides and lipid peroxidation in membranes (Halliwell, 1987).

1.4.3 Role of ROS in metabolism and redox signalling

On the other side, ROS participate in biosynthetic processes and act as secondary messenger regulating growth and development as well as stress responses (Foyer et al. 1997). The presence of H_2O_2 was shown to be necessary for tobacco protoplast division (de Marco and Roubelakis-Angelakis, 1996) and for leaf elongation (Rodríguez et al., 2002). ROS regulation of cell expansion is mediated by the activation of Ca^+ channels (Foreman et al., 2003). In ripening pear fruit cell walls, evidence that hydroxyl radicals attack cell wall polysaccharides suggests ROS participation in wall softening during maturation (Fry et al., 2001). Auxin-

induced elongation growth was shown to be inhibited by hydroxyl radical scavengers (Schopfer, 2001). The generation of ROS in the embryo axis as well as in the seed coat from soybean and radish during germination (Boveris et al., 1984; Puntarulo et al., 1988; Schopfer et al., 2001) was proposed to protect the emerging embryo against invasion by parasitic organisms by activating defense reactions (Schopfer et al., 2001). On the other hand, ROS are key mediators of pathogen-induced programmed cell death participating in a signal transduction pathway leading to the induction of defense responses against pathogens and cell death (Grant and Loake, 2000).

Nitric oxide is involved in plant growth and acts as second messenger in ultraviolet B signalling inhibiting mesocotyl elongation (Zhang et al., 2003) and as signalling molecule in defense responses and regulation of cell death (Grant and Loake, 2000).

The participation of ROS in redox signaling requires interactions between ROS and antioxidant. The latter include non-enzymatic scavengers (metabolites of low molecular weight; see below) and detoxifying enzymes that operate in the different cellular compartments (Noctor and Foyer, 1998). The homeostasis between ROS and antioxidants maintains the redox poise in plant cells. The lifetime and the specificity of the ROS signal is determined by the antioxidants, which continuously process ROS (Foyer, 2005). Photosynthesis, photorespiration, respiration, carbon metabolism and response to oxidative stress for environmental changes are partly controlled by interactiones ROS-antioxidants (Noctor et al., 2000; Dietz, 2003; Apel and Hirt, 2004; Foyer, 2005).

1.5 Antioxidative systems in plant cells

Oxidative stress is defined as the increase of ROS to levels that disrupt the cellular redox homeostasis. Some ROS can diffuse from the site of production, as H_2O_2, while others such as $O_2^{\cdot -}$, cannot cross biological membranes. Consequently, subcellular compartmentation of defense mechanisms is required for efficient removal of ROS at their generation sites. Every subcellular compartment has an own protection system consisting in specialized antioxidative enzymes such as catalase, ascorbate peroxidases, glutathione peroxidase,

glutathione reductase, MDHAR, DHAR, SODs, the Prx family and metabolites of low molecular weight such as ascorbate, glutathione and tocopherol.

1.5.1 Antioxidative enzymes overview

SOD

Superoxide dismutases (SOD) are the first line of defense against oxiradical-mediated damage. They catalize the disproportionation of $O_2^{\cdot -}$ into H_2O_2 and O_2 (Fridovich, 1986; Halliwell and Gutteridge, 2000). Plant SOD are metallo-enzymes, distributed in many cellular compartments. In Arabidopsis, the SOD family consists of three CuZnSOD, which are localized in plastids (CSD2, At2g28190), the cytosol (CSD1, At1G08830) and pressumably in the peroxisomes (CSD3, At5g18100), three plastidic FeSOD (FSD1, At4g25100; FSD2, At5g51100; FSD3, At5g23310) and one mitochondrial MnSOD (MSD1, At3g10920; Kliebenstein et al., 1998). Studies with chloroplastic CuZnSOD knock down plants suggested that the supression of this enzyme, which is attached to the thylakoid membrane at the vicinity of PSI (Asada, 1999) reduces the activity of the water-water-cycle (Rizhsky et al., 2003).

Catalase

Several enzymes are involved in detoxifying H_2O_2 and catalases are the most abundant. The catalase gene family in Arabidopsis consists of three genes (CAT1, AT4g35090; CAT2, At1g20630; and CAT3, At1g20620) encoding individual subunits, which form at least six isoforms (McClung, 1997). All of them are present in peroxisomes. Catalases are tetrameric, heme-containing enzymes. They catalyze the reduction of H_2O_2 to molecular oxygen and water. Their substrate affinity (K_m = 1.1 M) is low compared to other peroxidases. However, their very high reaction rates, allow removal of the bulk, but not a specific reduction of small amounts of H_2O_2. They are distributed in a tissue-specific manner in the plant, depending on the type of peroxisomes formed (e.g., glyoxysomes, geroxysomes). The common nomenclature for catalases in different plant species divides them in three categories: class I catalases are highly expressed in leaves, light dependent and remove H_2O_2 during photorespiration (CAT2); class II

(CAT1) are mainly found in vascular tissues; and class III (CAT3) are predominantly linked to glyoxysomes of seeds and young seedlings (Dat et al., 2000).

Enzymes of the ascorbate-glutathione cycle

In the ascorbate-glutathione cycle (Halliwell-Foyer cycle; Noctor et al., 2000) APx uses ascorbate as electron donor to reduce H_2O_2 to water (2 Asc + H_2O_2 → 2 MDHA + 2 H_2O). The monodehydroascorbate (MDHA) disproportionates spontaneously to ascorbate and dehydroascorbate (DHA) or is regenarated to ascorbate by the NAD(P)H-dependent monodehydroascorbate reductase (MDHA + NAD(P)H → Asc + $NAD(P)^+$), respectively. The re-reduction of DHA to ascorbate is coupled to oxidation of glutathione by dehydroascorbate reductase (DHA + 2GSH → Asc + GSSG), which is regenerated by the NAD(P)H-dependent glutathione reductase (GSSG + NAD(P)H → 2 GSH + $NAD(P)^+$). Enzymes of this cycle are present in the cytosol, mitochondria, peroxisomes and in the stroma and thylakoid lumen of chloroplasts (Chew et al., 2003; Jiménez et al., 1997; Kieselbach et al., 2000; Shigeoka et al., 2002). In Arabidopsis, chloroplasts contain at least three different isozymes of APx: a thylakoid bound APx (At1g77490), a lumenal APx (At4g09010) and a stromal APx (At4g08390). The latter, however, is dually targeted to the stroma and mitochondria (Chew et al., 2003). The cytosol contains two stress inducible APx (APx1, At1g07890) and (APx2, At3g09640) (Karpinski et al., 1999; Panchuk et al., 2002, Mittler et al., 2004). APx uses protophorphyrin as prostetic group. Three partially conserved amino acids, participate in hydrogen bonding and five amino acid residues that are conserved in all classes, are essential for the heme binding (Shigeoka et al., 2002).

Peroxiredoxins

The peroxiredoxin (Prx) family contains peroxidases with broad substrate specificity. In contrast to APx, which are specific for H_2O_2, the Prx can reduce also alkylhydroperoxides, small organic hydroperoxides up to complex lipid peroxides, to water or the corresponding alcohol (König et al., 2003; Dietz, 2003a) and hydroxyl radicals (Lim et al., 1993). Their capacity to reduce peroxinitrite was shown in bacteria and mammals (Dubuisson et al., 2004). The

Prx do not have redox cofactors such as metals or prosthetic groups. Their catalytic activity is based on conserved cysteine residues (Chae et al., 1994). Their regeneration cycle is considered to take place by intra- or inter-molecular thiol-disulfide-reactions using small thiols, such as thioredoxins, glutaredoxins, and glutathione as electron donors (Dietz, 2003a). In plants, like in many other organisms, four subgroups of Prx can be distinguished according to the number and position of one or two conserved cysteine residues in the primary structure: 1Cys-Prx, 2Cys-Prx, type II Prx and PrxQ, which are localized in various cell compartments. The Arabidopsis Prx family consists of 10 members: one 1Cys-Prx (At1g48130), which is localized in the cytosol or the nucleous (Stacy et al., 1999), two 2Cys-Prx (2Cys-PrxA, At3g11630 and 2Cys-PrxB, At5g06290; Baier and Dietz, 1999; Dietz et al., 2002) and one PrxQ (At3g26060; Lamkemeyer et al., 2006), which are chloroplastic and five type II Prx (Horling et al., 2003; Brehélin et al., 2003; Dietz et al., 2003; Finkemeier et al., 2005) distributed in cytosol (PrxIIB, At1g65980; PrxIIC, At1g65970; and PrxIID, At1g60740), chloroplast (PrxIIE, At3g52960) and mitochondria (PrxIIF, At3g06050). In the genome, an open reading frame was found for PrxIIA (At1g65990), which is considered to be a pseudogene (Horling et al., 2003).

Other peroxidases

Other important peroxidases are located in the cytosol, in vacuoles and in the cell wall. Their activity is often detected with the artificial substrate guaicol (Asada, 1992). Other examples are the glutathione peroxidases, which have been described in plants. Their special isoforms are capable to detoxify organic hydroperoxides and phospholipid peroxides. They have been localized in the cytosol, in chloroplasts, in mitochondria and in the endoplasmatic reticulum (Rodriguez Milla et al., 2003) and putatively in peroxisome (Churin et al., 1999)

1.5.2 Non enzymatic antioxidants

Tocopherols

Tocopherols are lipophilic antioxidants synthesized by all plants, some algae and cyanobacteria. There are four types of tocopherols (α, β, γ and δ) in plants, which

differ only in the number and positions of methyl substituents on the chromanol ring (Fryer, 1992). Tocopherols are amphipathic molecules, with the hydrophobic tail. They are associated with membrane lipids. The polar head groups remain at the membrane surface. In plants, they are synthesized in plastids (Fryer, 1992; Arango and Heise, 1998). They accumulate in various tissues to different degrees. Highest levels are typically found in seeds (Falk eta al., 2003). With their desaturated C=C-bonds, tocopherols participate in the quenching of singlet oxygen. Often they act as recyclable chain reaction terminators for polyunsaturated fatty acid radicals, which are generated by lipid oxidation (Fukuzawa et al., 1982; Girotti, 1998; Liebler, 1993). Tocopherol levels increase in photosynthetic tissues in response to a variety of abiotic stresses (Munne-Bosch and Alegre, 2002), protect the seeds from lipid oxidation during storage, improving their longevity and prevent lipid peroxidation during germination (Sattler et al., 2004). Arabidopsis mutants lacking tocopherol cyclase (*vte*-1) showed a normal phenotype under optimal growth conditions but decreased chlorophyll contents and quantum yield of PSII during photooxidative stress, demonstrating the central role of tocopherols in scavenging singlet oxygen in PSII (Porfirova et al., 2002).

Ascorbate

Ascorbic acid is the most abundant low molecular weight antioxidant in aqueous compartments of plants. Ascorbate is synthesized in mitochondria and accumulates to very high concentrations in leaves, fruits, and actively growing tissues (Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000). Its biosynthesis is controlled by respiration (Millar et al., 2003) It is one of the most important buffers protecting cell constituents against the high oxidative load that accompanies rapid metabolism in plants. The concentration range found in leaves of different species varies from 2-20 µmol g^{-1} fw (Wildi and Lutz, 1996). The pool is highly reduced (>90%) in leaves under normal conditions (Law et al, 1983). Ascorbate is a major primary antioxidant, reacting directly with hydrogen peroxide, hydroxyl radicals, superoxide, and singlet oxygen (Buettner and Juerkiewicz, 1996) and is implicated in the regeneration of α-tocopheryl radicals

produced when α-tocopherol reduces lipid peroxyl radicals (Padh, 1990). Lumenal ascorbate acts as electron donor to photosystem II (Ivanov et al., 2001) and also is a cofactor of violaxanthine de-epoxidase involved in zeaxanthine-dependent dissipation of excess excitation energy, a component of non-photochemical quenching (Niyogi, 1999). Ascorbate is the major and probably the only antioxidant buffer in the apoplast protecting the plasmalemma from oxidative damage (Pignocci and Foyer, 2003). Apoplastic ascorbate represents 10% of the total content in leaves (Noctor and Foyer, 1998).

Studies of the regulation of gene expression in the ascorbate deficient *vtc*-1 mutant, revealed differential expression of 171 from 8300 transcripts analyzed (Pastori et al., 2003). 32% of these genes were implicated in signalling control and developmental processes. On the other hand, genes involved in response to biotic stress, such as pathogenesis-related proteins and other lytic enzymes were induced by low ascorbate contents (Pastori t al., 2003). However, no genes coding for antioxidative enzymes were upregulated in the vtc-1 mutants (Pastori t al., 2003). Work on the liverwort *Riccia fluitans* and in leaf slices of *Arabidopsis thaliana* showed decreased transcript amounts of 2Cys-Prx to a very low level after addition of ascorbic acid (Horling et al., 2001, 2003).

Glutathione

Glutathione is the predominant pool of nonprotein-thiol in plant cells. This thiol-tripeptide reacts directly with ROS under oxidation of the sulphydryl groups of the cysteinyl residues. In addition, it participates in reactions catalyzed by glutathione peroxidase and glutathione-S-transferase (Cummins et al., 1999). Glutathione is the reductant in the regeneration of ascorbate (ascorbate-glutathione-cycle; Foyer et al., 1997), reduces glutaredoxins (Foyer et al., 1997), plays an important role in detoxification and sequestration of xenobiotics under formation of glutathione-conjugates and protects protein thiols from irreversible oxidation by mixed disulfide formation, i.e., glutathionylation (Noctor et al., 2002).

Studies with the mutants rax1-1 and cad2-1, both of them presenting low glutathione levels showed that the GSH concentration alone can influence the

cellular redox state and possibly, redox-sensitive regulators (Ball et al., 2004, Noctor et al., 2002). Downregulation of the antioxidative genes Fe- and Cu/ZnSOD and MDHAR and increased expression of the stress-inducible APx2 were observed. However, mutants were no more affected by oxidative stress than the wild type under the tested conditions, indicating that glutathione can modulate the activity of signalling proteins independent on ROS levels (Ball et al., 2004). Two mechanisms for glutathione modulating action were proposed: first glutathione changes the redox state of thiol-groups of proteins that acts as redox cofactors, altering either the activity or redox state of regulatory proteins, and secondly by S-glutathione to target Cys-residues (Klatt and Lamas, 2000; Paget and Buttner, 2003).

In addition to these antioxidants, other important hydrophilic and hydrophobic antioxidants such as carotenoids, lipoic acids, flavonoids and various alkaloids and phenolic compounds are present in plant cells (Dietz, 2003b; Larson, 1988).

1.5.3 Evidence of antioxidative systems in seeds and seedling development

During seed development and during the period of seedling establishment ROS generation takes place in a context of photosynthesis, fatty acid synthesis and lipid mobilisation. The high risk of lipid peroxidation requires a coordinated induction of the antioxidative mechanisms.

Less is known about the antioxidative systems in the seeds. The major part of the studies is focused on the acquisition of desiccation tolerance (Golovina et al, 1998, 2001; Buitink et al, 2000; Manfre et al., 2006). In the late maturity stage, seeds become tolerant to desiccation by replacement of water through molecules that form hydrogen bonds (Hoekstra et al., 2001). A redistribution of amphiphilic molecules from cytoplasm to lipid phase take place (Buitink et al., 2000). Di- and oligosaccharides and proteins, such as late embryogenesis abundant proteins (LEAs) and heat shock proteins, confer protection to cells during dehydration (Hoekstra et al., 2001).

The involvement of antioxidative mechanisms in the maturation of orthodox seeds is poorly documented, with studies performed only in *Vicia faba* (Arrigoni et al.,

1992), bean (Bailly et al., 2001), sunflower (Bailly et al., 2004) and *Triticum durum* (de Gara et al., 2003). Some of these studies showed the changes in the redox state of ascorbate and glutathione and in the activities of the enzymes, responsible for the recycling of their oxidized forms, during seed development (Arrigoni et al., 1992; de Gara et al., 2003). Their results demonstrated high activity of SOD, APx, MDHAR and GR during the first stages of seed development, but a strong decrease to minimal levels as maturity is reached (Arrigoni et al., 1992; de Gara et al., 2003; Bailly et al., 2001). Meanwhile, catalase activity increased strongly reaching a maximum in the desiccation stage (Bailly et al., 2001, 2004; de Gara et al., 2003). In *Triticum durum* low activity of catalase was observed including completely dehydrated kernels.

In the context of Prx, only the nuclear 1Cys-Prx in barley and Arabidopsis, was analyzed in seeds (Haleskås et al., 1998, 2003a, 2003b; Stacy et al., 1999; Manevich et al, 2002). 1Cys-Prx expression is seed specific. It is first detected in embryo at the bent cotyledon stage. The transcriptional activity increases strongly in the late stage of development. Expression was observed in the embryo and in a layer attached to the inside of the seed coat (Fig. 3). Its function is associated with protection against desiccation-induced free-radical damage.

In seedlings, regulation of antioxidant enzymes has been analyzed in context of salt or water stress (Selote et al., 2004; Menezes-Benavente et al., 2004). It was shown that catalase activity increases in maize seedlings under salt stress, but no significant changes in total SOD activity are observed despite the transcript accumulation (Menezes-Benavente et al., 2004). The effects of ABA on ROS production and antioxidative systems were e.g. studied in leaves of maize seedlings (Jiang and Zang, 2001). Treatments with low ABA concentrations (10-100 μM) induces an antioxidative defense response, but higher concentrations (1 mM ABA) induces excessive ROS generation leading to oxidative damage (Jiang and Zang, 2001). ROS generation in the embryonic axis of germinating seeds at the onset of germination suggests a risk of oxidative damage at that stage of development (Puntarulo et al., 1988, 1991; Schopfer et al., 2001). Some evidence of an active antioxidant system was reported. In radish seeds peroxidases were activated already after imbibition (Schopfer et al. 2001) and ascorbate

biosynthesis started in *Vicia faba* within the first hours after imbibition (Arrigoni et al. 1992).

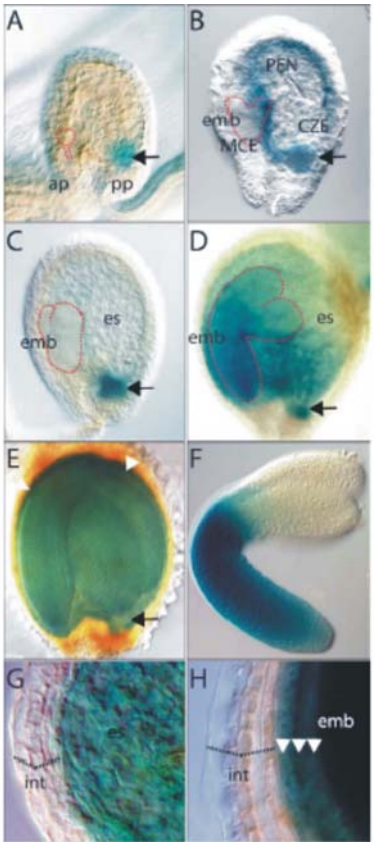

Fig. 3: *AtPER1::GUS* expression in embryo and endosperm development. Differential interference contrast (DIC) micrographs of whole-mount cleared preparations of GUS-stained *AtPER1::GUS* seeds. In A–D, the embryo is indicated by a red dotted line. A. *AtPER1::GUS* expression in chalazal cyst (arrow) in ovule at the embryo globular stage. Note that the embryo is not in focal plane. ap, anterior pole; pp, posterior pole. B. *AtPER1::GUS* expression in the chalazal cyst (arrow) and the endosperm of ovule at the embryo heart stage. Note that the endosperm expression lines the seed coat in chalazal endosperm (CZE) and peripheral endosperm (PEN), but not in micropylar endosperm (MCE). C. *AtPER1::GUS* expression in the chalazal cyst (arrow) of an ovule at the embryo early torpedo stage. D. *AtPER1::GUS* expression in the chalazal cyst (arrow), in chalazal, peripheral and micropylar endosperm (es) and the embryo (emb) in early bent cotyledon stage ovule. E. *AtPER1::GUS* expression in mature bent cotyledon stage seed. Note expression in aleurone (arrowheads). F. *AtPER1::GUS* expression in mature bent cotyledon stage embryo. G. Detail of *AtPER1::GUS* expression in endosperm (es) of late torpedo stage ovule. Note there was no expression in integuments and seed coat (int). H. Detail of *At-PER1::GUS* expression in aleurone (arrowheads) and embryo (emb) of bent cotyledon stage ovule. Note that there was no expression in integuments and seed coat (int). (by Haslekas et al. 2003a)

In soybean it was shown that the α-tocopherol content increased in embryonic axes upon imbibition and post-germination under oxidative stress (Simontacchi et al., 1993). However, a systematic analysis of the expression of antioxidant enzymes in seedlings is missing so far despite the importance of regulation of the antioxidative defense system upon germination and seedling establishment.

1.6 Aim of the present work

The present work aims at understanding the regulation of the antioxidant defence system during the two most critical stages of development of oil seed plants, namely the transition from lipid based heterotrophic growth to photoautotrophic metabolism in seedlings and the development of siliques during the lipid storage phase of seeds. Special focus is given to peroxiredoxins, which are involved in detoxification of alkyl hydroperoxides.

The first part of the study compares seedlings grown in continuous light conditions and day/night cycles in the presence or absence of external carbohydrate source. The induction of antioxidative enzymes was analyzed in the context of ROS patterns, photosynthetical performance and content and redox state of ascorbate.

The second part of the study focusses on the regulation of peroxiredoxins in *Arabidopsis thaliana* during silique development (from early morphogenesis stage and during seed maturity) in the context of the photosynthetical performance of the silique wall. The information taken from the study on Arabidopsis was transferred to the oilseed crop *Brassica napus*, whose agricultural importance and physiological properties classify it as an alternative model organism for genomic and molecular approaches.

2. Materials and Methods

2.1 Plant Material

2.1.1 Seedlings

Seedlings of *Arabidopsis thaliana* var. Col-0 and *Arabidopsis* reporter gene plants expressing GUS under control of the ICL (ICL::GUS, Penfield et al., 2004) and 2Cys-PrxA promoter (2Cys-PrxA, Baier et al., 2004) were grown on filter paper (surface weight 85 g cm^{-2}, thickness 0.17 mm; Macherey-Nagel, Karlsruhe). After two days cold treatment the seeds were transferred to a growth chamber and cultivated either at a day and night cycles of 14 h at a photosynthetically active radiation of 90 µmol quanta m^{-2} s^{-1} at 21°C and 10 hr dark at 19°C or in continuous light conditions (120 µmol quanta m^{-2} s^{-1}, 22°C). The seedlings were daily irrigated with tap water (15 ppm NO_3^-, 25 ppm Cl^-, 35 ppm SO_4^{2-}, 171 ppm HCO_3^-, 11 ppm Na^+, 1.8 ppm K^+, 69 ppm Ca^{2+}, 2.5 ppm Mg^{2+}, 0.92 ppm PO_4^{3-}; pH 7.6, conductivity 405 µS cm^{-1}; water hardness: 10.3 °dH). Beginning 1.5 days after radicle emergence the seedlings were harvested in the morning 1 h after onset of illumination and in the evening 1h before the end of the illumination period, immediately immerse in liquid nitrogen, and stored at -80°C until analysis. For sucrose treatment, the seedlings were watered with 1% (w/v) sucrose (in tap water). The sucrose treatment was started 12 h after radicle emergence to avoid a delay of germination by sucrose.

For experiments with two week old seedlings, plants were cultivated in petri dishes on solid MS medium (0.43 % (w/v) MS medium (Duchefa, Haarlem, Netherlands), 0.44 % (w/v) Phytagel (Sigma, Steinheim), pH 5.7, autoclaved) at day/night-cycles as described above. For sterilization, the seeds were treated with ethanol-SDS solution (70 % (v/v) ethanol, 0.1 % (w/v) SDS) for 5 min and afterwards with bleach-SDS solution (20 % (v/v) chlorbleach (Glorix, Lever Fabergé), 0.1 % (w/v) SDS) for 15 min. Subsequently, the seeds were washed 4 to 6 times with steril water for 5 min. Finally they were sown under sterile conditions on sterile plates containing the solid MS media.

2.1.2 Siliques

Arabidopsis thaliana var. Col-0, GUS reporter gene line for 2Cys-Prx A and *Brassica napus* were sown on soil culture (1/3 autoclaved soil, 1/3 perlite, 1/3 vermiculite) and placed into a dark cold room for two days at 4°C. Afterwards they were grown in a growth chamber at 50 % humidity and in light/dark cycles of 10 h (120 µmol quanta m^{-2} s^{-1}, 22°C) / 14 h (20°C). After flowering, the siliques were harvested according to their developmental stage, immediately immersed in liquid nitrogen, and stored at -80°C. For histochemical analysis of GUS-reporter gene activity, the plants were used immediately after harvesting.

2.2 Extraction of Plant Material for Ascorbate Assay and Determination of Pheophytin Content

2.2.1 Measurement of reduced and total ascorbate (according to Foyer et al.,1983)

100 mg frozen plant material was extracted in 650 µL ice-cold 1M $HClO_4$ (crude extract). After 5 min centrifugation at maximal speed (ca. 10000 x g) at 4°C, 400 µL of the supernatant were transferred into 200 µL of 1 M HEPES/KOH buffer (pH 7.5). The pH of the solution was adjusted to about pH 5.0 with 5 M K_2CO_3. The samples were centrifugated at maximal speed at 4°C for 5 min to remove the formed precipitates. From the supernatant the contents of reduced and total ascorbate were quantified spectrophotometrically.

Reduced ascorbate was measured from 100 µL of the supernatant in 900 µL of 0.1 M sodium phosphate buffer (pH 5.6) by monitoring a decrease in A_{265} in the presence of 5 units of ascorbate oxidase (Sigma, Deisenhofen). For determination of the total ascorbate content, the oxidized ascorbate pool was reduced with 50 mM DTT in four volumes of 0.1 M sodium phosphate (pH 7.5). After 30 min of incubation on ice, the ascorbate content was quantified as described for reduced ascorbate.

2.2.2 Determination of pheophytin content

From the homogenates of plant material in 1 M $HClO_4$ 100 µL samples were transferred into 900 µL of 80 % (v/v) acetone and extracted in the dark at -20°C. The pheophytin contents were quantified spectrophotometrically from the clear supernatant obtained after centrifugation at maximal speed for 5 min. According to Vernon (1960) the pheophytin content was calculated from the absorbance at 655 and 666 nm by the following equation:

$$\mu g \text{ Pheo } mL^{-1} = (6.75 \times A_{666}) + (26.03 \times A_{655})$$

2.3 Extraction of Plant Material for Ascorbate Peroxidase Assay and Determination of Chlorophyll Content

2.3.1 Ascorbate peroxidase assay

Ascorbate peroxidase activity was determined as described by Hossain and Asada (1984). 50 mg frozen plant material was extracted in 125 µL extraction buffer (100 mM Hepes - NaOH pH 7.6). After 5 min centrifugation at maximal speed the supernatant was transferred to a fresh reaction tube. 50 µL extract were added to 1 mL assay-buffer (50 mM Hepes - NaOH pH 7.6) and 50 µL 5 mM ascorbate. The reaction was started after addition of 100 mL 3 mM H_2O_2. Spectrophotometrical measurements were carried out at 290 nm ($\varepsilon_{\text{ascorbate}} = 2.8\ mM^{-1}\ cm^{-1}$). Activity of ascorbate peroxidase was standardized on the protein and/or the chlorophyll contents. Protein contents were determined by the BioRad assay as described on section 2.7.2.

