

# Hypoxia – a phenomenon which shapes seed metabolism

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## Zusammenfassung

Die menschliche Ernährung basiert ganz wesentlich auf der Nutzung von Samen unserer Kulturpflanzen. Die Untersuchung von Prozessen, die sowohl die Quantität als auch die Qualität von Lipiden, Proteinen und Kohlenhydraten beeinflussen können, ist somit von grundlegender Bedeutung. Die vorliegende Arbeit beschreibt das Auftreten von Sauerstoffmangelsituationen (Hypoxie) während der Samenentwicklung und wie sich diese auf den Metabolismus der Samen auswirken. Mit Hilfe von sauerstoffsensitiven Mikrosensoren und Sensorfolien wurde erstmals die Konzentration und die Verteilung von Sauerstoff in sich entwickelnden (und keimenden) Samen von agronomisch wichtigen Kulturpflanzen vermessen. Samenhypoxie ist ein natürlicher Zustand, der sowohl entwicklungs- als auch umweltabhängig ist. *In vitro* Experimente sowie Versuche mit transgenen Modellpflanzen konnten zeigen, daß Stickstoffmonoxid an der Regulation von Respiration und endogener Sauerstoffbalance in Samen beteiligt ist. Die Energiebedürftigkeit der jeweiligen Biosynthesewege bedingt, dass die Akkumulation von Proteinen und Lipiden stärker durch Hypoxie limitiert ist als die von Stärke. Die Regulation des zentralen Stoffwechsels durch Hypoxie kombiniert transkriptionelle und posttranskriptionelle Mechanismen. So konnte gezeigt werden wie im Samen auftretende Hypoxie zu Anpassungen in der lokalen metabolischen Aktivität führt. Die daraus resultierende Akklimatisation ist für die Nutzungseffizienz von Kohlenstoff, Stickstoff und Energie vorteilhaft. Es kann folglich geschlussfolgert werden, dass die innerhalb der Samen entstehende Hypoxie den Stoffwechsel in charakteristischer Weise prägen kann. Die Mechanismen der Anpassung sind Teil des natürlichen Entwicklungsprozesses der Samen.

## Summary

Both the human and domesticated animal diets are heavily dependent on the carbohydrate, oil and protein accumulated in the seeds of crop plants, and seeds also provide raw materials for a number of industrial products. Thus, a knowledge of the factors which affect the quantity and quality of the seed is of great relevance. I describe here how oxygen deficiency (hypoxia) is a commonplace occurrence in the seed, and detail its effect on restricting respiration, central metabolism and storage product synthesis. Microsensors and planar sensor foils have been adapted to elaborate a profiling procedure of the concentration and distribution of oxygen in the developing (and the germinating) seed of a range of crop species. This endogenous hypoxia is both developmentally and environmentally regulated, with the steady state oxygen level being balanced by a process possibly involving nitric oxide. The *in vivo* accumulation of both lipids and proteins is limited by the low prevailing oxygen level in the seed, but that of the energetically cheaper storage compound starch is little affected. The control over this restriction comprises a combination of transcriptional, translational and post-translational mechanisms. Localized hypoxia is dealt with by the developing seed by instigating a series of local adjustments to their metabolic activity. The resulting acclimation to hypoxia may be beneficial in terms of the seed's carbon, nitrogen and energy-use efficiency. The global picture is therefore one where endogenous hypoxia shapes the seed's metabolism in a balanced manner, and that the induced acclimation is a part of the normal process of seed maturation.

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## Part A: Hypoxia – a phenomenon which shapes seed metabolism

### 1. Introduction and the aims of research

The modern atmosphere contains approximately 21 kPa oxygen, but this level has fluctuated from 15 to 35 kPa over the course of the past 550 million years, during which time the vascular plants invaded the land surface. This variation is held to have been an important evolutionary driver of the architecture and metabolism of plants (Raven, 1991; Berner, 1999), but at the same time, plants also can be exposed to significant short-term changes in the supply of oxygen in their immediate environment, most notably during soil waterlogging, which is thought to be responsible for substantial losses in crop production every year. The rate of diffusion of a gas through a liquid medium is four orders of magnitude slower than through air, so waterlogging rapidly induces hypoxia and eventually even anoxia in the root. The former state limits mitochondrial ATP production (oxidative phosphorylation), whereas the latter suppresses respiration altogether, since no oxygen is available.

The hypoxic state comes about whenever the capacity for oxygen diffusion is restricted, so that the concentration of oxygen available falls below the level required for regular cellular metabolism. These conditions apply most frequently in the root and other below ground organs, but can sometimes also obtain in aerial organs such as the stem or the fruit (for review see [8]<sup>1</sup>). With respect to developing seeds, there have long been a number of reports assuming the existence of oxygen-depleted zones, indicated by the presence of enzymatically active alcohol dehydrogenases and the release of ethanol (Wager, 1974; Boyle & Yeung, 1983), and also by the developmental decline in the cytosolic pH (Gambhir et al., 1997). Soybean seeds provided with supplemental oxygen respond by an increase in respiratory activity, and demonstrate a characteristic change in the ratio of adenine nucleotides present (Gale, 1974; Shelp et al., 1995). The correlation between seed number and size and the prevailing level of gaseous oxygen in *Arabidopsis thaliana*, rice, soybean and wheat (for review see [8]) indicates that substantial oxygen concentration gradients are necessary to force the diffusion of adequate quantities of oxygen into the seed. Although these observations can at best provide only indirect evidence, nevertheless they have engendered the hypothesis that hypoxia is a frequently encountered state at specific stages of seed development (particularly in large-seeded species). However, until recently there has been no means of directly measuring a seed's oxygen status, and thus no solid evidence as to how hypoxia affects seed metabolism.

