# *Arabidopsis thaliana* aldehyde dehydrogenases ALDH3H1 and ALDH3I1: cysteine mutations, *S*-nitrosylation and esterase activity

**Dissertation**

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Jawor, Polen

Bonn, 2015

**Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn**

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**Tag der Promotion: 02.09.2015**
**Erscheinungsjahr: 2016**

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# List of Abbreviations

| | |
|---|---|
| **% (v/v)** | Volume percentage |
| **% (w/v)** | Weight percentage |
| **A** | Adenine |
| **ABA** | Abscisic acid |
| **Ala** | Alanine |
| **ALDH** | Aldehyde dehydrogenase |
| **APS** | Ammonium persulfate |
| **At** | *Arabidopsis thaliana* |
| **Biotin-HPDP** | *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide |
| **bp** | Base pair |
| **BSA** | Bovine serum albumin |
| **β-Me** | β-mercaptoethanol |
| **BST** | Biotin switch technique |
| **C** | Cytosine |
| **CAM** | Crassulacean acid metabolism |
| **CaMV** | Cauliflower mosaic virus |
| **cDNA** | Complementary DNA |
| **Cp** | *Craterostigma plantagineum* |
| **Cys** | Cysteine |
| **DMF** | *N*,*N*-dimethylformamid |

| | |
|---|---|
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxyribonucleotide triphosphate |
| **DTNB** | 5,5'-dithio-bis-(2-nitrobenzoic acid) |
| **DTT** | Dithiothreitol |
| ***E.coli*** | *Escherichia coli* |
| **EDTA** | Ethylenediaminetetraacetic acid |
| ***g*** | Acceleration |
| **g** | Gram |
| **G** | Guanine |
| **GAPDH** | Glyceraldehyde-3-phosphate dehydrogenase |
| **GSH** | Glutathione |
| **GSNO** | *S*-nitroso-L-glutathione |
| **GSNOR** | *S*-nitrosoglutathione reductase |
| **GSSG** | Glutathione disulphide |
| **GTN** | Glyceryl trinitrate/ nitroglycerin |
| **h** | Hour |
| **HEN** | HEPES-EDTA-Neocuproine |
| **HEPES** | 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid |
| **His** | Histidine |
| **HNE** | 4-hydroxy-trans-2-nonenal |
| **IgG** | Class G immunoglobulin |

| | |
|---|---|
| **IMAC** | Immobilized-Metal Ion Affinity Chromatography |
| **IPTG** | Isopropyl ß-D-1-thiogalactopyranoside |
| **KAN** | Kanamycin sulphate |
| **kb** | Kilobase |
| **kDa** | Kilodalton |
| **λ** | Wavelength |
| **LB** | Luria-Bertani medium |
| **LEA** | Late Embryogenesis Abundant |
| **M** | Molar, moles per liter |
| **mA** | Miliamperes |
| **MDA** | Malondialdehyde |
| **min** | Minute |
| **ml** | Mililiter |
| **MMTS** | Methyl methanethiosulfonate |
| **MS** | Murashige and Skoog |
| **MW** | Molecular weight |
| **NAD** | Nicotinamide adenine dinucleotide |
| **NEM** | N-ethylmaleimide |
| **Ni** | Nickel |
| **nm** | Nanometers |
| **NO** | Nitric oxide |
| **NOS** | Nitric oxide synthase |

| | |
|---|---|
| **OD** | Optical density |
| **Os** | *Oryza sativa* |
| **Ox-PTM** | Oxidative posttranslational modification |
| **PAGE** | Polyacrylaide gel electrophoresis |
| **PBS** | Phosphate-Buffered Saline |
| **PCR** | Polymerase Chain Reaction |
| **Pfu** | *Pyrococcus furiosus* |
| **pI** | Isoelectric point |
| **PMSF** | Phenylmethylsulphonyl fluoride |
| ***p*-NPA** | *p*-nitrophenyl acetate |
| **RNase** | Ribonuclease |
| **RNS** | Reactive nitrogen species |
| **ROS** | Reactive oxygen species |
| **RSS** | Reactive sulphur species |
| **rpm** | Revolutions per minute |
| **RuBisCO** | Ribulose-1,5-bisphosphate carboxylase/oxygenase |
| **SDS** | Sodium dodecyl sulphate |
| **SDS-PAGE** | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| **Ser** | Serine |
| **SNAP** | *S*-nitroso-N-acetylpenicillamine |
| **SNO** | *S*-nitrosothiol |
| **SNP** | Sodium nitroprusside |

| | |
|---|---|
| **SOC** | Super Optimal broth with Catabolite repression |
| **T** | Thymine |
| **$T_a$** | Annealing temperature |
| **$T_m$** | Melting temperature |
| **TAE** | Tris-acetate-EDTA |
| **Taq** | *Thermophilus aquaticus* |
| **TBS** | Tris-Buffered Saline |
| **TCA** | Trichloroacetic acid |
| **TE** | Tris-EDTA |
| **TEMED** | N,N,N′,N′-tetramethylethane-1,2-diamine |
| **Tris** | Tris(hydroxymethyl)aminomethane |
| **Triton X-100** | Polyoxyethylene octyl phenyl ether |
| **Tween-20** | Polyethylene glycol sorbitan monolaurate |
| **U** | Unit |
| **UV** | Ultraviolet |
| **V** | Volts |
| **Val** | Valine |
| **Vp** | *Vitis pseudoreticulata* |

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# 1. Introduction

## 1.1. The importance of water

Around 70 percent of the earth's surface is covered by water. Water and temperature have a big impact on the distribution of plants. It has been suggested that the water is the most available and the most important substance on earth. Furthermore, the quantity of water available has an effect on kinds and density of vegetation occurring on various places on the earth's surface. Nowadays, it has been shown that the availability of water is a factor that limits not only the growth of plants, but can also influence the growth and the development of cities and industries (Kramer and Boyer 1995).

### 1.1.1. The role of water in plant growth and development

Water plays an important role in all physiological processes in plants. In non-woody plants the biomass of leaves and roots include 80 to 95 percent of water (Hirt and Shinozaki 2004). Many physiological processes, occurring in plants, depend on the water supply in a direct or indirect manner. For instance, a decrease in water content causes inhibition of photosynthesis and usually reduces the rate of respiration. Insufficient water supply contributes to loss of turgor and wilting, closure of stomata and may also disrupt other metabolic processes taking place in plants. It has been shown that disorganization of the protoplasm in plant cells is caused by the excessive loss of water. The death of most plants is a consequence of the continued dehydration (Kramer and Boyer 1995). The importance of water for plants can be summarized under four general points (Jenks and Hasegawa 2005):

**1. Constituent:** Water is a main compound of plant cells, because the water content exceeds 90% of the fresh weight in most herbaceous plants (Jenks and Hasegawa 2005).

**2. Solvent:** Water is a good solvent for chemical compounds. Due to unique biophysical properties (high enthalpy of vaporization and high surface tension), water occurs in the liquid state in a wide temperature range and dissolves many ions, minerals and hydrophilic molecules (Jenks and Hasegawa 2005).

**3. Reactant:** Water participates in many biochemical reactions. For instance, water occurs as a reactant in photosynthesis and serves as the primary electron donor (Jenks and Hasegawa 2005). Furthermore, water plays an important role as a ligand in chemical reactions (Rand 1992).

**4. Maintenance of turgor:** Water participates in maintaining cell turgor. Turgor enables cell enlargement and growth. In plant cells turgor is responsible for the opening and closing of stomata and the movements of leaves, flowers and petals (Kramer and Boyer 1995).

## 1.1.2. Water supply and water demand for agriculture

The extreme events such as droughts and floods occur more frequently and are more intense due to climate change. It has a negative influence on food production. The main challenge of agriculture is sufficient supply of food which satisfies a growing population. The higher demand for food production needs more water. It has been estimated that between now and 2050 worldwide demand for additional food is 60% (FAO 2011).

The agricultural production of the world has increased between 2.5 and 3 times during the last 50 years. At the same time the cultivated area has been extended only by 12%. Agriculture is the biggest consumer of water, accounting for almost 70% of all water used. In developing countries the water consumption in agriculture is up to 95%. In agriculture, irrigation requires the biggest quantities of water (FAO 2012).

Water availability is one of the major factors that limits the plant productivity (Boyer 1982), and regulates the distribution of plant species (Jenks and Hasegawa 2005). The arid and semiarid areas account for over 35% of the world's land surface. Over 35% of the world's land surface is considered to be arid or semiarid. Some agricultural regions that were affected by drought showed yield losses of up to 50% or more (Jenks and Hasegawa 2005).

## 1.2. Plant stressors

The living organisms, including plants, demonstrate the high level of organization and participate in complex and multiple interactions with the environment. Plants are exposed to a wide range of environmental factors that occur both in nature and in the agricultural fields (Zlatev and Lidon 2012). The environmental factors can be of abiotic and biotic nature. Abiotic stress factors such as heat, cold, drought, salinity, high light intensity, ozone ($O_3$) and nutrient stress have a huge impact on plant growth and yield under field conditions. Plants must generate the mechanisms that let them to defend themselves from attack by pests and pathogens, including fungi, bacteria, viruses, nematodes, and herbivorous insects (Hammond-Kosack and Jones 2000; Atkinson and Urwin 2012). The influence of the environment on the performance of the plants is determined both by the strength and duration of the environmental factors and by genetic properties of the plant (Zlatev and Lidon 2012). Plants have generated a specific and unique stress response mechanism that is activated when the plants are subjected to a combination of multiple stresses (Rhizhsky *et al.,* 2004). Stress represents both negative and positive effects on plants. Moreover, the stress factors may contribute to improved resistance and adaptive evolution (Larcher 1980).

## 1.3. Mechanisms of adaptation in response to water deficit

Water deficit is mainly considered as lack of water but in several cases might also be caused by high salinity of soil. Many environmental factors like low and high temperature or high salinity can also contribute to water deficit and simultaneously have negative influence on plant productivity (Hirt and Shinozaki 2004). For instance, the plants that are exposed to unfavourable conditions show the reduction in crop yield up to 69% (Boyer 1982; Bray *et al.,* 2000; Wang *et al.,* 2003). The genotype determines the ability of the plant to survive and manage in environments with insufficient water supply. Moreover, plant growth and development depend on many additional factors such as the duration of the water deficit, the intensity of stress imposition, and the developmental stage of the plant during the stress exposition (Bray *et al.,* 2000). The responses to water deficit occur at the molecular, metabolic, physiological and developmental levels and these responses are initiated by a sensing mechanism (Bray 1993). Some plants show the

resistance to water deficit that may result from the ability to tolerate water deficit by these plants or from the presence of mechanisms that allow avoiding the water deficit. Some species, such desert ephemerals are able to avoid drought by adaptation to very short wet periods. These organisms complete their life cycle when water is available (Bray *et al.,* 2000). Other plants avoid water deficit by the development of a large root system. Long roots facilitate the access to water from the soil. Avoidance of water deficit may also be achieved by using mechanisms that accumulate moisture in their fleshly tissues (desert succulents).

Some plants such as Crassulacean Acid Metabolism (CAM) plants have improved water use efficiency (Ting 1985). In CAM plants photosynthesis is modified. At night when the temperature is lower and evaporation is reduced, the stomata are opened and $CO_2$ can easily diffuse into the cytoplasm. $CO_2$ is stored as malic acid. During the day, the stomata of CAM plants are closed and malic acid is transported into chloroplasts. During the photosynthesis, the malic acid is used by CAM plants as a building unit of more complex organic molecules (Buchanan *et al.,* 2000). This mechanism promotes the use of less water (Bray 2001). Furthermore, many cacti and other stem-succulent plants occurring in the desert regions show columnar growth, have no leaves and possess vertically-erect, green trunks. These physiological adaptations allow maximizing light interception in the early morning and in the evening, but at the same time avoiding the excessive heat generated by midday sun. The overheating may cause the damages in the plant tissues, or even kill the plants (Zavala-Hurtado *et al.,* 1998). The resurrection plants and mosses have the biochemical and morphological mechanisms that allow them to tolerate dehydration (Bray 2001). The resurrection plants have the ability to tolerate an extreme dehydration. The water content in the vegetative tissue of resurrection plants after dehydration might reach 2%. It is spectacular that the resurrection plants might activate the metabolism and growth after rehydration. To survive in unfavourable conditions, the resurrection plants developed several molecular and metabolic mechanisms which allow them to adapt to the long-lasting lack of water (Bartels *et al.,* 1990; Piatkowski *et al.,* 1990). For instance, *Craterostigma plantagineum* belongs to Linderniaceae and is a resurrection plant indicating desiccation tolerance. It has been reported that *C. plantagineum* shows drastic changes in specific gene expression at dehydration and rehydration stages. For instance, the transcripts abundantly present during dehydration disappear at rehydration stage (Bartels and Salamini 2001). Moreover, the carbohydrate

metabolism of *C. plantagineum* is changed in response to an extreme loss of water. The high level of octulose is decreasing upon dehydration, whereas sucrose is accumulating. Contrary, upon rehydration sucrose level declines and the level of octulose is increasing (Bianchi *et al.,* 1991). Another example of a resurrection plant is *Selaginella lepidophylla* belonging to the spike-moss family. Selaginella is able to survive an extreme loss of water due to the accumulation of trehalose. This sugar has the protective properties, including the stabilization of proteins and the preservation of cellular membranes (Adams *et al.,* 1990).

## 1.4. Aldehydes and aldehyde dehydrogenases

Aldehydes are organic compounds which possess a characteristic functional formyl group. Aldehydes may be generated from both endogenous and exogenous precursors during physiological processes. One of these processes is the biotransformation of organic compounds, including amino acids, proteins, carbohydrates, vitamins and lipids (Vasiliou *et al.,* 2000; Vasiliou *et al.,* 2004; O'Brien *et al.,* 2005). For instance, amino acid catabolism generates several aldehyde intermediates such as phenyl acetate. Some aldehydes are derived from exogenous sources, including the metabolism of xenobiotics (Lindahl 1992). Xenobiotics are defined as the foreign chemical compounds, that are present in organisms, but they are not naturally generated by these organisms. With the respect to plants, xenobiotics include pesticides and herbicides. The aldehydes are highly reactive molecules due to the electrophilic nature of their carbonyl group (Lindahl 1992). α,β-unsaturated aldehydes such as 4-hydroxy-2-nonenal (4-HNE) also contain two additional reactive groups: a double bond at the ß-carbon and a hydroxyl group at carbon 4, that participate in reactions (Esterbauer *et al.,* 1991). Aldehydes have a long half-life. Some aldehydes such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) are derived from lipid peroxidation (LPO), defined as the oxidative degradation of cellular membrane lipids (Esterbauer *et al.,* 1991). These aldehydes are harmful to cells at various levels. Aldehydes react with the wide range of compounds present in cells, including glutathione (GSH), nucleic acids, and protein amino acids. Adducts, formed as the result of these reactions, lead to impaired cellular homeostasis, enzyme inactivation, DNA damage, and cell death (Nadkarni and Sayre 1995; Brooks and Theruvathu 2005). Aldehydes are molecules widely distributed in plants that participate in the different physiological processes. For instance, the best characterized metabolites of the

hydroperoxide lyase (HPL) branch are green leafy volatiles, which predominantly consist of $C_6$-aldehydes ((Z)-3-hexenal and n-hexanal) and their respective derivatives (Matsui 2006), were suggested to be involved in the resistance of potatoes to the aphid *Myzus persicae* (Vancanneyt *et al.,* 2001). However, volatile aldehydes are also components of the flavours of fruits in several plant species (Hatanaka *et al.,* 1987). It has been shown that aldehydes may participate in plant defence responses against pests and pathogens. For instance, hexanal and 3-hexenal possess a potent antimicrobial activity (Deng *et al.,* 1993) and are able to reduce aphid fecundity *in vitro* (Hildebrand *et al.,* 1993). Furthermore, it has been suggested that hexanal and 3-hexenal are involved in the regulation of defence-gene expression (Bate and Rothstein 1998). It has been reported that some aldehydes may function as signalling molecules (Weber *et al*., 2004).