2.3.2 Determination of chlorophyll content

Prior to centrifugation, aliquots (10 µL) of crude homogenates of plant material in 1 M $HClO_4$ were transfered to 990 µL of 80 % (v/v) acetone. Following extraction of the pigments in the dark at -20°C, the chlorophyll contents were assayed in the clear supernatant obtained after centrifugation for 5 min at maximal speed at $A_{663.6}$ and $A_{646.6}$ and calculated according to Porra (2002) by the following equation:

$$\text{Total Chlorophyll } [\mu g\ mL^{-1}] = 17.76 \times A_{646.6} + 7.34 \times A_{663.6}$$

2.4 Isolation of RNA from Plant Material

2.4.1 Isolation and Purification of RNA

Lysis buffer	100 mM	Tris-HCl (pH 8.5 - 9.0)
	25 mM	EDTA
	25 mM	EGTA
	100 mM	β-Mercaptoethanol
	2 % (w/v)	SDS

Total RNA was isolated from 200 - 300 mg plant material. This was ground to a fine powder in liquid N_2 and homogenized in 500 µL lysis buffer, 500 µL Roti®-Aqua-Phenol (Carl Roth GmbH + Co, Karlsruhe) and 300 µL chloroform (chloroform:isoamylalcohol (24:1)). After 10 min centrifugation at maximal speed the supernatant was first extracted with 500 µL Roti®-Aqua-Phenol and 500 µL chloroform and afterwards with 1 mL chloroform. The RNA was precipitated from the aqueous phase by addition of 500 µL isopropanol for 1 hr at 4°C and sedimented by centrifugation. The pellet containing the RNA was washed with 70 % ethanol and disolved in 200 µL DEPC-Water (0.1% v/v DEPC). For further purification the RNA was precipitated with 20 µL 3 M sodium acetate and 600 µL ethanol at -20 °C for at least 3 hr. After centrifugation the RNA was washed with 70 % ethanol and disolved in 25 - 50 µL DEPC-water. From silique samples, the RNA was precipitated with 8 M LiCl instead of 3 M sodium acetate. In this case, following the isopropanol precipitation, the pellet was resuspended in 375 µL TE buffer (10 mM Tris, 1 mM EDTA). After adding 125 µL 8 M LiCl, the samples were incubated at 4°C for two hours. After centrifugation, the pellet was washed with ethanol and resuspended in 25 µL DEPC-Water. With RNA samples of the oldest siliques this step was repeated once to obtain a better RNA quality.

2.4.2 Determination of RNA concentration

For quantification of the RNA content the samples were diluted 1:500 in DEPC-treated water. The absorption of the samples was measured at 230, 260, 280 and 320 nm using the Gene Quant Spectrophotometer (Amersham-Pharmacia-Biotech, Freiburg). One A_{260} corresponds to 40 µg mL^{-1} RNA. The purity of the sample

was calculated from the $(A_{260}–A_{320})/(A_{280}-A_{320})$ and $(A_{260}-A_{320})/(A_{230}-A_{320})$ ratios, which indicate contaminations with aromatic metabolites and proteins. For values higher than 1.6 the RNA purity was considered acceptable.

2.4.3 Electrophoretic separation of RNA (MOPS-formaldehyde gel)

DEPC-H_2O	100 µL	DEPC for 1 L distilled water mixed under agitation
		Autoclaved prior to use
10x MOPS-buffer	200 mM	MOPS, pH 7.0
	50 mM	Na-acetate
	10 mM	EDTA
RNA-loading buffer	50 mM	Tris-HCl, pH 8.0
	10 mM	EDTA
	50.0 % (v/v)	glycerol in water
	0.4 % (w/v)	bromophenol blue
	0.4 % (w/v)	xylene cyanol FF

For quality control the RNA was analyzed in MOPS-formaldehyde gels with RNase-free devices. 1 % agarose was melted in 1x MOPS-buffer (10x MOPS-buffer diluted with DEPC-H_2O). After cooling down to about 60°C 2 mL formaldehyde were added. For solidification, the gel medium was poured on horizontal gel trays equiped with polystyrol combs to form vertical gel loading pockets. The polymerized gels were transferred into a RNase-free gel tank containing 1x MOPS-buffer.

Samples containing 20-25 µg RNA in 26 µL were mixed with 18 µL formaldehyde [18 % (v/v)] and 5 µL 10x MOPS-buffer. After heating the samples at 65°C for 5 min they were immediately transferred on ice. Prior to loading, 2 µL RNA-loading buffer, 1 µL ethidiumbromide (EtBr; 10 mg mL^{-1}) and 25 µL formamide were added.

The RNA was separated on the gels at 120 volts for 1h. The fluorogramme was documented photographically in a UV-light box (312 nm; INTAS, Göttingen) using a CCD-camera.

2.5 cDNA-First Strand Synthesis

2.5.1 DNase digestion

Contaminating DNA was removed during 25 min incubation at 25°C by adding 1 µL 10x DNase I Reaction Buffer (RQ1 DNase 10x Reaction Buffer, Promega, Mannheim) and 1 µL DNase (RQ1 RNase-free DNase, 1 U/µL, Promega, Mannheim) to 3 µg RNA in 8 µL sterile distilled water. To stop the DNase reaction 1 µL 25 µM EDTA was added. After 20 min incubation at 70°C, as an additional inactivation step, the samples were frozen shortly in fluid nitrogen.

2.5.2 First strand cDNA synthesis reaction

cDNA was synthesized from DNase-treated RNA with M-MLV reverse transcriptase H(-) Point (Promega, Madison, Wisconsin). For 30 µL reaction 2 µL OligodT_{12}N (0.5 µg/µL), 2 µL sterile distilled water and 1.5 µL dNTPs (each 10 mM dATP, dCTP, dGTP, dTTP) were added to 11 µL DNase digested samples and incubated at 70°C for 10 min. After 5 min on ice, 6 µL 5x reverse transcriptase-buffer (M-MLV RT 5x Reaction Buffer, Promega, Mannheim), 1.5 µL RNase-Inhibitor (RNasin®Plus, 40U/µL, Promega, Madison, Wisconsin, USA) and 4.5 µL sterile distilled water were added to the samples, mixed and incubated for 5 min at 37°C followed by an incubation for 50 min at 37°C after addition of 1 µL reverse transcriptase (M-MLV Reverse Transcriptase, 200 U/µL, Promega, Mannheim). The reaction was stopped by 15 min incubation at 70°C. The cDNA was stored at -20°C.

2.6 Polymerase chain reaction (PCR)

10x Taq-buffer	200 mM	Tris-HCl, pH 8.4
	500 mM	KCl
	15 mM	$MgSO_4$
	5 mM	$MgCl_2$

2.6.1 Composition of PCR reaction

Polymerase chain reaction (PCR) was used to amplify the number of copies of specific DNA sequences *in vitro*. In the experiments presented in this work, the heat-stable Taq DNA polymerase was used for catalyzing the polymerase reaction. The general program is depicted in Table 1. The number of cycles was optimized for each gene to yield optimal contrast between samples in the fluorogrammes of subsequently performed EtBr-agarose gel electrophoresis. For a typical PCR reaction the cDNA prepared as described in 2.5.2 were used as template in a 30 µL sample containing 1x Taq-buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM of each primer (reverse and forward) and 1U Taq-polymerase.

Cycles	Reaction step	Temperature (°C)	Time (min)
1	Denaturation	94	2
28 - 42	Denaturation	94	0.5
	Primer-annealing step	50-58	0.5
	Polymerization	72	0.75-1
1	Polymerization	72	2

Table 1: General RT-PCR-Program

2.6.2 Separation of DNA by agarose gel electrophoresis

50x TAE-buffer	40 mM	Tris-acetate, pH 7.5
	1 mM	EDTA

5x DNA-loading buffer	0.25 % (w/v)	Bromophenolblue
	0.25 % (w/v)	Xylenecyanol
	30 % (v/v)	Glycerol

PCR products were separated electrophoretically on EtBr containing agarose gels. In dependence on fragment size 1 - 2 % (w/v) agarose (Sea Kem LE Agarose, Cambrex Bio Science Rockland, Rockland, UK) were melted in TAE buffer. To the liquid gel medium 0.5 µg mL^{-1} EtBr were added. Prior to loading, an appropiate amount of 5x DNA-loading buffer was added to each cDNA sample. The DNA samples were separated at 100 V in 1x TAE-buffer. For estimation of the fragment sizes 1kb DNA-ladder (Invitrogen, Karlsruhe) was separated in parallel. The detection of DNA was carried out in a UV-light box (312 nm; INTAS, Göttingen) and documented electronically with a CCD-camera.

2.6.3 Semiquatitative RT-PCR

The cDNA samples were standardized on actin-2 transcript amount (At5g09810) using gene specific primers in a PCR (Apendix A). Actin-2 is almost constitutively expressed in plant cells. It is part of the cytoskeletton in which it forms the filaments that stabilize the cell structure. By the standardization on actin-2 transcript amounts, equal product band intesities in a PCR reaction approximately correspond to equal cDNA concentrations. For quantification, the band intensities were calculated with Gelscan 2.0 (Science Group, BioSciTec, Marburg) from the gray scale in the digital images of the fluorogrammes after separation of the PCR products on ethidium bromide-TAE-gels.

2.7 Protein isolation and analysis

2.7.1 Protein isolation

Extraction buffer	20 mM	Tris-HCl, pH 7.5
	2 mM	$MgCl_2$

Proteins were extracted from 100 mg frozen plant material in reaction tubes containing 500 µL extraction buffer by grinding with a micro pistil. After 10 min centrifugation at maximal speed, the supernatant was transferred to fresh reaction tubes in 50-100 µL aliquots. The extracts were either directly used or stored at -20°C.

2.7.2 Protein quantification

The protein concentration of samples was determined spectrophotometrically after adding 10 µL protein extract to 790 µL distilled water and 200 µL BioRad-Reagenz (BioRad Protein Assay, BioRad Laboratories GmbH, München) and reading after 5 min the absorption at 595 nm, which depends on binding of Coomassie brilliant blue G250 to proteins. From the absorption, the protein content was quantified using standard curves with 1-10 µg BSA (Albumin Bovine Fraction V, Serva, Heidelberg).

2.7.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations proteins migrate approximately according to their molecular mass. The anionic detergent sodium dodecyl sulfate (SDS) denatures proteins by wrapping the hydrophobic tail around the polypeptide backbone. For almost all proteins, SDS binds at a ratio of approximately 1.4 g SDS per gram of protein. This gives proteins a negative net charge proportional to the length of the polypeptide chain. The proteins can be totally unfolded when a reducing agent such as dithiothreitol (DTT) is added, which reduces disulphide bonds.

The proteins were separated in a discontinuous system, in which protein mobility is intermediate between the mobility of the buffer ion of the same charge (usually negative) in the stacking gel and in the buffer tank. The proteins concentrate in a very thin zone, called the stack, between the leading ion and the trailing ion. When the proteins reach the border between the stacking and the separating gel, due to a pH or an ion change, the proteins become the trailing ion and separate on the gel.

Preparation of the gels

Reservoir buffer	1.44 % (w/v)	Glycine
	0.3 % (w/v)	Tris
	0.1 % (w/v)	SDS
6% Stacking gel	25 % (v/v)	1.5 M Tris-HCl, pH 6.8
	5.84 % (w/v)	Acrylamide
	0.16 % (w/v)	Bisacrylamide
	0.04 % (w/v)	AMPS
	0.03 % (w/v)	TEMED
12.5 % Separation gel	25 % (v/v)	1.5 M Tris-HCl, pH 8.8
	12.17 % (w/v)	Acrylamide
	0.33 % (w/v)	Bisacrylamide
	0.04 % (w/v)	AMPS
	0.03 % (v/v)	TEMED

The freshly prepared separation gel solution was filled between two glass plates. The rest of the space was filled with distilled water to avoid contact with oxygen, which can inhibit polymerization. After polymerization of the separating gel the overlaying water was discarded and the stacking gel solution was poured onto the separating gel. A comb was inserted to form the pockets for the samples.

Samples preparation and electrophoresis

Protein-loading buffer	125 mM	Tris-HCl, pH 6.8
	10 mM	DTT
	2.3 % (w/v)	SDS
	10 % (v/v)	Glycerol
	0.01 % (w/v)	Bromophenol blue

Protein samples were mixed with one volume protein-loading buffer and incubated 5 min at 95°C for denaturation. Afterwards the samples were transferred immediately on ice to prevent secondary structure formation.

The samples standardized on protein amount were loaded on the gel and separated electrophoretically at 40 mA. For estimation of the molecular masses the molecular mass protein marker SeaBlue (Invitrogen, Karlsruhe) was separated in the same gel in parallel.

2.7.4 Coomassie staining of proteins on polyacrylamide gels

Coomassie staining of proteins separated on polyacrylamide gels was performed to control the protein amounts to be transferred by Western blotting or to control gel loading.

Coomassie stain solution	50 % (v/v)	Methanol
	0.1 % (w/v)	Coomassie brilliant blue
	2 % (v/v)	Acetic acid

Destain solution	40 % (v/v)	Methanol
	5 % (v/v)	Acetic acid

Following electrophoretic separation of the proteins (2.7.3.2), the polyacrylamide gels were incubated in Coomassie stain solution for 1 h minimum. During this time Coomassie brilliant blue binds to the proteins. To remove unbound

Coomassie brilliant blue the gels were subsequently incubated in destain solution until the protein bands were optimally contrasted.

2.7.5 Western Blotting

10x Anode buffer	300 mM	Tris, pH 10.4
	15 % (v/v)	Methanol

1x Cathode buffer	40 mM	ε-Aminocaproic acid
	20 mM	Tris, pH 9.4
	15 % (v/v)	Methanol

Inmediately after separation, the protein gels for Western-blotting were transferred on nitrocellulose membrans (Protran®, Nitrocellulose Transfer Membrane, Schleicher & Schuell, Bioscience, Dassel), whose protein binding sites were activated through the 20 % methanol of the blot buffer.

Between six layers of filter paper (Gel-Blotting Paper, GB002, Schleicher & Schuell, Dassel), which were soaked either in anode or cathode buffer, the nitrocellulose membrane and the gel were piled up. For the electrophoretic transfer, the current was set to 2 mA cm^{-2} of the membran. For the Prx (17 and 24 kDa) the transfer time was 35 min.

2.7.6 Immunodetection with specific antibodies

TBS buffer	25 mM	Tris-HCl, pH 7.5
	150 mM	NaCl

Blocking solution	1% (w/v)	Milk powder in TBS

Detection of 2Cys-Prx, PrxIIC, PrxIIF and PrxQ on nitrocellulose membranes was performed with polyclonal rabbit antibodies (Table 2). The antibody against 2Cys-Prx, which was raised against barley 2Cys-Prx fused to glutathione-S-transferase (Baier and Dietz, 1997) recognizes both isomers, i.e. 2Cys-PrxA and 2Cys-PrxB, the antibody against PrxIIC (Horling et al., 2003) recognizes also the highly homologous proteins PrxIIB, PrxIID and PrxIIE, while PrxIIF is specifically detected only by the PrxIIF antibody (Finkemeier et al., 2005; Table 2).

Primary antibody		Heterologous expressed in *E. coli*	
BasFus	Rabbit-anti-2Cys-Prx	*Hordeum vulgare*	Baier and Dietz, 1997 Horling et al., 2003
*At*PrxIIC	Rabbit-anti-PrxIIC	*Arabidopsis thaliana*	Finkemeier et al.,
*At*PrxIIF	Rabbit-anti-PrxIIF	*Arabidopsis thaliana*	2005
*At*PrxQ	Rabbit-anti-PrxQ	*Arabidopsis thaliana*	Lamkemeyer et al., 2006

Table 2: List of antibodies against Prx

Nitrocellulose membranes with the transferred proteins were blocked for at least 30 min at room temperature with 1% (w/v) milk powder (Magermilch-Pulver, Lasana, Humana Milchunion eG, Herford) to avoid unspecific binding of the antibodies. Subsequently the membranes were incubated over night at 4°C in antibody solutions (2Cys-Prx 1:8000; Prx IIC 1:5000; Prx Q 1:8000; Prx IIF 1:8000) prepared in fresh blocking buffer. The detection of the primary antibodies was carried out by chemoluminescence (Sec. 2.7.7) and/or the NBT/BCIP method (2.7.8).

2.7.7 Detection of proteins by chemoluminescence

TBST buffer	25 mM	Tris-HCl, pH 7.5
	150 mM	NaCl
	0.05 % (v/v)	Tween20

Blocking solution	1 % (w/v)	Milk powder in TBST

For the detection of the proteins by chemoluminescence blocking of the membranes and incubation with the first antibodies were carried out with TBST buffer. After incubation with the first antibodies the membrane were washed 3 times for 5 min with TBST and incubated 1 h with the secondary antibody (Super Signal® Goat against rabbit-IgG-Antibody, Horseradish peroxidase, Pierce Biotechnology Inc., Perbio Science Company, Rockford, UK; 1:5000 in blocking solution) at room temperature.

After incubation with the secondary antibody, the membrane was washed 3 times for 5 min with TBST and incubated for 10 min in the chemiluminescent substrate (Super Signal® West Pico Chemiluminiscent Substrate, Pierce Biotechnology Inc.,

Perbio Science Company, Rockford, UK). For detection of the fluorescence signals X-Ray films (X-Ray Retina, Fotochemische Werke GmbH, Berlin) were exposed to the membranes and developed in one volume developer solution per 3 volumes water (Kodak X-Ray Developer, Kodak, Paris, France) and fixed in one volume fixer solution per 3 volumes water (Kodak X-Ray Fixer, Kodak, Paris, France).

2.7.8 Detection of proteins with NBT/BCIP

Carbonate buffer	100 mM	$NaHCO_3$, pH 9.8
	1 mM	$MgCl_2$
NBT solution	30 mg/mL	NBT in 70% Dimethylformamide
BCIP solution	15 mg/mL	BCIP in Dimethylformamide
Detection solution	0.05 % (v/v)	NBT solution in carbonate buffer
	0.05 % (v/v)	BCIP solution in carbonate buffer

After incubation with the first antibodies the membranes were washed 3 times for 5 min with blocking solution and incubated for 3 h with the secondary antibody (Goat against rabbit IgG, alkaline phosphatase-conjugated, Sigma, Steinheim; 1:3000 in blocking solution) at room temperature.

Subsequently the membranes were washed 3 times for 5 min with TBS and equilibrated for 1 min in carbonate buffer. For detection they were incubated in fresh detection solution until visibility of the signals. 5-Bromo-4-chloro-3-indolylphosphate (BCIP) is dephosphorylated by the alkaline phosphatase bound to the secondary antidody. The $BCI^{\cdot}$ radical reacts afterwards with p-nitroblue-tetrazoliumchloride (NBT) and forms a purple precipitate. To stop the reaction the membranes were washed several times with tap water.

2.8 Determination of $O_2^{\cdot-}$

1x PBS-buffer, pH 6.9	137 mM	NaCl
	2.7 mM	KCl
	10 mM	Na_2HPO_4
	2 mM	KH_2PO_4

Destain solution	1 vol	ethanol
	5 vol	chloroform
	0.15 % (v/v)	trichloracetic acid

$O_2^{\cdot-}$ were stained i*n situ* with nitroblue tetrazolium (NBT) according to Jabs *et al*. (1996) directly or after 2 h dark treatment. The samples were incubated in 5 mg/mL NBT prepared in PBS buffer pH 6.9 in the dark for 4 hr at room temperature. With superoxide NBT forms a dark blue insoluble formazan compound (Flohe and Otting, 1984; Beyer and Fridovich, 1987). For better visibility of the precipitate chlorophyll was removed by several washing steps in destain solution.

2.9 Determination of GUS Activity

2.9.1 Histochemical GUS assay (according to Jefferson et al., 1987)

GUS staining solution	10 mg	X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) dissolved in 200 µL dimethylformamide
	200 mg	Triton X-100
	40 mL	5 mM $K_4Fe(CN)_6$ / $K_3Fe(CN)_6$
	20 mL	0.5 M Na_2HPO_4 / NaH_2PO_4 pH 7.2
	ad to 200 mL	distilled water

The plant material was infiltrated three times for one minute each with GUS-staining solution and incubated in the dark at 37°C. The solution was removed as soon as the optimal staining intensity was reached. The plant material was washed with water and treated with 80 % ethanol to remove chlorophyll.

2.9.2 Quantitative GUS assay (according to Abel and Theologis, 1998)

Extraction buffer	50 mM	Na_2HPO_4 / NaH_2PO_4 pH 7.0
	10 mM	DTT
	0.1 % (v/v)	Triton X-100
	0.1 % (w/v)	SDS
	10 mM	EDTA

Reaction buffer 20 mg p-nitrophenyl-ß-D-glucoronide in 20 mL extraction buffer

50 mg plant material was extracted in 250 µL extraction buffer and centrifuged by maximal speed for 5 min. 10 to 50 µL extract were added to 500 µL reaction buffer and incubated at 37°C for 60 min. The reaction was stopped by adding 500 µL 400 mM Na_2CO_3. The absorption was determined by 405 nm (ε = 18300 Δ Abs L mol^{-1} cm^{-1}).

2.10 Chlorophyll-a fluorescence parameters

Chlorophyll-a fluorescence of PSII was determined with the Mini PAM setup (Walz, Effeltrich) using a modulated measuring beam of < 0.1 µmol quanta m^{-2} s^{-1}, an actinic light intensity of 120 µmol quanta m^{-2} s^{-1} (Fs), and a saturating beam of about 5000 µmol quanta m^{-2} s^{-1}, which closes all PSII reaction centres and allows determination of the maximum fluorescence level F_M. Each measurement was carried out in the dark with the Mini PAM as unique source of light to avoid interferences. Prior to 4.5 min analysis of the chlorophyll fluorescence pattern, seedlings were kept in the dark for 30 min.

Fluorescence parameters in siliques were determined using the PAM-Control setup (Walz, Effeltrich) with the same illumination program described above. Whole plants were dark-adapted for 30 min and the measurements were also carried out in darkness.

Photosynthetic parameters as maximal photosynthetic yield ($\Phi PSII_{max}$), photosynthetic yield (ΦPSII), coefficient of photochemical quenching (qP) and non-photochemical quenching (NPQ) were calculated according to Schreiber and Bilger (1993; equations in Apendix B).

2.11 Transmission electron microscopy

Leaf segments of developing seedlings of *Arabidopsis thaliana* were cut into pieces of an approximate area of 1 mm^2. The leaf pieces were fixed in 5 % (v/v) glutaraldehyde (Serva, Heidelberg) in buffer (50 mM KH_2PO_4 / Na_2HPO_4, pH 7.0) for 1h. A second fixation step was conducted for 1 h at room temperature in 2 % (v/v) osmium tetroxide (OsO_4) in distilled water. The tissue was dehydrated in a graded acetone series and embedded in transmit resin (TAAB Laboratories Equipment, Berkshire, UK). Ultrathin sections of 60 nm were cut with a diamond knife (DuPont) on an Ultracut microtome (Reichert Ultracut E) and placed onto 400 mesh gold grids. Samples were counterstained with 0.1 % (w/v) uranyl acetate (5 sec) followed by 2 % (w/v) lead citrate (5 sec). Preparations were examined with Hitachi H500 electron microscope at 75 kV.

2.12 Fluorescence microscopy

Sections of entire siliques were cut by hand and embryo, seed coat and silique wall chlorophyll autofluorescence were obtained using an Axioskop microscope (Zeiss, Jena) equipped with a 100 W mercury arc epi-illumination, a CCD-camera (Sony) and a fluorescein filter set (Zeiss, Jena). The images were digitally processed using the software Axiovision (Zeiss, Jena).

2.13 Confocal microscopy

Sections of entire siliques were cut by hand and embryo, seed coat and silique wall were examined for chlorophyll distribution with a confocal microscope (Leica SP2, Heidelberg). Pigment autofluorescence was analyzed by obtaining emission spectra in the range 485-700 nm 30 nm bandwidth and 50 steps, excited with an argon-ion-laser at 458 nm. For the measurements a double dichroic mirror DD458/514 (Leica, Heidelberg) and an oil-inmersion objective with 20-fold magnification (Leica HC PL Apo CS 20.0*0.70 Imm/CorUV) were used. The scan speed was 400 Hz, the image resolution 1024 x 1024 pixels and the pinhole diameter 100 µm. 12 bit scans were performed to achieve a high signal to noise ratio.

For images, the autofluorescence signal was detected in the ranges 510-600 nm and 600 -700 nm using two active photomultipliers. Images were processed with Leica Confocal Software and Zeiss Image Browser.

3. Results

Seedling establishment

Following root emergence, post-germinative growth continues with expansion and greening of the cotyledons. In parallel a massive reorganisation of metabolism takes place and photoautotrophic growth takes over from metabolization of storage lipids. Here, redox control and antioxidant defence during seedling development were studied between 1.5 and 5 days after radicle emergence (DARE) focussing specifically on regulation of the expression of peroxiredoxins and genes encoding proteins of the Halliwell-Asada-cycle. To modulate transition from lipid to carbohydrate metabolism and carbohydrate availability, the seedlings were either grown in continuous light or in day and night cycles in the presence and absence of 1 % sucrose as external carbohydrate source. Sugar was applied first 12 hours after radicle emergence to the media to prevent delay in the germination (Dekkers et al., 2004), but ensuring a slower mobilization of the rest of the stored lipids.

3.1.1 ICL promoter activity as indicator for lipid mobilization

In Arabidopsis, germination is followed by a period of about two days of heterotrophic growth, which is supported by seed storage reserves (Eastmond and Graham, 2001). By the end of this period seedlings must be able to continue their development photoautotrophically. One of the main pathways involved in lipid breakdown is the glyoxylate cycle with isocitrate lyase (ICL) and malate synthase (MLS) as key enzymes. ICL transcription is induced during seed germination and peaks about two days after imbibition (Eastmond and Graham, 2001). Plants expressing a chimeric ICL::GUS reporter gene construct (Penfield et al., 2004) were used to monitor the regulation of lipid breakdown during seedling establishment in continuous light in presence and absence of 1% (w/v) sucrose, respectively. At 1.5 DARE the ICL promoter activity was higher in seedlings in presence of sucrose than in those grown in the absence of sucrose demonstrating the carbohydrate mediated inhibition of lipid mobilization (To et al., 2002; Eastmond et al., 2000). Between 1.5 and 3 DARE ICL-driven GUS activity of

sugar-free grown seedlings slowly decreased with manifestation of the photoautotrophic growth (Fig 4) and rapidly declined in seedlings grown on sucrose-supplemented medium. At 4 DARE promoter activities were very low and indistinguishable between the treatments.

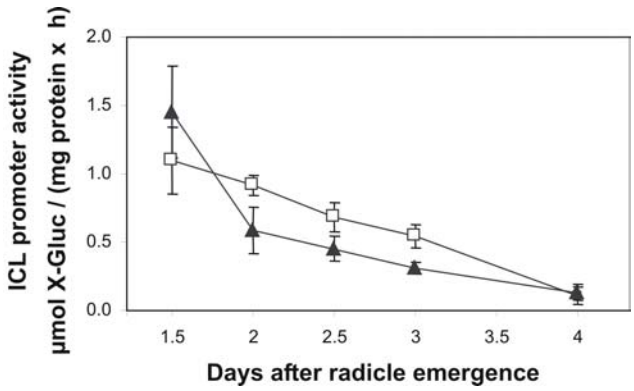

Fig. 4: **Isocitrate lyase promoter activity as indicator of lipid metabolization.** ICL:GUS activity in seedlings grown in day/night rhythms in the (▲) presence and (□) absence of 1 % sucrose (mean ± SD; n = 3)

3.1.2 Cellular ultrastructure: chloroplasts

During early seedling development, following cell division the proplastids of the meristems undergo a quick programme of division and morphological changes (Mullet, 1988). Upon germination, chloroplasts become photosynthetically active. With development of primary leaves the cotyledons loose their function as source organs within days. Here Arabidopsis seedlings were analyzed for chloroplast morphology between 1.5 and 5 DARE in continuous light and in day-and-night cycles in the presence and absence of sucrose by transmission electron microscopy after osmium-tetroxide staining (Fig. 5).

At the end of the second light period after radical emergence (1.5 DARE) in the still expanding mesophyll cells chloroplasts were fully developed in all plants tested. In continuous light the inner chloroplast membrane system had formed several stroma thylakoids and grana stacks with 4-8 grana layers. Randomly, dark stained plastoglobuli were observed, but no transitory starch. In contrast, in discontinuous light in the evening of the second DARE (1.5 DARE) regularly small starch granules were observed. In the presence of sucrose, per chloroplast 3

- 5 starch granules were formed, while only 2 - 3 slightly smaller granules were observed in the absence of sucrose. The size of the plastids was similar in all samples.