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<sup>1</sup> Numbers in square parentheses refer to own publications accompanying this Habilitationsschrift (as listed on page 21 and 22).

The significance of studying the occurrence and effects of hypoxia in the developing seed rests on the importance of the seed to both human and animal nutrition. If, as is believed, endogenous hypoxia restricts respiration, storage product accumulation and in the final basis, seed yield, some priority needs to be given to measures aimed at alleviating the constraints imposed by hypoxia, and/or to avoid it altogether. A detailed knowledge of the factors determining the severity and localization of hypoxia is needed to formulate a realistic strategy to achieve these objectives.

My research programme directed at defining the “where” and “when” of hypoxia in the developing seed has required the solving of a range of scientific and technical issues:

- A procedure was needed to determine the oxygen profile within a living seed, so that a quantitative analysis of oxygen concentration and distribution could be carried out.
- The factors which most heavily influenced the oxygen status of the seed needed to be identified.
- An understanding was necessary regarding the mechanisms both via which the endogenous steady state oxygen level is controlled *in vivo*, and via which the detrimental effects of hypoxia on seed development are avoided.
- A quantification of the effect of oxygen availability on gene expression, enzymatic activity, metabolite pool sizes and metabolic fluxes was required, as these processes determine the rate of accumulation of seed biomass and individual storage products.

The developing seed (in the case of the cereals, the caryopsis) of crop species representative of the major types (legumes, cereals, oilseeds) and of the model plant *A. thaliana* have provided the experimental subject of most of the relevant research. While the major focus has lain with the developing seed, some attention has also been given to an analysis of the oxygen status of the germinating seed.

## **2. Oxygen concentration and distribution in the seed**

### **2.1 A method for the spatial resolution of oxygen concentration in the developing seed**

Oxygen-sensitive microsensors enable the precise measurement of oxygen concentrations within a localized region, such as the root and (in legumes) the root nodule (Bowling, 1973; Witty et al., 1987; Armstrong et al., 1994). Most of the devices currently available are based on miniaturized Clark-type electrodes (Revsbech & Jørgensen, 1986; Armstrong et al., 2009), which can deliver a spatial resolution at the low  $\mu\text{m}$  scale. Increasingly, however, this technology is being replaced by optical oxygen microsensors (micro-optodes) based on fibre optic materials (Klimant et al., 1995). Notably, the spatial resolution achievable using micro-optodes is lower ( $\sim 30\text{-}50\mu\text{m}$ ) than is provided by miniaturized Clark-type electrodes, but there are also a number of advantages [7]. In our standardized procedure, a micro-manipulator is used to drive a micro-optode into the seed in a series of timed steps, thereby capturing the local oxygen concentration along a transect [7]. Following this, the seed is dissected to relate the measured oxygen concentrations with each of the structurally distinct zones within the seed (i.e., seed coat, vacuole, embryo and endosperm).

In most recent work, we are developing a novel oxygen sensing approach, with the aim of generating a bi-dimensional distribution of oxygen concentration [4]. In this approach, an optical sensor foil (i.e. the planar optode) is attached to the surface of the sample, and is designed to convert the light signal into an oxygen measurement. Since a single image captures an array of sensor points, the system can capture the oxygen distribution over a specific area at a resolution level of  $\sim 2\mu\text{m}$ . While similar approaches have been described in the literature (Liebsch et al., 2000; Glud et al., 2005; Kühn & Polerecky, 2008), the system we have developed represents a significant improvement with respect to spatial resolution, handling and image processing, and thus to its overall ease of use. The planar sensor system has been used, for example, to monitor the oxygen status within a complex root system over a prolonged time period, and also has proven informative when applied to seed, stem or tuber cross-sections.

### **2.2 Oxygen profiles in the developing seed of major crop species**

The microsensor approach has allowed for the acquisition of seed oxygen concentration profiles from barley [17], wheat (unpublished data), maize [13], broad bean [20,22], soybean [15], pea [20,22], oilseed rape [8] and sunflower [12] (Fig. 1). Despite the wide variation in morphology, size, pigmentation, major storage products, etc. displayed by these seeds, in all of them the oxygen level drops markedly towards the seed's interior, reaching a steady state concentration of  $\sim 1\text{-}20\mu\text{M}$  when measurements are made in the dark. This concentration appears low enough to partially inhibit respiratory activity in the seed (see §5.1), and

indicates that seed hypoxia is a commonplace occurrence in the developing seeds of our major crops.