It is very important to control the level of aldehydes and maintain the balance between physiologically essential and harmful levels (Kotchoni *et al.,* 2006). There are two mechanisms for detoxification of the aldehydes:

1) Reduction of aldehydes to primary alcohols is catalysed by aldo-keto reductases (Oberschall *et al.,* 2000). For instance, the enzymatic activity of the recombinant protein from rice (OsAKR1) is able to reduce toxic aldehydes including methylglyoxal (MG), that is generated during glycolysis and malondialdehyde (MDA), that is produced during lipid peroxidation (Turóczy *et al.,* 2011).

2) The irreversible oxidation of excessive aldehydes to their corresponding carboxylic acids is catalysed by aldehyde dehydrogenases (ALDHs): (E.C 1.2.1).

ALDHs are oxidoreductases which use $NAD^+$ or $NADP^+$ as cofactor (Yoshida *et al.,* 1998; Perozich *et al.,* 1999). Some ALDHs are non-specific and use a wide range of substrates (aliphatic and aromatic aldehydes), but other classes of ALDHs have high substrate specificity such as betaine aldehyde dehydrogenase (BADH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Skibbe *et al.,* 2002).

ALDHs are present in both prokaryotic and eukaryotic organisms (Sophos and Vasiliou 2003) and localised in different subcellular compartments including cytosol, plastids, mitochondria, nucleus, endoplasmic reticulum, microsomes, and peroxisomes (Marchiti *et al.,* 2008). This differential localisation proves their functional specialisation. ALDH isozymes, with the exception of cytosolic proteins, contain the sequences (Braun *et al.,* 1987), which after translocation or import to specific organelles are removed

(mitochondrial and chloroplast targeting sequences) or can remain intact (microsomal and nuclear sequences) (Vasiliou *et al.*, 1996; Pappa *et al.*, 2005).

In 1998 the ALDH Gene Nomenclature Committee (AGNC) determined the criteria for naming and classifying the different ALDHs (Vasiliou *et al.*, 1999). This system was formed on the basis of the divergent evolution and amino acid sequence similarity. Figure 1 presents the standardized ALDH nomenclature system. Enzymes having 40% identity of amino acid sequences constitute a family. Enzymes with more than 60% similarity of amino acid sequences constitute a protein subfamily (Kirch *et al.*, 2004). According to nomenclature developed by the AGNC the first number in the name of ALDH indicates "family'', and the first letter denotes a "subfamily", while the final number determines an individual gene within a subfamily (Skibbe *et al.*, 2002).

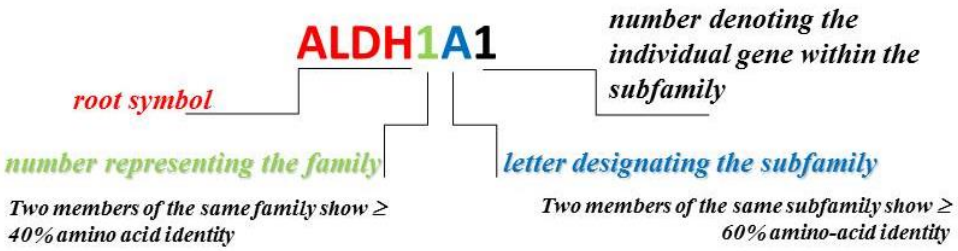



**Figure 1: The standardized ALDH nomenclature system based on divergent evolution.**

Plant ALDHs have been classified into 14 distinct protein families, where seven families (ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH11 and ALDH18) have the orthologues in mammalians, and seven families (ALDH10, ALDH12, ALDH19, ALDH21, ALDH22, ALDH23 and ALDH 24) are specific for plants (Zhang *et al.*, 2012; Brocker *et al.*, 2013;). Human ALDHs comprise 19 homologous enzymes which are classified into 11 families and four sub-families (Vasiliou and Nebert 2005). Table 1 shows the number of ALDH family members identified in plant and mammalian species and indicates common ALDH family members for plants and mammalians.

**Table 1: ALDH family members identified in plants and mammalians**

plant specific (7)

have homologues in mammalians (7)

mammalian specific (10)

| | | | | | | | | | | | | | | | | | | | | | | | | | Σ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mammalians | *R. norvegicus* | 7 | 1 | 1 | 1 | - | - | - | 1 | - | - | 1 | 3 | 1 | 1 | 1 | - | 1 | - | - | - | - | - | - | - | 19 |
| | *M. musculus* | 7 | 1 | 1 | 1 | - | - | - | 1 | - | - | 1 | 4 | 1 | 1 | 1 | - | 1 | - | - | - | - | - | - | - | 20 |
| | *H. sapiens* | 6 | 1 | 1 | 1 | - | - | - | 1 | - | - | 1 | 4 | 1 | 1 | 1 | - | 1 | - | - | - | - | - | - | - | 19 |
| | ***FAMILIES*** | **1** | **4** | **8** | **9** | **13** | **14** | **15** | **16** | **17** | **20** | **2** | **3** | **5** | **6** | **7** | **11** | **18** | **10** | **12** | **19** | **21** | **22** | **23** | **24** | |
| plants | *A. thaliana* | - | - | - | - | - | - | - | - | - | - | 3 | 3 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | - | - | 1 | - | - | 16 |
| | *C. reinhardtii* | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 | 1 | - | 1 | 1 | 1 | 1 | - | - | 1 | - | 1 | 9 |
| | *O. sativa* | - | - | - | - | - | - | - | - | - | - | 5 | 5 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | - | - | 1 | - | - | 20 |
| | *P. patens* | - | - | - | - | - | - | - | - | - | - | 2 | 5 | 2 | 1 | 1 | 5 | 1 | 1 | 1 | - | 1 | - | 1 | - | 21 |
| | *P. trichocarpa* | - | - | - | - | - | - | - | - | - | - | 4 | 6 | 1 | 4 | 2 | 3 | 2 | 2 | 1 | - | - | 1 | - | - | 26 |
| | *S. moellindorffi* | - | - | - | - | - | - | - | - | - | - | 6 | 2 | 1 | 1 | 1 | 6 | 1 | 1 | 1 | - | 1 | 1 | 2 | - | 24 |
| | *S. bicolor* | - | - | - | - | - | - | - | - | - | - | 5 | 4 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | - | - | 1 | - | - | 19 |
| | *V. vinifera* | - | - | - | - | - | - | - | - | - | - | 5 | 4 | 3 | 3 | 2 | 2 | 2 | 2 | 1 | - | - | 1 | - | - | 25 |
| | *Z. mays* | - | - | - | - | - | - | - | - | - | - | 6 | 5 | 2 | 1 | 1 | 1 | 2 | 2 | 1 | - | - | 1 | - | - | 22 |

Sequence alignments of ALDH genes from different organisms revealed the identification of three characteristic amino acid motifs present in ALDH proteins (Perozich *et al.*, 1999):

a) The ALDH glutamic acid active site signature sequence MELGGNA

b) A glycine-rich motif (GxGxxG) corresponding to the $NAD(P)^+$-binding fingerprint

c) The catalytic thiol from a cysteine residue

The residues participating in enzyme catalysis and those involved in coenzyme binding are highly conserved (Hempel *et al.*, 1997).

Crystal structures of ALDHs from different organisms were solved. Each subunit of ALDH protein contains three domains: the catalytic domain, the $NAD(P)^+$ cofactor-binding domain, consisting of a Rossmann fold which is made up of five stranded open α/β domains, and the oligomerization domain (Liu *et al.*, 1997). Figure 2 shows the structure of a single subunit of ALDH from *V. harveyi*. The interface of these three domains (catalytic domain, cofactor-binding domain and oligomerization domain) forms the funnel passage that leads to the catalytic pocket. The amino acid residues derived from all three domains (catalytic domain, cofactor-binding domain and oligomerization domain), which are localised in the upper part of the funnel, are supposed to be responsible for ALDH specificity. The lower part of the funnel is composed of highly

conserved amino acid residues derived from both cofactor binding and catalytic domains, which form the catalytic site (Liu *et al.,* 1997; Steinmetz *et al.,* 1997). Subunits are linked by intra-molecular hydrogen bonds leading to the formation of enzymatically active homodimers for ALDH families 3 and 10 (Liu *et al.,* 1997; Ahvazi *et al.,* 2000; Kopečny *et al.,* 2013) and homotetramers for ALDH families 1, 2, 7 and 9 (Steinmetz *et al.,* 1997; Johansson *et al.,* 1998; Moore *et al.,* 1998; Cobessi *et al.,* 1999).

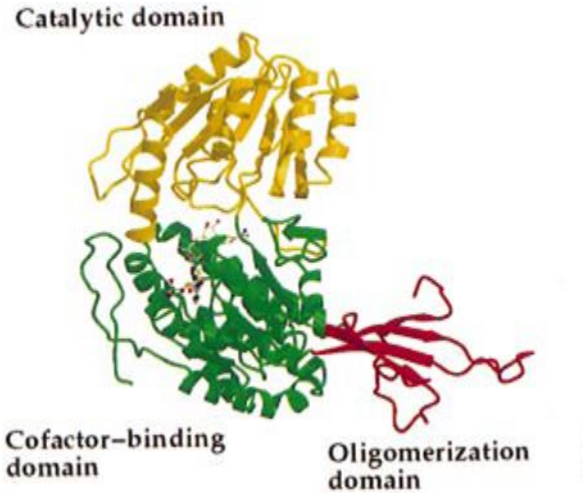



**Figure 2: The structure of the NADP$^+$-specific aldehyde dehydrogenase from *Vibrio harveyi***

A single subunit of the ALDH enzyme is presented. The catalytic domain is shown in yellow, the cofactor-binding domain is shown in green and the oligomerization domain is shown in red. Ref. Ahvazi *et al.* (2000).

ALDHs may be bifunctional enzymes. It has been reported that human liver aldehyde dehydrogenase (ALDH2) has also esterase activity, apart from dehydrogenase (Sidhu and Blair 1975). The conversion of *p*-nitrophenyl acetate (*p*-NPA) is specific for hydrolases. The oxidation of the aldehydes and the hydrolysis of *p*-nitrophenyl acetate by horse liver aldehyde dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase take place at the same catalytic centres (Alfonzo and Apitz-Castro 1971; Feldman and Weiner 1972). The *A. thaliana* isoforms ALDH3H1 and ALDH3I1 also show the ability to hydrolyse *p*-NPA (Pandey 2013).

## 1.4.1. ALDHs in plants

In plants, ALDH enzymes are involved in several biosynthetic pathways. During the last years, revealing their physiological significance has become an important topic. The mitochondrial RF2/ALDH2B2 gene from maize was the first gene identified in plants (Liu *et al.*, 2001). It functions as nuclear restorer required for male fertility (Cui *et al.*, 1996). The first plant ALDH protein which was isolated and characterized comes from spinach (ALDH10). ALDH10 is betaine aldehyde dehydrogenase (BADH) involved in the synthesis of glycine betaine that serves as osmoprotectant in plants (Weretilnyk and Hanson 1989). ALDH2C4, occurring in *A. thaliana*, participates in ferulic acid and sinapic acid biosynthesis (Nair *et al.*, 2004). In *A. thaliana*, the ALDH2B4 enzyme that is localised in the mitochondria participates in the pyruvate dehydrogenase bypass pathway (PDH bypass) (Wei *et al.*, 2009). The data from Chinese wild grapevine have shown that the ectopic expression of VpALDH2B4 confers tolerance to salt stress and enhances resistance to mildew pathogens (Wen *et al.*, 2012). It has been reported that the expression patterns of ALDH genes corresponding to protein members of families 2, 3, 5, 7 and 10 are up-regulated when they are subjected to a variety of abiotic stress conditions including ultraviolet radiation, dehydration, high salinity, low temperature and heat shock (Weretilnyk and Hanson 1989; Deuschle *et al.*, 2001; Kirch *et al.*, 2001; Bouché *et al.*, 2003; Tsuji *et al.*, 2003; Kirch *et al.*, 2005; Shin *et al.*, 2009). The relevance of these genes in stress protection has been confirmed by analysis of transgenic plants. Plant ALDHs are involved in the protection against abiotic stress. It has been confirmed by the analyses of ALDH3I1 and ALDH7B4 T-DNA knockout (KO) mutants from *A. thaliana*. Mutant lines of ALDH3I1 and ALDH7B4 which contained T-DNA insertion were more sensitive to abiotic stress (dehydration and salinity) compared to wild-type plants (Kotchoni *et al.*, 2006). The expression of ALDH proteins affects also the flavour of fragrant rice strains such as Jasmine and Basmati (Bradbury *et al.*, 2008; Sakthivel *et al.*, 2009) or soybean (Arikit *et al.*, 2011). Two-acetyl 1-pyrroline is a compound responsible for the flavour in the fragrant rice strains (Bradbury *et al.*, 2008; Sakthivel *et al.*, 2009). ALDH enzymes, for instance *Oryza sativa* ALDH7B, play a role in seed development and maturation (Shin *et al.*, 2009). Mutated OsALDH7B caused the accumulation of oryzamutaic acid A in rice. The excess of this acid leads to the production of yellow-coloured endosperms (Shin *et al.*, 2009; Shen *et al.*, 2012). The members of ALDH

family 12 in plants are involved in the proline and arginine degradation (Brocker *et al.*, 2013).

## 1.4.2. Aldehyde dehydrogenases in *A. thaliana*

The *A. thaliana* genome contains 16 genes that encode members of 10 ALDH protein families: ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18 and novel protein family ALDH22 (Brocker *et al.*, 2013). Family 10 and family 18 are encoded by two genes, whereas family 2 and family 3 are encoded by three genes. The remaining six families (5, 6, 7, 11, 12 and 22) are represented by single genes (Kirch *et al.*, 2004).

### 1.4.2.1. Family 3 aldehyde dehydrogenases

Protein members of family 3 (EC 1.2.1.5) are homodimeric enzymes. The *A. thaliana* family 3 has three ALDH isoforms: ALDH3F1, ALDH3I1 and ALDH3H1 which share 60-76% amino acid identity with ALDH3 from *C. plantagineum* (Cp-ALDH) (Kirch *et al.*, 2004). ALDH3I1 is localised in the chloroplast, whereas ALDH3F1 and ALDH3H1 are found in the cytosol. Protein localization to the chloroplast results from the presence of a chloroplast-targeting peptide (Kirch *et al.*, 2004; Kotchoni *et al.*, 2006). The isoforms of family 3 have different expression patterns. For instance, ALDH3I1 is abundantly expressed in leaves and the expression increases after induction by exogenous abscisic acid (ABA), dehydration, high salinity, oxidants ($H_2O_2$ and paraquat), and heavy metals (Kirch *et al.*, 2001; Sunkar *et al.*, 2003; Stiti *et al.*, 2011). Moreover, it has been suggested that ALDH3I1 is involved in response to oxidative stress (Kirch *et al.*, 2001; Sunkar *et al.*, 2003). ALDH3H1 is expressed at low levels in leaves, but it is activated in roots in response to osmotic stress or ABA treatment. ALDH3F1 expression remained unchanged after stress treatment (Kirch *et al*., 2004).

Members of ALDH family 3 (ALDH3H1, ALDH3I1) catalyse the conversion of medium to long-chain aliphatic aldehydes, but long-chain aldehydes are preferred. Their affinities to unsaturated aldehydes are lower than to saturated aldehydes. Both enzymes are not able to oxidize aromatic aldehydes (Stiti *et al.*, 2011).