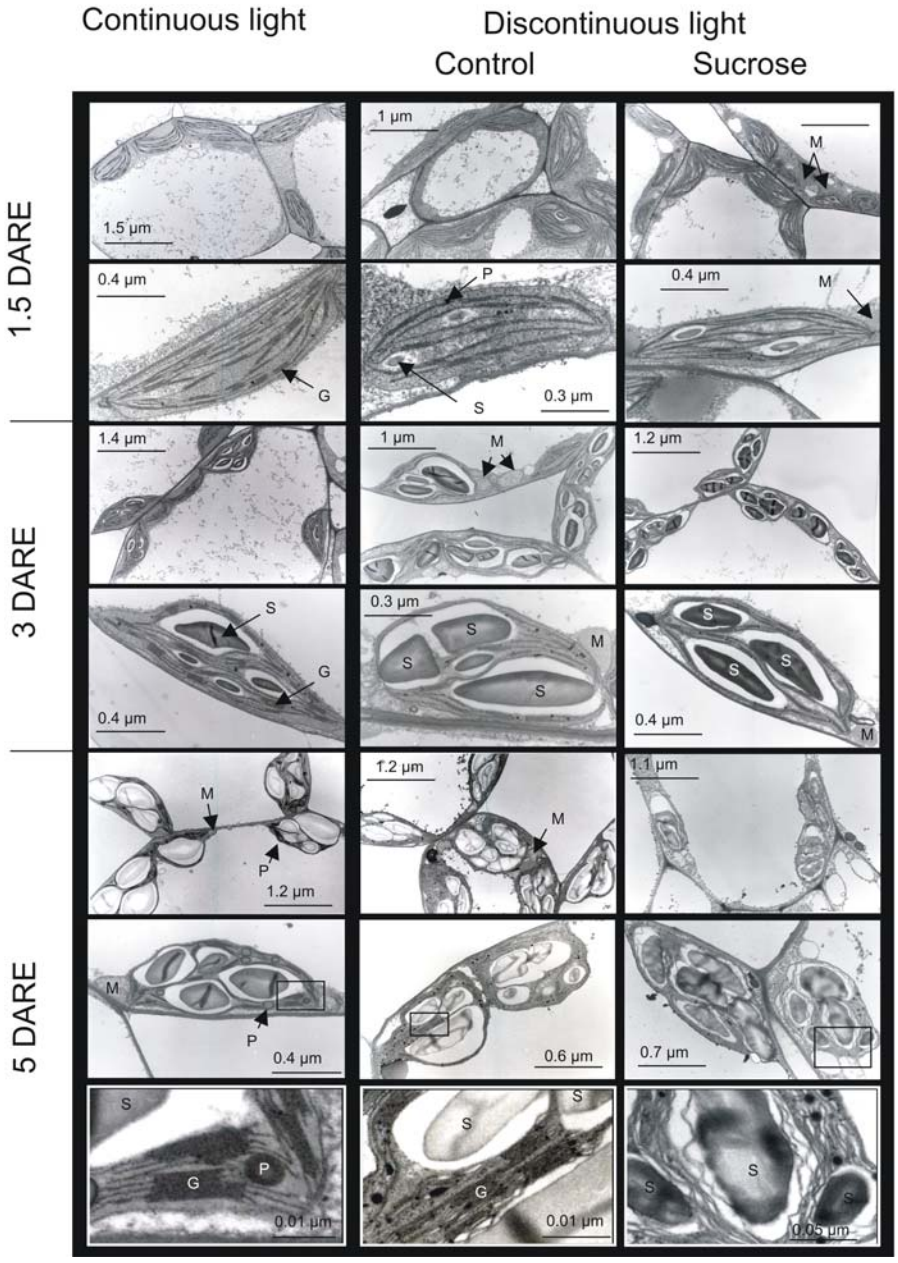

Fig. 5: **Chloroplast ultrastructure.** 1.5, 3 and 5 DARE in seedlings grown in continuous light or in 14 h day / 10 h night-cycles in the presence and absence of sucrose, respectively. G: Grana stacks; M: mitochondrion; P: plastoglobulus; s: starch granule.

During further development cotyledons become sink organs for carbohydrate storage. To compare the chloroplasts under conditions where excess starch

accumulates, the chloroplast ultra-structure was compared in leaf material harvested in the morning of the third and fifth DARE. Under all three conditions analyzed, excess starch was observed. The number of starch granules per chloroplast was similar. However, in continuous light at 3 DARE the starch granules were smaller. In discontinuous light, irrespective of the sucrose availability, the chloroplasts contained starch granules of similar size. In addition, similar numbers of stroma and grana thylakoids were observed. However, the grana stacks were slightly thinner by 1 - 2 layers in the presence of sucrose. Similar to 1.5 DARE strongest grana stacking was observed in continuous light.

At 5 DARE the size of the starch granules was increased in all samples. In contrast to 3 DARE, the form of the starch granules even determined the shape of the plastids. In the chloroplast stroma, in continuous light round shaped membrane structures were formed regularly demonstrating thylakoid reorganisation. In discontinuous light in the absence of sucrose grana stacks with 8 - 10 thylakoid layers were observed and first signs of thylakoid reorganisation and degradation was shown, while the presence of sucrose impaired the regular structure of the thylakoid structures.

3.1.3 Chlorophyll accumulation in Arabidopsis seedlings

To monitor chloroplast development, 1.5 – 5 DARE accumulation of chlorophylls was quantified from the pheophytin contents in acidic extracts. In higher plants, chlorophyll biosynthesis is controlled by feed-back inhibition (Meskauskiene and Apel, 2002). Therefore, chlorophyll contents also indicate the extent of expression and assembly of chlorophyll binding proteins.

In continuous light, the chlorophyll content constantly increased with age from 115.1 ± 16.0 µg g^{-1} fw 1.5 DARE to 225.5 ± 18.5 µg g^{-1} fw 5 DARE. In seedlings grown in discontinuous light, the chlorophyll content was only 60.8 ± 8.9 µg g^{-1} fw 1.5 DARE (52.8 % of the content in continuous light) in absence of sucrose and 49.7 ± 7.5 µg g^{-1} fw in it presence. During further development in the absence of sucrose chlorophyll content doubled up to 2.5 DARE and was 4-fold higher than the initial value at 5 DARE (254.0 ± 31.0 µg g^{-1} fw). This increase exceeded slightly that of seedlings grown in continuous light from 3 DARE onwards (Fig

6). Chlorophyll accumulation within the first 2.5 DARE under external application of 1 % (w/v) sucrose was similar to control seedlings (Fig. 6). However, later on chlorophyll accumulation was hardly affected and was adjusted to contents around 120 µg g^{-1} fw.

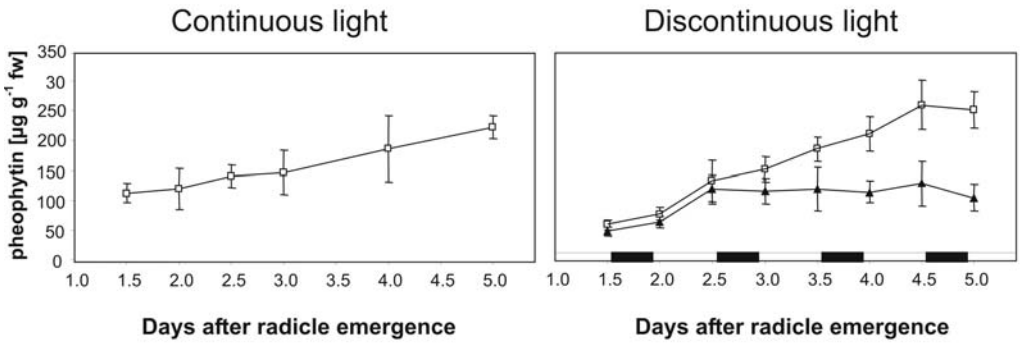

Fig. 6: **Chlorophyll contents during seedling development.** Chlorophyll equivalents per fresh weight in seedlings grown in continuous light and in 14 h day / 10 h night-cycles in the presence (▲) and absence (□) of 1 % sucrose, respectively, as measured by quantifying pheophytin in acidic extracts. This method was employed in order to use the extracts for metabolite analysis in parallel. The plant material was harvested 1 h after on-set of light and 1 h before on-set of darkness. (mean ± SD; n = 6)

3.1.4 Photosynthetic performance of seedlings

Establishment of the photosynthetic apparatus during the first days of seedling development and the efficiency of photosynthetic electron transport were monitored by chlorophyll-a fluorescence of PSII between 1.5 – 5 DARE and the quantum yield (photosynthetic efficiency) of PSII (ΦPSII), photosynthetic quenching (qP) and non-photosynthetic quenching (NPQ) were determined (Fig 7).

Chlorophyll-a fluorescence provides information on photosynthetic electron transport and its response to environmental stress (Schreiber, 1983; Schreiber et al., 1986, Baker, 1991). At room temperature, the fluorescence yield from chlorophylls associated with PSII is much greater than that emanating from PSI chlorophylls and can be determined more specifically (Schreiber, 1983).

The PSII maximum efficiency ($\Phi PSII_{max}$) of dark-adapted material demonstrates the efficiency with which light absorbed by the pigment matrix associated with

PSII is used to drive stable photochemistry when all PSII centres are in an open state. The determination is based on the fact that the first saturating pulse after the dark adaption closes all PSII centres giving rise to maximum fluorescence yield.
Much of the non-photochemical quenching is linked to the formation of the trans-thylakoid pH gradient and acidification of the thylakoid lumen (Schreiber, 1983; Schreiber et al., 1986). While photochemical quenching is caused by charge separation at PSII reaction centres, non-photochemical quenching is due to a set of non-radiative de-excitation processes in PSII (Maxwell and Johnson, 2000).
In dark-adapted plant material grown in continuous light, beginning 2 DARE the initial quantum yield ($\Phi PSII_{max}$) varied between 0.659 and 0.704 (Fig 7 and 8). A maximum was reached 3 DARE (0.704 ± 0.017). A significantly lower $\Phi PSII_{max}$ of 0.413 ± 0.165 was observed 1 DARE indicating that at this early state of seedling development the photosynthetic complexes were less efficiently arranged (Fig 8). One day after germination, cotyledons were not completely expanded, while cotyledons of two day old seedlings were green and fully developed. From 2 DARE onwards light induction of PSII was similar to that in five day old seedlings. Upon the first 4.5 min of illumination, the steady state quantum yields ($\Phi PSII_{4.5\ min}$) reached values between 0.481 ± 0.106 and 0.577 ± 0.045 in 2 to 4 day old seedling corresponding to 73 – 82 % of $\Phi PSII_{max}$ observed after dark adaptation. In older seedlings, it decreased again and was around 0.522 at 7 DARE. In 1 day old seedlings, ΦPSII only slowly increased upon illumination. After 4.5 min it was 0.281 ± 0.188, which corresponded to only 68 % of the already very low $\Phi PSII_{max}$.
The steady state qP gradually increased up to 5 DARE from 0.805 ± 0.161 (1 DARE) to 0.992 ± 0.007 (5 DARE; Fig 8). NPQ was low at 1 DARE and constantly high later in development (Fig 7 and 8).
In discontinuous light, seedlings were analyzed for their chlorophyll-a fluorescence pattern up to 5 DARE. $\Phi PSII_{max}$ steadily increased between 1.5 and 5 DARE from 0.636 ± 0.015 to 0.754 ± 0.006 (Fig 8). No difference in $\Phi PSII_{max}$, as well as in $\Phi PSII_{4.5\ min}$, were observed between seedlings grown on 1 % sucrose or without extra carbohydrates, but in the $\Phi PSII_{2min}$, at the beginning of the steady

state. The steady state was reached slower in seedlings cultivated on exogenous sucrose.

While under constant light only the youngest seedlings (1 DARE) showed limitations in activation of qP and NPQ, in day/night-cycles, under control conditions steady state levels of qP ($qP_{4.5min}$; Fig 8) slightly decreased with age. qP of seedlings grown on sucrose presented a similar pattern to control seedlings after 4.5 min.

Independent of age and sucrose availability, NPQ was activated in all seedling populations tested and was increasing with their development. However, the initial induction of qP and NPQ in seedlings grown on sugar media determined 75 s after on-set of light was decreased compared to the control (Fig 8).

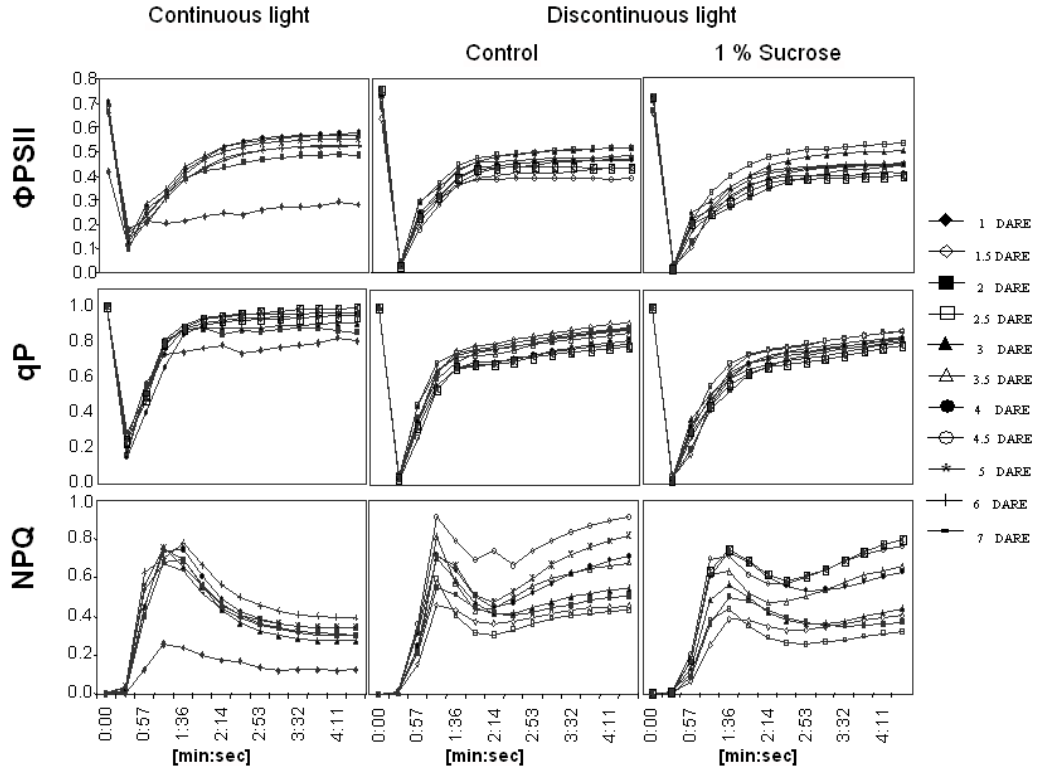

Fig. 7: **Induction curve of PS II measured by chlorophyll-a fluorescence.** Effective quantum yield of PSII (ΦPSII), photoquemical quench (qP) and non-photochemical quench (NPQ) after 30 min dark adaptation of seedlings cultivated at (A) constant light (190 µmol quanta m^{-2} s^{-1}, 22°C), and at a light regime of 14 h illumination (120 µmol quanta m^{-2} s^{-1}, 21°C) and 10 h darkness (B) control and (C) in presence of 1% sucrose. Seedlings cultivated on day/night conditions were harvested and 1 h (2, 3, 4 and 5 days after germination) and 13 h after onset of illumination (1.5, 2.5, 3.5 and 4.5 days after germination); (mean ± SD; n = 5).

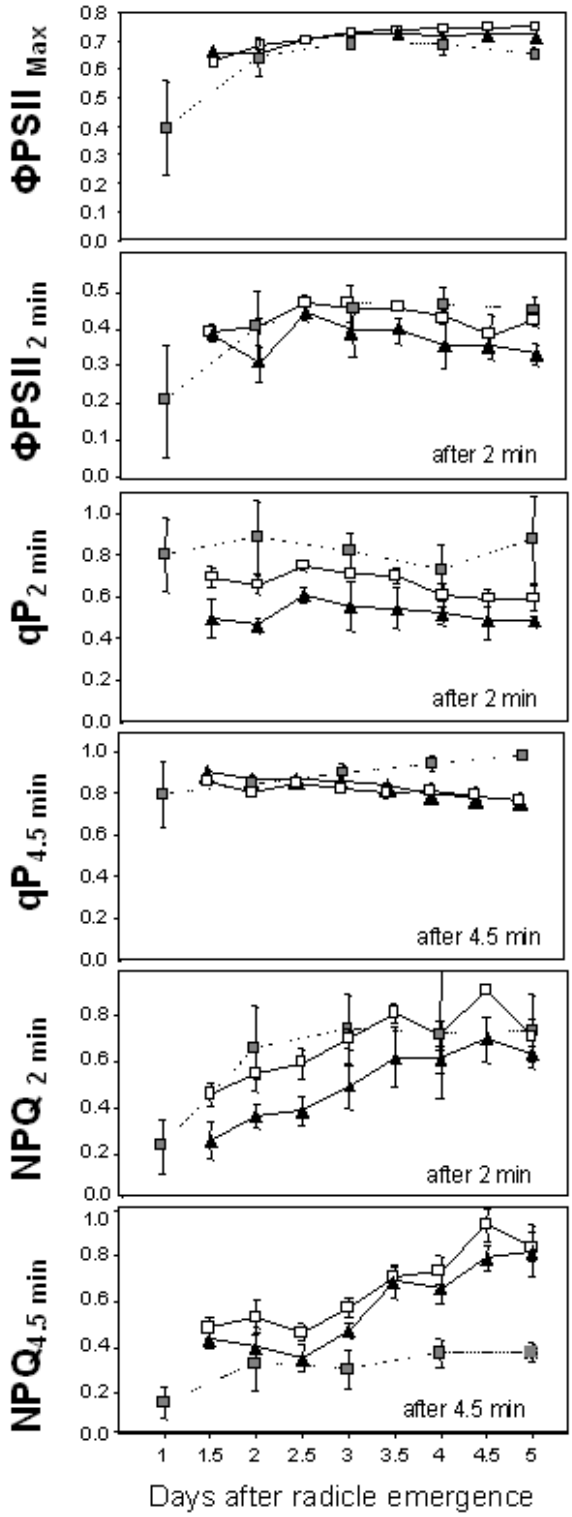

Fig. 8: **Age-dependent changes in photosynthetic parameters.** The quantum yield (ΦPSII) and the photochemical (qP) and non-photochemical quench (NPQ) observed 2 min or 4.5 min, respectively, after on-set of light (continuous light: dotted line; absence of sucrose: squares; 1 % sucrose: triangles); (mean ± SD; n = 6)

3.1.5 Presence of ROS in early stages of seedling and leaf development

Metabolic processes, from lipid mobilization to establishment of photoautotrophic seedlings are subjected to the production of ROS. In addition, expanding organs such as embryonic axes (Puntarulo et al., 1988), growing roots (Jon et al., 2001) and germinating seeds (Schopfer et al., 2001) generate ROS. ROS present in the apoplast are postulated to be implicated in leaf elongation and in promoting cell wall biosynthesis (Rodriguez et al., 2002). H_2O_2 is one type of ROS that is involved in many metabolic processes and participates also as secondary messenger regulating growth and development and in stress responses (Foyer et al., 1997). H_2O_2 and superoxide radical are generated mainly in the Mehler reaction and in photorespiration (Foyer and Noctor, 2000). H_2O_2 accumulation has to be antagonized by antioxidant mechanisms to prevent its conversion to hydroxyl radical via Fenton-type reactions. Hydroxyl radicals cause lipid peroxidation, protein degradation and modification and DNA damage such as strand breakage (Fridovich, 1986). Cellular imbalances in redox homeostasis, e.g. singlet oxygen formation by photoactivation of pigments during the assembly process of the photosynthetic membrane (Hideg et al., 2001; Hutin et al., 2003) or increased Mehler reaction activity due to insufficient coupling between photosynthetic electron transport and electron consuming chloroplast metabolism, can support ROS production ultimately leading to oxidative stress.

Differences in the formation of ROS, as $O_2^{\cdot-}$, can be visualized by NBT-staining. In the presence of superoxide ($O_2^{\cdot-}$) a deep blue formazane precipitate is formed (Maly et al., 1989). In 1.5 DARE seedlings incubated for 2 h in the dark prior to staining the $O_2^{\cdot-}$ was mainly detected in the roots (Fig 9G). In cotyledons only weakly stained patches were observed. In 12 h older seedlings (Fig 9 H), root staining was decreased and accumulation of superoxide anions were detected in the cotyledones, especially near the edges. From 3 DARE onwards, in the roots only in the central cylinder decreasing amounts of $O_2^{\cdot-}$ were detectable, while the staining in the hypocotyl and cotyledons further increased (Fig 9 I-L). In plants stained without dark adaption, stronger NBT staining was observed in the cotyledons of any age (Fig 9 A-F) with an homogeneous distribution across the cotyledon area. High amounts of $O_2^{\cdot-}$ in the central cylinder of the roots were still

detectable until 4 DARE (Fig 9 A-E) and dissapeared at 5 DARE (Fig 9 F). The hypocotyl was stronger stained compared to seedlings with dark adaption. The difference between light and dark treated seedlings was strongest between 1.5 and 3 DARE suggesting a strong impact of light-dependent metabolism on the generation of ROS by photosynthetic activity. In two weeks, strong superoxid accumulation was observed in the primary leaves, but was only detectable along the veins in the youngest leaves (Fig 9 M).

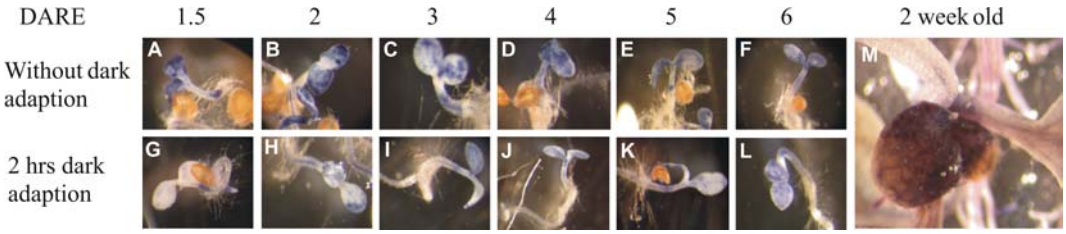

Fig. 9: **Staining of seedlings for superoxide anions.** The staining was performed with NBT with 1.5 day to 2 week old *Arabidopsis* seedlings grown in day/night-cycles in the absence of sucrose. The seedlings A-F were stained directly, while the seedlings G-L were stained after 2 h dark adaptation. M shows the developing primary leaves of a 2 week old plant. For the figure, seedlings with representative staining patterns were selected from at least 20 seedlings stained.

3.1.6 Content and Redox State of Ascorbate

Ascorbic acid is the most abundant low-molecular-weight hydrophilic antioxidant in plants, reacting directly with hydrogen peroxide, hydroxyl radicals, superoxide, and singlet oxygen (Buettner and Juerkiewicz, 1996) and is involved in the regeneration of α-tocopherol radicals (Padh, 1990). It is one of the most important buffers protecting against the high oxidative load that accompanies rapid metabolism (Noctor and Foyer, 1998). The concentration range found in leaves of different species varies from 2-20 µmol g^{-1} fw (Wildi and Lutz, 1996). The pool is highly reduced (>90%) in leaves under normal growth conditions (Law et al, 1983). Therefore, the redox state of the ascorbate pool is often used as indicator to monitor signs of oxidative stress.

During seedling development, the ascorbate availability was around 3 µmol g^{-1} fw and 4 µmol g^{-1} fw in seedlings grown on constant light and on light/dark periods, respectively (Fig 10). In continuous light total ascorbate remained relative

constant from 1.5 to 5 DARE (Fig 10 B), while in day/night cycles in absence of sucrose, it slightly decreased from 4.3 ± 1.3 to 3.7 ± 0.9 µmol g^{-1} fw between 1.5 and 4.5 DARE (Fig 10 D). In the presence of 1 % sucrose, a high variability of the ascorbate content was observed with levels oscillating between 4.4 and 3.7 µmol g^{-1} fw in the first 4.5 DARE. Seedlings at 5 DARE showed the lowest value of 2.9 µmol g^{-1} fw (Fig 10 D).

With the chlorophyll content constantly increasing throughout development (Fig 6) the ascorbate content per µg pheophytin (which reflects chlorophyll in acidic extracts) decreased along the days in all conditions studied (Fig 10 A-B). While the ascorbate content only slowly decreased between 1.5 DARE and 5 DARE in constant light from 25.2 ± 5.4 µmol mg^{-1} pheophytin to 12.7 ± 2.0 µmol mg^{-1} pheophytin (Fig 10 A), in discontinuous light the ascorbate contents dropped between 1.5 and 3 DARE from 73.6 ± 19.3 to 25.8 ± 4.7 µmol mg^{-1} pheophytin and 88.9 ± 28.3 to 41.7 ± 12.0 µmol mg^{-1} pheophytin in the absence and presence of sucrose (Fig 10 B). At that point, chlorophyll content duplicated the initial value in both cases. With further development the ascorbate content in the absence of sucrose decreased to levels similar to those of seedlings kept in continuous light (16.4 ± 5.4µmol mg^{-1} pheophytin). In the seedlings treated with 1 % sucrose the ascorbate content relative to pheophytin remained around 35 µmol/mg pheophytin up to 3 DARE, demonstrating that the ascorbate content is controlled independent from chlorophyll accumulation.

In continous light, the ascorbate pool was highly reduced (> 86 %) throughout seedling development (Fig 10 E). In diurnal light cycles in presence and absence of sucrose the redox state of ascorbate was severely affected and strongly oxidized, specially in the youngest seedlings (41 – 46 %; Fig 10 F). A constant increase was observed in control seedlings from 2 DARE onwards reaching a reduction state of 71 % 4.5 DARE. 5 DARE a slight decrease to 61.2 % was observed, which may reflect first signs of redox imbalances caused by cotyledon senescence.

In the presence of sucrose reduced ascorbate was slightly higher in the first 2.5 DARE compared to those in its abscence suggesting a positive influence of the presence of exogenous sugar on early development. Afterwards, the redox state

strongly decreased with a minimal value of 32 % at 3 DARE. The redox state was stabilized to aproximatively 55 % reduced in the following days and increased further till 5 DARE (78 %) indicating an inclination to balance metabolic imbalances.

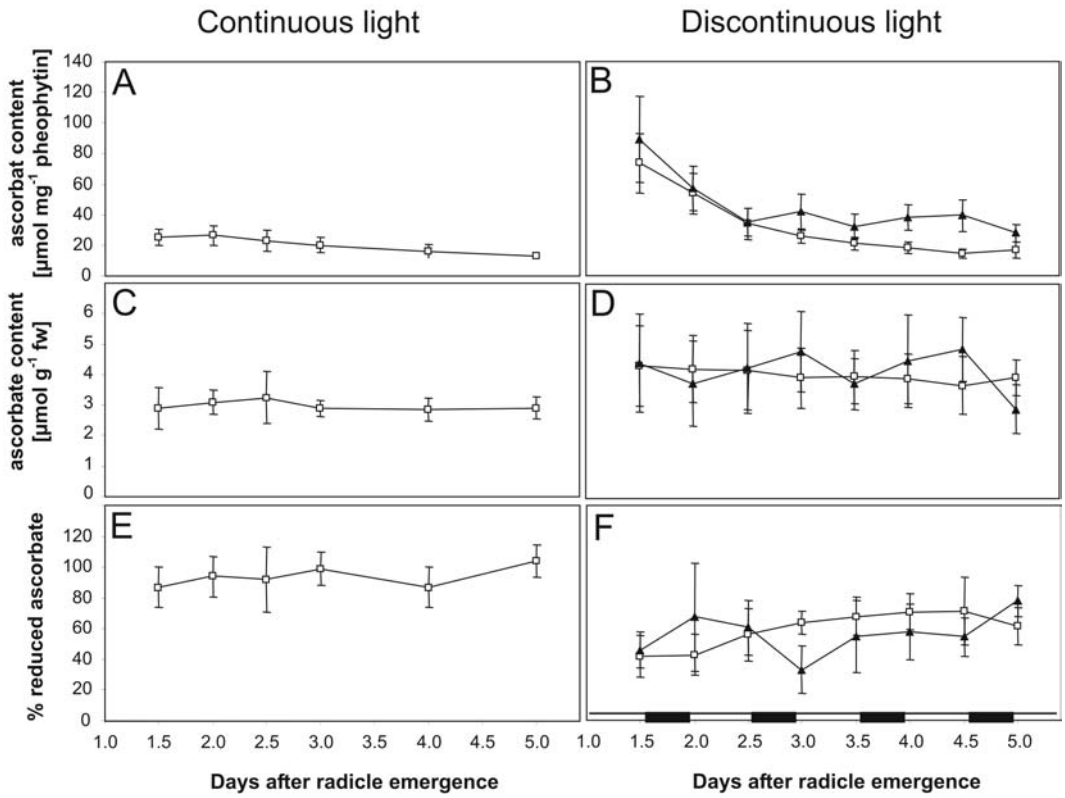

Fig. 10: **The ascorbate content and redox state.** 1.5 – 5 DARE seedlings grown in continuous and discontinuous light (14 h light / 10 h dark) in the absence (□) and presence (▲) of 1 % sucrose were extracted and used for determination of (A, B) the ascorbate content standardized on the pheophytin content, (C, D) the ascorbate content standardized on fresh weight and (E, F) the redox state of ascorbate (mean ± SD; n = 6). Non-overlapping SD-bars indicate statistical significance in Students T-test at $p < 0.05$.