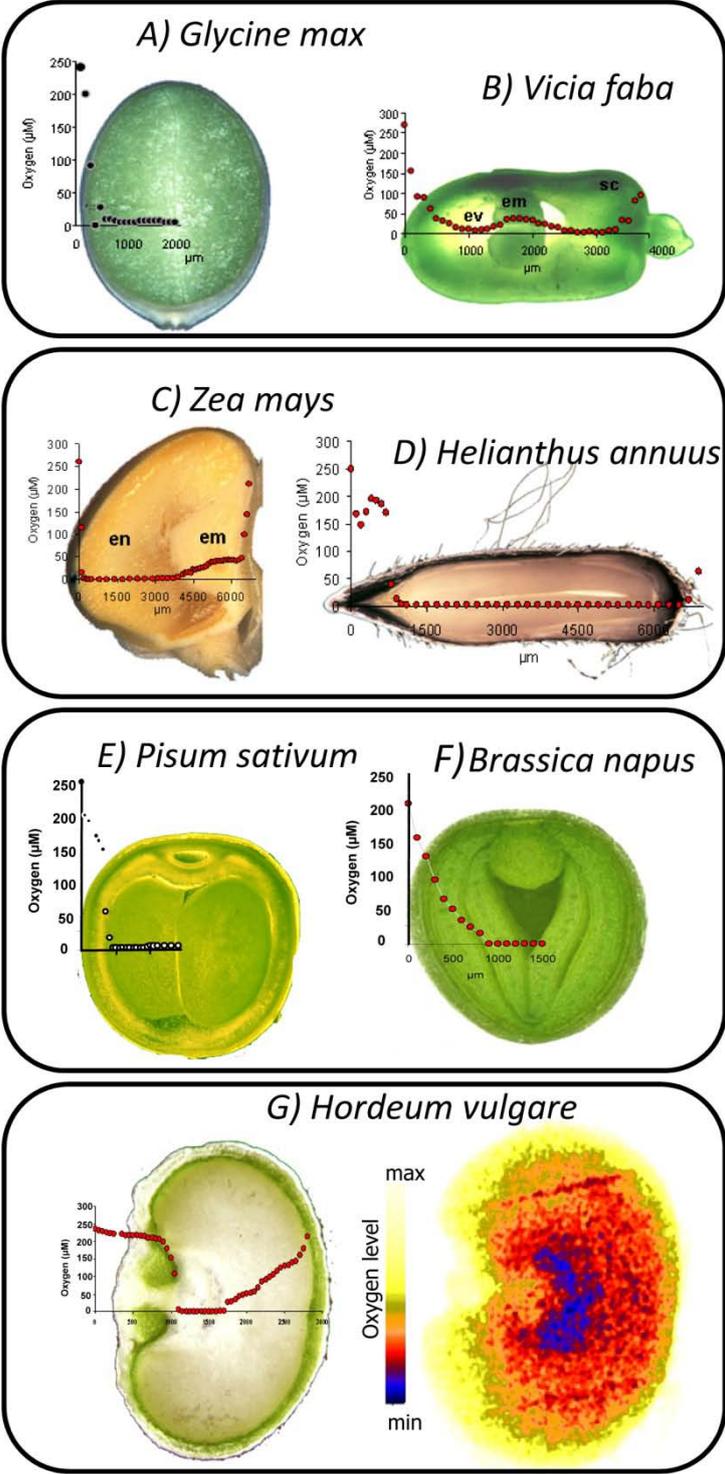


Figure 1: Characteristic oxygen concentration profiles measured in seeds of major crops. A,C-F: Measurements under non-lit conditions; B: Measurements under lit-conditions; G: comparison of oxygen distribution in a barley caryopsis measured under non-lit conditions using a needle-type microsensor (left) versus a sensor foil (right; oxygen level is color-coded).

The slope of the oxygen gradient depends on the thickness of surface layers and in particular, the seed coat, and thus is quite species-specific. Where photosynthesis takes place within the embryo itself (as for example in legumes and oilseed rape), the mean oxygen level responds positively to light, since the photosynthetic reaction generates oxygen. In these seeds, the oxygen concentration is lowest within the endospermal liquid, while in the embryo proper, it is boosted by illumination [11,15,20]. In cereal caryopses, the major storage organ is the starchy endosperm (rather than the cotyledon), a structure which is enveloped by a chlorenchymatic layer within the pericarp (except in maize). Very little assimilate is stored within the embryo. When measurements are made in the dark, the oxygen concentration declines only marginally across the pericarp, but typically drops dramatically in the central endosperm. When the same measurements are made in the light, the gradient is altered, but still the central endosperm remains hypoxic [17].