ALDH3I1 has a conserved valine residue in the coenzyme binding site and uses both $NAD^+$ and $NADP^+$ as coenzymes, whereas ALDH3H1 uses only $NAD^+$. Isoleucine 200 is a key residue responsible for the $NAD^+$-dependence and it is localized at a central position in the coenzyme-binding site, opposite to the negatively charged glutamate. It was previously shown that 2'hydroxyl of the adenine ribose of $NAD^+$ is bound to the glutamate residue (Perozich *et al.,* 2000; Stiti *et al.,* 2011). Stiti *et al.* (2014) showed that amino acids localized at positions 149, 178 and 200 in ALDH3H1 are critical for cofactor specificity ($NAD^+$ and $NADP^+$). Substitutions of these three amino acids made it possible that ALDH3H1 enzyme was able to use $NADP^+$ besides $NAD^+$. In the double mutant E149T/I200V of ALDH3H1 dual coenzyme specificity was demonstrated. The triple mutant E149T/V178R/I200V of ALDH3H1 showed a preference for using $NADP^+$ with almost 7-fold higher catalytic efficiency compared to the reaction in which $NAD^+$ was used (Stiti *et al.,* 2014). Cysteine mutant analyses showed that Cys-253 in ALDH3H1 and Cys-316 in ALDH3I1 are the catalytic cysteines which are performing the nucleophilic attack on the carbonyl group of the aldehyde substrate, whereas N-terminal cysteines, Cys-45 in ALDH3H1 and Cys-114 in ALDH3I1, are the redox-responsive residues mediating the dimerization via disulfide bonds under oxidizing conditions (Stiti *et al.,* 2011).

### 1.4.2.2. Family 7 aldehyde dehydrogenases

The members of family 7 are also known as α-aminoadipic semialdehyde (α-AASA) dehydrogenases or antiquitins (Brocker *et al.,* 2013). Proteins belonging to ALDH family 7 are highly conserved among plants and animals. A comparative analysis of amino acid sequences derived from animal and plant species shows about 60% sequence identity (Brocker *et al.,* 2010). ALDH7B1 from garden pea (*Pisum sativum*) previously known as protein 26g was the first ALDH7 protein discovered in plants. This enzyme is expressed upon dehydration, low temperature, heat shock and ABA treatment (Guerrero *et al.,* 1990). The

*A. thaliana* genome contains the gene ALDH7B4 that encodes a turgor responsive ALDH (ALDH7B4) (Guerrero *et al.,* 1990). The ALDH7B4 protein is localised in the cytosol (Kotchoni *et al.,* 2006) and is accumulated in plants exposed to salt stress, dehydration, ABA, $H_2O_2$ treatment or heavy metals. ALDH7B4 from *A. thaliana*, apart from its role

as detoxifying enzyme, was found to be an efficient reactive oxygen species (ROS) scavenger and involved in the inhibition of lipid peroxidation (Kotchoni *et al.*, 2006). Transgenic *Arabidopsis* plants over-expressing the ALDH7B4 gene under the control of the CaMV 35S promoter showed reduced lipid peroxidation under drought and salt stress. Moreover, the over-expression of ALDH7B4 gene in transgenic plants indicated better growth and higher germination rates in exposure to salt stress. The transgenic lines are more tolerant to high salinity (Kotchoni *et al.*, 2006). The ALDH7B4 promoter is responsive to wounding in addition to salt and dehydration (Missihoun *et al.*, 2014). It has been suggested that ALDH7B4 is involved in pathogen defence since ALDH7B4 is strongly induced by pathogens on the transcriptional level (Zimmermann *et al.*, 2004).

## 1.5. Nitric oxide (NO) as a signalling molecule in plant stress responses

Nitric oxide (NO) is a neutral, gaseous free radical with relatively short half-life (in biological systems 3-5s) (Henry *et al.*, 1997; Tuteja *et al.*, 2004). It has a relatively small molecular weight (30 g/mol) and belongs to the group of molecules composed of two atoms. Due to its lipophilic properties nitric oxide diffuses easily through biological membranes and cytoplasm.

Nitric oxide is a bioactive signalling molecule first described in mammals, in which it is involved in several physiological processes such as relaxation of smooth muscles, neural communication, and immune regulation, inhibition of platelet adhesion and aggregation or apoptosis (Schmidt and Walter 1994; Furchgott 1995). There are several reports which show that NO also plays important roles in plants including cellular processes such as plant growth and development (germination of seeds and flowering), respiratory metabolism, ripening of fruit and senescence of organs (Leshem *et al.*, 1998; Durner and Klessig 1999; Garcia-Matta and Lammatina 2001; Zottini *et al.*, 2002; Hung and Kao 2003; Lammatina *et al.*, 2003; Prado *et al.*, 2004), formation of xylem tissue, programmed cell death, pathogen defence, stomatal closure, and geotropism (Lamattina *et al.*, 2003; Neill *et al.*, 2003; Delledonne 2005; Lamotte *et al.*, 2005) as well as plant responses to biotic (disease infection) and abiotic stressors (drought, salt, heat) (Arasimowicz and Floryszczak-Wieczorek 2007).

## 1.5.1. NO biosynthesis and removal in plants

### 1.5.1.1. NO biosynthesis in plants

In animals, the enzyme responsible for NO biosynthesis is called nitric oxide synthase (NOS) (Arasimowicz and Floryszak-Wieczorek 2007). Four isoforms of this enzyme have been identified: neuronal NOS (nNOS), inducible NOS in macrophages (iNOS), endothelial NOS (eNOS) and mitochondrial NOS (mtNOS) (Nathan and Xie 1994, Tatoyan and Giulivi 1998). In an active form as a homodimer, NOS is responsible for the oxidation of L-arginine to L-citrulline and NO. When the reduced form of L-arginine is not available in animal cells, NOS produces both superoxide anion ($O_2^{\bullet-}$) and NO, leading to the generation of peroxynitrite ($ONOO^-$) (Xia *et al.*, 1998).

A gene encoding a NOS-like protein (AtNOS1) was isolated from *A. thaliana*. This protein is involved in growth and hormonal signalling (Guo *et al.,* 2003). There are any similarities between genes encoding AtNOS1 protein and NOS isoforms occurring in animals (Zemojtel *et al.,* 2006). Further studies showed that AtNOS1 is a member of the GTP-binding family. It has been proposed to rename the AtNOS1 gene and call it AtNOA1-nitric oxide associated 1 (Crawford *et al.,* 2006). Recently, it was shown that plant NOS (AtNOA1) has no NOS activity (Moreau *et al.,* 2008), but it was proposed that it is a chloroplast-targeted GTPase which is involved in proper ribosome assembly and stability (Flores-Pérez *et al.,* 2008).

Nitrate reductase (NR) is also known as a main source for NO biosynthesis (Kaiser *et al.,* 2002). The primary function of this enzyme in plants is nitrogen ($N_2$) assimilation by converting nitrate ($NO_3^-$) to nitrite ($NO_2^-$) (Neil *et al.*, 2008). Nitrate reductase is responsible for one electron transfer from NAD(P)H to ($NO_2^-$), resulting in NO production (Dean and Harper 1986). The generation of NO derived from the activity of NR is documented in many plant species, for example: cucumber (De la Haba *et al.*, 2001), sunflower, spinach, maize (Rockel *et al.*, 2002), *A. thaliana* (Desikan *et al.*, 2002), and wheat (Xu and Zhao 2003).

The other endogenous source of NO in plants is nitrite: NO-oxidoreductase (Ni-NOR). It is a protein which is localised in the plasma membrane and identified only in tobacco roots (Stöhr *et al*., 2001). This enzyme uses a cytochrome *c* as an electron donor (Stöhr and Stremlau 2006). Ni-NOR catalyses the reduction of nitrate to nitrite in the apoplastic

space and then part of nitrite can be reduced to NO by another enzyme (for instance nitrate reductase).

NO can also be synthesized non-enzymatically following the reaction between nitrogen oxides and plant metabolites. It has been reported that NO may be produced by the reduction of $NO_2^-$. This reaction is catalysed by carotenoids and light (Wojtaszek 2000). The light-mediated, non-enzymatic conversion of $NO_2^-$ to NO at acid pH has been demonstrated in the apoplast of barley aleurone layer (Bethke *et al.*, 2004). Figure 3 shows the possible pathways of NO biosynthesis in plants.

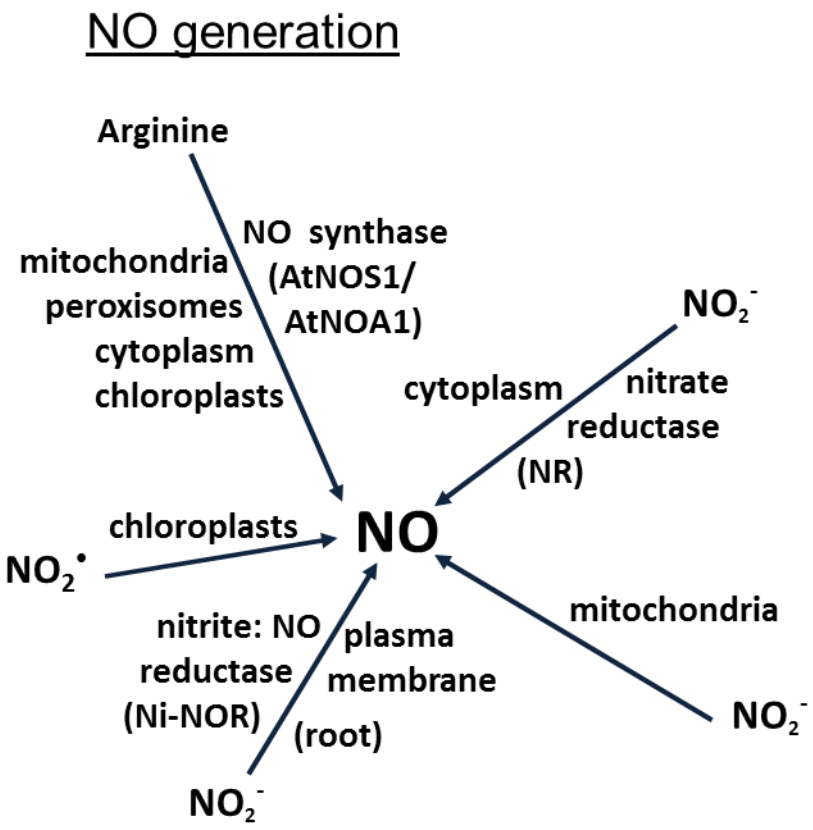



**Figure 3: The routes of nitric oxide biosynthesis in plants.**

Ref. Neil *et al.* (2008).

### 1.5.1.2. NO removal in plants

Nitric oxide is a biologically active molecule and can be rapidly metabolized or removed (Neil *et al.,* 2008). NO as a free radical is unstable and can react with oxygen ($O_2$), leading to the formation of the following oxides $NO_2^{\bullet}$, $N_2O_3$ and $N_2O_4$, which may react with cellular amines and thiols. During reaction with oxygen, nitric oxide can be also converted to nitrate and nitrite (Wendehenne *et al.,* 2001). Nitric oxide is often produced simultaneously with superoxide anion radicals ($O_2^{\bullet}$) in the same subcellular compartments in plants. Superoxide ($O_2^{\bullet}$) and nitric oxide (NO) can react together resulting in production of peroxynitrite ($ONOO^-$) (Delledonne *et al.,* 2001). In the physiological pH range peroxynitrite is unstable, but can easily diffuse through biological membranes due to a long half-life (approximately 1s) causing serious damage to cell structures (Wendehenne *et al.,* 2001). Furthermore, peroxynitrite can provoke the nitration of tyrosine residues both *in vivo* and *in vitro*, and this reaction may serve as a regulatory mechanism for protein activity (Sakamoto *et al.,* 2004).

Nitric oxide radical ($NO^{\bullet}$) may readily react with transition metals, especially haem iron and iron-sulphur centres of proteins (Stamler *et al.,* 1992). Haem-containing proteins, such as non-symbiotic haemoglobins (nsHbs) may react with NO to form nitrate as reported for *Arabidopsis*, barley and alfalfa (Perazzoli *et al.,* 2004; Perazzoli *et al.,* 2006). Moreover, the nitrosonium ion ($NO^+$) may react with sulphur, iron, nitrogen and carbon of organic compounds due to its electrophilic character (Popova and Tuan 2010).

Under physiological conditions the free NO radical may be converted to various other reactive nitrogen species (RNS). Nitrosonium cation ($NO^+$) is produced upon one electron oxidation of $NO^{\bullet}$, whereas the reduction of $NO^{\bullet}$ causes the production of a nitroxyl anion ($NO^-$) (Stamler *et al.,* 1992; Wojtaszek 2000).

The thiol group of cysteine residues in proteins and a single thiol group of glutathione (GSH) are modified by NO during a reversible process which is named *S*-nitrosylation (Neil *et al.,* 2008).

The typical concentrations of GSH range from 2 to 3 mM in plant cells (Ball *et al.,* 2004). The reaction between NO and GSH results in the formation of *S*-nitrosoglutathione (GSNO). GSNO is an endogenous nitrosothiol and a source of bioavailable NO. Protein *S*-nitrosylation can be mediated by GSNO produced by *S*-nitrosylation of GSH by NO (Wang *et al.,* 2006). It has been suggested that GSNO serves as a mobile reservoir and storage form of NO (Neil *et al.,* 2008). In plant cells, GSNO may be metabolised to

glutathione disulphide (GSSG) and ammonium ($NH_3$) by GSNO reductase (Diaz *et al.*, 2003). Figure 4 shows the possible pathways of NO removal in plants.

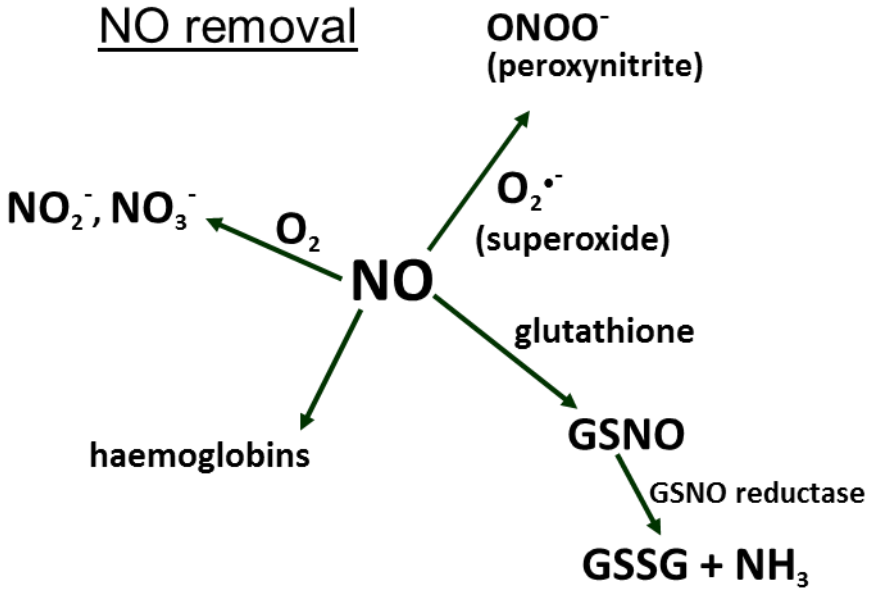



**Figure 4: The routes of nitric oxide removal in plants.**
Ref. Neil *et al.* (2008).

## 1.5.2. The effects of nitric oxide in response to drought stress

Nitric oxide, as an effective antioxidant, can have the beneficial effects on plants. It was demonstrated that NO, as potential oxidant, has also harmful effects on plants mostly due to the capacity to injure membranes, proteins and nucleic acids in plant cells. The impact of NO depends on its concentration and location in cells (Qiao and Fan 2008). Higher concentrations of nitric oxide impair leaf expansion, change thylakoid viscosity, inhibit shoot and root growth, photosynthetic electron transport, damage DNA and cause cell death (Leshem *et al.*, 1997, 1998; Pedroso *et al.*, 2000). The exposure of plants to the different abiotic stress conditions mostly induces NO production (Qiao and Fan 2008). Nitric oxide is responsible for the regulation of plant responses to stressors like high and low temperature, heavy metals, salinity, drought and oxidative stress (Garcia-Matta and

Lammatina 2001; Zhao *et al.,* 2001; Uhida *et al.,* 2002; Kopyra and Gwóźdź 2003; Zhao *et al.,* 2004).