3.1.7 Transcript abundance for antioxidant enzymes

The transcript regulation of antioxidative enzymes during the post-germinative growth of the seedlings was studied. Special attention was given to the Prx family. These enzymes have the property to reduce H_2O_2 and a wide range of alkyl hydroperoxides, including complex lipid peroxides (König et al., 2003; Rouhier et al., 2004). Prx are considered as candidates for antioxidant protection during the

transition from lipid mobilization to photoautotrophism in the seedlings and during the storage of oil reserves in the siliques. Prx induction was compared to the transcript regulation of enzymes participating in the Halliwell-Asada-cycle (water-water-cycle; Asada, 1999).

In actin-2 normalized cDNA samples, the transcript amounts of twelve antioxidant enzymes (Csd2, stromal and thylakoid-bound APx, MDHAR, 2Cys-PrxA and 2Cys-PrxB, type II Prx and PrxQ) were analyzed by semi-quantitative RT-PCR in seedlings cultivated in continuous light or in discontinuous light in presence and absence of sucrose. Transcripts of 2Cys-PrxA, 2Cys-PrxB, stromal and thylakoid-bound APx and Csd2 are routinely amplified with similar cycle numbers in 8 to10 days old seedlings and mature leaves (Baier et al., 2000). During early seedling development the cycle numbers for analysis of gene expression had to be increased for some genes. Stromal and thylakoid-bound APx so as MDHAR were amplified with 7-11 cycles more than those needed for actin, suggesting very low relative expression for these genes, while the transcript abundance of most peroxiredoxins and CuZn-superoxide dismutase Csd2 were higher.

In continuous light, the transcript level of mitochondrial PrxIIF, the two 2Cys-Prx , PrxQ, PrxIIE and thylakoid-bound APx were only slightly modulated between 1.5 and 5 DARE, while a transient increase (that turned stronger since 3 DARE) in the relative transcript abundance of stromal APx, MDHAR, Csd2, PrxIIB, PrxIIC and PrxIID were observed suggesting a major reorganization of gene expression (Fig 11).

In discontinuous light in absence of sugar, the transcript levels of all genes encoding chloroplast antioxidant enzymes followed the diurnal light pattern with higher transcript amounts in the evening than in the morning. Strongest expression was observed up to 2.5 DARE. In the third and fourth dark period after germination the transcript levels were maintained very low. Compared to the other genes for antioxidant enzymes, Csd2 showed generally only weak fluctuation during day and night periods suggesting lower light-dependent regulation (Fig 12).

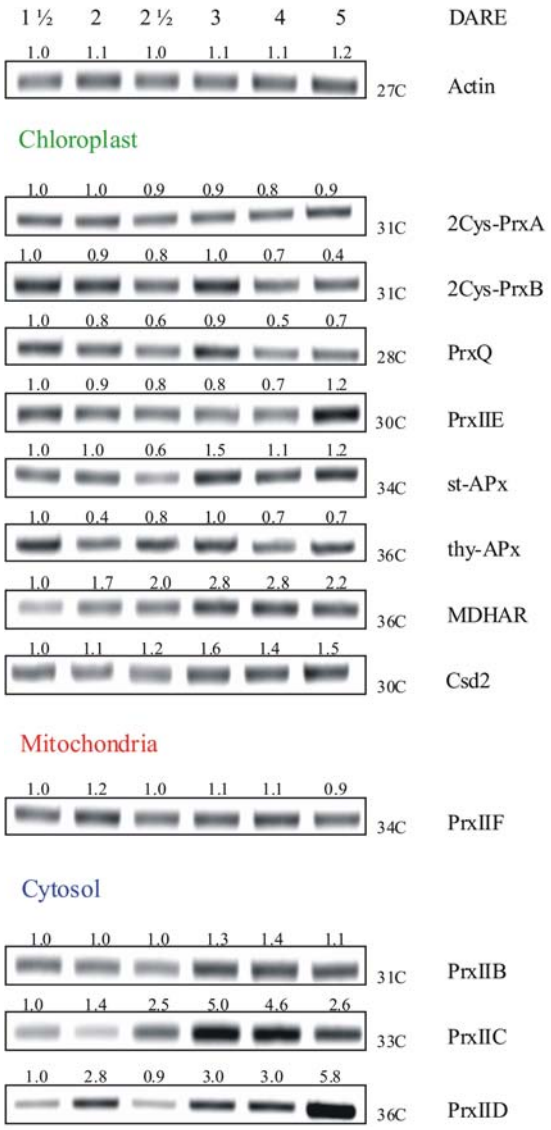

Fig. 11: Transcript levels of chloroplastic, cytosolic and mitochondrial antioxidative enzymes during early seedling development in continuous light. The transcripts were amplified from cDNA samples standardized on actin-2 transcript amounts and separated on agarose gels. For better reproducibility the grey scale of the fluorograms of the ethidium bromide stained gels was inverted. From three biological and at least two technical replicates the relative transcript abundances were calculated. The numbers above the lanes give the means relative to the signal intensity 1.5 DARE. The numbers on the right hand side give the gene-specific cycle numbers for transcript amplification.

In seedlings cultivated on 1 % sucrose, induction of sAPx during the day was lost, while for all other nuclear encoded antioxidant enzymes gene expression was similar compared to sugar free media. The transcript level of mitochondrial PrxIIF showed a similar pattern as most genes for chloroplast antioxidant enzymes. The

transcript levels were higher in the evening than in the morning. On 1% sucrose slightly higher transcript levels and a lower amplitude of regulation were observed than without additional sugars (Fig 12).

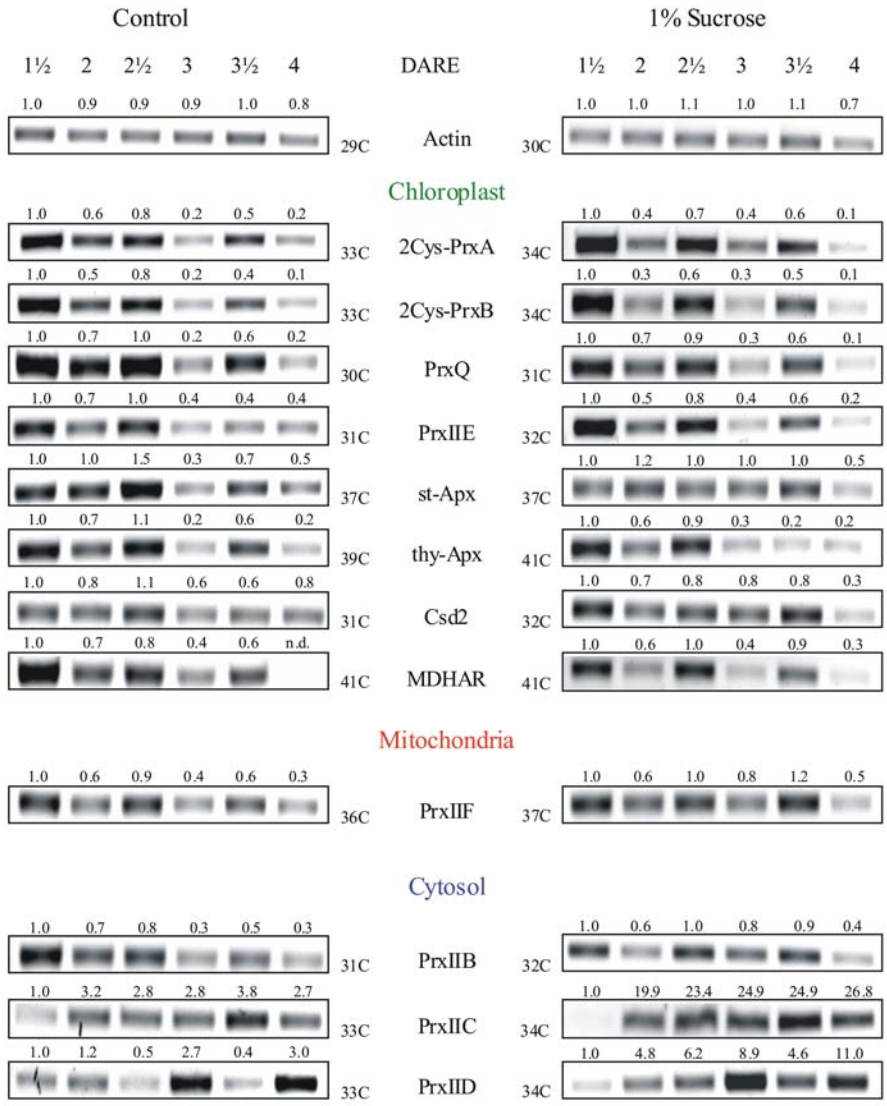

Fig. 12: **Transcript levels of chloroplastic, cytosolic and mitochondrial antioxidative enzymes during early seedling development in 14 h day / 10 h night-cycles in the presence and absence of 1 % sucrose**. The transcripts were amplified from cDNA samples standardized on actin-2 transcript amounts and separated on agarose gels. For better reproducibility the grey scale of the fluorograms of the ethidium bromide stained gels was inverted. From three biological and at least two technical replicates the relative transcript abundance was calculated. The numbers above the lanes give the means relative to the signal intensity 1.5 DARE.

In contrast to the wide coordination of gene expression observed for chloroplast antioxidant enzymes, the transcript abundance of the three analysed cytosolic

peroxiredoxins was individually regulated (Fig 12). PrxIIB transcript abundance followed the pattern of the organellar antioxidant enzymes. In contrast, for PrxIID onset of diurnal variation started only on the third day and then showed higher expression in the night than during the day. Without extra carbohydrates at 2 DARE the transcript level was similar to that observed at 1.5 DARE. An obvious diurnal modulation started the next day with a decrease of transcript abundance. On 1% sucrose, the transcript level accumulated up to the morning of the fourth day (3 DARE) and decreased for the first time during the forth day (3.5 DARE). Like in constant light, the transcript level of PrxIIC increased with age. In response to external sucrose, expression increased stronger during the second night (1.5 – 2 DARE).

3.1.8 Ascorbate peroxidase activity

To analyze how the fluctuations of APx transcript abundance are reflected in the enzyme activity, the activity was quantified in extracts from seedlings harvested 1.5 – 5 DARE (Fig. 13). In continuous light, APx activity increased up to 3 DARE stronger than the chlorophyll content and from 3 DARE onwards like the chlorophyll content (Fig 13 A and C). Except 2 DARE, when a strong induction of chloroplast proteins took place in the enlarging cotyledons, the specific activity was constant during development.

In discontinuous light, from 2 DARE onwards the specific activity of APx was higher in the evenings than in the mornings demonstrating that the activity pattern follows the diurnal variations in transcript abundance (Fig 13 B and D). Also consistent with transcript abundance regulation, the specific activity was low at 1.5 DARE. Relative to the chlorophyll content, which strongly increased between 1.5 to 5 DARE (Fig 13 D), high variability in the Apx : chlorophyll ratio was observed. However, the trend line linking the means of the determined values indicates a relative increase in Apx activity during the days and decrease in the nights from 2.5 DARE onwards.

In presence of sucrose, the specific activity followed the pattern observed in continuous light with the absolute activities 1.5-fold higher at 2.5 – 3 DARE. After 3 DARE, in contrast to continuous light, the specific APx activity slightly

increased to about 2-fold that of seedlings grown in continuous light. In contrast to seedlings grown in the absence of sucrose hardly any diurnal fluctuations in the specific APx activity were observed. However, related to the chlorophyll content, which was maintained at a constant level in the presence of sucrose, 3.5 and 4.5 DARE higher Apx activities were observed at the end of the light periods than in the morning hours (3, 4 and 5 DARE) demonstrating that the Apx activity fluctuates irrespective of the stagnation in chlorophyll accumulation.

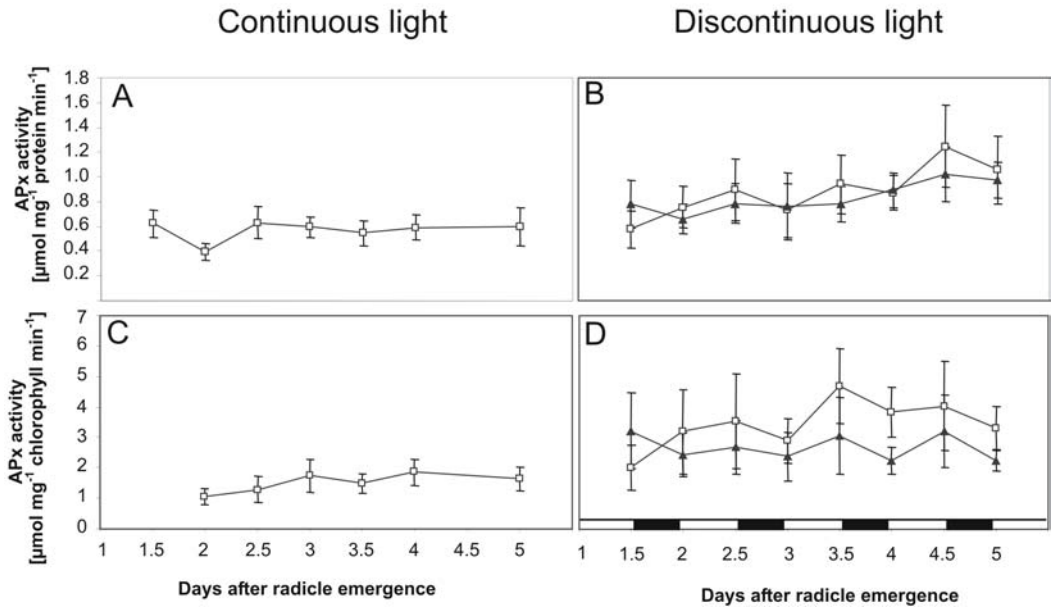

Fig. 13: **Activity of ascorbate peroxidase** (APx). 1.5 – 5 DARE in continuous light and in 14 h day / 10 h night cycles in the presence (▲) and absence (□) of sucrose standardized on protein (A) and chlorophyll content (B), respectively. (mean ± SD; n = 6). Non-overlapping SD-bars indicate statistical significance in Students T-test at $p < 0.05$.

3.1.9 Protein amounts of peroxiredoxin

To analyze the impact of gene expression regulation on the antioxidant potential, in addition to RT-PCR analysis, the protein levels of the peroxiredoxins were quantified by Western-Blot analysis (Fig 14). In constant light, in samples standardized on the same protein amounts, the protein levels of PrxIIF, PrxQ and the type II Prx were detected with antibodies generated with heterologously expressed protein from *Arabidopsis thaliana*, while the 2Cys-Prx was detected with the antibody generated with heterologously expressed protein from *Hordeum*

vulgare. Type II Prx detected with the antibody against PrxIIC recognize PrxIIB, PrxIIC, PrxIID and with significantly less affinity PrxIIE. The protein amounts of all these Prx were unchanged between 1.5 and 5 DARE.

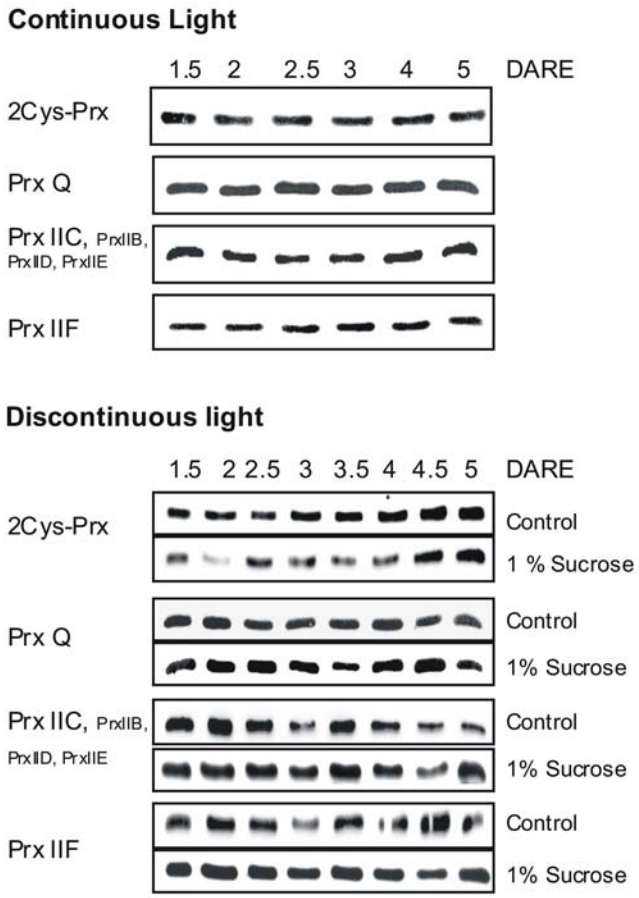

Fig. 14: **Peroxiredoxin protein amounts during seedling development**. Seedlings were grown in continuous and discontinuous light with 14 h day / 10 h night-cycles. Representative Western Blots from 3 parallel experiments are shown. Equal protein amounts were loaded in each lane.

In seedlings grown in day/night cycles, 2Cys-Prx protein amounts increased with time showing a similar diurnal pattern as on the transcript level. In the evenings of the third, forth and fifth day higher protein amounts were detected than in the morning. Day/night fluctuations are no more appreciable in the protein expression of Prx Q. In older seedlings a trend towards stronger accumulation of PrxIIF in the morning was observed, while the PrxIIC protein amounts slightly decreased after 3.5 DARE.

Application of sugar resulted in higher accumulation of 2Cys-Prx during the light phase. While the morning protein amounts still steadily increased with age,

especially at 1.5 and 2.5 DARE in the evening higher protein amounts were observed. Compared to growth in the absence of sugar, for the type II Prx diurnal variations were almost lost. PrxQ had the same behaviour in presence and absence of sugar.

3.1.10 Histochemical localisation of GUS::2Cys-PrxA promoter activity

Histochemical localisation of GUS::2Cys-PrxA promoter activity in seedlings of *Arabidopsis thaliana* grown under constant light between 1 to 6 DARE was performed according to Jefferson et al. (1987). Upon 1 DARE seedlings presented strong activity in the cotyledons and central cylinder, supporting the RT-PCR and protein expression data (Fig. 15). Stronger staining was shown at the edges of the cotyledons in 1 and 2 day old seedlings ressembling the distribution of $O_2^{\cdot -}$ shown by NBT-staining of seedlings (Fig. 9). Upon 3 DARE hypocotyl elongation was accompanied by high 2Cys-PrxA promoter activity. After 3 DARE the activity in the central cylinder decreased. With root elongation 2Cys-PrxA activity disappeared in this tissue. The apical meristem region was free from staining in all developmental stages analyzed.

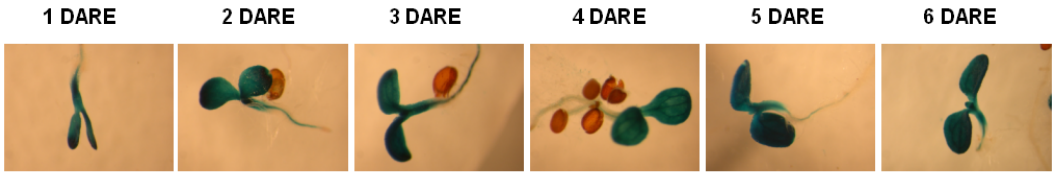

Fig. 15: **Histochemical localisation of GUS::2Cys-PrxA promotor activity in seedlings from *Arabidopsis thaliana* between 1 to 6 DARE.** Seedlings were grown in continuous light. Control tissue without reporter gene does not show any blue staining under these conditions.

3.2 Silique and seed development in the model plant *Arabidopsis thaliana*

Studies in developing seeds of several oilseeds plants (Asokanthan et al., 1997; King et al., 1998; Borisjuk et al., 2005; da Sylva et al., 1997) have indicated that photosynthetic activity and accumulation of storage reserves overlap during early seed growth. Lipid biosynthesis is known to be strongly energy-dependent and

stimulated by light (Neuhaus and Emes, 2000; Rawsthorn, 2002). Leaves represent the principal photosynthetically organ in plants, but at the initiation of reproductive growth, a rapid increase in flower-bearing branches from the shoot apical meristem takes place. The photosynthetic leaf area declines then quickly because of senescence (Pechan and Morgan, 1985), thereby an important source of assimilates is lost at a time when seeds have a great import demand. At this time, only the seeds developing first have started to accumulate storage products. In the absence of leaves, photosynthesis in the silique wall is the main source of assimilates. It may contribute up to 50 to 60 % of final plant dry weight (Lewis and Thurling, 1994). Previous reports indicate that the silique wall on chlorophyll basis has a photosynthetic capacity similar to or greater than that of the leaf, but a 75 to 80 % lower chlorophyll content reduces photosynthesis per unit area (King et al., 1998). On the other hand, significant photosynthetic electron transport takes place in embryos of oilseed plants, although the photoassimilation of CO_2 is very low in comparison to leaves (Asokanthan et al., 1997).

In the present work, the expression of the Prx was studied during silique development in *Arabidopsis thaliana*. A comparison was carried out with the transcript level regulation of enzymes forming the Halliwell-Asada-cycle in chloroplasts.

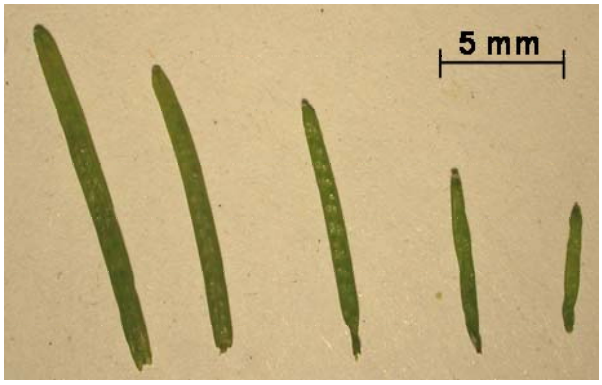

Fig. 16: Siliques of five developmental states from *Arabidopsis thaliana* were used for the experiments.

Green siliques from five developmental stages in *Arabidopsis thaliana* (Fig 16) were collected for the quantification on transcript and/or protein levels relative to

photosynthetic activity. The siliques corresponded to stages between early embryogenesis and close to the end of the maturity stage (Fig 2) and were standardized by size. With the same criteria, siliques were chosen for photosynthetic parameters measurements *in planta*.

3.2.1 Photosynthetical performance of siliques

The efficiency of photosynthetic electron transport in siliques of *Arabidopsis thaliana* was monitored by chlorophyll-a fluorescence of PSII in the five developmental stages (S1 - S5; Fig 17). Induction of PSII-fluorescence was analyzed by dark adapted plants determining the quantum yield (photosynthetical efficiency) of PSII (ΦPSII), photosynthetical quenching (qP) and non-photosynthetical quenching (NPQ).

The induction curves of the five siliques ages showed similar patterns with $\Phi PSII_{max}$ between 0.781 ± 0.020 and 0.797 ± 0.027. Activation of the ΦPSII steady state (with values between 0.534 ± 0.042 and 0.594 ± 0.030) was reached with the third illumination pulse (1.11 min). After 5 min of illumination the steady state quantum yields reached values between 0.628 ± 0.041 and 0.565 ± 0.039 in S2 to S5 corresponding to 80 % and 71 % of $\Phi PSII_{max}$ observed after dark adaptation, respectively.

The qP curves representing the reduction state of the plastoquinone pool showed also high similarity in the siliques at different developmental stages. Transiently qP-values between 0.609 ± 0.107 (S1) and 0.465 ± 0.072 (S5) were reached at 1.11 min. After 5 min of illumination qP values fluctuated between 0.840 ± 0.073 (S1) and 0.778 ± 0.039 (S5) with a maximum of 0.879 ± 0.064 at S2.

NPQ remained low in all developmental stages with high variability (0.459 ± 0.265 for S1 and 0.319 ± 0.105 for S5 after 5 min of illumination). The youngest siliques showed slightly higher values than the subsequent stages, which showed similar patterns. Significant differences were not detected.

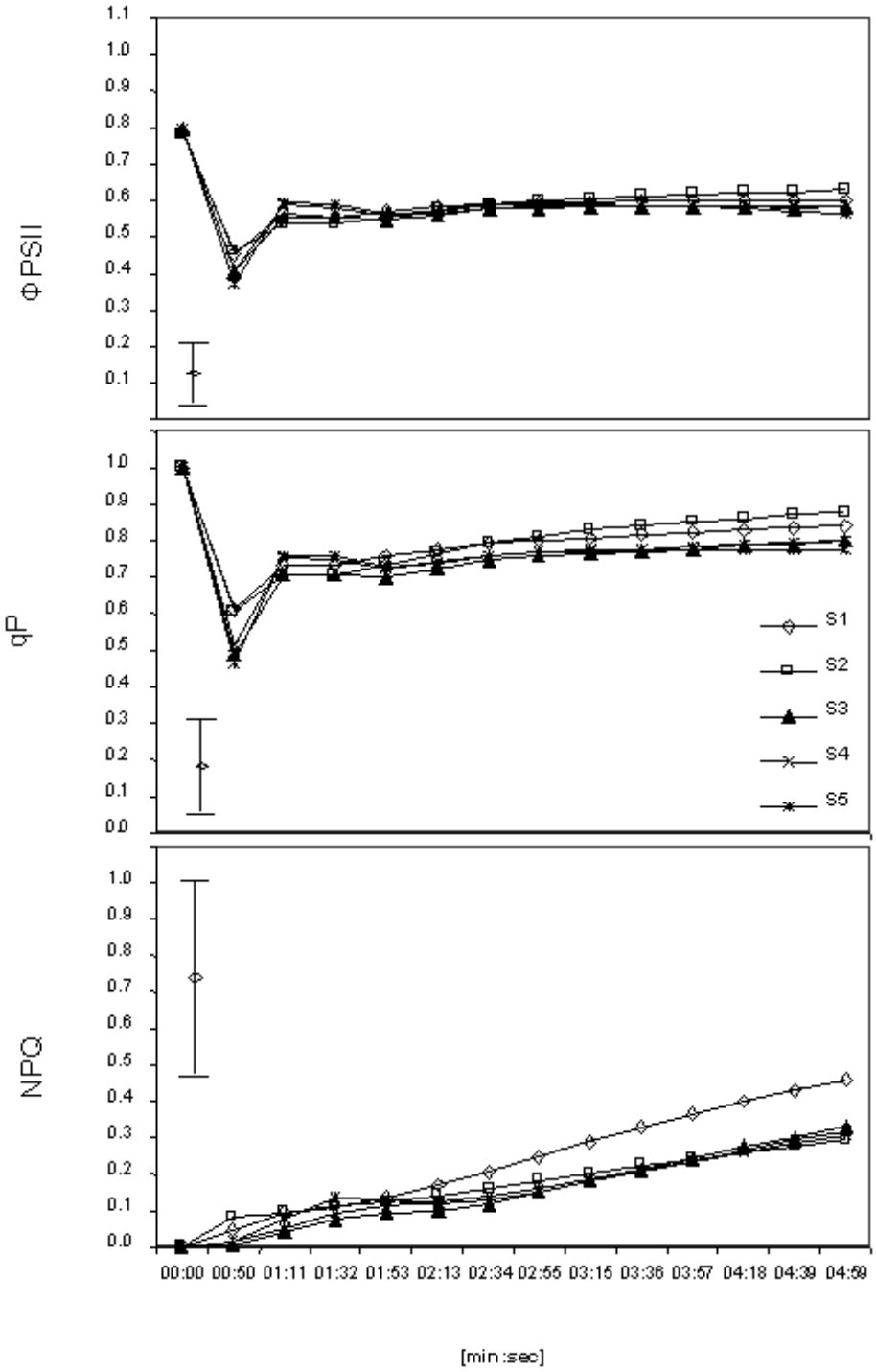

Fig. 17: **Induction curves of PSII** measured by chlorophyll-a fluorescence after 30 min dark adaptation of the plants in five developmental stages of siliques (as defined in see Fig.....). (A) Effective quantum yield of PSII (ΦPSII), (B) photochemical quench (qP) and (C) non-photochemical quench (NPQ); (mean ± SD; n = 15). The inset on the left hand side shows the maximal calculated SD.