### **2.3 Anatomical and other features favouring seed hypoxia**

Certain structural features of the developing seed can severely hamper their gas exchange capability. First, the outermost layers of the seed (the cuticle, the dense epidermal or compressed cells of the pericarp and the seed coat) form a sealed space separated from the external environment. The outer epidermal layers of the dicotyledenous seed coat, for example, have no functional stomata (Geisler & Sack, 2002), while the monocotyledenous caryopsis typically has only few stomata (Cochrane & Duffus, 1979). Second, many species form lipid-containing or suberized cell layers in their seed (Freeman and Palmer, 1983). The barrier function of certain structures within the sunflower seed has been analysed in [12]. A major block to diffusive oxygen uptake is represented by the very thin lipid-rich membrane covering the oil-storing embryo, while the much thicker multiple layers of the pericarp and attached seed coat show a poor level of control over gas exchange. This finding emphasizes the importance of chemical composition in addition to tissue thickness as determinants of oxygen diffusibility. Thus, even the very small *A. thaliana* seed can experience hypoxia, with just a small reduction in the concentration of ambient oxygen being sufficient to induce a hypoxic response (Gibon et al., 2002). Third, seeds lack any dedicated gas circulation system, such as the aerenchyma present in certain vegetative tissues (Justin & Armstrong, 1987). Fourth, the level of porosity (i.e., the relative volume of unfilled space) is quite low in the seed, as demonstrated by Cloetens et al. (2006) for *A. thaliana*. Synchrotron X-ray tomography applied to the seeds of oilseed rape showed the presence of clear tissue-specific differences in the interconnectivity of pores (P. Verboven, pers. comm.); at present it is uncertain whether there is any genetic basis for the formation of this weak network. Note that in the root, the conformation of the intercellular air space can be influenced by a number of environmental stresses, with implications for gas exchange and respiration (van Heerden

et al., 2008). The overall conclusion is that the developing seed is a compact organ which may include structures able to impede gas exchange. Given that the developing seed is an actively respiring organ [3,20], the seed's interior region experiences hypoxia over a major proportion of its development.

## **2.4 Hypoxia during germination**

Germination and early seedling growth have for a long time been the major subject of research focussed on seed hypoxia (Al-Ani et al., 1985; Corbineau & Come, 1995). The process of germination begins with the uptake of water (“imbibition”), very soon followed by the re-establishment of mitochondrial respiration, as shown by patterns of gene expression and the activity of tricarboxylic acid (TCA) cycle enzymes (Chao & Lin, 1996; Fait et al., 2006; Sreenivasulu et al., 2008). The conventional assumption is that the activation of respiration increases the oxygen demand of the seed, with a consequent downward pressure on its internal oxygen concentration, but the steady state oxygen levels in the germinating seed was not quantifiable before the application of our microsensor approach. This analysis demonstrated that the oxygen level in the non-imbibed mature seed is essentially in equilibrium with the atmosphere (~250 $\mu$ M), but that during imbibition it falls rapidly to below 3 $\mu$ M [3,10]. This decline is related to both a low rate of oxygen diffusion through the seed coat and to an increased level of respiration. Once the seed coat has been ruptured by the emerging radicle, the endogenous oxygen concentration is free to rise rapidly once again to the ambient level, underlining the importance of the seed coat as a barrier against gas diffusion (Corbineau & Come, 1995). Increasing the oxygen supply to the imbibed seed clearly increases the adenylate energy charge, and promotes a corresponding decrease in the release of fermentation-associated products [10]. These changes are well reflected at the level of transcription (Narsai et al., 2009). Experimentally induced seed hyperoxia may promote the growth of the young pea seedling, but has little apparent effect on their germination rate. It seems, therefore, that the imbibed seed has adapted itself to tolerate hypoxia, so that factors other than the rate of ATP generation control germination.

## **3. Determinants of the oxygen status of the developing seed**

### **3.1 Environmental factors**

In most dicotyledonous species, the major seed storage organ (the embryo) becomes green and photosynthetically active early in development. In soybean, ~10% of incident light is able to reach the embryo surface [15], and similar proportions apply in white lupin (Atkins & Flinn, 1978) and oilseed rape (Eastmond et al., 1996). Although this level of light is quite modest, nevertheless photosynthetic activity within the seed generates rapid fluctuations in the internal oxygen status [8,15,20,22]. In oilseed rape, the switch from light to dark induces a

particularly strong and rapid decline, moving from a hyperoxic ( $>700\mu\text{M}$ ) to a strongly hypoxic ( $<1\mu\text{M}$ ) state [11].

The plastids within the green embryo are somewhat specialized; their thylakoids contain chlorophyll-protein complexes similar to those present in leaf chloroplasts, but exhibit more granal stacking. As a result, they have been classified as photoheterotrophic organelles (Asokanthan et al., 1997; Saito et al., 1989). Their structure allows for a low-light saturation level with respect to photosynthetic electron transport [3,14,16]. The Rubisco to total chlorophyll ratio is characteristically low in the seed (Ruuska et al., 2004), resembling rather closely that found in the leaves of shade-loving plants. The photosynthetic activity affects not only the oxygen status of the seed, but also its energy/redox state, and consequently its biosynthetic flux (Browse & Slack, 1985; Ruuska et al., 2004; [3,15,20]). The chloroplasts of the cereal caryopsis enjoy a better light supply and accordingly are more photosynthetically active than their equivalents in the green dicotyledonous embryo [3]. Under light saturated conditions, the photosynthesis occurring in the pea seed is sufficient to supply ~50% of its oxygen demand. In contrast, in barley, the quantity of photosynthetic oxygen produced in a lit environment exceeds its respiratory requirements, so the immature barley caryopsis (as also is the case for other cereal grains, see Nutbeam & Duffus, 1978) is a net producer of oxygen. The spatial distribution of photosynthetic capability can affect both the oxygen gradient and local biosynthetic activity.