Drought is one of the main factors leading to the decrease in relative water content (RWC). Exogenous application of sodium nitroprusside (SNP) on detached wheat leaves and seedlings that are subjected to drought causes a significant reduction of water loss and a slight decrease of ion leakage. Moreover, SNP-treated wheat leaves show a significant decrease of transpiration rate (TR) (Garcia-Mata and Lammatina 2001).

Drought stress may contribute to the reduced transpiration. The rates of transpiration are regulated by several factors, including stomata closure. It has been reported that NO-treated leaf peels of *Tradescantia* sp., *Salpichroa organifolia* and *Vicia faba* showed a significant reduction in the stomatal opening. These results suggest that SNP as nitric oxide donor might enhance plant tolerance to drought stress (Garcia-Mata and Lammatina 2001).

During water deficit late embryogenesis abundant (LEA) proteins accumulate to high levels. It has been demonstrated that SNP-treated detached leaves from wheat seedlings exhibited an increased accumulation of the group 3 LEA transcript. These data suggest that nitric oxide may be involved in the signal transduction pathway of LEA gene expression in response to drought stress (Garcia-Mata and Lammatina 2001).

Water deficit induces a decrease in photosynthesis. Furthermore, drought stress plays a key role in the generation of high levels of ROS, resulting in lipid peroxidation. It has been reported that exogenous application of SNP enhanced photosynthesis and inhibited lipid peroxidation in leaves of wheat seedlings subjected to osmotic stress (Tan *et al.,* 2008).

Zhao *et al.* (2001) showed that ABA synthesis in wheat roots in response to water deficit may be induced by NO donors in the presence of reactive oxygen species (ROS). This is evidence for a synergistic effect between NO and ROS (Zhao *et al.,* 2001). Moreover, NO and ROS may participate in the leaf water maintenance in wheat seedlings by stimulating ABA biosynthesis (Xing *et al.,* 2004).

There is evidence that proline accumulation is accelerated by exogenous application of nitric oxide in wheat leaves exposed to osmotic stress (Tan *et al.,* 2008).

# 1.6. Production of reactive nitrogen species and cross-talk with reactive oxygen species

Plants are subjected to oxidative stress conditions which cause the production of reactive oxygen species (ROS) that reach excessive and imbalanced levels (Couturier *et al.,* 2013). ROS are continuously produced during oxidative cellular metabolism. These active oxygen molecules may undergo further reactions with nitrogen or sulphur compounds, to produce reactive nitrogen species (RNS) and reactive sulphur species (RSS), respectively (Lo Conte and Carroll 2012). Reactive oxygen species include superoxide radical ($O_2^{\bullet}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH\cdot$), and singlet oxygen ($^1O_2$), whereas reactive nitrogen species contain nitric oxide ($\cdot NO$), and derived molecules such as nitrogen dioxide ($NO_2$), dinitrogen trioxide ($N_2O_3$) and peroxynitrite ($ONOO^-$). *In vivo* peroxynitrite is formed via the reaction of superoxide and nitric oxide (Couturier *et al.,* 2013). Peroxynitrite is an evidence for the interplay between ROS and RNS. This reaction determines the steady-state level of ROS and RNS, and the different developmental, metabolic and defence pathways (Molassiotis and Fotopoulos 2011). Peroxynitrite is a much more reactive molecule compared with nitric oxide and superoxide. Upon crossing biological membranes peroxynitrite is able to interact with critical biomolecules, including proteins and DNA (Molassiotis and Fotopoulos 2011). Another evidence for a cross-link between RNS and ROS is the simultaneous presence of NO and $H_2O_2$ in various physiological processes. In some cases the production of these two molecules is mutually dependent. The best characterized interaction is the spatial and temporal production of nitric oxide and hydrogen peroxide in guard cells following ABA challenge (Bright *et al.,* 2006). There is also evidence on the interaction between ROS and RNS at the gene and protein level (Tanou *et al*., 2009). For instance high ozone ($O_3$) concentrations cause the production of ROS. Nitric oxide may modify signalling, hormone biosynthesis and gene expression in *A. thaliana* plants during $O_3$ exposure (Ahlfors *et al.,* 2009). The interaction between ROS and RNS is illustrated in Figure 5.

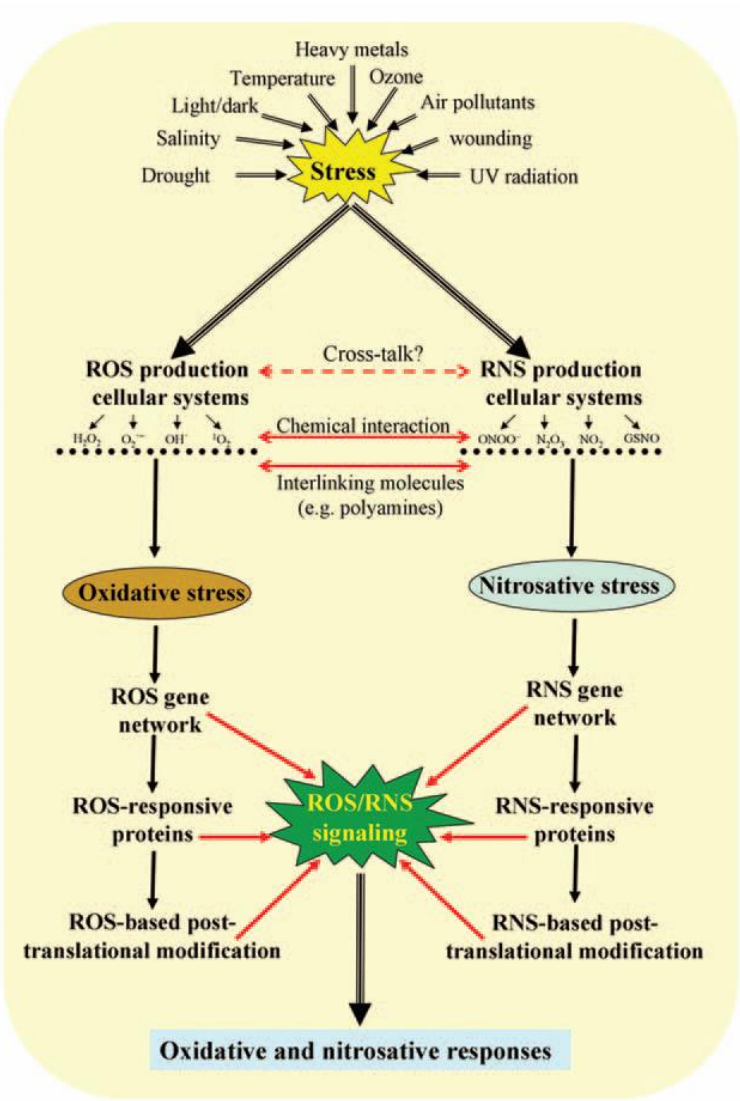



**Figure 5: The cross-talk of oxidative and nitrosative signalling in response to abiotic stress in plants.** Ref. Molassiotis and Fotopoulos (2011).

# 1.7. Posttranslational modifications of cysteine residues

Cysteines (Cys) are the least abundant (1-2%) amino acids and they are responsible for maintaining the stability of protein structures by stabilisation of hydrophobic interactions, and formation of disulphide bonds. Furthermore, cysteines play a key role in regulating proteins functions, participating in active sites of enzymes and binding to metals. Cys residues are major targets of reactive oxygen species and reactive nitrogen species. These reactions are named oxidative posttranslational modifications (Ox-PTMs) (Kim *et al.*, 2014). This is an important mechanism that may regulate protein structure and function. The reactivity of the individual Cys residue, its surrounding environment, and the

composition of the local redox-environment are factors responsible for the generation of an individual posttranslational modification (Chung *et al.*, 2013). Many posttranslational modifications are reactive and can convert the one into the other (Lo Conte and Carroll 2012).The side chain of a Cys residue contains a terminal thiol (-SH) as functional group. The thiol group contains sulphur which may assume a wide range of oxidation states (from -2 to +6) (Paulsen and Carroll 2013). The availability of different oxidation states permits the formation of a diverse range of Ox-PTMs, including reversible regulatory disulphide bonds, *S*-thiosulfinates, *S*-glutathionylation, sulfenic acid, *S*-nitrosylation and irreversible species such as sulfinic and sulfonic acids (Reddie and Carroll 2008; Roos and Messens 2011).

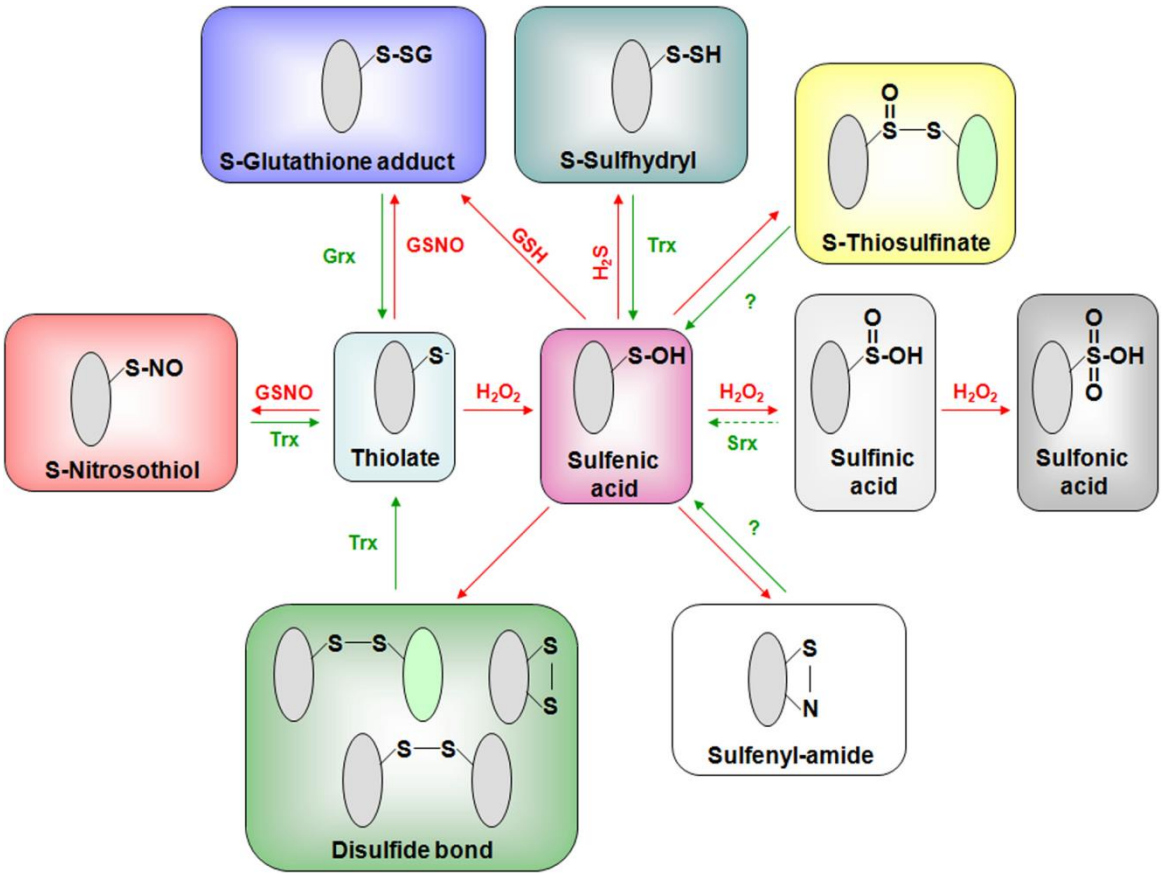



**Figure 6: Oxidative posttranslational modifications (Ox-PTMs) on cysteine residues and their reduction pathways.**

Ref. Couturier *et al*, (2013).

Figure 6 shows the possible modifications of protein thiols mediated by $H_2O_2$, ·NO and $H_2S$. The free cysteines have a pKa of about 8.3. Some proteins comprise deprotonated cysteines defined as reactive cysteines, which are more reactive due to a lower pKa, ranging from 3 to 7. The deprotonated form of cysteine is known as thiolate ($RS^-$) and is able to react faster with oxidants than thiol (R-SH) due to high nucleophilicity (Couturier *et al.,* 2013). Cysteine thiolate may be oxidized by $H_2O_2$. As a result of this reaction sulfenic acid is formed (-SOH), which is unstable and highly reactive. Due to these properties, sulfenic acid can react further with one or two additional molecules of $H_2O_2$, forming sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acids, respectively. Furthermore, sulfenic acid can react with the main nitrogen chain of neighbouring amino acids leading to production of thiosulfinate. In most cases, the sulfenic acid reacts with another thiolate from a protein cysteine. As a consequence of this reaction, an intra or inter-molecular disulphide bond is formed. Glutathione that occurs abundantly in cells may react with thiolate. This process is known as *S*-glutathionylation (Reddie and Caroll 2008).

Nitric oxide is not an effective oxidant or reducing agent under physiological conditions. NO-dependent amino acid modification mostly occurs as a secondary reaction after the oxidation of NO to nitrogen dioxide ($NO_2$), dinitrogen trioxide ($N_2O_3$) or peroxynitrite ($ONOO^-$) in the presence of oxygen, reactive oxygen species or metal centres (Broniowska and Hogg 2012). Transfer of nitric oxide to a sulphur atom on a cysteine residue to form *S*-nitrosothiol is known as *S*-nitrosylation. There are four major mechanisms to generate *S*-nitrosothiols:

1) The oxidative pathway of *S*-nitrosylation

Formation of *S*-nitrosothiols is mediated by higher oxides of NO, such as nitrosonium cation ($NO^+$) and dinitrogen trioxide ($N_2O_3$). The nitrosonium cation directly attacks thiol at neutral pH, but it is unstable in water and is immediately hydrolysed to nitrite ($NO_2$) (Kettenhofen *et al.,* 2007), which can further react with ·NO to generate $N_2O_3$ (Goldstein *et al.,* 1996). $N_2O_3$ can directly react with a thiolate anion to yield *S*-nitrosothiols and nitrite. Moreover, $N_2O_3$ can also be formed by the condensation of nitrous acid ($HNO_2$) (Guikema *et al.,* 2005).

2) The radical pathway of *S*-nitrosylation

The major reaction between two radicals is the addition of ·NO to a thiyl radical ($RS^·$) leading to *S*-nitrosothiol formation (Madej *et al.,* 2008). The direct interaction of ·NO

with thiol results in generation of intermediate radicals (RSN·OH), which can be further oxidized to *S*-nitrosothiols and superoxide (Gow *et al.,* 1997). $\cdot NO_2$ is formed by a one-electron oxidation of ·NO and is involved in conversion of thiols to thiyl radicals (Jourd'heuil *et al.,* 2003). Moreover, *S*-nitrosothiols may also be formed during direct reaction between peroxynitrite and thiolate anion (van der Vliet *et al.,* 1998) or through thiyl radicals (Goldstein *et al.,* 1996; Keszler *et al.,* 2010).

3) Metal catalysed *S*-nitrosylation

The main sites of action of nitric oxide are haem groups, containing iron in the prosthetic group (Brandish *et al.,* 1998). Proteins comprising iron in the prosthetic group react fast with nitric oxide due to a reversible electron transfer between these two molecules. There is evidence that iron and copper are involved in *S*-nitrosothiol generation via one-electron oxidation of a thiol to a thiyl radical or by the formation of NO-metal complexes (Staubauer *et al.,* 1999).