3.2.2 Chlorophyll fluorescence images of seeds

Chlorophyll accumulation in Arabidopsis embryos and in the seed coat was visualized by fluorescence microcopy at an excitation wavelength of 450-500 nm (Fig 18 A, D, G, J). For higher detailed resolution and sensitivity the fluorescence emission spectrum was analyzed across sequential thin sections of the siliques using confocal microscopy (Fig 18 B-C, E-F, H-I, K-L).

Seeds from the S1 stage corresponded to an early embryo morphogenic stage (Fig 18 A-C). Chlorophyll accumulated in the seed coat (Fig 18 A; Fig 19 B). Spectral resolution of the emitted light demonstrated that in the seed coat chlorophyll fluorescence was overlaid by an emission in the green-yellow (small peak by 550 nm) region of the spectrum (Fig 18 B). Beeckman et al. (2000) reported the presence of a pigment from lipid nature in the epidermis of the inner integument during early embryogenesis that dissapeared from the late torpedo stage onwards. A relation between this observation and the short wavelenght maximum detected in the seed coat from S1 could be supposed. At S1 stage, the embryo was not detected by fluorescence microscopy. At that developmental stage the inner integuments had a curved shape (Beeckman et al., 2000). The cells, tentatively identified to belong to the inner integuments were highly vacuolized and contained green fluorescent material (Fig 18 C).

S3 siliques showed embryos in the torpedo stage (Fig 18 D-F). High chlorophyll emission in both embryo and seed coat was observed, suggesting photosynthetic activity of both tissues at that point (Fig 18 D; Fig 19 A-B). The seed coat started to present small dome-like structures that were clearly identified in S5 seed coats (green round shaped structures in Fig 18 K). The appearance of starch accumulation regions with the shape of pyramidal small domes in the torpedo stage indicates the onset of mucilage formation in the epidermis (Beeckman et al., 2000). S5 seeds presented embryos in the bent cotyledonary stage (Fig 18 G-I). In the seeds, only the embryo showed strong chlorophyll fluorescence. In the seed coats, emission was only observed in the green-yellow region (Fig 18 J-L), indicating that chlorophyll was already degraded.

In the embryo chlorophyll accumulated in shoot and cotyledons (yellow in Fig 18 E and H by the overlay of red chlorophyll-fluorescence and green cell wall

fluorescence), but not in the axial and radicle meristems, where plastids mainly exist as proplastids. Consistent with the photosynthetical performance in the siliques described in Section 3.2.1, the chlorophyll fluorescence emission was constant in the fluorogrammes of the silique wall during all stages of development (S1-S5; Fig 19 C).

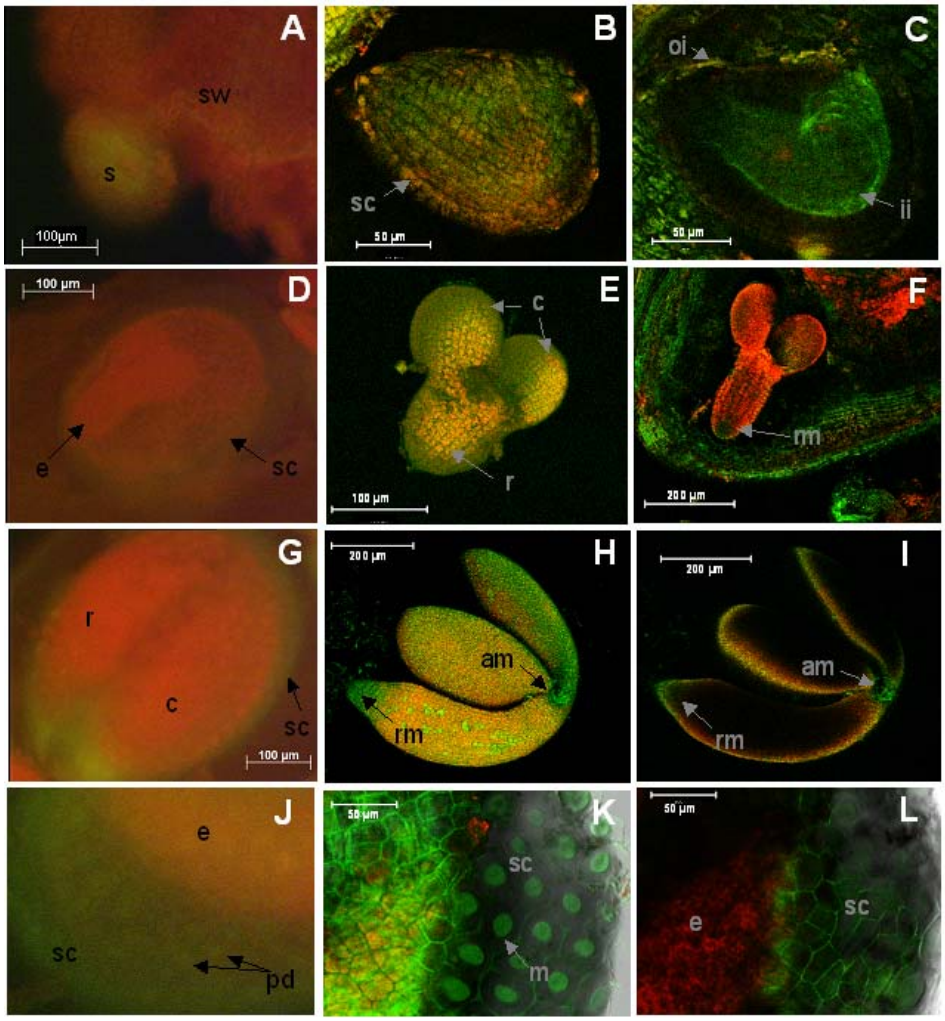

Fig. 18: **Developmental changes in chlorophyll emission in embryos and seed coats** of S1 (A-C), S3 (D-F) and S5 (G-L) stages. The red signal in the fluorometric images displays the chlorophyll fluorescence. A, D, G and J were obtained by fluorescence microscopy. B, E and H are calculated projections of image stacks obtained by confocal microscopy. C, F, I, K and L are single optical sections obtained by confocal microscopy. Figures A to C show seeds in an early embryogenic stage; D to F correspond to embryos at the torpedo stage; G-I show embryos and J-L, seed coats of a bent-cotyledonary stage. In the projection (E and H) the red chlorophyll fluorescence is overlaid by a strong green fluorescence of the outer cell layer. S: seed; sw: silique wall; sc: seed coat; oi: outer integument; ii: inner integument; e: embryo; r: root; rm; root meristem; am; axial meristem; pd: pyramidal domes; m: mucilage.

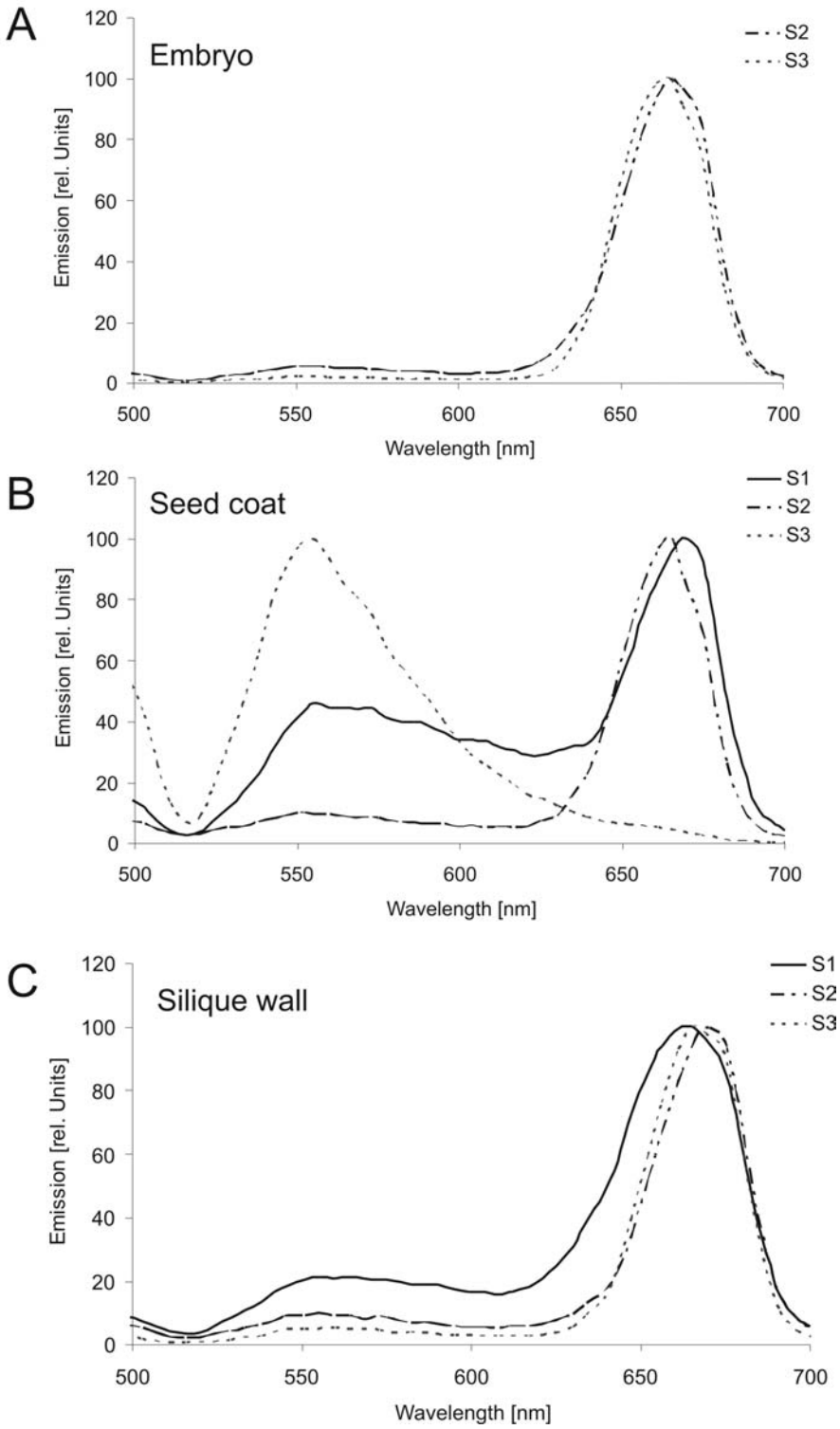

Fig. 19: **Quantification of the fluorescence** from (A) embryo, (B) seed coat and (C) silique wall of siliques from the S1, S3 and S5 developmental stages. As absolute fluorescence emission intensities cannot be compared between the specimen, the maximum intensities were normalized to 100% in each case.

3.2.3 Transcript abundance for antioxidant enzymes

In actin-2 normalized cDNA samples, the transcript amounts of twelve antioxidant enzymes (Csd2, stromal and thylakoid-bounded APx, MDHAR, 2Cys-PrxA and 2Cys-PrxB, the type II Prx and PrxQ) and one enzyme related to lipid metabolism (ICL) were analyzed by semi-quantitative RT-PCR in siliques in relation to silique development and compared with the transcripts in mature leaves (Fig 20).

The transcript amounts of all chloroplastic Prx as of Csd2 and MDHAR showed very strong signals in young siliques. The signal intensity was higher than in mature leaves for most of the genes. The expression intensity decreased with silique development. In older siliques, the transcript levels of most chloroplastic enzymes decreased to values between 60 % and 30 % of the levels in the youngest siliques. In contrast, the transcript levels of PrxIIE showed a slight decrease between S1 and S3, but a strong increase in the old pods, indicating a protection role in the mature seeds independent on photosynthesis. Both APx, stromal and thylakoid-bound APx, showed also high transcript levels in young siliques. It was constantly high until the middle stage (S3) and decreased abruptly in the oldest stage (S5). However, the signals of both APx remained slightly higher in mature leaves than in the pods (Fig 20).

The mitochondrial PrxIIF was constitutively expressed in mature leaves and siliques irrespective of age (Fig 20). The cytosolic Prx were independently regulated indicating specific roles according to different metabolic situations. PrxIIB showed a pattern similar to PrxIIF, while the PrxIIC transcript levels followed the pattern of the chloroplastic enzymes with high levels in young siliques and decreasing contents with development. Generally, the transcripts levels in actin standardized samples were higher than in leaves. PrxIID remained relative constant in all stages and around 50% higher than in leaves.

ICL was used as marker for lipid metabolism. As expected, ICL transcript was only detected in the oldest siliques correlating with the activation of the glyoxylate cycle at the end of the maturation stage (Fig 20; Chia et al., 2005).

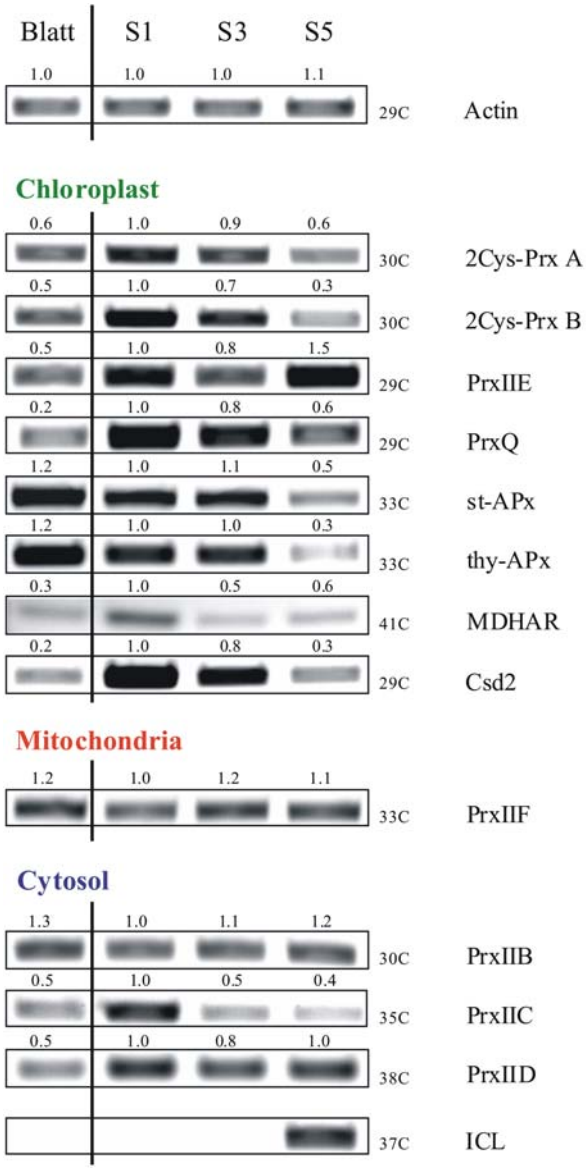

Fig. 20: **Transcript levels of one marker enzyme for lipid metabolism and 12 chloroplastic, cytosolic and mitochondrial antioxidative enzymes during silique development.** The transcripts were amplified from cDNA samples standardized on actin-2 transcript amounts and separated on agarose gels. For better representation the grey scale of the fluorograms of the ethidium bromide stained gels was inverted. From three biological and at least two technical replicates the relative transcript abundance was calculated. The numbers above the lanes give the mean values relative to the signal intensity S1.

3.2.4 Histochemical localisation of GUS::2Cys-PrxA promotor activity

GUS::2Cys-PrxA promotor activity was shown in all green tissues in adult Arabidopsis plants (Fig 21). In leaves, strong staining on the whole area was

shown with exception of trichomes. This staining decreased dramatically in senescent leaves indicating lose of activity with the destruction of the photosynthetic machinery. Intense activity on the upper part of the sepals was shown in close flowers. With opening of the sepals the intensity decrease. A slight staining was appreciated in petals and filaments, but high activity was observed in the upper part of the ovary directly under the stigma. In siliques, the activity decreased with development and was only possible to observe in the carpels. The seed coat was free from stain in all seed stages. No activity was detected in the embryo.

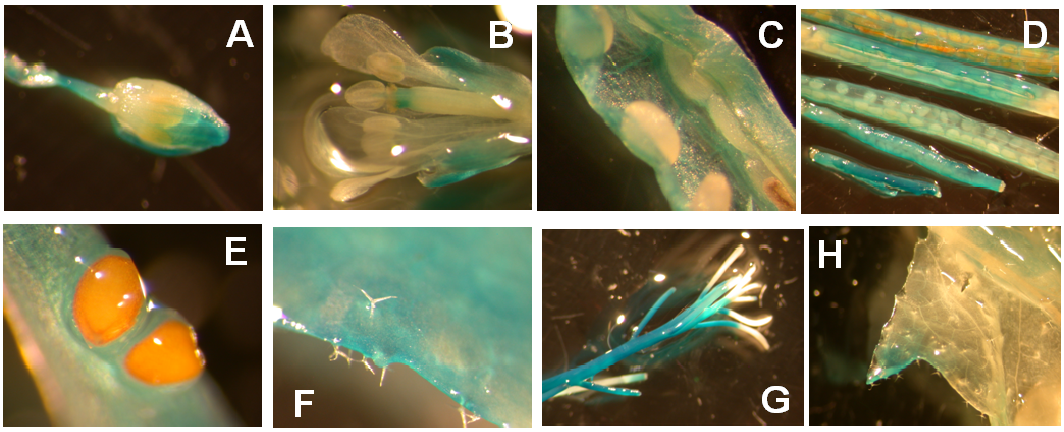

Fig. 21: **Histochemical localisation of GUS::2Cys-PrxA promoter activity in different tissues from *Arabidopsis thaliana*.** (A) Closed flower; (B) open flower; (C) silique from the S3 developmental state; (D) five developmental states of siliques; (E) silique from the S5 developmental state; (F) mature leaf; (G) shoot; (H) senescent leaf. Similar staining patterns were seen in 6 experiments.

3.2.5 Protein amounts of peroxiredoxin in *Arabidopsis thaliana*

The protein expression of the peroxiredoxins in different tissues of *Arabidopsis thaliana* was analyzed immunochemically by Western-Blotting. These analysis enabled the study of the tissue dependent accumulation of various peroxiredoxins. Here, antibodies against *Hv*Bas1, *At*PrxIIC, *At*PrxIIF and *At*PrxQ were used.

Chloroplastic peroxiredoxins like 2Cys-Prx and PrxQ were detected in all green tissues and in flowers, but not in roots (Fig 22). Nevertheless, low levels of 2Cys-Prx were reported in roots (Finkemeier et al., 2005). According to that,

histochemical localisation in *At*2Cys-Prx::GUS reporter plants showed promoter activity in the central cylinder of the roots and the shoots in seedlings.

The chloroplastic peroxiredoxins 2Cys-Prx and PrxQ showed a similar pattern of protein distribution (Fig 22). Highest amounts were observed in leaves. In siliques the protein abundance decreased progressively with development as the transcript levels. With progressing development, seeds gained weight and accumulated proteins. The ratio seed / silique increased. This suggests a correlation between the decrease of 2Cys-Prx and PrxQ protein amounts with silique age and the increment of the ratio seed / silique wall.

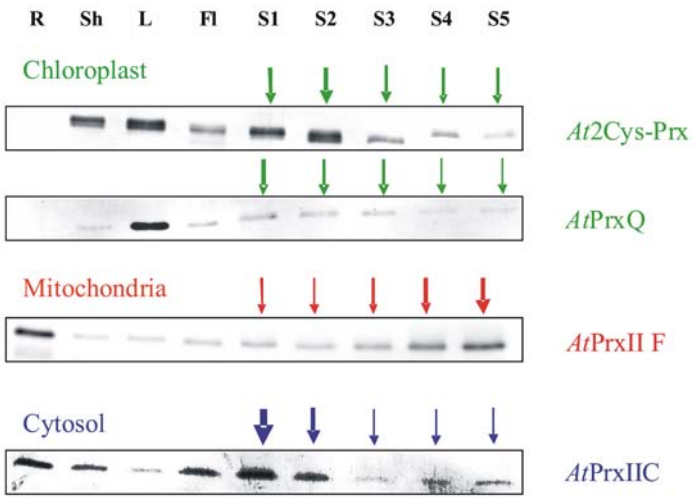

Fig. 22: **Peroxiredoxin protein amounts in different tissues from *Arabidopsis thaliana***. R: root; Sh: shoot; L: leaf; Fl: flower; S1: youngest siliques to S5: oldest siliques. The figure shows representative Western Blots from 3 parallel experiments. Equal protein amounts were loaded in each lane.

Type II Prx recognized by the antibody against the cytosolic PrxIIC, namely PrxIIB, PrxIID and PrxIIE, showed high expression especially in roots, flowers and young siliques (S1 and S2), but only low protein levels in leaves (Fig 22; Horling et al., 2002; Brehélin et al., 2003). The protein amounts decreased strongly at S3 and remained low until S5, which corresponds with the transcript pattern for PrxIIC (Fig 20). However, the transcript levels of the other type II Prx remained constant (PrxIIB and PrxIID) or increased with development (PrxIIE; Fig 20). Thus, a smaller contribution of the latter enzymes to the protein amounts detected by the antibody against *At*PrxIIC can be supposed.

The mitochondrial PrxIIF was present in all samples with high levels in roots (Finkemeyer et al., 2005) and in old siliques (Fig 22). Contrary to the other peroxiredoxins, the protein amounts increased gradually with silique development, suggesting a correlation with increasing respiration rates during maturation.

3.3 The peroxiredoxin family in *Brassica napus*

Another aim of this study was to collect information about the peroxiredoxin gene family in *Brassica* species. *Brassica*-related species are of great agricultural importance. The improvement of crop quality and harvesting yields of Brassica species since long is the interest of plant breeders and for the biotechnology industry. A better understanding of the mechanisms related to prevent oxidative stress is expected to ease the conventional or genetic manipulation of the cultivars. The studies carried out in the present work in the model plant *Arabidopsis thaliana* were compared with the induction at protein level of the genes coding for the Prx family in its close relative *Brassica napus*.

3.3.1 Search for peroxiredoxin genes in Brassica species

In order to identify Prx genes in Brassica species, a search with known Arabidopsis sequences was conducted in available internet resources. Alignments were carried out using NCBI BlastP (www.ncbi.nih.gov/BLAST/). This comparison on the protein level supports the conclusion that the antibodies directed against Arabidopsis thaliana peroxiredoxin proteins (except for 2Cys-Prx, which was directed against BasFus, a 2Cys-Prx, of Hordeum vulgare sp. vulgare; Baier, 1996) are sufficiently specific to be employed in protein analysis of Brassica species.

Highly conserved sequences were observed for 1Cys-Prx, both 2Cys-Prx and cytosolic and chloroplastic type II Prx in *Arabidopsis*, *Brassica napus* and *Brassica rapa*. No information was found in relation to PrxQ, which is a nuclear encoded chloroplast Prx found in many dicot species (Kong et al., 2000; Lamkemeyer et al., 2006).