Temperature represents a second environmental factor which reversibly affects the oxygen status of the seed. In the developing sunflower seed, a negative relationship obtains between the internal oxygen level and the external temperature [12]. As the temperature is raised, so the oxygen concentration falls rapidly, dropping below  $0.2\mu\text{M}$  when the temperature reaches  $40^{\circ}\text{C}$ . High temperatures both increase the rate of respiration and decrease the amount of dissolved oxygen in the cell sap (the effect of temperature on oxygen diffusibility is only small (Ho et al., 2007)). The temperature dependence of oxygen concentration can regulate the *in vivo* activity of oxygen-demanding enzymes, e.g. during fatty acid synthesis in oilseeds [12].

### **3.2 The influence of developmental stage**

The three major factors affecting the oxygen status of the seed are its respiratory activity (endogenous oxygen demand), its photosynthetic activity (endogenous oxygen provision) and its diffusive resistance to gas exchange. All three of these vary during seed development in a species-specific manner. In cereals (barley and maize), the mean oxygen level in the endosperm falls between the pre-storage and the major storage stage, a change which is accompanied by a decreasing cell energy status, characteristic changes in metabolite pattern and the induction of fermentation activity (MacNicol & Jacobsen, 2001; [13,17]). Thus during the main storage phase of cereal seeds, conditions become increasingly hypoxic and

energy-limited. In legumes (pea, broad bean and soybean), seed oxygen concentrations remain low during early stages of development. The later shift to a higher concentration corresponds to the cessation of ethanolic fermentation. Thus unlike in the cereal caryopsis, hypoxia in the legume seed peaks early during development [15,20,22]. In sunflower, the seed oxygen concentration also follows a clear developmental pattern. The lowest level is seen between ten to 25 days after flowering [12], coinciding with the period of peak oil accumulation (Luthra et al., 1991), a process which has a high energy/respiratory demand.

#### **4. The role of nitric oxide and non-symbiotic haemoglobin in the developing seed**

Despite the presence of hypoxia, the level of fermentation by-products such as lactate and ethanol remains rather low in the seed, indicating the almost avoidance of fermentation and anoxia *in vivo*. Photosynthetically induced or experimentally manipulated changes in the oxygen supply are typically compensated for by shifts in the respiratory oxygen consumption of the developing seed (Vigeolas et al., 2003; Van Dongen et al., 2004; [12,13,15]). The inference is that the seed possesses mechanisms to sense and balance oxygen, enabling a precise and tight control over its molecular and physiological response to hypoxia. Low oxygen signalling in plants has recently been shown to operate via the post-translational regulation of transcription factors (Gibbs et al., 2011; Licausi et al., 2011). It appears that the protein stability of the group VII ERF transcription factors is oxygen-dependent, and that this stability shift can regulate downstream signalling networks involved in the acclimation response to hypoxia (Sasidharan & Mustroph, 2011). However, what remains to be shown is whether hypoxia triggers protein oxidation directly (via an oxygen-dependent enzymatic mechanism) or indirectly (via changes in cellular homeostasis, pH, redox state, etc.; Bailey-Serres & Voeselek, 2008). While this sort of signalling triggers the hypoxic responses through changes in gene expression, it cannot regulate the instantaneous changes in respiratory/metabolic fluxes. Rather, we hypothesised that, just as in mammalian cells (Moncada & Erusalimsky, 2002; Hagen et al., 2003), nitric oxide acts as a rapid response signalling molecule. Nitric oxide is sensitive to oxygen, and inhibits mitochondrial respiration via its reversible binding to COX (cytochrome C oxidase) (Yamasaki et al., 2001; Zottini et al., 2002).

We have demonstrated that endogenous nitric oxide levels in seeds are oxygen dependent, and that nitric oxide modulates respiratory oxygen consumption and thereby the steady state oxygen level in the seed [10,11]. The rapidity of nitric oxide accumulation in seeds, attributed to both its *de novo* synthesis and an increase in its stability, mediates the short-term adaptive response of the seed to hypoxia. Its dynamic nature is of particular significance, because the instant repression of respiratory oxygen demand avoids the risk of anoxia and subsequent fermentation. Oxygen shortage enhances the accumulation of nitrite (Botrel et al., 1996),

which then provides a substrate for nitric oxide synthesis in the seed [11]. Nitrite is used as an alternative electron acceptor by plant mitochondria exposed to hypoxia (Stoimenova et al., 2007; [10]), a capability which has perhaps been retained over the course of mitochondrial evolution. There is further evidence that nitric oxide mediates responses which comprise an essential part of the oxygen signalling pathway (for a review, see [8,11]).

To test our hypotheses regarding the role of nitric oxide, we applied a transgenic approach in which the non-symbiotic haemoglobin *AtHb1* gene was over-expressed in the *A. thaliana* seed [5]. The *AtHb1*-encoded protein is known to scavenge nitric oxide, and thus represents the means to manipulate the seed's endogenous nitric oxide level [9]. Global transcript profiling of the seeds/silques of plants grown under standard growing conditions revealed that genes related to ABA synthesis and signalling, receptor-like kinase- and MAP kinase-mediated signalling pathways, WRKY transcription factors and reactive oxygen species metabolism were all induced. The over-expression of *AtHb1* shifted the seed's metabolism to an energy-saving mode, with the most prominent alterations occurring in cell wall metabolism. The induced changes in transcription and metabolism together indicated a pre-adaptation to hypoxic stress. Notably, the mature seeds formed by transgenic plants were heavier than those formed by the wild type, demonstrating the beneficial effects of the seed-specific over-expression of *AtHb1*. Under experimentally-induced hypoxic stress conditions, the transgenic seeds were better able to maintain lower levels of endogenous nitric oxide and to maintain both a higher respiration rate and a higher energy status than the wild type. These observations were consistent with our initial hypothesis, and confirmed the beneficial effect of non-symbiotic haemoglobins under hypoxia (Hunt et al., 2002; Vigeolas et al., 2011). Preliminary as yet unpublished data collected from pea transformants carrying the same construct suggest that the seed-specific over-expression of *AtHb1* induced a higher rate of respiration and some alterations in metabolic activity. However, no increase on seed size or yield has been associated with the expression of the transgene, indicating that the constraints acting in the pea seed may differ from those acting in *A. thaliana*.