4) *Trans*-nitrosylation

*Trans*-nitrosylation is the most important reaction of *S*-nitrosothiol inside a cell (Arnelle and Stamler 1995). During this reaction a nitrosyl moiety can be transferred from a donor (*S*-nitrosocysteine or *S*-nitrosylated protein) to an acceptor cysteine thiol (Cys-to-Cys transfer). *S*-nitrosoglutathione as the major physiological NO donor is involved in mediating *trans*-nitrosylation (Dahm *et al.,* 2006). Furthermore, it has been suggested that *trans*-nitrosylation between cellular proteins plays an important role in cell signalling pathways (Nakamura and Lipton 2013).

## 1.7.1. *S*-Nitrosylation

Most proteins have cysteine residues. Over 95% of proteins contain cysteines (Shiio and Aebersold 2006), but the affinity of this amino acid residue to nitric oxide can vary widely among proteins (Kovacs and Lindermayr 2013). The specificity of *S*-nitrosylation is determined by several factors, such as local pH, redox state, or the presence of metal ions ($Mg^{2+}$ or $Ca^{2+}$). These factors control thiol accessibility or reactivity (Hess *et al.,* 2001). *S*-nitrosothiol formation is favoured in more ionisable cysteines, such as those surrounded by acidic or basic amino acids. This observation prompted the development of an acid-based motif for protein *S*-nitrosylation (Stamler *et al.,* 1997). The acid-base motif

is comprised of flanking acidic (D,E) and basic (R,H,K) residues surrounding the reactive thiol of cysteine [KRHDE]-C-[DE]. This motif can suppress or facilitate the formation of nucleophilic thiolate ($RS^-$) (Kovacs and Lindermayr 2013). The other factor, which influences *S*-nitrosothiol formation, is the low p$K_a$ of cysteine (Hess *et al.,* 2005). The presence of aromatic side chains and interaction with Cys thiols promote the formation of thiolate anions. A thiolate anion is more reactive compared to thiol and this enhances the possibility for NO modifications (Britto *et al.,* 2002). *S*-nitrosylated cysteines were found in hydrophobic pockets of proteins (Greco *et al.,* 2006). The hydrophobic environment can facilitate the direct transport and stabilization of the radicals to form *S*-nitrosylating species (Nedospasov *et al.,* 2000). In addition, secondary structure analysis reveals that endogenous *S*-nitrosylation may locate to α-helices (Doulias *et al.,* 2010), and NO donor-treated *S*-nitrosylation may locate to β-sheets (Chen *et al.,* 2010).

## 1.7.2. *S*-Glutathionylation

Glutathione (GSH) may be an electron donor for oxidative species and can form mixed disulphide bonds with available Cys. GSH is maintained at milimolar concentrations inside cells (Meister and Anderson 1983). Glutathione occurs in two different redox states: the reduced form (GSH) and the oxidized form (GSSG). *S*-glutathionylation appears at relatively high GSH/GSSG ratios and can take place through two possible mechanisms: first, thiol-disulphide exchange of GSSG with a thiolate or second, condensation of GSH with sulfenic acid (Paulsen and Carroll 2013). Cellular levels of *S*-glutathionylated proteins increase during oxidative stress. It has been proposed that *S*-glutathionylation can prevent the damages derived from irreversible oxidative modifications and protect critical Cys residues from the negative effects of these modifications (Thomas *et al.,* 1995; Hill and Bhatnagar 2012). The activity of several enzymes can be activated or inhibited by *S*-glutathionylation, such as plant glycine decarboxylase (Palmieri *et al.,* 2010). Protein-DNA and protein-protein interactions are also modified by *S*-glutathionylation (Paulsen and Carroll 2013).

### 1.7.3. Oxidation-formation of disulphide bonds

Disulphide bonds (RS-SR[1]) are formed between the thiyl radicals of two independent free thiols that are in close proximity, either within a protein or between proteins termed intra or intermolecular disulphide bonds, respectively. Disulphides can also be formed by reaction with sulfenic acid. In the first step, sulfenic acid is converted to a thiyl radical, in the presence of high concentrations of reactive oxygen species (ROS). Then, the thiyl radical reacts with thiolates to form a disulphide bond (Wardman and von Sonntag 1995; Roos and Messens 2011). The efficiency of disulphide bond formation is higher for an intramolecular disulphide than for an intermolecular disulphide (Lo Conte and Carroll 2012). Due to thiol-disulphide exchange, many enzymes can be activated (Thomas *et al.,* 1995). Moreover, disulphide bonds are often involved in protein folding or complex formation and induce a static protein conformation. It has been suggested that some disulphide bonds are dynamic and can affect changes in protein structure and function (Braakman and Bulleid 2011).

## 1.8. The methods used for detection and identification of *S*-nitrosylated proteins

The detection of *S*-nitrosylated proteins is a challenge due to the low level and dynamic features of *S*-nitrosylated proteins. The products of *S*-nitrosylation, known as *S*-nitrosothiols (SNO), are unstable due to the reactive nature of the S-N bond. The bond energy of *S*-nitrosylation (S-NO) is 29 kcal/mol (Koppenol 1998), which is significantly weaker than other posttranslational modifications, for instance disulphide bonds (S-S, 62 kcal/mol) (Jursic 1997).

*S*-nitrosothiols can easily undergo decomposition catalysed by light, metal ions (especially copper), enzymes, or reducing reagents, such as ascorbate and glutathione, to form NO and thiyl radicals ($RS^{\bullet}$). S-NO bonds are sensitive also to tertiary and quaternary changes in protein structure and cellular protein location (Pawloski *et al.,* 2001). The time needed for the extraction of *S*-nitrosylated protein from an intact cell and the assessment of the S-NO bond can dramatically affect results (Gow and Stamler 1998).

New methods were developed for identification and quantification of SNO-proteins and SNO-sites that enabled studying protein *S*-nitrosylation in complex biological systems

(Foster 2011). These methods, presented in Table 2, can be classified into three categories. X-ray crystallography, ultraviolet/visible (UV-vis) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS) and SNO-specific antibodies are the biophysical techniques that belong to the first class and enable direct detection of a NO-modified thiol. These methods are suitable for the characterization of single purified SNO-proteins and generally have low-sensitivity (Foster 2011). The methods belonging to the second class include Saville assay, diaminofluorescein (DAF) assay, gas chromatography-mass spectrometry (GC-MS), photolysis chemiluminescence, reductive chemiluminescence and NO electrode measurements. These methods enable the detection of SNO-proteins due to chemical reduction or photolytic breakdown of *S*-nitrosothiol and are suitable for quantifying total amounts of endogenous SNO-proteins in biological mixtures. Analysis of a single *S*-nitrosylated protein in complex mixtures by the second class methods is limited (Foster 2011). Biotin switch technique (BST), *S*-nitrosothiol capture (SNOCAP), SNO-site identification (SNO-SID) and resin-assisted capture of SNO proteins (SNO-RAC) constitute the third class of methods. In these techniques the *S*-nitrosylated Cys thiol contains a tag that enables the detection of SNO-proteins. The methods of the third class are suitable for the enrichment, identification and relative quantification of SNO-proteins from complex mixtures but also for the identification of SNO-sites (Foster 2011).

**Table 2: Methods for detection and characterization of *S*-nitrosylated proteins**

Ref. Foster (2011).

| Class 1: Detection of intact SNO | Class 2: Detection of SNO-derived nitrite and NO | Class 3: Labeling of SNO-derived Cys thiol |
|---|---|---|
| X-ray crystallography | Saville assay | Biotin switch technique (BST) |
| UV-vis spectroscopy | DAF-2 assay | SNO-site identification (SNO-SID) |
| NMR spectroscopy | GC-MS | S-nitrosothiol capture (SNOCAP) |
| Mass spectrometry | Photolysis chemiluminescence | Resin-assisted capture of SNO-Proteins (SNO-RAC) |
| SNO- specific antibodies | Reductive chemiluminescence | Spin trapping after UV photolysis |
| | NO electrode | Organomercurial-binding |
| | | Phosphine-based ligation |

## 1.8.1. Biotin Switch Technique (BST)

The biotin switch technique (BST) is the most widely used indirect technique for isolation and detection of *S*-nitrosylated proteins extracted from cells (Jaffrey *et al.*, 2001; Jaffrey, 2005). It allows to detect only high (nmol/mg protein) levels of intracellular *S*-nitrosothiols (Zhang *et al.*, 2005). In *A. thaliana*, this method enabled the identification of 63 proteins from GSNO-treated cell cultures and 52 proteins from NO-treated leaves (Lindermayr *et al.*, 2005). The list of identified proteins includes stress-related proteins (Cu/Zn-superoxide dismutase, Hsp 90), signalling/regulating proteins (elongation factor EF-2), redox-related proteins (glutathione peroxidase), cytoskeleton proteins (annexin, actin 2/7) and metabolic enzymes (aconitase, ATP synthase CF1 α- and β-chain, and glyceraldehyde-3-phosphate dehydrogenase) (Lindermayr *et al.*, 2005). About 60% of the identified proteins were already detected in animals as *S*-nitrosylated or *S*-glutathionylated proteins.

# 1.9. Objectives of the study

An objective of this study was to investigate the effect of NO-mediated posttranslational modification on *A. thaliana* stress-responsive aldehyde dehydrogenases (ALDHs) including ALDH3I1, ALDH3H1 and ALDH7B4. The investigations were composed of the following tasks.

1) Modulation of recombinant aldehyde dehydrogenases (ALDH3H1 and ALDH3I1) by two different nitric oxide donors and comparison of the *S*-nitrosylation patterns.

2) Comparison of the effects of nitric oxide donors on dehydrogenase and esterase activity.

3) Exploration of reversibility of *S*-nitrosylation. For this purpose the *S*-nitrosylated enzymes (ALDH3H1 and ALDH3I1) were treated with two different reducing reagents.

4) Detection of *in vivo* and *in vitro S*-nitrosylation

5) Identification of cysteine residue(s) involved in the process of *S*-nitrosylation. For this purpose Cys mutants were used.

6) Identification of SNO-sites involved in *S*-nitrosylation by mass spectrometry

7) Over-expression and purification of the recombinant ALDH7B4 protein

# 2. Materials and methods

## 2.1. Plant material

In this work *A. thaliana* ecotype Col-0 was used as the subject of study.

### 2.1.1. Sterilisation methods of plant seeds

Two different methods for surface sterilisation of *A. thaliana* seeds were used.

#### 2.1.1.1. Sterilisation method using ethanol

Seeds were first placed into sterile Eppendorf tubes. Then they were washed with 70% (v/v) ethanol for 30 s and the ethanol was poured off. In the second step the seeds were treated with 70% (v/v) ethanol and centrifuged for 10 min at maximum speed. In the next step the ethanol was poured off and the seeds were finally rinsed three to four times with sterile distilled $H_2O$. The sterilised seeds were placed on plates containing MS-agar.

#### 2.1.1.2. Sterilisation method using sodium hypochlorite

Seeds were first placed into sterile Eppendorf tubes. The seeds were washed with 70% (v/v) ethanol for 2 min. Then the alcohol was eliminated and seeds were treated with sterilisation solution containing 7% (v/v) sodium hypochlorite (Carl Roth; Karlsruhe, Germany) and 0.1% (w/v) SDS for 10 min, continuously rotating. In the next step the sterilisation solution was poured off and seeds were rinsed four times with sterile distilled $H_2O$. Afterwards the sterilised seeds were placed on plates containing MS-agar.

## 2.1.2. Root culture growth conditions

Seeds of *A. thaliana* ecotype Col-0 were sterilized using sodium hypochlorite method (2.1.1.2) and then forty seeds were sown on plates containing MS-agar. The plates were pre-incubated in a cold room at 4ºC for three days and then transferred to a growth chamber (22ºC; light/dark photoperiod of 8/16 h) for germination. Then one week later, when the seedlings reached a certain size, they were transferred to Erlenmeyer flasks containing 50 ml of liquid MS media. Twenty seedlings were cultivated in each flask. To avoid the exposure to light, the Erlenmeyer flasks were wrapped with aluminium foil and shaken at 120 rpm. The root culture was checked every week for any contamination. Part of the root culture was dried and the material was frozen in liquid nitrogen before being stored at -80ºC as a control sample. Another part of the root culture was subjected to nitric oxide treatment (2.1.3).

## 2.1.3. Nitrosative treatment of *A. thaliana* roots in liquid culture

Root cultures of *A. thaliana* were subjected to nitric oxide treatment adding 100 µM GSNO (*S*-nitrosoglutathione). The treatment lasted 4 hours. GSNO concentration was adjusted to a volume of 50 ml of liquid MS media by adding correct volume from stock solution of 1 mM. After GSNO treatment, the roots were dried and frozen in liquid nitrogen before being stored at -80ºC until further use.

# 2.2. Bacteria

## 2.2.1. Bacteria strains

- ***Escherichia coli* DH10B (Lorrow and Jessee, 1990)**

  Genotype: F $^{-}$ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80d lacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara, leu)7697 araD139 galU gal K nupG rpsL $\lambda^{-}$

This strain of *E. coli* was used as a tool for cloning experiments especially for multiplying recombinant plasmids and subsequent sequencing of the inserts.

- ***Escherichia coli* BL21 (DE3) (Pharmacia, Freiburg)**

  Genotype: F $^{-}$ ompT hsdSB ($r_B^{-}$, $m_B^{-}$) gal dcm, λDE3(lacl, lacUV5-T7 gene1, ind1, sam7, nim 5)

  This strain of *E. coli* was used for transformed in order to over-express the desired recombinant ALDH protein.

## 2.2.2. Growing conditions of bacteria cultures

All *E. coli* strains were incubated at 37ºC. Solid LB-agar plates with bacteria were transferred in an incubator with adequate aerobic conditions. Liquid cultures were grown on a shaker at 200 rpm. Proper antibiotic was added to culture to insure the selection of transformed bacteria.

## 2.2.3. Preparation of competent *E. coli* cells

Transformation is a process of gene transfer. Competent bacteria should be able to take up genetic material (plasmid DNA) from the environment and incorporate it into the cell. The transfer process occurs through bacterial cell membrane. During a quick heat shock treatment a thermal gradient is generated which allows free, extra-chromosomal DNA (such as plasmids) to enter the cell.

**Rubidium chloride method for competent cells**

Small aliquot of *E.coli* strain DH10b or BL21 (DE3) cells from a glycerol stock was plated on LB-agar plates and grown overnight at 37ºC. Next day a single colony was picked and transferred to a tube containing 3 ml of liquid LB-media. This pre-culture was grown at 37ºC, overnight on a shaker at 200 rpm. Two ml of pre-culture were taken and incubated into 200 ml of fresh liquid LB-media in Erlenmeyer flask. The bacteria culture was grown at the conditions mentioned before, until $OD_{600}$ reached a value between 0.5 and 0.6.

The obtained bacteria culture was cooled down on ice for 15 min and divided into four 50 ml Falcon-tubes. Each 50 ml of cell suspension was centrifuged at 3,000 rpm at 4ºC for 12 min. The resulting pellets were gently resuspended in 10 ml cold TFB1 buffer and incubated on ice for 5 min. The mixtures were centrifuged at the same conditions as before. The supernatants were discarded and the cell pellets were washed using 10 ml of TFB1 buffer then centrifuged at 2,000 rpm at 4ºC for 10 min. The obtained supernatants were discarded and final cell pellets were gently resuspended in 2 ml TBF2 buffer. Resuspensions were aliquoted in 50 µl samples, which were immediately frozen in liquid nitrogen and stored at -80ºC until further use.

| | |
|---|---|
| **TFB1 buffer:** | 30 mM KAc, 100 mM RbCl, 10 mM $CaCl_2 \cdot 2H_2O$, 50 mM $MnCl_2 \cdot 4H_2O$, 15% (v/v) glycerol; pH was adjusted to 5.8 with acetic acid, filter sterilized, stored at 4ºC |
| **TFB2 buffer:** | 10 mM MOPS, 75 mM $CaCl_2 \cdot 2H_2O$, 10 mM RbCl, 15% (v/v) glycerol; pH was adjusted to 6.5 with KOH, filter sterilized, stored at 4ºC |

### 2.2.4. Transformation of bacteria using heat-shock method

An aliquot of competent cells (2.2.3) was thawed slowly on the ice. Five µl of plasmid DNA was added to 45 µL of competent cells suspension and then carefully mixed. The mixture was incubated on ice for 20 min. Then the tubes were transferred to a heating block at 42ºC for 50 s and returned to ice for 2 min. In the next step, 450 µL of SOC-media was added to and incubated for 1 hour at 37ºC with continuous shaking 180 rpm. The obtained bacteria cells suspension was spread on LB-agar plates, containing the required antibiotic for selection. The plats were incubated overnight at 37ºC. A single positive colony was picked and inoculated in 3 ml liquid LB-media supplemented with the proper antibiotic. This pre-culture was used for plasmid isolation and sequencing or for large scale over-expression and purification of the desired recombinant protein.