The Prx antioxidant (AAF61460), a 1Cys-Prx, protein sequence from *Brassica napus* reported by Gaoth et al. (1999) showed 90 % identity (94 % similarity) with the 1Cys-Prx (At1g48130) from *Arabidopsis thaliana* (Fig 23).

```
Arabidopsis thaliana 1CysPrx      MPGITLGDTVPNLEVETTHDKFKLHDYFANSWTVLFSHPGDFTPVCTTEL 50
Brassica napus Prx antioxidant    MPGITLGDTVPNLEVETTHKNFKLHDYFADSWTVLFSHPGDFTPVCTTEL 50
                                  ********************.:********:********************

Arabidopsis thaliana 1CysPrx      GAMAKYAHEFDKRGVKLLGLSCDDVQSHKDWIKDIEAFNHGSKVNYPIIA 100
Brassica napus Prx antioxidant    GAMGKYAHEFEQRGVKLLGLSCDDIQSHKDWIPDIEAFTPGSKVTYPIIA 100
                                  ***.******::************:******** *****. ****.*****

Arabidopsis thaliana 1CysPrx      DPNKEIIPQLNMIDPIENGPSRALHIVGPDSKIKLSFLYPSTTGRNMDEV 150
Brassica napus Prx antioxidant    DPNKEIIPQLNMIDPIENGPSRALHVVGPDCKIKLSFLYPSTTGRNMDEV 150
                                  *************************:****.********************

Arabidopsis thaliana 1CysPrx      LRALDSLLMASKHNNKIATPVNWKPDQPVVISPAVSDEEAKKMFPQGFKT 200
Brassica napus Prx antioxidant    LRALDSLLMAAKHKNKIATPVNWKPDEPVVISPAVSDEEAKKLFPQGFKT 200
                                  **********:**:************:****************:*******

Arabidopsis thaliana 1CysPrx      ADLPSKKGYLRHTEVS 216
Brassica napus Prx antioxidant    AKLPSKKGYLRVADVS 216
                                  *.********* ::**
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Fig. 23: **Amino acid sequence alignment of 1Cys-Prx from *Arabidopsis thaliana* with Prx antioxidant (1Cys-Prx) from *Brassica napus*.** The conserved cysteine residues are marked yellow, identical aminoacids are shaded in grey. Asterisk show identical amino acids, double points indicate conserved substitutions and single points indicate semi-conserved substitutions. The full length 1Cys-Prx amino acid sequences for Arabidopsis and Brassica were used.

The protein sequence of 2Cys-Prx (AAG30570) from *Brassica napus* reported by D Alessio et al. (2000) showed 95 % identity (98 % similarity; starting with position 69 of the protein sequence) with the 2Cys-PrxA (At3g11630) from the position 65 of the protein sequence, which corresponds approximately to the sequence of the mature protein (Fig 24). 96 % identity (98 % similarity) was shown between *Brassica napus* (starting with position 57 from the protein sequence) and 2Cys-PrxB (At5g06290) from *Arabidopsis thaliana* from position 60 (Fig 24). In that case, only 37.5 % of the differences were located in the mature protein, demonstrating highest homology with 2Cys-PrxB.

The *Brassica rapa* 2Cys-Prx precursor (AAF00001) reported by Cheong et al. (1999) exhibited 91 % identity (92 % similarity; starting with position 65) with *At*2Cys-PrxA from position 67 and 89 % (92 % similarity; starting with position 59) with the *At*2Cys-PrxB from position 64. This isogene thus showed higher similarity to the 2Cys-PrxA (Table 3).

The alignment of the two 2Cys-Prx of *Arabidopsis thaliana* and the 2Cys-Prx of the *Brassica* species also included the 2Cys-Prx, Bas1 (CAA84396) of *Hordeum*

vulgare sp *vulgare* (Baier, 1996). High similarity was shown between the proteins of the Brassicaceae species and that of the Triticeae, demonstrating that 2Cys-Prx is highly conserved during evolution.

Fig. 24: Amino acid sequence alignment of 2Cys-PrxA, 2Cys-PrxB from *Arabidopsis thaliana*, 2Cys-Prx from *Brassica napus*, 2Cys-Prx precursor from *Brassica rapa* and BAS1 from *Hordeum vulgare* sp *vulgare*. The conserved cysteine residues are marked yellow, identical amino acids are shaded in grey. Asterisk show identical aminoacids, double points indicate conserved sustitutions and single points indicate semi-conserved substitutions. The full lenght 2Cys-Prx amino acid sequences for Arabidopsis and Brassica were used.

The group of cytosolic type II Prx (PrxIIB, At1g65980; PrxIIC, At1g65970; PrxIID, At1g60740) in *Arabidopsis thaliana* showed highly conserved regions in their protein sequences (Fig 25), with 94 % - 98 % identity among them. The active centers of the chloroplastic Prx IIE (At3g52960) and the cytosolic Prx are highly conserved (Fig 25). The alignment revealed a 60% identity. The type II Prx from *Brassica rapa* (*Br*PrxII; AAD33602) reported by Choi et al. (1999) presented 91 % to 96 % identity to the cytosolic type II Prx group from

Arabidopsis thaliana considering the whole protein and a 60 % identity (75 % similarity; starting with position 2) with the chloroplastic *At*PrxIIE from position 71. The highest similarity was seen with the *At*PrxIIBn (Table 3). The type II Prx gene of *Brassica rapa* does not encoded a transit peptide.

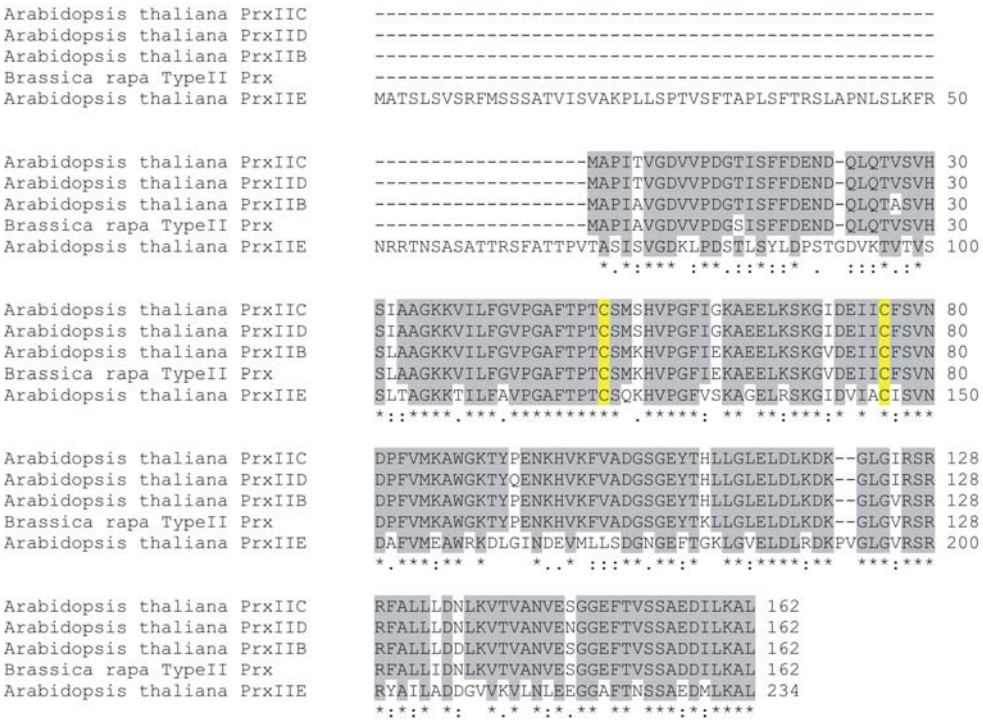

Fig. 25: Amino acid sequence alignment of PrxIIB, PrxIIC, PrxIID and PrxIIE from *Arabidopsis thaliana* with Type II Prx from *Brassica rapa*. The conserved cysteine residues are marked yellow, identical amino acids are shaded in grey. Asterisk show identical amino acids, double points indicate conserved sustitutions and single points indicate semi-conserved substitutions. The full length amino acid sequences for Arabidopsis and Brassica were used.

The mitochondrial *At*PrxIIF showed only 37 % to 39 % identity with the rest of the Type II Prx. The same 37 % identity (59 % similarity; from position 71 in the *Arabidopsis* protein sequence) was observed with the type II Prx from *Brassica rapa* from position 21. An alignment between *At*PrxIIF and the *Br*PrxII revealed a conserved area close to each cysteines, but a otherwise low similarity (Fig 26).

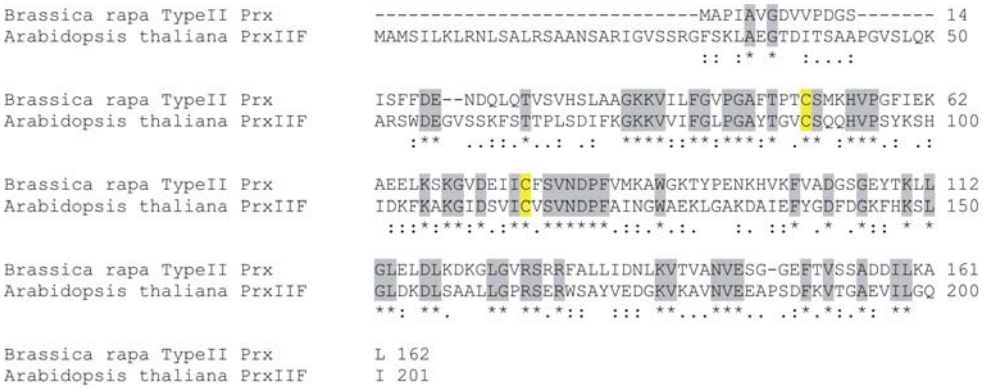

Fig. 26: **Amino acid sequence alignment of PrxIIF** from *Arabidopsis thaliana* with type II Prx from *Brassica rapa*. The conserved cysteine residues are marked yellow, identical amino acids are shaded in grey. Asterisk show identical amino acids, double points indicate conserved sustitutions and single points indicate semi-conserved substitutions. The full length amino acid sequences for Arabidopsis and Brassica were used.

3.3.2 Specificity and cross-reactivity of antibodies

The high similarity of the amino acid sequences encoding putative cytosolic and plastidic type II Prx of *Arabidopsis* and *Brassica* type II Prx described in Section 3.3.1 implied that the antibody against *At*PrxIIC could represent a useful tool to study *Brassica* species (Table 3).

	Brassica species			*Arabidopsis thaliana*			
	Lenght (aa)	**Molecular mass (kDa)**	**Homologue**	**Identity / Similarity (%)**	**MIPS number**	**Lenght (aa)**	**Molecular mass (kDa)**
Prx antiox	216	23.9	1Cys-Prx	90 / 94	At1g48130	216	24.1
Bn2Cys-Prx	183 (270)	20.5 (29.5)	2Cys-PrxA	95 / 98	At3g11630	183 (266)	20.5 (29.1)
Br2Cys-Prx	187 (273)	20.7 (29.4)	2Cys-PrxA	91 / 92	At3g11630	183 (266)	20.5 (29.1)
Bn2Cys-Prx	183 (270)	20.5 (29.5)	2Cys-PrxB	96 / 98	At5g06290	183 (271)	20.5 (29.6)
Br2Cys-Prx	187 (273)	20.7 (29.4)	2Cys-PrxB	89 / 92	At5g06290	183 (271)	20.5 (29.6)
BrPrxII	162	17.4	PrxIIB	96 / 98	At1g65980	162	17.4
BrPrxII	162	17.4	PrxIIC	93 / 97	At1g65970	162	17.4
BrPrxII	162	17.4	PrxIID	92 / 96	At1g60740	162	17.4
BrPrxII	162	17.4	PrxIIE	60/ 75	At3g52960	164 (234)	17.3 (24.7)
BrPrxII	162	17.4	PrxIIF	37 / 59	At3g06050	171 (199)	18.3 (21.2)
-	-	-	PrxQ	-	At3g26060	159 (216)	17.5 (23.6)

Table 3: Comparison of the Prx in *Brassica napus* and *Brassica rapa* with the Prx in *Arabidosis thaliana*

In contrast, the mitochondrial *At*PrxIIF, is only distantly related to the rest of the Type II Prx group in *Arabidopsis* (Table 3). Consequently, the antidody against *A*tPrxIIF shows high specificity (Finkemeyer et al., 2005), suggesting that it could also recognize a *Bn*PrxIIF specifically. The homologies in the protein sequences of the Bas1 protein, a 2Cys-Prx, from *Hordeum vulgare* sp. *vulgare*, 2Cys-PrxA and 2Cys-PrxB from Arabidopsis and 2Cys-Prx from *Brassica napus* (Fig 24) let assume also specificity from *Hv*BAS1 to recognize *Bn*2Cys-Prx.

3.3.3 Protein amounts of peroxiredoxin in different tissues in *Brassica napus*

Prx protein abundance in *Brassica napus* was studied in three developmental stages of leaves (L1 for young leaves, L2 for mature leaves and L3 for senescent leaves) and four silique developmental stages (S1 for the youngest state and S4 for the oldest ones in the maturity stage) in addition to roots, shoots and flowers (Fig 27).

The protein levels of 2Cys-Prx were similar in young and mature leaves and significantly higher in leaves than in the other tissues. Only in senescent leaves, degradation of the protein was observed. The flowers only showed a low protein level compared to green tissues. In siliques the protein amounts decreased with development.

PrxIIC was detected in all tissues with exception of senescent leaves. Flowers showed higher expression than leaves suggesting a preferential function in reproductive organs (Brehélin et al., 2003). Also in siliques a decreasing signal during development was observed.

Antibodies against *At*PrxIIF and *At*PrxQ were successfully used in Brassica, although no genomic or cDNA sequences for these enzymes have been reported to date (Section 3.3.1). The detection of proteins with similar tissue specificity with the same molecular mass as in *Arabidopsis thaliana* demonstrated the existence of a putative mitochondrial type II Prx and a PrxQ-like protein in *Brassica napus* (Fig 27).

*At*PrxIIF antibody recognized a protein, which is highly expressed in roots, leaves, flowers and siliques (Fig 27). A lower signal intensity was observed only in shoots. Differently to Arabidopsis, the protein amounts during silique development remained constant, indicating a constitutive expression in siliques.
Irrespective of the lack of homologous sequences in the Brassica databases (Sec. 3.3.1), *At*PrxQ antibody detected a protein with the same expression pattern as PrxQ in *Arabidopsis thaliana*. This protein was only detected in green tissues, specially in leaves and young siliques. The expression in the siliques decreased strongly with development.

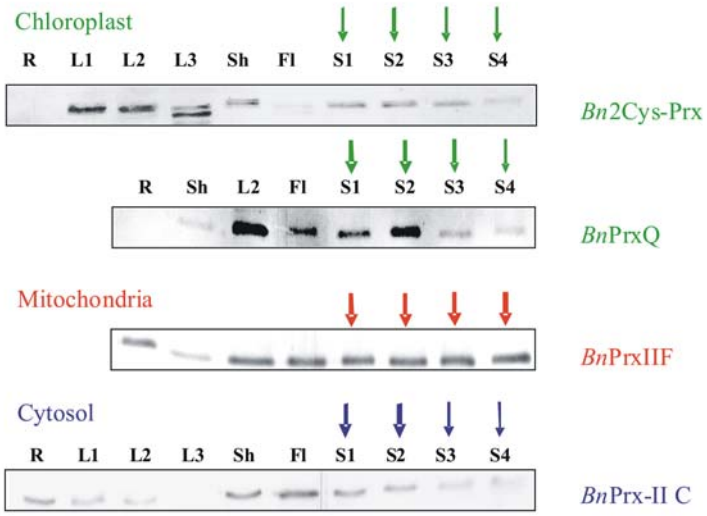

Fig. 27: **Peroxiredoxin protein amounts in different tissues from *Brassica napus***. R: root; Sh: shoot; L1: young leaves; L2: mature leaves; L3: senescent leaves; Fl: flower; S1: youngest siliques to S5: oldest siliques. Representative Western Blots from 2 parallel experiments are shown. Equal protein amounts were loaded in each lane.

3.3.4 Expression of peroxiredoxins in silique walls and seeds

For a better understanding of the role of the peroxiredoxins during the various stages of silique development it was of special interest to differenciate the expression of seeds and silique walls (carpels; Fig 28). Siliques at stage S3 and S5 of Arabidopsis and at S3 of Brassica were separated into silique walls and seeds. For further analysis the S5-seeds of Arabidopsis and S3-seeds of Brassica were compared for Prx protein levels. No signal in seeds from Arabidopsis was

detected for the chloroplastic 2Cys-Prx and PrxQ, while in Brassica, 2Cys-Prx was detected in seeds. The PrxQ protein amounts in carpels decreased strongly from S3 to S5, but was constant for 2Cys-Prx. This observation suggests that PrxQ is present only in carpels. Consequently, when the silique walls start to senescence, the protein stopped to be synthesized and possibly was degradated. The decrease in the expression of 2Cys-Prx in the whole silique during development and the issue that this protein accumulate in green tissues points at specific expression in young seeds. PrxIIC was present in both carpels and seeds. The expression is similar in carpels and seeds of the same stage, suggesting that the protein amount decreased in both organs at the same time. In contrast, PrxIIF was strongly expressed in green and non-green seeds. The protein levels in the carpels of S3 and S5 were constant and in the seeds was slightly higher in Arabidopsis, suggesting that the increment in the expression of this enzyme is related to a major accumulation in the seed during maturation.

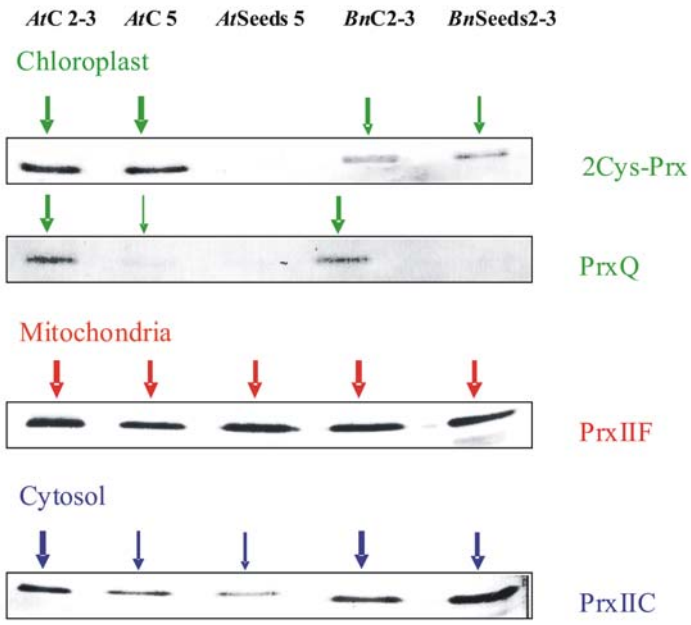

Fig. 28: **Peroxiredoxin protein amounts in silique walls and seeds from *Arabidopsis thaliana* and *Brassica napus***. AtSW2-3 and AtSW5: Carpels from the S2-S3 developmental state and from the S5 developmental state from siliques of *Arabidopsis thaliana*, respectively; AtSeed5: Seeds from the S5 developmental state of *Arabidopsis thaliana*; BnSW2-3 and BnSeeds2-3: Carpels and seeds from the S2-S3 developmental state and from the S5 developmental state from siliques of *Brassica napus*, respectively. Equal protein amounts were loaded in each lane.

4. Discussion

4.1 Seedling development

In oil seed plants, after the onset of germination, seedling establishment depends on the efficient metabolization of the storage lipids, which provide the carbon skeletons and energy necessary for growth until photoautotrophic metabolism is established (Bewley and Black, 1994). *Arabidopsis thaliana* seeds store lipids in the form of TGA in oil bodies in the cotyledons, which are progressively transformed into photosynthetic organs (Eastmond and Graham, 2001).

Seedling development involves generation of ROS, produced mainly by growth, lipid mobilization, respiration and photosynthetical activity (Puntarulo et al., 1988; Corpas et al., 2001; Foyer and Noctor, 2000). Redox control is neccesary to avoid damage of membranes and for protection of the meristematic cells.

The regulation of antioxidative mechanisms during early post-germinative growth in context to light and dark metabolism and under delay of lipid mobilization by sucrose was studied in the present work. The ascorbate contents, the cellular redox state and the induction of genes of the Halliwell-Asada cycle and of the Prx scavenging system were correlated with parameters that characterized the developmental grade of the seedlings.

4.1.1 Chloroplast morphologic changes and chlorophyll accumulation as indicators of photoautotrophic development

The differentiation from proplastids in meristematic cells (or from etioplasts in seedlings grown in the dark) into photosynthetically competent chloroplasts requires the coordinated synthesis of lipids and proteins for the formation of thylakoid membranes and of pigments, such as chlorophylls and carotenoids (Vothknecht and Westhoff, 2001), whose biosynthesis is regulated by the exposure to white-light (Woitsch and Römer, 2003).

In seedlings grown in continuous light, the chlorophyll rapidly increased to 115 ± 16 μmol g^{-1} fw at 1.5 DARE (Fig 6), subsequently doubling during the next 3.5 days. In absence of sucrose, in discontinuous light at 1.5 DARE the chlorophyll content only was 53 % that of seedlings grown in continuous light. Within the

next 3.5 days the chlorophyll content increased 4.2-fold. This increase even over-compensated the initial delay. Thus, chlorophyll accumulation stimulated by constant light was balanced once photoautotrophic metabolism had been established. At 5 DARE, seedlings reserves are usually exhausted (Eastmond et al., 2000) as indicated here by the extremely low activity of the ICL promoter at 4 DARE in absence of sugars (Fig 4). Consistent with the chlorophyll accumulation profile, 1.5 DARE the chloroplast ultrastructural analysis demonstrated high organization of the thylakoids in seedlings grown under continuous light. The thylakoid membrane was more developed than in seedlings grown under discontinuous light conditions, under which fewer thylakoids were observed per grana (Fig 5).

It was shown that accumulation of carbohydrates in plant cells repress genes coding for proteins involved in photosynthesis (Krapp et al., 1993; Dijkwel et al., 1996; To et al., 2003). High sugar availability decreases expression of chlorophyll-a/b-binding proteins (Krapp et al., 1993; To et al., 2003). Low availability of chlorophyll-binding proteins subsequently slows down chlorophyll accumulation by feed-back inhibition of chlorophyll biosynthesis (Meskauskien and Apel, 2002). Jang and Sheen (1994) reported that in protoplasts from mature Arabidopsis leaves the maize *cab*-promoter is significantly suppressed within 3 h in 1 mM and 10 mM glucose. Here, chlorophyll accumulation in seedlings grown in presence of 28 mM sucrose, was inhibited from 3 DARE onwards (Fig 6). In parallel to accumulation of transitory starch, less grana stacking and earlier disorganization of the thylakoid structure were observed at 5 DARE compared to seedlings grown in discontinuous light in the absence of sucrose (Fig 5).

Plants grown under day/night cycles retain part of the photoassimilates in the form of starch in leaves during the day and remobilize it in the night to support respiration and metabolism (Geiger and Servaites, 1994). Starch grains were found in seedlings grown under continuous light from 3 DARE onwards, indicating that starch synthesis takes place in the light and responds to changes of the sink-source balance. The accumulation of excess starch in the cotyledons in the presence demonstrated limited export of carbohydrates from the chloroplast and may result from saturation of extra-plastidic sinks for photosynthates.

4.1.2 Development of the photosynthetic membrane

Chlorophyll-a fluorescence kinetics provide information on the efficiency of light energy conversion and photochemistry in the PSII reaction centres (Schreiber and Bilger, 1993). In dark adapted seedlings the maximum quantum yields ($\Phi PSII_{max}$) were lowest at 1 DARE in seedlings grown under constant light. At that point of development cotyledons start to expand and to green (Fig 7). $\Phi PSII_{max}$ at 1 DARE was also lower than 1.5 DARE in seedlings grown on discontinuous light. Until the third DARE the photosynthetic membrane developed. Light/dark cycles promoted thylakoid development as indicated by a higher ΦPSII (Fig 7).

In discontinuous light, higher $\Phi PSII_{2min}$ in the mornings up to 2 DARE, but similar $\Phi PSII_{2min}$ in the evenings demonstrated that sucrose application significally inhibited morning photosynthesis. However, the impact of sucrose on ΦPSII was less pronounced than on the chlorophyll content (Fig 8). Consequently, sucrose mainly affected the amount of functional units. The kinetics of ΦPSII activation partly depend on the size of the light-harvesting antenna (Tyystjärvi et al., 1999) and the pool size and reduction state of electron carriers (Stirbet et al., 1998), whose expression is well known to be suppressed by externally applied sugar (Krapp et al., 1993; Dijkwel et al., 1996).

Photochemical quenching (qP) demonstrates the efficiency by which photosynthetic processes are activated (Schreiber and Bilger, 1993). In constant light, it was activated stronger in seedlings of all ages than in day/night cycles. In addition, sugar application decreased the qP consistent with a sugar-dependent inhibition of the Calvin-Cycle (Stitt et al., 1987), which is the major sink for electrons from the photosynthetic electron transport chain (Dietz and Heber, 1984).

Developmental regulation of NPQ mainly correlated with the age-dependent differences in ΦPSII. Between 3 and 3.5 DARE the long-term component of NPQ especially increased in discontinuous light, while in continuous light the age-dependent differences resulted in a weak increase of NPQ at 2 DARE onwards. Characterization of the *npq4*- and *npq1*-mutants (Li et al., 2002; Niyogi et al., 1998) demonstrated that activation of NPQ depends on sensing of the thylakoid lumen pH by PsbS and violaxanthin de-epoxidation. In young seedlings low

chlorophyll availability limits photosynthetic electron transport and restricts membrane energisation and NPQ activation. However, the developmental difference in NPQ between seedlings in presence and absence of sucrose is unlinked to differences in chlorophyll accumulation, which only started 3 DARE (Fig 8). It points at a direct sugar-mediated limitation of NPQ in early seedling development, as e.g. through insufficient thylakoid acidification.

4.1.3 Evidence of ROS accumulation in expanding tissues during post-germinative growth

In the presence of trace amounts of Fe or Cu $O_2^{\cdot -}$ and H_2O_2 initiates reaction sequences which form highly reactive hydroxyl radicals (Thompson et al., 1987). The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids and destruction of proteins (Asada and Takahashi, 1987; Bowler et al., 1992; Halliwell, 1987). The peroxidation of lipids can be initiated by free radicals (Girotti, 1985; Thompson et al., 1987). During expansion of cotyledons, which are the main oil storage organs in Arabidopsis, and establishment of the photosynthetically machinery β-oxidation is still active (Eastmond and Graham, 2001) increasing the generation of ROS in presence of light. NBT-staining of Arabidopsis seedlings (Fig 9) revealed increased rates of superoxide formation in the light at 1.5 and 2 DARE. In roots at 1.5 and 2 DARE the superoxide accumulation correlates with high cell wall biosynthesis (Rodriguez et al., 2002). The cytotoxic $O_2^{\cdot -}$ (Jabs et al., 1996) were detected in patch-like patterns all over the cotyledons while later during development highest $O_2^{\cdot -}$ levels were observed in the hypocotyls and along the veins in both dark adapted seedlings and those without dark adaption (Fig 9).

The release of ROS in germinating seeds was proposed to be an active and beneficial biological reaction taking place in healthy developing seedlings (Puntarulo et al., 1988; Schopfer et al., 2001). Peroxidase was active where ROS were generated. It is suggested that they act as a constitutive defense reaction against infection by microorganisms (Schopfer et al., 2001). Participation of ROS was proposed to regulate cell expansion through modulation of Ca^{2+} channel activity (Foreman et al., 2003) or affecting the reological properties of cell walls

(Cosgrove, 1999) thereby contributing both to wall loosening and stiffening (Schopfer 1996, 2001). However, the risk of oxidative stress due to imbalances between production and scavenging of ROS exists and requires an efficient and coordinated regulation of the antioxidative scavenging system.

4.1.4 Changes in the redox state during post-germinative growth

In plants, ascorbate plays a pivotal role by acting as a redox buffer as well as a sensor of metabolic changes by redox reactions (Pastori and Foyer, 2002; Foyer and Noctor, 2003). In addition, ascorbate is involved in plant development, with levels and redox state indicating developmental stages and conditions (Arrigoni et al., 1992; De Gara et al., 2003; de Pinto and De Gara, 2004). In *Vicia faba* and *Triticum durum* it was observed that the ascorbate content decreased during seed and kernel maturation, respectively, and the redox state changed from reduced to oxidized pool during maturation (Arrigoni et al., 1992; De Gara et al., 1997). It was demonstrated that angiosperm seeds contain a moderate amount of dehydroascorbate and DHAR activity, which might be enough to suppport the ascorbate demand for cell division and expansion after imbibition until its *de novo* biosynthesis takes place (Liso et al., 1984; Cittero et al., 1994; Córdoba-Pedregosa et al., 1996). Ascorbate biosynthesis and expression of ascorbate peroxidase was shown to increase during the first 48 h after germination in wheat according to the recovery of oxidative metabolism and subsequently requirement of ROS elimination (De Gara et al., 1997).

Here, in the developmental period of Arabidopsis seedlings 1.5 to 5 DARE presented stable ascorbate contents on a fresh weight basis in both continuous and discontinuous light in sucrose-free media (Fig 10 C, D). The apparent decrease of asorbate content on pheophytin basis is the consequence of the rapidly increment of chlorophyll accumulation (Fig 10 A, B). On constant light, seedlings of all ages revealed a highly reduced redox state indicating non-stressed growth conditions (Law et al., 1983), despite the presence of high ROS presence (Fig 10 E, F). In parallel, the total ascorbate peroxidase activity was stable from 2.5 DARE onwards. It can be concluded that the scavenging systems are balanced with the production of ROS in seedlings grown on constant light.

On discontinuous light, the ascorbate contents were higher than in continuous light and decreased slightly from 1.5 to 4.5 DARE (Fig 10A-D). The redox state was severely oxidized 1.5 DARE in abscence of sucrose (41 %) increasing transiently during the next 3 DARE reaching a 71 % of reduced ascorbate. Consistently, the total ascorbate peroxidase activity increased during development with some fluctuations around 2.5 DARE. The redox state on day/night cycles (Fig 10 F) correlates with the slower development of the membrane system (light-harvesting system) of the photosynthetic apparatus (Fig 8) indicating major exposure to oxidative stress by incomplete NPQ activation, which basically indicates the thermal dissipation of excess absorbed light energy in the light-harvesting antenna of PSII via xanthophyll cycle and depends on ascorbate availability (Niyogi et al., 2005).

In presence of sucrose, ascorbate contents randomly fluctuated during development. The ascorbate redox state at 1.5 DARE was similar (45 %) to that in seedlings on sugar-free media. 12 h later the redox state showed considerable improvement as compared to non-treated seedlings, probably reflectives the positive influence of sucrose. However from 3 DARE onwards, the redox state was severely oxidized (Fig 10 E, F). Total APx activity increased with development and was only slightly lower in seedlings in the presence than in absence of sugar (Fig 13 B and D), demonstrating, that the imbalances were produced by oxidative stress and not by repression of APx. Lipid mobilization was affected in seedlings by the presence of sucrose as indicated by the lower activity of the ICL promoter 2 to 4 DARE compared to control seedlings (Fig 4). The excess of carbon sources induced extra starch accumulation in the chloroplast from 3 DARE and early senescence as indicated by the disorganization of the thylakoids at 5 DARE (Fig 5). It can be concluded, that the redox buffering capacity of the ascorbate scavenging system is not sufficient to protect chloroplasts that are subjected to arrested development caused by exogenous carbohydrates during photoheterotrophic growth.