## **5. Relevance of endogenous hypoxia for seed growth and metabolism**

### **5.1 Adjustments in respiratory metabolism**

The respiratory activity in both the developing and germinating seed is limited by low prevailing oxygen levels (oilseed rape: Vigeolas et al., 2003; wheat: van Dongen et al., 2004; soybean: [11,15]; maize: [13]; pea: [10]; barley: [1]). Experimentally-induced increases in oxygen availability can stimulate respiratory fluxes, energy provision and eventually the energy status of cells. Likewise, developmentally or light regime induced changes in mean oxygen levels can alter the ATP/ADP ratio, a consequence supported by proteomic data

collected from developing maize and rice caryopses, where the expression of glycolytic/fermentative enzymes replaces that of TCA cycle ones as development progresses and oxygen concentration falls (Mechin et al., 2007; Xu et al., 2008). The distribution of ATP in the seed also responds to variation in the oxygen supply [13,15].

The general mechanisms underlying the effect of hypoxia on energy metabolism have been well described (Geigenberger 2003; Bailey-Serres & Voeselek, 2008; Mustroph et al., 2010; Narsai et al., 2011). Cells optimize the provision and usage of energy by reorganizing metabolic fluxes, involving shifts in TCA cycle activity, accelerated glycolysis, the increasing use of pyrophosphate as an energy donor in place of ATP, the induction of fermentation, etc. A peculiarity of seeds is the almost complete avoidance of fermentation: the levels of lactate and ethanol remain low during seed development, and quite strong reductions in the external oxygen supply are necessary to increase these levels (Vigeolas et al., 2003; [20]). This suggests the existence of seed-specific mechanisms to constrain fermentation<sup>2</sup>.

The minimum oxygen levels detected in the seed are typically higher than needed to saturate the respiratory enzyme COX (*in vitro*:  $K_m \sim 140\text{nM}$ ; Armstrong & Beckett, 2011). This fact initially engendered some doubt regarding the mechanisms via which respiratory flux is restricted in seeds. However, there may exist an intracellular oxygen gradient, which would imply that the oxygen level measured by the microsensor is not the same as that prevailing within the mitochondrion. Additionally, it is unclear whether the *in vitro* estimates of  $K_m$  apply *in vivo*, because the nitric oxide inhibition of COX is competitive with oxygen, and thus increases the effective oxygen  $K_m$  under *in vivo* conditions (Brown, 1995; Moncada & Erusalimsky, 2002; [10,11]). This modulation of  $K_m$  might explain the onset of respiratory restrictions at the oxygen levels prevailing in the seed (Geigenberger, 2003; [20])<sup>3</sup>.

## 5.2 Hypoxic constraints on central and storage metabolism

In general, hypoxia induces various changes to the transcriptome, proteome and metabolome, which together imply a reconfiguration of metabolism. Mustroph et al. (2009) identified a core set of 49 translated hypoxia-induced mRNAs in 21 different *A. thaliana* cell populations, of which 35 were also up-regulated in the *A. thaliana* seed [5]. Thus there appears to be a consistent adaptation strategy to hypoxia across various tissues/organs. The induction of the glyoxylate cycle in combination with lipid degradation seems to be seed-

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<sup>2</sup> Seeds can (transiently) synthesize large quantities of alanine, a molecule regarded as an alternative fermentation product (see §6.1).

<sup>3</sup> As demonstrated by Armstrong & Becket (2011), the degree and distribution of respiratory oxygen demand and diffusive resistance can both play a major role in determining the sufficiency of the oxygen supply. Care must therefore be taken when interpreting the onset of respiratory restrictions at relatively high oxygen concentrations.

specific. Under hypoxia, seeds induce the expression of genes involved in sugar, amino acid, oligopeptide and general nutrient (aquaporin) transport. This induction is accompanied by a series of changes to metabolic flux and metabolite levels, for example a switch from invertase to sucrose synthase, the metabolism of sugars such as trehalose-6-P (a signalling molecule affecting carbon partitioning; Paul et al., 2008; Mustroph et al., 2010), amino acids such as alanine (Vigeolas et al., 2003; [1,5,15,20]) and various reactive oxygen species [5]. Though less understood, hypoxia-induced metabolic changes involve cross-talk between hormones (in particular ethylene<sup>4</sup>) and other regulatory factors. We have demonstrated that hypoxia in the *A. thaliana* seed leads to a strongly induced metabolism/signalling of hormones (including abscisic acid, ethylene, jasmonate, salicylic acid and gibberellic acid), along with the activation of several transcription factors (*WRKY*, *MYB*, *bZIP* and *AP2-EREBP*). Similar responses have been recorded in vegetative plant organs (Mustroph et al., 2009).