### 2.2.5. Preparation of bacterial glycerol stocks

Bacteria are stocked in glycerol for long-term storage in order to keep the strain and possible reuse of the carried plasmids. For this reason a single bacterial colony with previously sequenced plasmid was picked and grown over-night at 37ºC in 3 ml of liquid LB-medium containing proper antibiotic. One millilitre of over-night culture was mixed vigorously with 1 ml of autoclaved 100% (v/v) glycerol. The obtained mixture was divided into four cryogenic tubes. The tubes were immediately frozen in liquid nitrogen and stored at -80ºC until further use.

## 2.3. Materials

### 2.3.1. Chemicals

The chemicals with appropriate purity used in this work were provided by specialised companies including:

- Amersham Pharmacia (Braunschweig, Germany)
- Applichem GmbH (Darmstadt, Germany)
- Bio-Rad (München, Germany)
- Boehringer (Mannheim, Germany)

- Carl-Roth GmbH (Karlsruhe, Germany)
- Enzo Life Sciences GmbH (Lörrach, Germany)
- Fluka (Buchs, Switzerland)
- GE Healthcare (Piscataway, NJ, USA)
- Merck (Darmstadt, Germany)
- New England Biolabs (Beverly, MA, USA)
- Roche (Mannheim, Germany)
- Serva (Heidelberg, Germany)
- Sigma Aldrich Chemie GmbH (München, Germany)
- Stratagene (Heidelberg, Germany)
- Thermo Scientific Pierce (Rockford, IL, USA)
- Whatmann (Maidstone, UK)

## 2.3.2. Enzymes and markers

- Restriction enzymes and their corresponding buffers were purchased from Thermo Fisher Scientific Biosciences GmbH (St. Leon-Rot, Germany).
- The DNA marker (1 kb ladder) was purchased from Thermo Fisher Scientific Biosciences GmbH (St. Leon-Rot, Germany), New England Biolabs (Beverly, MA, USA) and Thermo Scientific (Rockford, IL, USA).
- Unstained Protein Molecular Weight Marker was purchased from Thermo Scientific (Rockford, IL, USA)
- DNA polymerases for PCR were purchased from: Stratagene, Heidelberg; Germany; (*Pfu* Turbo polymerase), Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot; Germany; (*Taq* polymerase) and Thermo Scientific Rockford; IL; USA (Phusion Hot Start polymerase)
- 6x DNA loading dye Thermo Fisher Scientific Biosciences GmbH (St. Leon-Rot, Germany)
- dNTPs Thermo Fisher Scientific Biosciences GmbH (St. Leon-Rot, Germany)

## 2.3.3. Kits

Plasmid DNA was purified, according to manufacturer's specifications with kits delivered by the following companies:

- GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany)
- Genopure Plasmid Midi Kit (Roche, Mannheim, Germany)
- NucleoBond® Xtra Midi/Maxi Kit (Macherey-Nagel GmbH, Düren, Germany)
- Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany)

DNA fragments were isolated from agarose gels using High Pure PCR Cleanup Micro Kit (Roche, Mannheim, Germany).

## 2.3.4. Databases and software

Online databases and software used in this study are listed below:

**Table 3: List of databases and software**

| Name | Application | Address |
|---|---|---|
| **ClustalW2** | Multiple alignment of nucleotide or amino acid sequences | www.ebi.ac.uk/Tools/msa/clustal0/<br>The European Bioinformatics Institute (EMBL-EBI) |
| **Double digest** | Determination of a buffer suitable for restriction with two enzymes at the same time | www.thermoscientificbio.com/webtools/doubledigest |
| **ExPASY Compute $M_w$ tool** | Molecular weight of protein | http://expasy.org/tools/pi_tod.html |
| **Microsoft Office 2010** | Calculation and writing of text | |

| | | |
|---|---|---|
| **OligoCalc** | Calculation of $T_m$ and GC values of oligonucleotides | http://www.basic.northwestern.edu/biotools/oligocalc.html |
| **Predict cysteine *S*-nitrosylation sites in proteins iSNO-PseAAC** | Prediction of S-nitrosylated cysteines in amino acid sequence | www.app.aporc.org/iSNO-PseAAC |
| **Primer 3** | Designing primers | http://frodo.wi.mit.edu/primer3/ |
| **QuickChange Primer Design** | Designing primers for site-directed mutagenesis | www.genomis.agilent.com |
| **Reverse Complement** | Reverse complement of a nucleotide sequence | http://bioinformatics.org/sms/rev_comp.html |
| **Vector NTI Advance™ 10** | Sequence analysis, cloning strategies, modification of plasmid vector maps | Invitrogen, Carlsbad; CA; USA |

## 2.3.5. Equipment: machines and other devices

**Table 4: List of devices**

| Device | Name | Company |
|---|---|---|
| **Balance** | BL 1500S<br>BP 61S | Sartorius, Göttingen; GER |
| | ABJ 120-4M | Kern and Sohn GmbH, Eichbehörde; Baden-Württemberg; GER |
| **Centrifuge** | Microcentrifuge 5415D<br>Microcentrifuge 5417R<br>Centrifuge 5810R | Eppendorf, Hamburg; GER |
| | Sorvall RC5C Plus | Du Pont, Bad Homburg; GER |
| **Chemiluminescence detector** | Intelligent Dark Box II | FUJIFILM Corporation, Tokyo; JPN |
| **Electroporator** | Gene Pulser II | Bio-Rad, Hercules; CA; USA |
| **Heating block** | HLC heat block | DITABIS, Pforzheim; GER |
| | QBT digital block heater | Grant Instruments Itd, Shepreth; UK |
| **Hotplate stirrer** | HTS-1003 | LMS Laboratory and Medical Supplies, Tokyo; JPN |
| **Imaging system** | Typhoon 9200 | Amersham Biosciences, Piscatway; NJ; USA |

| | | |
|---|---|---|
| **Power supply** | Electrophoresis power supply EV243 | PEQLAB Biotechnologie; Erlangen; GER |
| **Protein Blotting cell** | Criterion blotter | Bio-Rad, Hercules; CA; USA |
| **Rotation shaker** | Innova 4000 incubator shaker | New Brunswick Scientific, Edison; NJ; USA |
| | Incubator shaker G25 | |
| | Chromate® U | B. Braun Biotech Inc., Allentown; PA; USA |
| **Scanner** | Image Scanner III | GE Healthcare; Piscataway; NJ; USA |
| **SDS-PAGE** | SDS-PAGE Minigel system | Biometra, Göttingen; GER |
| **Sonicator** | Ultrasonic Processor UP200S | Hielscher- Ultrasound Technology, Teltow; GER |
| **Spectrophotometer** | Smart Spec 3000 | Bio-Rad, Hercules; CA; USA |
| | Bio Spec-nano Spectrophotometer for Life Science | Shimadzu Biotech, Tokyo; JPN |
| | Ultrospec 2000 UV/Visible Spectrophotometer | Amersham Pharmacia Biotech, Freiburg; GER |
| **T3- thermocycler** | PCR-Cycler PTC200 | MJ Research, Walthman; |

| | | |
|---|---|---|
| | | USA |
| **Vortex** | Vortex Mixer UZUSIO VTX-3000L | LMS Laboratory and Medical Supplies, Tokyo; JPN |

## 2.3.6. Membrane

Protein transfer (Western blot) was performed on nitrocellulose membrane Protran BA-85 0.45 µm (Whatmann, Maidstone, UK).

## 2.3.7. Antibodies

For the immunodetection of proteins the following antibodies were used:

Primary antibodies:

- Monospecific antibody against ALDH3H1 purified from crude serum (BioGenes GmbH, Berlin; Germany)
- Monospecific antibody against ALDH3I1 purified from crude serum (BioGenes GmbH, Berlin; Germany)

Secondary antibodies:

- Monoclonal anti-biotin-peroxidase antibody produced in mouse BN-34 (Sigma-Aldrich; St. Louis; MO, USA)
- Anti-rabbit IgG from donkey directly coupled to horseradish peroxidase (HRP) (Sigma-Aldrich; St. Louis; MO, USA)

## 2.3.8. Vectors

In this work the following plasmid vectors were used. Detailed sequence and features of plasmid vectors are provided in the appendix.

- pET28a (+) an expression vector (Novagen, Darmstadt; Germany). This vector was used for His-tagged protein over-expression
- pJET 1.2 a cloning vector (Fermentas, Burlington; Canada). This vector was used to clone PCR products
- pNtap 289 a binary expression vector (Stratagene, Amsterdam; The Netherlands). This vector was used to clone a gene with affinity tags adjacent to the 5' end of the gene

## 2.3.9. Primers

All primers were synthesized by Sigma-Aldrich (Munich; Germany) and diluted in sterile water to a final concentration 100 µM and stored at -20ºC.

**Table 5: Primer list**

| Primer name | Sequence (5'→3') | Application |
|---|---|---|
| **Fwd3I1Ala114Val** | GATTGATGAGAAGGAGAAAGTCATCACCGAAGCTTTGTATC | Site-directed mutagenesis of $ALDH3I1_{Ala114Val}$ |
| **Rev3I1Ala114Val** | GATACAAAGCTTCGGTGATGACTTTCTCCTTCTCATCAATC | |
| **ALDH6-fwd-123** | CTGAGTGAGATTGGGCTGAC | Colony PCR of ALDH7B4 |
| **ALDH6-rev-706** | GCTACTAGCTTGGTCATTGCG | |
| **$T_7$ promoter_pET28a** | TAATACGACTCACTATAGGG | Sequencing of ALDH7B4 after cloning |
| **$T_7$terminator_pET28a** | GCTAGTTATTGCTCAGCGG | |

## 2.3.10. Media, buffers and solutions

All media were autoclaved at 120ºC at 1.5 bars for 20 min. Antibiotics and vitamins were added to the media after autoclaving to avoid their degradation though high temperature. Media containing antibiotics were stored at 4°C until further use.

| | |
|---|---|
| **LB-medium (per litre)** | 10 g tryptone; 10 g NaCl; 5 g yeast extract; adjust pH to 7.5; for solid medium add 15 g agar |
| **MS-medium (per litre)** | 4.6 g MS-salts; 20 g sucrose; 1 ml vitamin stock solution; adjust to 5.8; for solid medium add 8 g agar |
| **SOC-medium (per litre)** | 20 g tryptone; 5 g selected yeast extract; 10 mM NaCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$; 20 mM glucose; 2.5 mM KCl |
| **50x TAE buffer** | 2 M Tris base; 100 mM EDTA; adjust pH to 8.0 with glacial acetic acid |
| **1x TE buffer** | 10 mM Tris-HCl; 1 mM EDTA; adjust pH to 8.0 |

### 2.3.10.1. Additives for media

| | |
|---|---|
| **RNAse A** | 10 mg/ml RNAse A in mili-Q sterile water; aliquots are stored at -20ºC until further use |
| **IPTG (stock solution)** | 100 mM in sterile water; filter sterilize; aliquots are stored at -20ºC until further use working solution: 1:100 dilution in LB-medium |
| **Vitamin stock solution (Roots growth culture)** | 1 mg/ml glycine; 0.5 mg/ml niacin; 0.5 mg/ml pyridoxine-HCl; 5 mg/ml thiamine-HCl; 0.25 mg/ml folic acid; 0.5 mg/ml biotin; aliquots are stored at 4ºC until further use |

# 2.4. Extraction of nucleic acids

## 2.4.1. Extraction of plasmid DNA from *E. coli*

Two ml of an overnight bacterial culture were centrifuged for 2 min at maximum speed 13,200 rpm. The supernatant was removed. The pellet was resuspended in 250 µl of resuspension buffer P1 by vortexing. Two hundred-fifty microliters of lysis buffer P2 was added to the resuspended cells and mixed gently by inversion. The mixture was incubated at room temperature for 5 min. In this step, the solution became viscous and slightly clear. Three hundred-fifty microliters of neutralization buffer P3 was added to the lysate and mixed gently by inversion then centrifuged at maximum speed 13,200 rpm for 10 min at room temperature. The supernatant was transferred to a new tube containing 700 µl of isopropanol. The mixture was cooled on ice for 15 min before centrifugation for 30 min at maximum speed 13,200 rpm at room temperature. The resulting pellet was washed with pre-chilled 70% (v/v) ethanol and resupended in the desired volume with TE buffer.

| | |
|---|---|
| **Buffer P1: resuspension buffer** | 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A; stored at 4 °C after adding RNase A |
| **Buffer P2: lysis buffer** | 200 mM NaOH; 1% (w/v) SDS |
| **Buffer P3: neutralization buffer** | 3 M potassium acetate, pH 5.5 |
| **TE buffer** | 10 mM Tris-HCl, pH 7.5; 1 mM EDTA |

## 2.4.2. Purification of plasmid DNA using mini-prep columns

The plasmids were purified using plasmid mini-prep kits GeneJET™ (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot; Germany) or Qiaprep Spin (Qiagen, Hilden; Germany). To purifiy the plasmids for big scale, Genopure Plasmid Midi Kit (Roche, Mannheim; Germany) and NucleoBond® Xtra Midi/Maxi Kit (Macherey-Nagel GmbH, Düren; Germany) were used. All kits were used as described in the data-sheets provided by the supplier.

## 2.4.3. Qualitative estimation of DNA by agarose gel electrophoresis

The concentration of DNA was spectrophotometrically determined by measuring the absorbance at 260 nm and 280 nm. A value of $A_{260}$=1 approximately corresponds to 50 µg/µl for DNA solution. To estimate the purity of DNA sample, the ratio $A_{260}/A_{280}$ was measured. For a pure DNA solution, the value of ratio should be between 1.8 and 2.0, a lower value indicates contamination of DNA samples with proteins or phenolic compounds.

Agarose gel electrophoresis was used as the standard method for testing the quality and quantity of DNA. DNA molecules were separated on horizontal electrophoresis with concentration of agarose ranging from 1% to 2% (w/v) of agarose. The used gels were prepared in 1x TAE buffer containing 1:1000 (v/v) ethidium bromide (10 mg/ml). Ethidium bromide intercalates with double strand of DNA and can be visualised by excitation through UV-light (245nm). The samples were loaded in 1x loading dye (see above). DNA-ladder (1 kb) (Thermo Fisher Scientific Biosciences GmbH; St. Leon-Rot; Germany) was loaded as reference. The electrophoresis was performed in 1xTAE buffer at 80-100 V and 400 mA.

| | |
|---|---|
| **10x loading dye** | 0.25% (w/v) bromophenol blue; 30% (v/v) glycerol in 1x TAE buffer; 0.25% (w/v) xylene cyanol |

### 2.4.4. Purification of DNA fragments from agarose gel

Products obtained after PCR reactions were isolated after electrophoresis using the High Pure PCR Cleanup Micro Kit (Roche, Mannheim; Germany) or NucleoSpin® Extract II Kit (Macherey-Nagel GmbH, Düren; Germany). The kit removes primers, salts, unincorporated nucleotides and the DNA polymerase. All these compounds may inhibit subsequent enzymatic reactions such as sequencing or sub-cloning the PCR products. The extraction and purification were done upon excising the DNA bands from the agarose gel according to the instructions of the kit manufacturer.