4.1.5 Antioxidant expression during seedling establishment

The expression of Prx and enzymes of the Halliwell-Asada cycle was studied during the period of seedling establishment under constant light and during day/night cycles. In addition, the regulation of these genes was analyzed under the effect of external carbohydrate supply, which prolongated the heterotrophic growth.

4.1.5.1 Expression of chloroplastic antioxidative genes

Growth under constant light conditions

In continuous light most genes for antioxidant enzymes were rapidly induced after germination. The transcript levels for chloroplast peroxiredoxins were maximal already 1.5 DARE (Fig 11). In parallel to high superoxide accumulation in young seedlings (as shown by the NBT-staining; Fig 9) Csd2 transcripts were high 1.5 DARE and increased transiently during further development (Fig 11). In contrast, the transcript levels for the stromal APx and MDHAR remained low until 3 DARE.

In the chloroplasts the water-water cycle mantains an electron flow through the photosynthetic apparatus even when CO_2 availability and Calvin cycle activity are limited or inhibited (Asada, 1999; Mittler, 2002), which prevents photoxidative damage. Increasing the expression of the thylakoid-attached CuZnSOD (Csd2) or thylakoid-bound APx enhances abiotic stress tolerance in transgenic plants (Yabuta et al., 2002). A recent study with Csd2 knock down lines of *Arabidopsis thaliana* (KD-SOD) demonstrated that the water-water-cycle pathway is essential for protection of chloroplasts even in absence of environmental stress (Rizhsky et al., 2003). The KD-SOD plants showed suppressed rates of photosynthesis and lower chlorophyll levels than wild type. Decreased Csd2 expression resulted in the induction of two transcripts encoding FeSOD (FSD1 and FSD3) and two transcripts encoding catalase (CAT1 and CAT3; Rizhsky et al., 2003). The induction was not able to compensate the suppression of Csd2. In contrast, transcripts encoding thylakoid-bound APx, 2Cys-PrxB, chloroplastic and cytosolic glutathione reductase ferredoxin-$NADP^+$ reductase and PSI subunit I were suppressed (Rizhsky et al., 2003). This co-suppression suggested that

transcripts encoding different members of the cycle are regulated coordinatly as a pathway (Rizhsky et al., 2003).

Here, the relative cycle numbers used for amplification of transcripts of Csd2 in seedlings in a RT-PCR-reaction in relation to actin was in the range of the chloroplastic Prx, indicating similar relative expression. Meanwhile, the relative expression of the two APx and MDHAR were lower. Unlike APx, the peroxiredoxins are able to reduce also alkyl hydroperoxides to their corresponding alcohols and water (König et al., 2002; Horling et al., 2003). In differentiating chloroplasts massive biosynthesis of fatty acids takes place (Appelquist et al., 1968) and high portions of fatty acids are desaturated (Harwood, 1980; Miége et al., 1999). In parallel, chlorophyll-binding proteins accumulate in the thylakoid membrane (Mullet, 1988).

Consistent with the observation that 2Cys-Prx antisense Arabidopsis showed delayed development (Baier and Dietz, 1999), the high expression of peroxiredoxins in young seedlings, reflected by high transcript and protein levels (Fig 11,12 and 14), suggest importance of this pathway in seedling development. Putative points of action are the control of fatty acid or oxylipid metabolism and the detoxification of H_2O_2 and alkyl hydroperoxides. Histochemical localization of promoter activity in *At*2Cys-PrxA reporter lines showed strong staining of seedlings between 1 and 6 DARE (Fig 15). Strong activity was shown in cotyledons especially on their edges in 1 and 2 d old seedlings resembling the distribution of $O_2^{\cdot-}$ shown by NBT-staining of seedlings (Fig 9). Staining in the central cylinder demonstrates 2Cys-PrxA promoter activity also in non differentiated plastids.

It can be concluded, that during the differentiation of chloroplasts and maturation of the photoautotrophic membrane, the induction of the ascorbate-independent water-water-cycle (Prx-dependent peroxide detoxification) is especially important for ROS scavenging, while the enzymes of the ascorbate-dependent water-water-cycle (Asada, 1999) become more prominent upon a developmental switch at 3 DARE. In addition, the increase in the transcript levels for enzymes dominating the antioxidant defence network in mature leaves correlates with the decrease in superoxide accumulation in the intercostal fields of the cotyledons (Fig 15)

suggesting that their expression is involved in providing the full protection against superoxide radicals. The subsequent decrease of the Prx transcript levels at 4 DARE may reflect feed-back regulation in response to the improved antioxidant protection that was also indicated by the increased APx activity (Fig 13).

Growth in day/night cycles

All tested chloroplastic genes showed significant day/night fluctuation after 2.5 DARE in presence as well as absence of sucrose. Only Csd2 presented weak diurnal fluctuations in seedlings on sugar-free media. The strong induction within the first 2.5 DARE of all these genes corresponds to the need of an efficient scavenging system able to overcome redox misbalances, which are indicated by the low redox state of ascorbate during the period of establishment of photoautotrophism. The APx activity on the protein basis reflected strongly the diurnal transcript amount regulation (Fig 12) demonstrating that the expressional regulation of the ascorbate-dependent water-water-cycle follows strong diurnal variations. However, the APx activity increased more strongly from 1.5 to 5 DARE, indicating accumulation of APx protein during development. The strongest difference between day and night was observed for MDHAR, especially at 4 DARE demonstrating strong light influence in full photoautotrophic plants (Fig 12).

In sucrose treated seedlings, down-regulation for the stromal APx and Csd2 during the night phase was only detected at 4 DARE. In contrast, the decreased transcript levels of the thylakoid-bound APx at 3 DARE indicated repression by exogenous sugar. Total APx activity was also lower than in the control seedlings reflecting the influence of the sucrose addition. At 3 DARE, chlorophyll stopped to accumulate in seedlings in the presence of sugars (Fig 6), while thylakoids desintegrated in chloroplasts at 5 DARE. The oxidative stress reflected also by the extremely oxidized redox state after 3 DARE in sugar-treated seedlings (Fig 9) could be also consequence of the repression of the APx scavenging system. The almost constitutive transcriptional induction of Csd2 might be due to high rates of superoxide generation. A possible reason for thylakoid-bound APx repression is overproduction of H_2O_2. Thylakoid-bound APx is the key enzyme for reduction of H_2O_2 and is attached with CuZnSOD on the PSI complex of the thylakoids

(Asada, 1999). The stromal APx is considered a "second defence" of the thylakoid system or for scavenging ROS in the stroma (Asada, 1999). It is postulated that the lack of diurnal fluctuations for stromal APx expression until 3.5 DARE (Fig 12) in presence of sucrose compensates the repression of the thylakoid-bound-APx, which was more exposed to inhibition.

In the case of the Prx, no difference in the transcript regulation was observed between control and sucrose-treated seedlings, suggesting a dominant role in the ROS scavenging system under stress conditions. Lower transcript induction of the 2Cys-Prx during the night resulted in lower protein levels in young seedlings. After the morning of day 2 (2 DARE), the diurnal variations in transcript abundance were barely reflected in the protein amount showing accumulation from 3 DARE onwards in presence and absence of exogenous sugar (Fig 12). Horling et al. (2003) reported strong down-regulation of the transcript of the chloroplastic 2Cys-PrxA, 2Cys-PrxB and PrxQ in leaf slices after 6 h from transferring from growth light conditions (120 µmol quanta m^{-2} s^{-1}) to low photon rates (10 µmol quanta m^{-2} s^{-1}). Only PrxIIE decreases to a lesser extent than the other chloroplastic Prx under these conditions (Horling et al., 2003). However, immunoblots of plants transferred from growth light to low light conditions using antibody against 2Cys-Prx of barley showed accumulation of protein in the next 72 hr after low light exposure (Horling et al., 2003). In addition, accumulation of 2Cys-Prx in senescent leaves was shown in previous reports (Horling et al., 2002). These observations are explained by the stability of the 2Cys-Prx protein, explaining the markedly increasing protein levels during development of the seedlings after 3 DARE (Fig 14). In contrast, PrxQ protein levels showed no variation during day and night and remained stable during development in presence and absence of sucrose (Fig 14). Protein stability during the night hours resulted in protein accumulation in combination with *de novo* synthesis during the day time. It can be concluded, that the chloroplastic Prxs dominate the protection of cells under oxidative stress when destruction of the chloroplastic organization takes place, avoiding the eventual permeation of ROS to other cell compartments.

4.1.5.2 Expression of cytosolic type II Prx

The cytosolic PrxIIC and PrxIID showed the same transcriptional regulation as the stromal APx and MDHAR, with high induction from 3 DARE onwards (Fig 11, 12). PrxIIB, in contrast, showed elevated relative expression and only slight up-regulation after 3 DARE, similar to Csd2 (Fig 11, 12). β-oxidation of fatty acids generates H_2O_2, which can permeate the glyoxysomal membrane producing superoxide radicals at the cytosolic site (del Río et al., 1998; López-Huertas et al., 1999). Horling et al. (2003) reported induction of PrxIIB in presence of butyl hydroperoxide, which is lipophilic and suggested a protective function near the plasma membrane. The protein content of the cytosolic and chloroplastic type II Prx was high and stable during seedling development (Fig 11, 12), suggesting that these Prx-isoforms play an important role in the cytosol, e.g. in membrane protection during lipid mobilization from the oilbodies.

The cytosolic Prx were differently regulated at transcriptional level. Protein levels of the type II Prx presented no correlation with the transcript amounts. PrxIIB followed the diurnal pattern of the chloroplast Prx (Fig 14), being in line with the light dependency shown by Horling et al. (2003), who described down-regulation 6 h after transfer from growth light conditions (120 µmol quanta m^{-2} s^{-1}) to low photon rates (10 µmol quanta m^{-2} s^{-1}).

PrxIIC was transiently induced and PrxIID showed the inverse light response after 3 DARE (Fig 11, 12). Previous reports indicate that PrxIIC is induced under stress conditions, remaining low under favourable conditions (Horling et al., 2002). Consistently, in the presence of sucrose, the induction of PrxIIC and PrxIID was 6-fold and 4-fold higher at 2 DARE than in control seedlings, respectively.

During lipid mobilization, the capacity for the flow of carbon through the decarboxylating side of the citric acid cycle is restricted, but during chloroplast biogenesis and the associated development of photosynthetic function, the capacity exists for operation of the complete citric acid cycle (Hill et al., 1991; Eastmond and Graham, 2001). Under low rates of lipid breakdown, oxygen uptake increases. Consequently, the rate of dark respiration increases in photosynthetic tissues (Azcón-Bieto et al., 1989; Hill et al., 1991). The up-regulation of PrxIID transcription coincides with the slow down of lipid

mobilization. At 3 DARE, seedlings presented accumulation of starch (Fig 5), indicating active carbon metabolism product of CO_2 fixation by photosynthesis. Starch degradation provides the carbon skeleton to support respiration in the dark. The protective role of PrxIID may be related to high rates of mitochondrial respiration, which implicates the operation of the complete Krebs cycle. It takes place only in absence or low activity of lipid mobilization either by establishment of photoautotrophism or by sugar repression (Hill et al., 1991; Eastmond and Graham, 2001; To et al., 2002).

4.1.5.3 Expression of the mitochondrial PrxIIF

The mitochondrial PrxIIF was highly expressed at transcriptional and protein level between 1.5 and 5 DARE (Fig 11, 12, 14). As shown recently by Finkemeier et al. (2005), Prx IIF, which is the only mitochondrial Prx, promotes root growth under cadmium stress, which strains antioxidant protection by the glutathione system (Dietz et al., 1999). The NBT-staining patterns of seedlings (Fig 9) showed that superoxide radicals are preferentially formed in roots at 1.5 and 2 DARE, when cell wall biosynthesis is maximal (Rodriguez et al., 2002). Furthermore, the respiratory chain and especially oxygen activation by the alternative oxidase provide sources for ROS (Turrens, 1997). The importance of Prx-mediated protection against respiratory ROS during seedling establishment was shown recently by enhanced growth inhibition of PrxIIF-knock out plants in the presence of the alternative oxidase-inhibitor salicylic hydroxamic acid (SHAM) (Finkemeier et al., 2005). Thus, high expression of PrxIIF in roots of young seedlings may protect against extracellular ROS accumulation due to cell wall biosynthesis and respiratory ROS.

Transcriptional induction of the mitochondrial PrxIIF showed a diurnal pattern similar to the cytosolic PrxIIB and the chloroplastic genes. This regulation could be explained by interorganellar communication via the light-dependent thioredoxin signal of chloroplasts through metabolites exported to the cytosol, i.e., malate and glyconate, which participates of mitochondrial metabolism (Balmer et al., 2004). These molecules were suppossed to reduce thioredoxin by means of NADP/thioredoxine reductase in mitochondria, thereby completing transmission of the light signal (Balmer et al., 2004). Thioredoxines are known to act as

electron donors in the regeneration of peoxiredoxins (Dietz, 2003a). No differences were observed in its regulation in presence and absence of sugars, indicating that its induction is independent of the increment in mitochondrial respiration. Slight diurnal fluctuations were observed on protein levels on control seeds. This fluctuation was lost in the presence of sugar. PrxIIF expression was independent from seedling development or stress conditions remaining high from very young stages onwards. These observations demonstrate its constitutive high importance for mitochondrial protection during seedling establishment.

4.1.7 Conclusion

Compared to mature leaves, the relatively high transcript levels of all Prx in seedlings suggests that Prx have the predominant function in peroxide detoxification during early seedling development while the ascorbate-dependent water-water-cycle gains importance later in development. Compared to ascorbate peroxidases, which are the major peroxidases of mature leaves (Asada, 1999), the catalytic activity of Prx is low (Horling et al., 2002; 2003; Finkemeier et al., 2005), but is partially compensated by the high enzyme amounts available (Dietz et al., 2006). High Prx levels during the transition from heterotrophic to autotrophic metabolism may protect from damage by H_2O_2 as well as lipid peroxides during the phase of rapid lipid metabolism (Girotti, 1985). It is concluded that Prx dominate the antioxidant protection of seedlings prior to full induction of the Halliwell-Asada-Cycle.

4.2 Silique and seed development

Developing siliques are photosynthetically active fruits. They consist of two closely interconnected organs: the seeds and the silique wall. Silique development can be divided in three phases: early embryogenesis, seed maturation and desiccation (Baud et al., 2002). From the early embryogenic stage to the end of maturity, a constant flux of nutrients takes place from the maternal tissues to the growing seeds. After floral initiation, the start of programmed leaf senescence is the primary event, which initiates redistribution of resources, such as nitrogen reassimilation products (Ye et al., 2000). With the rapid decline of functional leaf

area, the silique wall turns to be the principal source of photoassimilates. Work on *Brassica napus*, a close relative of Arabidopsis (Sec. 1.1.1), has shown that the rates of net CO_2 fixation can be as high as 35 % of that found in leaves on an area basis and equivalent on a chlorophyll basis (King et al. 1998). Consequently, silique wall is the major site of CO_2 fixation and O_2 evolution during seed development (King et al., 1997, 1998; Furbank et al., 2004). However, photosynthesis is also a major source of ROS (Foyer and Noctor, 2000), which demands for an effective control and scavenging of ROS.

In the present work, the expression of antioxidative enzymes was studied during silique development in *Arabidopsis thaliana*. Prx expression was compared with the induction of transcripts for enzymes involved in the Halliwell-Asada-cycle. In addition, the regulation pattern of the Prx protein levels was analyzed in the oilseed plant *Brassica napus*, whose economical importance and physiological properties have fostered its use as an alternative model organism.

4.2.1 Photosynthetic capacity of the siliques

Consistent with previous reports (King et al., 1997; Ruuska et al., 2002), a stable and high efficiency of the electron transport was observed in siliques until maturation. $\Phi PSII_{Max}$ was stable between the stages S1 and S5. After 5 min illumination, the steady state levels were adjusted between 70 % and 80 % of $\Phi PSII_{Max}$ in all developmental stages tested. The qP was activated rapidly and the NPQ values remained low, indicating high and stable photosynthetic competence from young to mature siliques and demonstrating the capacity of the silique wall to supply the seeds with photoassimilates according to the respective developmental requirement.

4.2.2 Transcript regulation of antioxidative enzymes during silique development

As shown in Section 4.2.1, the silique walls are highly photosynthetically active. In green tissues, the photosynthetical electron transport chain in chloroplasts is the main source of ROS production (Asada, 1994). In addition, high levels of ROS generation were shown to be associated with cell division and expansion of young

tissues (Puntarulo et al., 1988; Schopfer et al., 2001; Rodriguez et al., 2002). In developing seeds, repiration rates increase during fatty acid synthesis and with it, the risk of overreduction of the respiratory electron chains (Kang and Rawsthorn, 1994; Møller, 2001).

Prior to the analysis of the antioxidant defence sytems with the Prx and enzymes of the Halliwell-Asada-cycle, the developmental stage of older siliques was characterized by quantifying the transcript accumulation of ICL, a key enzyme of the glyoxylate cycle, which takes place during mobilization of the storage lipids in the mature seeds (Chia et al., 2005).

4.2.2.1 Determination of silique maturity stages by a lipid metabolism marker gene

Studies on *Brassica napus* reported mobilization of the storage lipids mainly during the desiccation stage. However, expression of enzymes of the β-oxidation, glyoxylate cycle and gluconeogenesis can be also observed in the period of fatty acid synthesis during seed maturation (Comai et al., 1989; Chia et al., 2005). Then, their activities are much lower than during germination or early seedling growth (Chia et al., 2005). ICL was first detected next to the end of the maturity stage in *Brassica napus* (Chia et al., 2005). Here, transcripts were detected exclusively in the S5 stage in siliques from *Arabidopsis thaliana*. Thus, the three chosen stages (S1, S3 and S5) covered silique development from early embryogenesis (S1) to the end of seed maturation (S5; Fig 16; Fig 2).

4.2.2.2 Transcript abundance of chloroplastic antioxidative enzymes

In RT-PCR, the relative cycle number used for amplification of transcripts for the chloroplastic genes from cDNA samples was very similar in leaves and siliques and close to the cycle number used for amplification of actin, indicating high relative expression. An exception was MDHAR, which required 12 cycles more than actin for amplification to similar levels, demonstrating that the MDHAR transcript is much less abundant in siliques and in leaves.

The water-water-cycle is one of the electron transport pathways controlling photosynthesis (Asada, 1999). Redox regulation of gene expression has been shown for the enzymes participating in this cycle, CuZnSOD, APx and MDHAR

(Asada, 1999). In this context, redox regulation is defined as transcript abundance regulation correlating with accumulation of ROS.

Gene regulation depends on the length of the day period, organ specificity and the developmental profile of siliques, seeds and seedlings. This is evident from genome-wide transcript analysis in public available 22K-cDNA-array data using Genevestigator (www.genevestigator.ethz.ch./at; Fig 29-31).

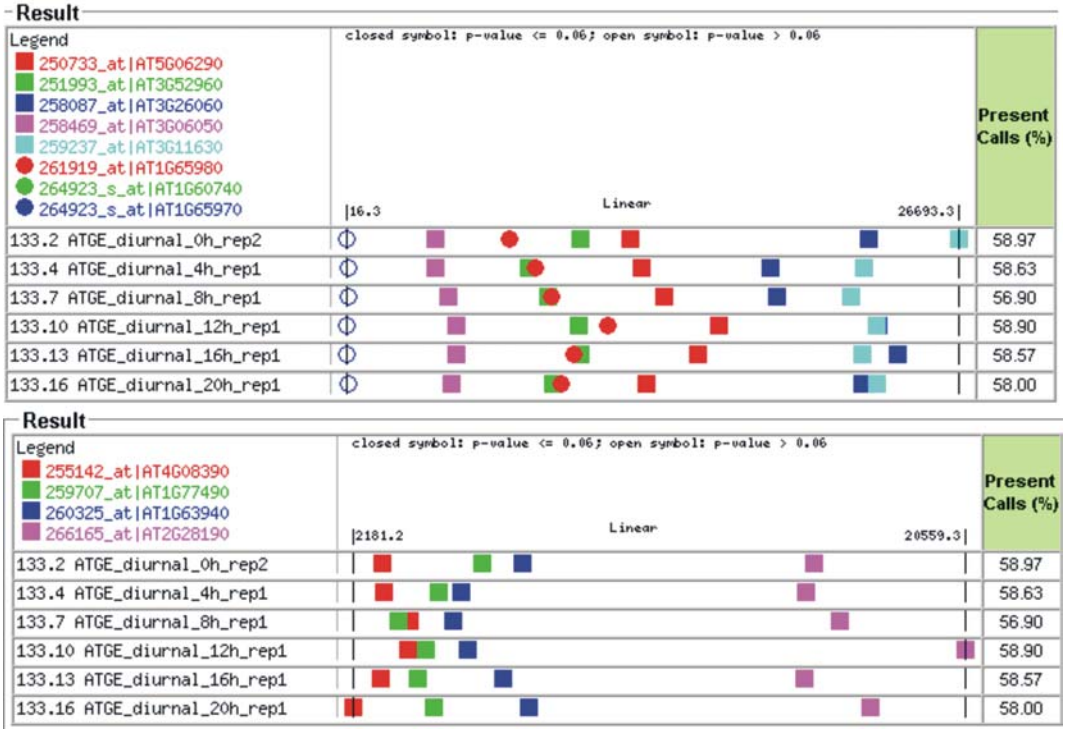

Fig. 29: **Changes of transcript levels in normal day-night cycle (AtGenExpress, Exp. 133).** Graphic display of the transcript abundance of the genes coding for enzymes involved in the Halliwell-Asada-cycle and of the members of the Prx family from *Arabidopsis thaliana* as detected by microarray hybridization (Genevestigator). 2Cys-PrxA, At3g11630; 2Cys-PrxB, At5g06290; PrxQ, At3g26060; PrxIIB, At1g65980; PrxIIC, At1g5970; PrxIID, At1g60740; PrxIIE, At3g5960; PrxIIF, At3g06050; Csd2, At2g28190; st APx, At4g08390; thy APx, At1g77490; MDHAR, At1g63940.

Transcript amounts of enzymes of the water-water-cycle were not seriously affected by the duration of the diurnal period in young plants (4 rossete leaves; Experiment 133, Mark Stitt Group, Golm, 2004; Fig 29). However all genes were induced in the different diurnal regimes with 2Cys-PrxA, PrxQ and Csd2 showing the strongest up-regulation. Organ specificity analysis (Experiment 90, Weisshaar

Lab., 2004) showed that PrxIIB was the enzyme with the strongest expression, which increased with development of the siliques of all ages. In seeds, this Prx was highly expressed with maximum transcript levels in the mature seeds. Csd2 showed also a strong induction in young siliques, mantaining a moderate up-regulation during maturation of the seeds (Experiment 90, Weisshaar Lab., 2004; Fig 30). The induction of the genes coding for enzymes of the Halliwell-Asada-cycle was developmentally regulated in seeds and siliques, presenting highest levels in the young organs. Thylakoid-bound APx was only weakly expressed in seeds, while stromal APx and MDHAR were more active (Experiment 90, Weisshaar Lab., 2004; Fig 31). Their transcript level declined during seed maturity.

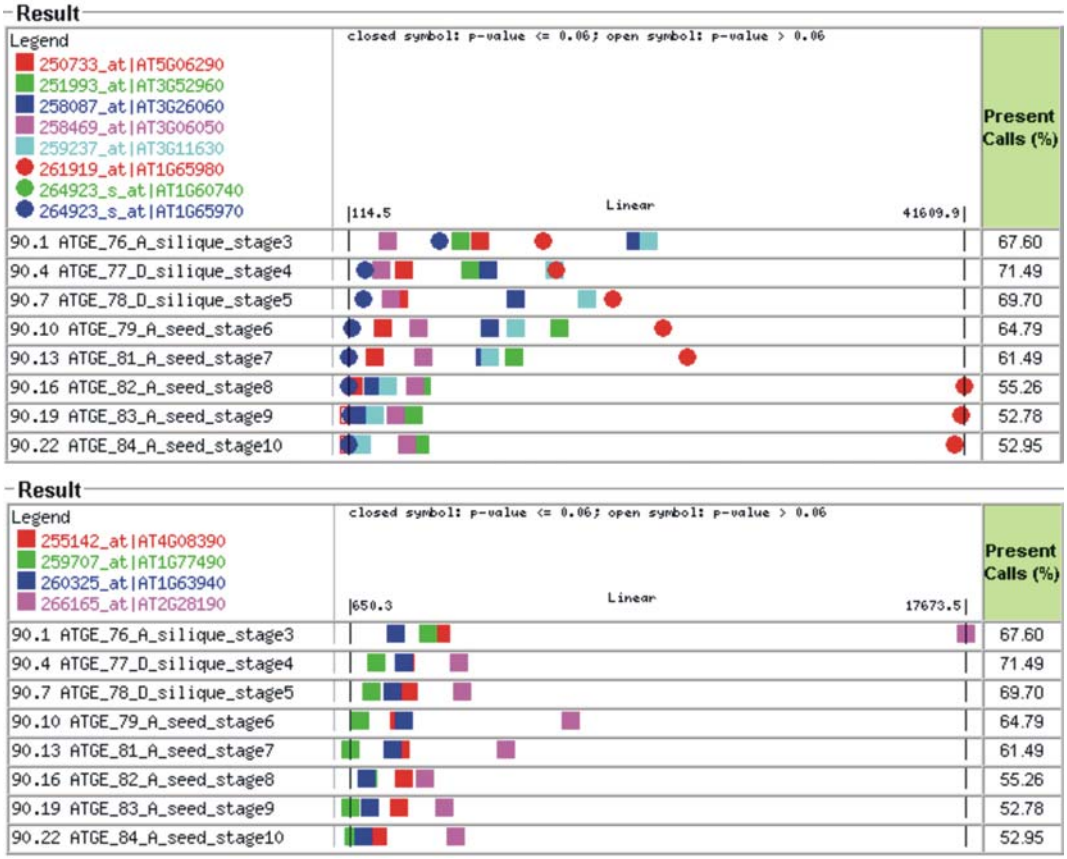

Fig. 30: **Seed and silique development (AtGenExpress, Exp. 90).** Graphic display of the expression of the genes coding for enzymes involved in the Halliwell-Asada-cycle and of the members of the Prx family from *Arabidopsis thaliana* as detected by microarray hybridization (Genevestigator). (2Cys-PrxA, At3g11630; 2Cys-PrxB, At5g06290; PrxQ, At3g26060; PrxIIB, At1g65980; PrxIIC, At1g5970; PrxIID, At1g60740; PrxIIE, At3g5960; PrxIIF, At3g06050; Csd2, At2g28190; st APx, At4g08390; thy APx, At1g77490; MDHAR, At1g63940)

Of the Prx, 2Cys-PrxA, PrxQ and PrxIIE showed strongest expression during seed and silique development. Their mRNA levels decreased during maturation. Conversely, PrxIIE maintained moderate expression in older seeds. Moderate to low transcript abundance was observed for PrxIIC only in siliques, but not in seeds (Experiment 90, Weisshaar Lab., 2004). In young seedlings, most of the Prx were up-regulated in green tissues (Experiment 87, Schmid et al., 2005). Highest transcript levels are indicated for 2Cys-PrxA and PrxQ. PrxIIC was observed only in roots, where PrxQ was absent. From the Halliwell-Asada-cycle genes, the highest transcript levels were shown by Csd2 in all tissues. Low expression of thylakoid-bound APx was observed in all tissues from young seedlings. In roots, it was absent, while in primary leaves was increased.

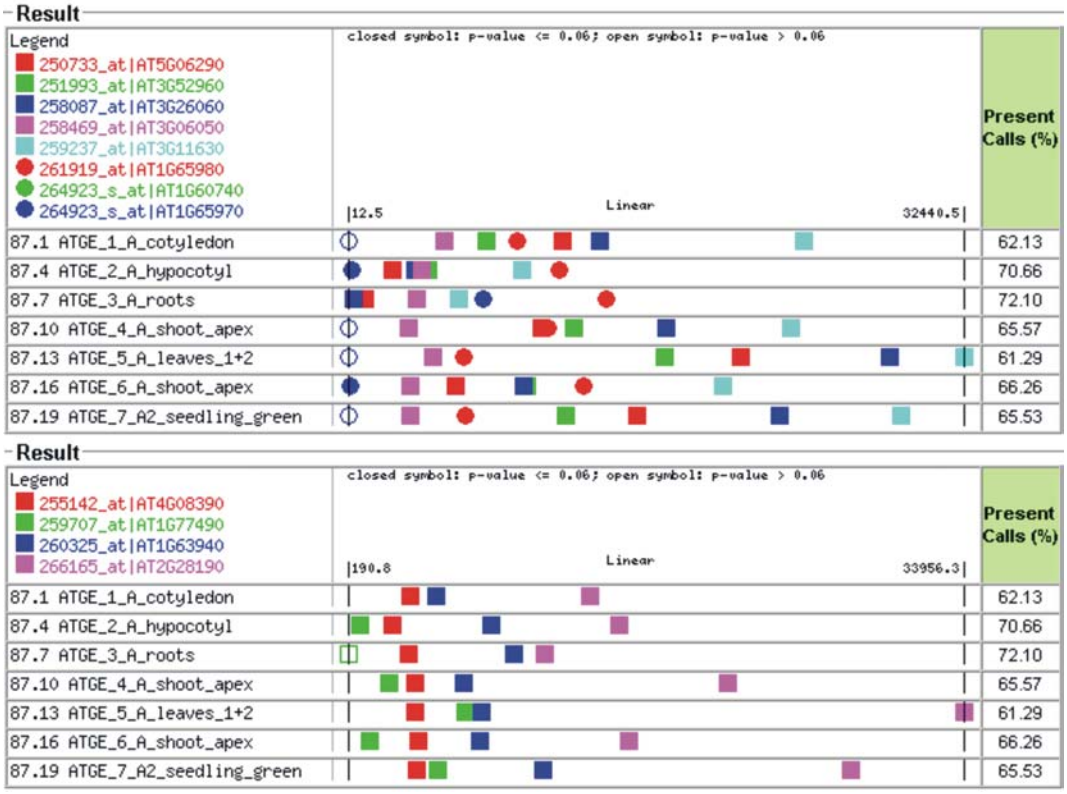

Fig. 31: **Baseline development I (AtgenExpress,Exp. 87)** Graphic display of the expression of the genes coding for enzymes involved in the Halliwell-Asada-cycle and of the members of the Prx family from *Arabidopsis thaliana* as detected by microarray hybridization (Genevestigator).. (2Cys-PrxA, At3g11630; 2Cys-PrxB, At5g06290; PrxQ, At3g26060; PrxIIB, At1g65980; PrxIIC, At1g5970; PrxIID, At1g60740; PrxIIE, At3g5960; PrxIIF, At3g06050; Csd2, At2g28190; st APx, At4g08390; thy APx, At1g77490; MDHAR, At1g63940)

In contrast, moderate up-regulation of the stromal Apx was present in all tissues. Irrespective from light time exposure, organ or tissue and developmental stage, the transcripts for the mitochondrial PrxIIF are constitutively available at similar levels (Experiments 87, 90, 133).

In the present work, the detailed gene-specific analysis for most chloroplastic antioxidant enzymes revealed higher transcripts amounts in S1 siliques than in leaves (Fig 20). Csd2 expression correlated with high generation of $O_2^{\cdot-}$. H_2O_2 produced by dismutation of $O_2^{\cdot-}$ can be scavenged by the two APx, the two 2Cys-Prx, PrxIIE and PrxQ. Most of these enzymes, together with MDHAR (which cover the last step of the water-water-cycle, regenerating the electron donors ascorbate) showed higher transcript abundance in the siliques than in leaves, especially at S1 (Fig 20). It has been reported that expanding organs such as embryonic axes (Puntarulo et al., 1988), growing roots (Jon et al., 2001), germinating seeds (Schopfer et al., 2001) and elongation zones of leaves (Rodriguez et al., 2002) generate ROS in the apoplast, where ROS is supposed to be necessary for cell wall growth (Schopfer et al., 2001; Rodriguez et al., 2002). In a converse manner, the main ROS source in mature leaves is photosynthesis (Foyer and Noctor, 2000). Consequently, gene induction may respond to the demand for antioxidant protection in young siliques, which are subjected to ROS generation by cell division and expansion. The genes for chloroplastic enzymes, with exception of both APx, decreased in siliques of the S3 stage, suggesting feed-back control. In siliques from the S1 and S3 stages the transcript abundances of the two APx remained constant (Fig. 20). During this period of development cell division and elongation take place in the siliques. Both processes utilize ascorbate (Liso et al., 1984, 1985), which influences DNA replication (Kerk and Feldman, 1995) and is the reductant for the hydroxylation of proline residues during extensin biosynthesis (Liso et al., 1985). High APx activity was reported in dividing cells and tissues undergoing differentiation (De Gara et al., 1996; de Pinto et al., 2000), whereas it decreases in senescent tissues (Borracino et al., 1994). Studies in *Triticum durum* seeds showed a highly reduced ascorbate pool at the beginning of kernel development, changing to an oxidized state during maturation (De Gara et al., 2003). The drop in the redox state of the ascorbate