Much research effort has been expended on understanding the restrictions which hypoxia imposes *in vivo* on the uptake and distribution of assimilates, and their subsequent conversion into storage products (Shelp et al., 1995; Vigeolas et al., 2003; van Dongen et al., 2004; [1,5,6,13,15,20]). The following major trends have emerged: (1) Phloem import into the seed is strongly coordinated by the low prevailing oxygen concentration. Elevated external oxygen levels or light-driven endogenous oxygen provision both can stimulate sugar uptake. (2) The distribution of sugars within the seed, in particular their partitioning between the cereal endosperm and embryo is affected by oxygen availability. (3) Sucrose degradation and its subsequent metabolic conversion are affected by endogenous hypoxia. (4) The rate of synthesis of storage products is determined by energy provision, and thus by oxygen availability. Consequently, the accumulation of storage proteins and oils is more restricted than that of the energetically cheaper starch. (5) Seed hypoxia may limit the *in vivo* activity of enzymes (which either directly or indirectly depend on oxygen availability), and thereby affect the composition of specific storage products. Thus, for example, it has been demonstrated that low endogenous oxygen levels in the sunflower seed regulate the activity of fatty acid desaturases (FAD2) and thereby the final composition of the storage oil [12]. The overall conclusion is that a number of mechanisms underlie the effect of endogenous hypoxia on central metabolism and in particular respiratory pathways acting in the seed. These restrictions can affect overall seed growth and biomass accumulation in a species-dependent

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<sup>4</sup> Enhanced ethylene synthesis under hypoxia is a well known phenomenon, often linked to the formation of tissues favouring gas transport (Armstrong et al., 1994). However, so far, this feature has only been described for vegetative plant organs such as the root, rhizome and stem, indicating that ethylene metabolism/signalling is differentially regulated in the seed.

manner and they likely provide the basis for the observation that both seed number and size in many plant species depend on the atmospheric oxygen level (for references see [8]). In this sense, experimentally-induced increases in oxygen supply can promote seed biomass (Goffman et al., 2005).

## **6. Making sense of the low oxygen environment in the seed**

### **6.1 Hypoxia in the seed is managed *in vivo* by local metabolic adjustments**

Recent research indicates that a proper understanding of the adjustments in seed metabolism to hypoxia needs to consider both the local features of the tissue and the local oxygen concentration. For example, a gradient of oxygen concentration (high at the periphery, low in the centre) is present in the pea seed. The genes encoding the legumin storage proteins are expressed throughout, but protein deposition is initiated at the periphery of the embryo, where ATP levels are locally up-regulated [21]. It has therefore been argued that the energy-demanding protein synthesis (the translation of legumin mRNA) is limited by the local availability of ATP, since the inner regions of the embryo are starved of oxygen/ATP and thus inhibited from synthesizing legumin. Hypoxic restrictions for protein synthesis were also shown for *Arabidopsis* seedlings (Branco-Price et al., 2008).

In a recent *in vivo* analysis based on  $^{13}\text{C}/^1\text{H}$  magnetic resonance imaging, we showed that in the developing barley caryopsis, sucrose passes via the well oxygenated crease vein and nucellar projection into the central endosperm [2], where an appreciable level of hypoxia obtains. The appreciable level of alanine amino transferase (Ala-AT) activity established locally synthesizes alanine [1], and thereby both avoids the accumulation of pyruvate and fermentation products, and provides a source of additional ATP. Nitrogen use efficiency is also raised by the re-utilization of the ammonia released by the deteriorating adjacent nucellar tissue (Radchuk et al., 2006). The cells are interconnected by multiple plasmodesmata, allowing for an energy-efficient movement of alanine along a concentration gradient towards the more peripheral endosperm cells. Since the peripheral cells are not in a hypoxic state, Ala-AT then acts to reconvert alanine back to pyruvate. Thus, the steady state level of alanine depends on the local conditions *in planta*<sup>5</sup>. It appears that the accumulation of alanine can represent a metabolic marker for localized hypoxia, at least in the developing seed – mirroring the situation in animal cells, where alanine synthesis is widely used as a diagnostic for certain hypoxia-related disorders (Nyblom et al., 2004).

Region-specific metabolism in the cereal endosperm has been further characterized using a combination of flux balance analysis and metabolite profiling [1]. The combined data suggest

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<sup>5</sup> Notably, none of the genes encoding enzymes involved in alanine metabolism nor the corresponding enzyme activities ( $V_{\max}$ ) respond to variation in the oxygen supply, indicating that a major part of flux control is handled post-translationally.

that in the central region of the cereal endosperm, the TCA cycle shifts to a non-cyclic mode, accompanied by elevated glycolytic flux but no significant induction of the Gaba shunt (as commonly observed in hypoxic roots). This may represent a seed-specific adaptation to the situation where oxygen deficiency is prolonged rather than transient as in the root. The proposed metabolic compartmentation of the endosperm is mirrored by the structure of the tissue, since the central part of the endosperm is composed largely of large cells, whereas those at the periphery are small. Although possibly coincidental, this pattern of cell size corresponds to the known promoting effect of hypoxia on cell expansion/elongation (Armstrong et al., 1994; Bailey-Serres & Voesenek, 2008). In summary, it appears that as a result of the architecture of the seed and of the non-uniform availability of sucrose and/or oxygen, constraints are not uniformly experienced. Any imbalances are balanced *in vivo* by local metabolic adjustments. We suggest that the metabolic compartmentation of the starchy endosperm provides a mechanism to ensure metabolic flexibility, and eventually contributes to the high carbon conversion efficiency shown by the cereal caryopsis (Alonso et al., 2011).