## 2.5. Cloning of DNA fragments

### 2.5.1. Primer design

DNA sequencing enables determination of the sequence of DNA fragments. This approach is similar to PCR reaction and requires a primer to start the chain reaction. The specific primers used for sequencing or PCR amplification were designed according to the following criteria:

1. The lengths of the primers should be in between 15 and 30 nucleotides
2. The G-C content of the primer should be relatively high. It has a direct relationship to the $T_m$. The percentage of G-C nucleotides should be about 50-60% of the primer sequence. The 3' end of the primer should finish with at least one G or C to promote annealing
3. The Primer ($T_m$) is important for the annealing phase of PCR and was calculated as follows: $T_m$= 4x (G+C) + 2x (A+T). The values of temperature should be between 60ºC and 65ºC. The difference between the forward and the reverse primer melting temperatures should be no more than 2ºC.
4. In case of mutagenesis, PCR was performed with the primers presenting mismatches comparing to the DNA template. This allows the introduction of novel restriction sites according to the cloning strategy.

## 2.5.2. Site-directed mutagenesis

Site-directed mutagenesis was used to modify single amino acids and subsequently generate mutated recombinant enzymes. The primers carrying the desired mutation were designed using a tool from Agilent Technologies (QuickChange Primer Design). Site-directed mutagenesis was performed using *Pfu* Turbo® DNA polymerase due to its high fidelity and DNA proof-reading ability. The PCR reaction mixture for site-directed mutagenesis is following:

18 µl milli Q dd$H_2O$

2.5 µl $R^+$ buffer (10x)

1 µl forward primer (10 µM)

1 µl reverse primer (10 µM)

1 µl plasmid dsDNA (2.5-25 ng)

0.5 µl *Pfu* Turbo® DNA polymerase (1.25 U/µl)

1 µl dNTP mix (5 mM)

The reaction was performed using the following PCR programme:

| | | | |
|---|---|---|---|
| Initial denaturation | 95ºC | 3 min | |
| Denaturation | 95ºC | 30 s | x 12 cycles |
| Annealing | 55ºC | 1 min | |
| Elongation | 68ºC | 8 min | |
| Storage | 4ºC | ∞ | |

The obtained PCR product was digested over-night at 37ºC using 1 µl of DpnI endonuclease. DpnI is a restriction enzyme which degrades methylated and hemimethylated DNA. It was used to digest the template of plasmid DNA and eliminate it from mutated DNA product. The final PCR-product was used in a bacteria transformation. Single colonies of the transferred bacteria were picked for further control by sequencing or digestion of the isolated plasmid DNA.

## 2.5.3. Polymerase chain reaction (PCR)

The polymerase chain reaction is a standard method in molecular biology used to amplify a DNA fragments. The PCR reaction mixture was prepared as below:

| **Reagents (stock concentration)** | **Final concentration** |
|---|---|
| $MgCl_2$ (25 mM) | 1.25 mM |
| dNTPs (10 mM) | 200 µM |
| Forward primer (100 µM) | 0.5 µM |
| Reverse primer (100 µM) | 0.5 µM |
| DNA template | 1-2 ng |
| *Taq* buffer (10x) | 1x |
| *Taq* DNA polymerase (2.5 U/µl) | 1 U |
| Milli Q water | up to the final volume of the PCR reaction mixture |

Standard final volume of PCR reaction mixture was 25 µl or 50 µl.

Reactions were mixed and PCR was performed in the PCR-Cycler PTC200 (MJ Research, Walthman; USA). The optimal number of PCR cycles and the annealing temperatures were determined empirically for each PCR reaction. A standard PCR programme followed:

| | | |
|---|---|---|
| Initial denaturation step | 94ºC | 5 min |
| Denaturation step | 94ºC | 30 s |
| Annealing step | $T_a$ | 30 s |
| Elongation step | 72ºC | 1-2 min |
| Final elongation step | 72ºC | 5 min |
| Storage | 4ºC | ∞ |

The annealing temperature ($T_a$) was set 5ºC below the melting temperature of the used primers ($T_m$). For primers with different $T_m$ values, the lower value is considered for the calculation of the $T_a$.

## 2.5.4. DNA digestion

Purified plasmid DNA was digested using one or two restriction enzymes. A double digestion was performed only when both restriction enzymes can be active in a common buffer; otherwise two digestions were performed sequentially. The reaction mixture was incubated usually at 37°C for 2-4 hours. Digested DNA fragments were visualized on agarose gels and compared with the size of the expected DNA sequences. Digestions were also used to generate compatible ends of PCR products and vectors for subsequent sub-cloning.

A typical digestion mixture contained the following compounds: 1 µg of plasmid DNA, reaction buffer 10x, 1.5 U of each restriction enzyme used per 1 µg of plasmid DNA. The volume of the final reaction must be at least ten times the volume of the enzyme(s) used for digestion.

## 2.5.5. DNA dephosphorylation

The linearized plasmid vector was dephosphorylated by Antarctic phosphatase (AnP) (New England Biolabs, Beverly, MA; USA) to prevent DNA recircularization during the ligation step. Phosphatase is an enzyme that removes the phosphates from both ends of the linear DNA to prevent self-ligation of plasmid DNA. The dephosphorylation reaction was comprised of 1 µl of 10x Antarctic Phosphatase Buffer, 1 µl of Antarctic Phosphatase (5 unit) and an adequate amount of plasmid. The mixture was filled up to 10 µl with sterile water. The reaction was incubated at 37ºC for 15 min for 5' extensions and 60 min for 3' extensions. The restriction enzyme was inactivated by heating at 70ºC for 5 min.

### 2.5.6. Ligation

The final step in the construction of recombinant plasmids is the insertion of double-stranded DNA into a compatibly digested vector. This reaction is catalyzed by T4 DNA ligase (Roche, Mannheim; Germany). This enzyme enhances the formation of covalent phosphodiester linkages, which permanently join the nucleotides together. The ligation reaction was performed in a 20 µl final volume comprising 2 µl 10x ligase buffer, 1 µl plasmid DNA vector, 1 µl T4 DNA ligase and Y µl of insert DNA. The volume was filled up to 20 µl with sterile water and incubated at 16ºC for 20 h. The amount of plasmid DNA in the reaction should be the third of the insert DNA to ensure an efficient ligation. The ligation product was used for bacterial transformation.

## 2.6. Extraction of proteins

### 2.6.1. Isolation of total proteins from root culture of *A. thaliana*

Total protein extracts were isolated from *A. thaliana* roots according to the procedure described by Jaffrey *et al.* (2001). The frozen roots were grounded in liquid nitrogen until obtaining a fine powder. The powder was transferred to 15 ml Falcon-tubes and resuspended in HEN buffer (250 mM Hepes-NaOH pH 7.7; 1 mM EDTA; 0.1 mM Neocuproine). The 1.2 ml of HEN buffer was added for each gram of cell powder. The extract was centrifuged at 2,000 x *g* at 4ºC for 10 min. The supernatant was carefully transferred to new Eppendorf tubes and the pellet was discarded. The extracted proteins were used for further assay.

### 2.6.2. Determination of protein concentration

Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Münich; Germany) or Roti®-Quant (Roth, Karlsruhe; Germany) protein quantitation assay according to Bradford (1976). The protein samples (5 or 10 µl) were diluted using ultrapure water up to 800 µl. Two-hundred microliters of Bradford reagent was added to the diluted protein samples and vortexed briefly until obtaining homogeneous solution. The samples were incubated at room temperature for 20 min before measuring the

absorbance at 595 nm. The blanking sample contains 5 or 10 µl of buffer instead of the protein sample. The amount of protein was estimated using a standard curve which was obtained by using a serial dilution of known concentrations of bovine serum albumin (BSA).

## 2.6.3. Over-expression of recombinant ALDHs in *E. coli* cells

Recombinant expression vectors pET-28_ALDH3H1 and pET-28_ALDH3I1 were used to express the corresponding recombinant proteins in *E. coli* BL21 (DE3). The glycerol stock of transformed bacteria was plated on a LB agar supplemented with kanamycin (50 µg/ml). The plates were incubated at 37°C overnight. Next day a single colony was picked from a plate and inoculated into a tube containing 3 ml of liquid LB medium supplemented with kanamycin (50 µg/ml). The pre-bacteria culture was incubated at 37ºC continuously shaking at 200 rpm for overnight. Next day 2 ml of overnight pre-culture was transferred into a 200 ml of LB medium supplemented with kanamycin and grown at 37ºC until $OD_{600}$ reached a value between 0.5 and 0.6. Then, the culture was placed in a dark room at 26ºC for 30 min with continuous shaking at 200 rpm. After this time, the expression of the protein was induced by adding 1 ml of 1 mM IPTG and cultivated for 3 hours at 26ºC in the dark. Bacteria were harvested by centrifugation at 4ºC at 5,300 rpm for 20 min. The obtained supernatant was discarded and the pellet was frozen at -20ºC. This pellet was used further for purification of recombinant ALDHs.

## 2.6.4. Purification of recombinant ALDHs by His-tag affinity chromatography

Soluble polyhistidine-tagged recombinant proteins expressed in *E. coli* were purified by immobilized metal affinity chromatography (IMAC) under native conditions. The pellets obtained after over-expression of recombinant ALDHs was used were thawed on the ice for 30 min then resuspended in 5 ml of buffer A by pipetting or vortexing. Lysozyme (1 mg/ml final concentration) was added to the resuspension solution and incubated on ice for 30 min. The cell suspension was sonicated for complete lysis. Sonication was used for disruption of the cell membrane. The process of sonication was repeated up to 6-7 times until the solution became transparent. During the sonication process, cell suspension was kept on ice to avoid warming up the cells. The solution resulting from sonication process

was centrifuged for 30 min at 4ºC at 14,000 rpm. The obtained supernatant was filtered through a sterile membrane (0.22 µm) to remove insoluble cell debris. The affinity gel was prepared for the purification. It was washed with sterile water to remove the rest of ethanol used for storage of the column and then charged with 5 volumes of $NiSO_4$ (10 mg/ml). Three volumes of sterile water and five volumes of buffer A were used for the equilibration of the column. The clear supernatant was loaded onto a previously equilibrated column and allowed to drain by gravitation. The flow through of the supernatant was collected and re-loaded on the column. Then the column was washed with 10 volumes of buffer A and 8 volumes of buffer B. The protein was eluted using 4 volumes of buffer C and the eluate was collected in 250 µl fractions. The eluted fractions were stored immediately on ice to prevent loss of enzyme activity. Protein concentration of each fraction was determined by the Bradford assay. The purity of eluted protein was verified by SDS-PAGE electrophoresis. In the case of the storage of the enzyme, the purified peak fractions of protein were stabilized by adding 1 mM PMSF; 0.5 mM NAD; 6 mM DTT and glycerol up to 50% (v/v) and were aliquoted and stored at -80 ºC. The aliquots of enzyme were used for further experiments.

After use, the column was cleaned with 2 volumes of sterile deionized water, 5 volumes of 6 M guanidine-HCl, pH 7.5; 3 volumes of sterile deionized water, 2 volumes of 30% (v/v) ethanol and then resuspended in 30% (v/v) ethanol for storage at 4ºC. The column was used up to 5 times and was recharged again with $NiSO_4$.

| | |
|---|---|
| **Buffer A:** | 50 mM HEPES-NaOH pH 7.4;<br>300 mM NaCl; 10% (v/v) glycerol;<br>0.1% (v/v) Triton X-100; 1 mM ß-Me<br>added freshly, 5 mM imidazole |
| **Buffer B:** | 50 mM HEPES-NaOH pH 7.4;<br>300 mM NaCl; 10% (v/v) glycerol;<br>0.1% (v/v) Triton X-100; 1 mM ß-Me<br>added freshly; 20 mM imidazole |
| **Buffer C:** | 50 mM HEPES-NaOH pH 7.4;<br>300 mM NaCl; 10% (v/v) glycerol;<br>0.1% (v/v) Triton X-100; 1 mM ß-Me<br>added freshly; 250 mM imidazole |

### 2.6.5. Purification of monospecific IgG antibodies against ALDH3H1 and ALDH3I1 from crude serum

The method described by Robinson *et al*. (1988) with the modifications introduced by Missihoun (2011) in his Ph.D. thesis was used to purify monospecific IgG antibodies against ALDH3H1 and ALDH3I1. The purified recombinant form of the protein was loaded on the gel and separated by SDS-PAGE electrophoresis. Then, the separated protein was immobilized onto a nitrocellulose membrane. The membrane was incubated with Ponceau S and the fragment containing the recombinant protein was cut into pieces of 1.5 cm length. Then, strips were incubated in Falcon-tubes containing 50 ml of blocking solution (1x TBS; supplemented with 4% (v/v) non-fat dry-milk powder) overnight at 4ºC with gentle shaking. The next day 0.5 ml of the crude serum was added to the blocking solution and the membrane strips were incubated for 1 h at room temperature. The blocking solution was removed and the membrane strips were washed for 20 min as follows: once using 1x TBST and twice using 1x TBS. After the last washing step, the membrane strips were soaked in 1 ml of 0.2 M glycine-HCl, pH 2.2 for 1 min with gentle shaking. The glycine solution was transferred to a new Eppendorf tube and stored. The membrane strips were neutralized with 666 µl of 1 M Tris-HCl, pH 8.8 for 10-20 s. Then Tris-HCl solution was poured into the glycine solution to neutralize the IgGs. The mixture of monospecific IgGs was added into 50 ml blocking solution and either used directly as a primary antibody or stored at -20ºC. The primary antibody could be used up to five times. The membrane strips were stored in 1x TBS; 0.2% sodium azide at 4ºC.

## 2.7. Electrophoresis of proteins

### 2.7.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE is a technique used for separation of negatively charged proteins based on their ability to move under an electrical current. Sodium dodecyl sulphate is added as detergent to remove secondary and tertiary protein structures. It maintains the proteins as polypeptide chains. The SDS coats the proteins proportionally to their molecular weight. The Laemmli system (Laemmli 1970) is the most used gel system for separating the

mixture of proteins. The gel consists of 4% (w/v) acrylamide stacking gel and 12% (w/v) acrylamide separating gel as described in table below. Samples were mixed with Laemmli buffer and heated at 95ºC for 5 min to denature the proteins before loading on gel. Electrophoresis was performed using 1x SDS-running buffer. The intensity of the current is 10 mA for the stacking gel and 20 mM for the separating gel. The protein ladder was provided by Thermo Fisher Scientific Biosciences (St. Leon-Rot; Germany). The unstained protein marker contains a mixture of seven native proteins: ß-galactosidase (*E. coli*; 116 kDa), bovine serum albumin (bovine plasma; 66.2 kDa), ovalbumin (chicken egg white; 45 kDa), lactate dehydrogenase (porcine muscle; 35 kDa), restriction endonuclease *Bsp*98I (*E. coli*; 25 kDa), ß-lactoglobulin (bovine milk; 18.4 kDa) and lysozyme (chicken egg white; 14.4 kDa). Electrophoresis was run until the dye front reached the bottom of the gel.