pool correlates with the decrease in the three APx isozymes activity, which is undetectable at the end of kernel maturity (desiccated stage; De Gara et al., 2003). In the present study, the constitutive expression of APx and the transient decrease of MDHAR transcript amounts during silique development indicates differences in straining of the ascorbate pool during development, which is seven to tenfold lower in expanding cells than in meristematic cells (Arrigoni et al., 1975). A similar drop in ascorbate availability may explain the decrease in MDHAR levels at S3, where cell elongation predominates over cell division (Fig 20).

Except PrxIIE, the chloroplastic enzymes showed a marked decrease in their transcript amounts at S5 reaching levels that were 40% to 70% lower than those at S1 (Fig 20). At the S5 stage siliques only slowly expanded compared to younger siliques. In that case, photosynthesis is the major ROS source. Transcript regulation correlates with the photooxidative requirements. Low expression was observed in siliques next to the end of the maturity stage (Fig 2). Consistent with the PSII yields, the siliques were at the end of maturity photosynthetically competent without signs of senescence (Fig 17). Senescence is a highly regulated and genetically controlled process (Smart, 1994; Buchanan-Wollaston, 1997). Chloroplasts initiates senescence by undergoing a transition to gerontoplasts (Parthier, 1988). The envelope membranes remain stable while thylakoid membranes are completely degraded. In later stages of senescence, when ROS scavenging systems are no longer sufficient to repair the damage caused by increasing ROS production, plastid envelopes loose their integrity and plastids degrade (Krupinska et al, 1998).

A strong increase of transcript abundance at S5 (150% of the levels at S1) was observed for PrxIIE, demonstrating independent transcript regulation (Fig20). Bréhelin et al. (2003) reported transcript and protein patterns of the type II Prx from *Arabidopsis thaliana* in several tissues. PrxIIE transcription was shown to take place mainly in green tissues and hardly in roots, with high predominance in buds, siliques, seeds and 10 days old seedlings (Bréhelin et al., 2003). A similar pattern was observed on the protein level. Histochemical localisation of GUS activity in *At*PrxIIE::GUS plants was also performed showing blue staining in stamen from young flowers, the embryo sac of young seeds and the albumen of

older seeds from green or yellow siliques, consistent with the observations in the present work. It is supposed that seed development in siliques of S5 stage influenced the transcript levels. At that stage, seeds cover the major volume of siliques. Because plastids of seeds differ from those in the silique wall, it is postulated that PrxIIE has a protective function that is not restricted to chloroplast, but is also present in elaioplasts in anther tapetum to prevent lipid peroxidation (Bréhelin et al., 2003). It is tempting to assume that PrxIIE has similar function in seeds, where it protects plastids subjected to differentiation.

4.2.2.3 Transcript abundance of the mitochondrial PrxIIF

Mitochondrial redox metabolism provides another important intracellular source of ROS (Section 1.4.1). PrxIIF is one of their important protective antioxidative systems (Finkemeier et al., 2005). Finkemeier et al. (2005) showed that PrxIIF expression is stimulated upon increased oxidative stress and that loss of PrxIIF function causes higher stress susceptibility. Here, like during early post-germinative growth (Fig 20) PrxIIF showed a constitutive expression between the stages S1 and S5 and in leaves (Fig 20). In seedlings, only slight fluctuations were observed upon day/night cycles (Fig 12; Section 3.1.7) demonstrating that PrxIIF transcript abundance is not regulated by developmental processes and that this gene is constitutively expressed in various tissues. However, in seedlings transcriptional regulation of PrxIIF was shown in response to diurnal fluctuations similarly to the chloroplastic antioxidant enzymes. It is concluded that the influence of light on PrxIIF regulation may be due to the light-dependent redox signals from the chloroplasts (Section 4.1.5.3).

4.2.2.4 Transcript abundance of the cytosolic Type II Prx

ROS in the form of O_2^- can be produced in the cytosol by permeation of nitric oxide and H_2O_2 through the peroxisomal membrane (Corpas et al., 2001). ROS scavenging systems in the cytosol are from highest importance especially in the seeds during the rapid oil storage period. At this stage, the risk for lipid peroxidation is highest. It has been reported that nascent oil bodies in young rape seed embryos may have only a surrounding phospholipid monolayer, but are still able to fuce together and hence grow in size (Murphy et al., 1989). The insertion into the oil body of its characteristic membrane proteins occurs towards the end of

seed development (Murphy et al., 1989). However, in siliques like in seedlings, the cytosolic PrxIIB, PrxIIC and PrxIID were regulated independently from each other pointing at responsiveness to different signals (Fig 11, 12, 20).

Bréhelin et al. (2003) detected PrxIIB transcript and protein in all green tissues and in roots from *Arabidopsis thaliana*, but preferentially in reproductive organs such as buds, flowers, siliques and seeds. In contrast, here PrxIIB showed a constitutive expression in siliques and mature leaves. Estimated from the relative cycle number necessary for amplification, similar DNA amounts by RT-PCR, transcript levels are similar like those for the chloroplastic enzymes (Fig 20). The difference between the observation in this work and that from Bréhelin et al. (2003) could be associated to the developmental stage of the siliques and seeds analyzed. Expression of PrxIIB may increase in older siliques, which are subject to dessication. On the other hand, Horling et al. (2003) reported induction of PrxIIB expression for leaves in presence of butyl hydroperoxide, which is a lipophilic peroxide and suggested a protective function on the plasma membrane. Consequently, in tissues subjected to dehydration, such as mature seeds, siliques in the desiccation stage and antheres membrane lipids are more exposed to lipid peroxidation, which could be counteracted by up-regulation of PrxIIB.

PrxIIC transcripts were markedly higher in S1 than in mature leaves (Fig 20). They decreased to steady state level in S3 and S5, while PrxIID showed a constitutive expression pattern in siliques, which was a stronger than in leaves (Fig 20). In both cases the relative transcript abundance was lower than those of the other Prxs, demonstrating weaker expression of PrxIIC and PrxIID in siliques. Previous reports suggest to a protective role of PrxIIC and PrxIID in pollen (Bréhelin et al., 2003), which are exposed to increasing free radical production during desiccation. In the case of young siliques of S1, the loss of moisture is low (Baud et al., 20002). Water content in *Arabidopsis* seeds decreases almost linearly during seed maturation. At the end of maturation, the water content reaches approximatly 50 %. This decrease is caused by a gradual accumulation of dry matter (Barlow, 1980). In the desiccation stage the dehydration process takes place, reducing the moisture content finally to ca. 7 % (Baud et al., 20002). Thus, induction of PrxIIC in young siliques cannot be related to protection of seeds

against dehydration. Horling et al. (2002) reported high efficiency for PrxIIC to decompose H_2O_2 *in vitro* and up-regulation under stress conditions in leaves. It has been suggested that any deviation from steady state activates the promoter. Consequently, the induction of PrxIIC, so as of the chloroplastic enzymes, in the young siliques (Fig 20) may be caused by redox or metabolic imbalances resulting from cell division, elongation and activation of metabolism. The decrease in S3 and S5 to levels similar to that found in leaves demonstrated down-regulation to steady state conditions and supports, consistently with Horling et al.´s previous hypothesis, that PrxIIC is only transiently activated by redox imbalances.

In the case of PrxIID, up-regulation in the early embryogenesis stage and during maturation is also not associated with dehydration process. Siliques from S1 stage to S5 stage had a high photosynthetic activity (Fig 17), which supplies carbohydrates to the seeds required for their optimal development (King et al., 1997). The constitutive pattern in the transcription in siliques from S1 to S5 (Fig 20) suggests induction by metabolic processes present in siliques but absent (or in a very low level) in leaves. The expression pattern of PrxIID in seedlings under day/night-cycles (Fig 12; Section 3.1.7) showed regulation on the transcript level in the dark periods indicating that the expressional response is related to sugar metabolism.

4.2.3 Prx expression in *Arabidopsis thaliana* and *Brassica napus*

Brassica napus is one of the most important members of the Brassica family due to its use in agriculture and industry. Since long, the research on Brassica species is from great interest for the biotechnology industry, but the information about their genomes is still very limited. Meanwhile, availability of the complete genome sequence of Arabidopsis is a helpfull tool to study gene regulation in oil seed plants.

Search in Brassica species sequence libraries for Prx was successful only for 1Cys-Prx, 2Cys-Prx and one type II Prx with high similarity (91 % to 96 %) to the cytosolic Prx and 60 % identity to the chloroplastic PrxIIE in *Arabidopsis thaliana* (Fig 23-26). No sequence information was found about a PrxQ or a PrxIIF in any Brassica species. Protein amounts were analyzed in both plants

using polyclonal antibodies against *At*PrxIIC (Horling et al., 2002), *At*PrxIIF (Finkemeier et al., 2005), *At*PrxQ (Lamkemeyer et al., 2006) and 2Cys-Prx (BasFus; Baier and Dietz, 1997). The existence of a PrxQ-like protein and of a mitochondrial Prx in *Brassica napus* enabled analysis of the Prx family in various tissues.

Prx protein amounts were compared in *Arabidopsis thaliana* and *Brassica napus* in various organs and during silique development (Fig 26-28). For better understanding of Prx expression in seeds, immunoblotting was performed with protein extracts from silique walls and seeds. Silique walls were obtained from a middle (S2-S3) and the last stage (S5) and seeds from the S5 stage of Arabidopsis and compared with carpels and seeds from a middle stage (S2-S3) in Brassica (Fig 28).

4.2.3.1 Organ specificity of chloroplastic Prxs

The chloroplastic 2Cys-Prx and PrxQ were detected in all green tissues, but not in roots in both plants, Arabidopsis and Brassica (Fig 26-27). Histochemical localisation of promoter activity using *At*2Cys-PrxA::GUS reporter gene lines showed transcription of 2Cys-PrxA in all green tissues, but also in the stigma of flowers (Fig 21) and in the central cylinder of seedlings (Fig 15), demonstrating expression in presence of other plastids. In siliques, the protein abundance of 2Cys-Prx and PrxQ decreased gradually with development. Highest accumulation of PrxQ was observed in leaves in both plants. In Arabidopsis, the high levels of both Prx in young siliques and the decrease after S3 was consistent with the observations on transcript level (Fig 20). However, the transcript levels in leaves were lower than in young siliques, while their protein amounts were higher, demonstrating post-transcriptional regulation. Baier and Dietz (1999) showed that 2Cys-Prx protein accumulates with leaf age. However, in siliques, 2Cys-Prx protein amounts decreased with age in *Arabidopsis thaliana* and *Brassica napus* (Fig 26, 27). A possible explanation could be the influence of seeds on total protein amount. Seeds grow and gain weight with development, reaching a significant quantity of the total protein in older siliques. Histochemical detection of promoter activity in *At*2Cys-PrxA::GUS plants showed transcription in the silique wall but not in the seed coats of siliques of all ages (Fig 21 C-E).

2Cys-Prx protein levels in Arabidopsis were similar in silique walls from S2-S3 and S5. 2Cys-Prx protein was absent from seeds in the S5 stage (Fig 28). In contrast, *in Brassica*, protein was detected in both silique walls and seeds. The chloroplastic 2Cys-Prx are induced in photosynthetically active tissues (Baier and Dietz, 1997). Seeds from Brassica are green and the seed coat is also photosynthetically active at that stage (Eastmond et al., 1996), while seeds from S5 in Arabidopsis have chlorophyll free seed coats (Fig 28). However, expression of 2Cys-Prx may also take place in the period of fatty acid synthesis in Arabidopsis seeds at a very low levels. Arabidopsis seeds are photosynthetically active. Several authors proposed a contribution of embryo photosynthesis to local metabolism rather than producing carbon for export as in source tissues. Three functions of seed photosynthesis have been postulated: 1) As an alternative pathway for NADPH and ATP production for the high demand in fatty acid synthesis (Eastmond et al., 1996, Asokanthan et al., 1997, Ruuska et al., 2004); 2) Prevention of anoxia inside the seeds when during seed coat development O_2 diffusion becomes limited, (Rolletschek et al., 2002; Vigeolas et al., 2003); 3) Improvement of biosynthetic efficiency of seeds by refixing respiratory CO_2 and the CO_2 released by fatty acid synthesis (King et al., 1998, Ruuska et al., 2004). Ruuska et al. (2002) reported high expression of genes involved in photosynthesis during this phase, suggesting that also 2Cys-Prx may be active.

Alternatively, explanations for the low contribution of Arabidopsis seeds to the total protein amounts in siliques from the maturity stage (from S2-S3 to S5) can be derived from the following studies. First, Eastmond et al. (1996) reported transmittance of light through the silique wall during *Brassica napus* embryo development with values between 29 and 34 %. This transmittance increased to 47 % only in desiccated seeds. In young siliques the transmitted light available for a seed is enriched with green light, which may be not effectively absorbed by chlorophyll (Ruuska et al., 2004). Therefore, the pigment composition shows shade adaptation (Ruuska et al., 2004). It is postulated that the low light availability inside the seeds keeps the expression of other chloroplastic enzymes low in comparison to the silique wall. This observations can also explain why

PrxQ protein was not detected in seeds of both plants. It can be assumed that PrxQ regulation was more sensitive to light availability.

Secondly, Baier et al. (2004) reported a positive correlation between the reduction state of NADPH/ $NADP^+$ and 2Cys-PrxA promotor activity in leaves. In high CO_2 and low O_2 the carboxylation of ribulose-1,5-biphosphate is favoured and consequently, the NADPH / $NADP^+$ ratio is low in the chloroplast resulting in a low 2Cys-PrxA promotor activity. Studies with siliques of *Brassica* plants growing under ambient oxygen concentration showed that oxygen is present at a concentration of only 0.8% [v/v] in the seeds (Vigeolas et al., 2003). It indicates that light-stimulated O_2 consumption, such as photorespiration or O_2 photoreduction are less efficient than respiratory activities (King et al., 1998). In addition the low endogenous O_2 concentration also suppresses photorespiratory and O_2 photoreduction reactions. Therefore, although photosynthetic activity was observed in seeds, ROS produced by the reductive power generated in the chloroplast can be assume to be minimal compared to that in the silique wall. Consistently, expression of 2Cys-Prx and PrxQ is less in seeds than in the silique wall.

PrxQ protein accumulation decreased from carpels in the S2-S3 stage to carpels in the S5 state, demonstrating that the decline during silique development was caused exclusively by the decrease of PrxQ-protein in the silique wall.

4.2.3.2 Organ specificity of the mitochondrial PrxIIF

The mitochondrial PrxIIF was detected in all tissues in both species. In *Arabidopsis*, PrxIIF showed the highest protein levels in roots and in the oldest investigated development stage of siliques (S5; Fig 26). Different to the transcript levels, the protein amounts increased transiently during siliques development, indicating post-transcriptional regulation, according to the increasing mitochondrial metabolism.

In Brassica, the PrxIIF protein levels were similar in the silique wall and in seeds (Fig 28). The protein amounts in silique walls decreased slightly from the middle to the last stage in Arabidopsis. The PrxIIF amounts in the seeds were higher than in the silique walls at the S5 stage and similar to those from the younger ones, consistent with the increase in total PrxIIF observed in the siliques during

development (Fig 26). Based on studies by Finkemeier et al. (2005) on PrxIIF expression in roots and leaves a correlation between respiration rates and post-transcriptional induction, or eventual protein accumulation is suggested. An increment of mitochondrial generation of H_2O_2 in the silique is expected. In the silique wall, CO_2 fixed by photosynthesis is mostly supplied as to sucrose (King et al., 1997), which is necessary for the metabolism in the silique wall and for support of seed growth (King et al 1997). The products of sucrose cleavage are converted to hexose-P entering the respiratory pathways via glycolysis to provide substrates and reducing power for growth and storage product synthesis (King et al 1997). Contrary, seeds are subjected to different metabolic processes demanding different respiration rates. The early embryo stages (S1 to S2) are dominated by a carbohydrate metabolism with consumption of hexoses and sucrose imported mainly from the silique wall to cover the demand for mitotical growth and cell elongation and accumulation from starch (Baud et al., 2002, King et al., 1997; Hill et al., 2003). Fatty acid biosynthesis starts during maturation, which involves high consumption of energy and carbon skeletons and increases the respiration rates (Kang and Rawsthorn, 1994). It can be concluded that the increment of PrxIIF during silique development is influenced by seed metabolism.

4.2.3.3 Organ specificity of cytosolic and chloroplastic type II Prx

Type II Prx (PrxIIB, PrxIIC, PrxIID and PrxIIE in Arabidopsis and a Type II Prx in Brassica) were recognized by the antibody against *At*PrxIIC. In Arabidopsis high protein levels were detected in roots, shoots, flowers and young tissues as siliques from S1 and S2 stages (Fig 26), while in *Brassica napus* the highest protein levels were observed in siliques from S1, S2 and young leaves (Fig 27). The protein amounts decreased in older siliques and in mature leaves in both plants, and were absent from senescent leaves (L3) in Brassica. Inconsistent with the suggestion of Brehélin et al. (2003) regarding a protective role of the cytosolic Prx against desiccation, here, accumulation of cytosolic and chloroplastic type II Prx is shown in Arabidopsis tissues, which are not subjected to dessication (Fig 26). Tissues as young siliques, roots and shoots have the capacity for cell division, elongation and differentiation. In roots, cell division takes place in apical meristem and in shoots, in meristem cylinders. In flowers, these processes take

place in the reproductive organs: ovules and pollination tubes. Whithout fully contradicting the hypothesis from Brehélin et al. (2003), it can be suggested that the induction of antioxidative systems such as the cytosolic Prx and the PrxIIE in young and/or meristematic tissues plays a role in protection of membranes against lipid peroxidation by developmentally triggered ROS sources. In addition, as postulated by Dietz (2003a) these enzymes may have a signalling function by antagonizing ROS-signals.

Consistent with the decreased accumulation during development, silique walls from *At*S2-S3 contained more protein than those in *At*S5. The protein levels were lower in older seeds than in the silique walls from the same stage in Arabidopsis, but younger seeds showed equivalent Prx levels as in Brassica. These results indicate that the protein levels decreased in both carpels and seeds with development, confirming the importance of these enzymes for the protection of young tissues.

4.2.4 Conclusion and perspectives

Siliques are exposed to high ROS production, especially in the early stages of development, requiring the coordinated action of different antioxidative systems such as the ascorbate-dependent water-water-cycle on the one hand, and the Prx on the other hand. Prx have a capacity to scavenge small organic hydroperoxides as well as with lower activity complex lipid peroxides. A dominant role of Prx can be proposed in the protection of siliques of oil storage plants.

The analysis of Prx expression in the silique wall and seeds, indicates that the importance of these enzymes is not restricted to the silique wall. On-going work demonstrates chlorophyll accumulation in young Arabidopsis embryos and in the seed coat (Fig 18). Seeds of the S1 stage are in an early embryogenesis stage showing chlorophyll accumulation only in the seed coat (Fig 18 A). In the S3 stage, chlorophylls accumulate also in the embryos, which are in the torpedo stage (Fig 18 E-F). Reports on canola seeds indicate that although testa and embryo in early and mid-cotyledonary stages contained both chlorophyll and were photosynthetically active, only the embryos essentially accounted for all of the photosynthetic and respiratory activity present in the seed (Eastmond et al., 1996).

Next to the end of maturation (S5), only the embryo contained high chlorophyll levels, while the seed coats lacked chlorophyll-a fluorescence and revealed emission only observed in the green-yellow region of the visible spectrum (Fig 19 A-B). This suggests accumulation of other pigments such as carotenoids and flavonoids. It is interesting to note that the pigment distribution in embryos of this stage was already differentiated: shoots and cotyledons accumulated chlorophyll, while it was abscent in the axial and radicle meristems. These observations demonstrate the dynamic evolution of the differentiation processes in the seeds and suggest an adaption of antioxidative systems. Immunolocalization of the chloroplastic, mitochondrial and cytosolic Prx has been initiated to study regulation of antioxidant enzymes with the seeds and especially in the embryos.

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Apendix A

Primers and annealing temperatures used for transcript quantification by RT-PCR:

Gene	**T_A**	**Gene code**	**Forward primer / reverse primer**
actin	55 °C	At5g09810	TACAACGAGCTTCGTGTTGC
			GGACAACGGAATCTCTCAGC
2-Cys PrxA	55 °C	At3g11630	TCAGCAAAGAGTACTTCTCAGCTATT
			GGGGACAAAGTGAGAATCAAA
2-Cys PrxB	55 °C	At5g06290	CCCCAAGCTCAGCAAAGAAT
			GCAGAAACAGGAGAAAGATGAGA
Csd2	50 °C	At2g28190	CTCCGTTCCTCTTTCAGC
			GCGTCAAGCCAATCACAC
PrxIIB	55 °C	At1g65980	AGCCAATGTCGAATCTGGTG
			CTTGGTTAATGTCTGAGAACAAGC
PrxIIC	55 °C	At1g65970	GTTGAATCTGGTGGCGAGTT
			GCCAAGCAAGTCAAACACAT
PrxIID	55 °C	At1g60740	GAAACATTATCATTCGCTTGTTG
			CAAGTCAATACACTTGCTTGTTGAT
PrxIIE	55 °C	At3g52960	TCTTGAAGAAGGAGGTGCTTT
			CAAATCCGATAATGATTCGTAGA
PrxIIF	55 °C	At3g06050	CCAGAGTTGCTGTTTCTTCTGA
			TGAAACTGTGGAAACAAAACAA
PrxQ	55 °C	At3g26060	CAGTTCCAGCCTGAGAAACA
			TGAAAATTCATACTCAACAGAAACAA
sAPx	*50* °C	At4g08390	TGGGGGCACATAATTTACTCA
			CGTTTCTCTAAAATCATAAGTCCTG
tAPx	50 °C	At1g77490	ATTTTGATTTCTCTTACCTACATACAT
			AAGGTGATTTTATTCAAGCAAAC
MDHAR	50 °C	At1g63940	TTGGAATTGGAGCTAAGC
			TTCTTCGACTGAAGATGC
ICL	58°C	At1g21440	GCAGCTACTCTGATCCAGTCG
			GTCTGCGATCTGTGCAAAGG

Apendix B

$$\Phi PSII = (F_M' - F_S) / F_M'$$

$$\Phi PSII_{max} = (F_M - F_S) / F_M$$

$$qP = Fq' / Fv'$$

$$NPQ = F_M / F_M' - 1$$

Fs : The fluorescence signal at any point between *Fo′* and *Fm′*.

Fm : The maximum fluorescence signal (when all PSII centres are in the closed state) from dark-adapted material.

Fm′ : The maximum fluorescence signal (when all PSII centres are in theclosed state) from light-adapted material.

Fo : The minimum fluorescence signal (when all PSII centres are in the open state) from dark-adapted material.

Fo′ : The minimum fluorescence signal (when all PSII centres are in the open state) from light-adapted material.

Fq′ : The difference between *Fs* measured immediately before a saturating pulse and *Fm′* measured at the peak of the same saturating pulse.

Fv : Variable fluorescence (difference between *Fo* and *Fm*) from dark-adapted material.

Fv′ : Variable fluorescence (difference between *Fo′* and *Fm′*.) from light-adapted material

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst angefertigt habe und nur die angegebenen Quellen und Hilfsmittel verwendet habe. Alle aus der Literatur ganz oder annähernd entnommenen Stellen habe ich als solche kenntlich gemacht.
Weiterhin erkläre ich, dass die vorliegende Dissertation weder vollständig noch teilweise einer anderen Fakultät mit dem Ziel vorgelegt worden ist, einen akademischen Titel zu erwerben. Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Universität Bielefeld.

Bielefeld, den 10. April 2006