Magnetic resonance imaging-based metabolite imaging has the great virtue of non-invasiveness, and so can be used to monitor physiological processes occurring *in vivo*. It allows for example the metabolic analysis of liquid endosperm in legume seeds, studying the effects of oxygenation on assimilate levels [6]. In the case of monitoring <sup>13</sup>C alanine synthesis, it was possible to identify which tissue in the barley caryopsis is the most susceptible to hypoxia [1]. The assay represents an *in vivo* alternative to the use of oxygen-sensitive microsensors. A number of possible applications for this approach in the general area of plant metabolism have been suggested by Borisjuk et al. (2012). For example, any labelled substrate can be provided to study the routes via which it is taken up by and distributed within the plant, and how finally it is either degraded or incorporated into storage molecules. It is believed that the combination of tools allowing *in vivo* observations [1], metabolic modelling [2] and *spatially-resolved* analysis of transcripts and metabolites (Schiebold et al., 2011) will greatly enhance our understanding on regulatory principles during seed development, involving the hypoxic acclimations in seed metabolism.

## **6.2 The potential benefits to seed metabolism of hypoxic acclimation**

The low internal oxygen concentration in the seed has a profound effect on storage product deposition, with clear implications for strategies aiming to increase seed yield. The question is whether molecular approaches can be employed to increase oxygen availability within the seed. One possibility could be to promote the photosynthetic capacity of the seed, which would not only deliver additional oxygen to its interior region, but would also stimulate the synthetic input of the plastids, benefitting cell metabolism, increasing the supply of energy

available for biosynthesis, and raising carbon use efficiency [14,18]. A series of considerations, however, argue against any approach taken to increase oxygen availability and gas exchange capability. The following side-effects of increasing oxygen levels in the seed are possible:

1. An increased oxygen supply would probably compromise the carbon conversion efficiency via the induction of respiration and/or a shift in assimilate partitioning (Goffman et al., 2005). This may be especially critical where source capacity (supply with sucrose and amino acids) is limiting.
2. The bioenergetic efficiency of mitochondria usually increases at low oxygen levels (Gnaiger et al., 2000). Thus, the low internal oxygen concentration present in the seed may help to promote carbon use efficiency.
3. Low oxygen levels help avoid high levels of reactive oxygen species (Simontacchi et al., 1995), which damage cellular structures, and require energy investment for repair. In maize, the expression level of genes associated with detoxification (encoding glutathione S-transferase, superoxide dismutases and ascorbate peroxidases) are known to fall during seed development (Mechin et al., 2007), consistent with the fall in oxygen availability.
4. Low internal oxygen levels in combination with high internal carbon dioxide levels can be expected to minimize photorespiration, thereby raising carbon use efficiency (Goffman et al., 2004).
5. The cuticle may reduce oxygen uptake, but it also restricts carbon dioxide loss. Carbon dioxide levels in seeds are high, so a low gas exchange capacity is helpful to promote the re-fixation of internally released carbon dioxide, which would otherwise escape from the seed. Thus increasing its permeability might have negative implications on the carbon economy of the seed (Schwender et al., 2004).
6. The cuticle also acts as a barrier against water loss, pathogen invasion and UV penetration. Weakening the barrier may therefore compromise some of these essential functions. Indeed, the deposition of an intact cuticle may be a prerequisite for proper development (Pruitt et al., 2000).

Overall, it may be advantageous for the seed to restrict its gas exchange. Even though the internal oxygen concentration falls to levels limiting respiration and storage metabolism, other considerations also come into play. As demonstrated for barley, hypoxia can be beneficial in certain regions of the developing caryopsis [1]. It remains to be seen whether attempts to increase oxygen availability will result in higher seed biomass and storage capacity *in vivo*, or whether some or all of the above-mentioned factors act to negate any gains made in crop productivity. In any case, a comprehensive understanding of the metabolism, structure and development of the seed is a prerequisite for initiating such approaches.

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## Part B: List of publications

1. **Rolletschek H**, Melkus G, Grafahrend-Belau E, Fuchs J, Heinzl N, Schreiber F, Jakob PM, Borisjuk L. (2011). Combined non-invasive imaging and modelling approaches reveal metabolic compartmentation in the barley endosperm. *Plant Cell* 23: 3041–3054.
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## **Eidesstattliche Erklärung**

Diese Habilitationsschrift wurde selbständig und ohne fremde Hilfe verfasst. Es wurden keine anderen als die angegebenen Hilfsmittel genutzt. Wörtlich bzw. inhaltlich übernommene Stellen wurden als Zitate mit entsprechendem Verweis gekennzeichnet.

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