**Table 6: Composition of the gels used for SDS-PAGE**

| **Stock solution** | **Separating gel** (12%; 6 ml) | **Stacking gel** (4%; 3 ml) |
|---|---|---|
| **$H_2O$** | 1.92 ml | 2.16 ml |
| **1.5 M Tris/HCl pH 8.8** | 1.65 ml | — |
| **1 M Tris/HCl pH 6.8** | — | 375 µl |
| **30% (v/v) Acrylamide** | 2.4 ml | 405 µl |
| **10% (w/v) SDS** | 60 µl | 30 µl |
| **TEMED** | 2.4 µl | 3 µl |
| **10% (w/v) APS** | 60 µl | 30 µl |

| | |
|---|---|
| **1x SDS-running buffer, pH 8.3** | 25 mM Tris; 192 mM Glycine; 0.1% (w/v) SDS pH was not adjusted |
| **Laemmli buffer** | 4% (w/v) SDS; 20% (v/v) Glycerol; 120 mM Tris pH 6.8; 0.2 M DTT; 0.01% (w/v) bromophenol blue |

## 2.7.2. Isoelectrofocusing

Isoelectrofocusing (IEF) is a method used for separation of proteins according to the differences in their isoelectric points (pI). The process consists of two steps: rehydration and equilibration. In the first step the protein pellets were resuspended in 125µl of rehydration buffer and then incubated at room temperature for 1h. After incubation the protein samples were centrifuged to remove insoluble material. The supernatant was placed in the middle of a strip holder channel. The IPG strip was put onto the sample, avoiding the introduction of air bubbles. The IPG strip and the protein sample were covered by IPG Cover Fluid to prevent evaporation during the rehydration process. The rehydration was performed during 16-17 h at 20ºC according to the following programme:

1) 14 h rehydration
2) 30 min 500 V
3) 30 min 1000 V
4) 100 min 5000 V

} 7 cm strip

It is important to maintain the temperature in the average of 20ºC, because temperatures higher than 37ºC can cause protein carbamylation, whereas temperatures lower than 10ºC can yield urea crystallization on the IPG gel.

| | |
|---|---|
| **IEF Rehydration buffer:** | 7 M urea; 2 M thiourea; 2% (w/v) CHAPS; 0.002% (w/v) bromophenol blue; 0.5% (v/v) IPG buffer pH 3-10 added freshly |

After IEF, the strips were equilibrated in two different buffers. Equilibration buffer I contains DTT which reduces sulfhydryl groups, whereas equilibration buffer II contains iodoacetamide which alkylates the obtained thiol groups of proteins. Then the strips were incubated with equilibration buffers for 15 min at room temperature.

| | |
|---|---|
| **Equilibration buffer I:** | 50 mM Tris/HCl, pH 6.8; 2% (w/v) SDS; 6 M urea; 30% (v/v) glycerol; 0,002% (w/v) bromophenol blue; 2 % (w/v) DTT |
| **Equilibration buffer II:** | 50 mM Tris/HCl, pH 6.8; 2% SDS (w/v); 6 M urea; 30% (v/v) glycerol; 0,002% (w/v) bromophenol blue; 2.5% (w/v) iodoacetamide |

After the equilibration step, the IPG strips were placed on the surface of separation in a SDS-PAGE gel and covered with IEF-melted agarose. The separation in the second dimension was performed until the bromophenol blue lane (visible during migration) reached the bottom of the separating gel.

| | |
|---|---|
| **IEF-agarose** | 0,5% (w/v) agarose; 0,002% (w/v) bromophenol blue in SDS-running buffer |

## 2.7.3. Staining of protein gels

After separation by SDS-PAGE electrophoresis, protein bands can be visualized using different methods for in-gel protein detection. In this work Coomassie blue staining and silver staining were used. Coomassie bluee staining has a detection range of 8-50 ng, whereas silver staining has a range of 1-2 ng.

### 2.7.3.1. Coomassie blue staining

The gels were stained with commercial solution Page Blue$^{TM}$ Protein Staining Solution (Fermentas, Vilnius; Lithuania). The gels obtained after electrophoresis were placed in a tank containing fixation solution for at least 1 h. Then, the fixation solution was removed and the gels were washed with distilled water (three times for 20 min each). Afterwards, Page Blue$^{TM}$ solution was added and the gels were stained for at least 2 h. The solution was removed. The gels were washed with distilled water (three times for 20 min each)

and the protein bands were visualized. Incubation and washing steps were performed at room temperature with constant shaking.

| | |
|---|---|
| **Fixation solution:** | 50% (v/v) methanol; 10% (v/v) acetic acid |

## 2.7.3.2. Silver staining

The gels were placed in a tank containing fixation solution and incubated at least 1 h with constant shaking. Mostly the fixation step was done over-night. Then, the fixation solution was removed. The gels were rinsed with distilled water and then incubated in an incubation solution for 2 h. The incubation solution was removed and gels were washed with distilled water (three times for 20 min each). Then, the gels were stained with silver solution for 30 min. In the last step, the protein bands were revealed with developing solution. The staining was stopped with stop solution.

| | |
|---|---|
| **Fixation solution:** | 50% (v/v) ethanol; 10% (v/v) acetic acid; filled with water up to 100 ml |
| **Incubation solution:** | 6.8 g sodium acetate; 0.2 g sodium thiosulfate; 30 ml ethanol; 1 ml 50% (v/v) glutardialdehyde; filled with water up to 100 ml |
| **Silver solution:** | 0.05 g silver nitrate; 15 µl 37% (v/v) formaldehyde; filled with water up to 50 ml |
| **Developing solution:** | 2.5 g sodium carbonate; 29 µl 37% (v/v) formaldehyde; filled with water up to 100 ml |
| **Stop solution:** | 0.8 g glycine; filled with water up to 200 ml |

## 2.7.4. Protein immunoblot

To immunologically detect the proteins of interest, the protein samples were analysed by SDS-PAGE electrophoresis and then transferred from the polyacrylamide gel onto a Protran nitrocellulose BA85 membrane. The proteins transferred to nitrocellulose are accessible for detection using antibodies. The transfer was carried out using a pre-chilled protein blot transfer buffer (PBTB) (Towbin *et al.*, 1979) and performed at 70 V for 1 h at 4ºC. After blotting the proteins were detected with a Ponceau Red solution. After confirmation of the protein transfer, the membrane was incubated in blocking solution overnight at 4ºC with constant shaking. The blocking step is carried out to reduce non-specific binding of the antibody with the membrane. Then the blocking solution was removed and the membrane was incubated with primary antibody for 1 h at room temperature in blocking. The concentration of the antibody depended on the type of the antibody used. After incubation with primary antibody the membrane was washed with 1x PBST as follows: once briefly and discarded, once 15 min and three times for 5 min. Subsequently, the membrane was incubated with the secondary antibody at a 1:5000 dilution anti-rabbit IgG coupled with horseradish peroxidase or 1:20000 dilution for monoclonal anti-biotin-peroxidase antibody produced in mouse in the blocking solution. After incubation with the secondary antibody the membrane was washed again as described above. For detection of the target protein-antibody complex the ECL Plus Western Blotting Detection kit (Amersham Biosciences, Little Chalfont Buckinghamshire; UK) was used. The secondary antibody coupled to horseradish peroxidase permits direct detection on the membrane by chemiluminescence under a CCD camera (Intelligent Dark Box II, Fujifilm Corporation; Tokyo; Japan). The reaction product produces luminescence in proportion with the amount of protein.

| | |
|---|---|
| **10x PBS, pH 7.4:** | 37 mM NaCl (80 g/l); 2.7 mM KCl (2 g/l); 8.1 mM $Na_2HPO_4$ (14.4 g/l); 1.47 mM $KH_2PO_4$ (2.4 g/l). Adjust pH with HCl |
| **PBST solution:** | 1x PBS + 0.1% (v/v) Tween 20 |
| **Protein-blot transfer buffer (PBTB):** | 25 mM Tris; 192 mM glycine; 20% (v/v) methanol. Do not adjust pH |

| | |
|---|---|
| **Blocking solution:** | 4% (w/v) non-fat dry-milk or 4% (w/v) BSA powder dissolved in PBST solution |

### 2.7.5. Ponceau-Red staining

Ponceau S is used to prepare a stain for rapid reversible detection of protein bands on the membrane. The Ponceau-Red staining was done after protein blotting. The membrane was stained for 10 min with Ponceau-Red solution with gentle shaking. The solution was rinsed and the membrane was then washed briefly with distilled water. Positions of the standard proteins from molecular weight ladder were marked with a pencil and the membrane was scanned. The membrane was used further for immunodetection.

| | |
|---|---|
| **Ponceau S solution:** | 0.2% (w/v) Ponceau S in 3% (w/v) TCA |

## 2.8. Process of *S*-nitrosylation

### 2.8.1. *In vitro S*-nitrosylation of recombinant ALDH3H1 and ALDH3I1 proteins

Freshly purified recombinant protein was *S*-nitrosylated by incubation with 500 µM GSNO or 500 µM SNP for 2.5 h at room temperature in the dark. The enzyme activity of the *S*-nitrosylated form was measured according to the enzymatic assay conditions. The *S*-nitrosylated enzyme was reduced by incubation with 10 mM DTT or GSH for 1.5 h at room temperature. The activity of the recovered enzyme was measured according to the enzymatic assay conditions. Simultaneously, the activity of the reduced enzyme was determined. The inhibition rate was calculated from the results.

The *S*-nitrosylated fraction of enzyme was used in further step of immunodetection.

## 2.8.2. Blocking of free sulfhydryl groups

*S*-nitrosylated protein was precipitated with an equal volume of 20% (v/v) TCA to remove the free nitric oxide donor from the protein solution. The NO donor may nitrosylate cysteines that are inaccessible after the proteins are denaturated in the subsequent steps. The sample was incubated on ice for 30 min and then centrifuged for 15 min at 4ºC at maximum speed. The supernatant was discarded and the pellet was washed twice with 300µl of pre-chilled acetone. The pellet was air-dried and resuspended in HEN buffer. Free thiols were blocked by incubation with MMTS or NEM in blocking solution. SDS is used as a protein denaturant to ensure the access of MMTS or NEM. This step was carried out at 50ºC for 1h with frequent vortexing. The sample was covered with aluminum foil to avoid light.

| | |
|---|---|
| **HEN buffer:** | 250 mM HEPES-NaOH pH 7.7; 1 mM EDTA; 0.1 mM Neocuproine |
| **Blocking solution (10 ml):** | HEN buffer (9 ml); 25% (w/v) (1 ml); 20 mM MMTS in DMF or 20 mM NEM in ethanol |

## 2.8.3. Biotinylation of *S*-nitrosothiols

Unreacted MMTS or NEM can compete with biotin-HPDP for thiol groups. The thiol-modifying agent used to block cysteine residues was removed from the protein solution by precipitation with an equal volume of 20% (v/v) TCA. The sample was incubated on ice for 30 min and then centrifuged for 15 min at 4ºC at maximum speed. The supernatant was discarded and the pellet was washed twice with 300 µl of pre-chilled acetone. The pellet was air-dried and resuspended in HEN buffer. Then the *S*-nitrosylated protein sample was treated with 1/3 volume of labelling solution for 1h at room temperature. At this step the sample was no longer protected from light. The biotinylated protein was detected by anti-biotin antibodies after Western blotting.

| | |
|---|---|
| **Labelling solution:** | 50 mM biotin-HPDP suspension in DMSO stored at -20ºC. Stock solution diluted to 4 mM with DMF |

### 2.8.4. Immunodetection of *S*-nitrosylated ALDH3H1 and ALDH3I1 proteins

SDS-PAGE electrophoresis was performed under non-reducing conditions to prevent non-specific reactions of biotin-HPDP. The membrane was blocked with 4% (w/v) BSA in 1xPBST and then incubated with specific monoclonal anti-biotin-peroxidase antibody in blocking solution for 1h at room temperature with gentle shaking. After incubation with secondary antibody the membrane was washed with 1x PBST as follows: once briefly and discarded, once for 15 min and three times for 5 min. The signal from the biotinylated protein was detected with the ECL detection system.

## 2.9. Enzymatic activity and assays

### 2.9.1. Dehydrogenase activity

Recombinant ALDH enzymes purified by affinity chromatography were used for enzymatic assays. The activity of ALDH enzymes was determined according to the optical Warburg test. Dehydrogenase activity was measured with a spectrophotometer at 340 nm by monitoring conversion of $NAD^+$ to NADH ($\varepsilon_{NADH}$= 6.22 $mM^{-1}$ $cm^{-1}$ at $\lambda$=340 nm). The reaction was performed at room temperature in a 1 ml quartz cuvette and the absorbance was recorded for 3-5 min at 15 s intervals. A standard reaction mixture contained 100 mM sodium pyrophosphate pH 8.0 (for ALDH 3H1) or pH 9.0 (for ALDH3I1), constant optimum concentration of NAD (2 mM) and variable concentration of aldehydes. The constant optimum concentration of aldehydes was used for measuring the enzyme activity of the *S*-nitrosylated form. Three independent repetitions were made for each enzymatic assay. The data are represented as mean±SEM. The enzymatic reaction was started by adding 1 µg of freshly purified enzyme or enzyme from glycerol stock to a standard reaction mixture. The solution was mixed, transferred to a quartz cuvette and placed in the spectrophotometer. According to the measured values

of absorbance, the kinetic parameters were determined using the Michaelis-Menten equation. One unit of aldehyde dehydrogenase was defined as the amount of enzyme needed to reduce 1 µmol of $NAD^+$ to NADH in 1 min.

## 2.9.2. Esterase activity

The buffer conditions for the esterase assay were optimized by Pandey (2013). According to the results obtained by Pandey, the esterase activity of ALDH was determinded using 25 mM HEPES pH 8.0 (for ALDH3I1) or pH 9.0 (for ALDH3H1). Recombinant ALDH enzymes purified by affinity chromatography were used for enzymatic assays. Esterase activity was measured spectrophotometrically following the hydrolysis of *p*-nitrophenyl acetate at 400 nm. The reaction was carried out in a cuvette containing 1 ml of 25 mM HEPES buffer pH 8.0 (or pH 9.0), 3 µl of 184 mM substrate *p*-nitrophenyl acetate and 1 µg of freshly purified enzyme. The enzymatic reaction was performed at room temperature and the absorbance was recorded for 2 min at 15 s intervals. Three independent repetitions were made for each enzymatic assay. The data are represented as mean±SEM. A molar extinction coefficient of $18.3x10^3$ at 400 nm for *p*-nitrophenolate was used to calculate the micromoles of *p*-nitrophenyl acetate hydrolyzed per minute. One unit of esterase activity was defined as the amount of esterase required to release 1 µmol of *p*-nitrophenyl under the assay conditions.

## 2.9.3. DTNB assay

DTNB known as Ellman's reagent (Ellman 1959) is used in colorimetric assays for quantification of free sulfhydryl groups. This compound has high specificity for -SH groups at neutral pH and allows to estimate the percentage of free sulfhydryl groups in *S*-nitrosylated enzymes with respect to the reduced form. Recombinant purified ALDH enzymes were *S*-nitrosylated by incubation with 500 µM nitric oxide donor (GSNO or SNP) at room temperature under dark conditions for 3h. The samples were collected at different time points and absorbance of these samples was measured at 412 nm. The enzymatic assay was performed as follows: first 2 µl of 20 mM DTNB was added to 18 µl of ALDH sample (*S*-nitrosylated or reduced), which was mixed with 780 µl of 100 mM potassium phosphate buffer pH 7.4 and incubated for 20 min at room temperature under

dark conditions. During this time the solution developed a yellow colour. After incubation, the absorbance of the released thiophenol anion ($TNB^-$) was measured spectrophotometrically at 412 nm. The obtained results are presented as the percentage of the remaining sulfhydryl groups in the *S*-nitrosylated enzyme compared to sulfhydryl groups in the reduced form of the enzyme.

# 3. Results

This section presents the outcomes of the experiments carried out in this work. The results obtained in this research are divided into four parts. In the first part the results of the location and substitution of Cys residues in amino acid sequences of ALDH enzymes (ALDH3H1 and ALDH3I1) are presented. This part contains the results of the purification of recombinant ALDH family 3 proteins and the corresponding Cys mutants. The second part includes the results showing the effects of *S*-nitrosylation on ALDH activities (dehydrogenase activity and esterase activity). The third part contains the results of the detection of *in vitro S*-nitrosylated ALDH3I1 enzyme and *S*-nitrosylated *in vivo* and *in vitro* ALDH3H1 enzyme. The final part includes the results of the over-expression and the purification of recombinant ALDH7B4 protein.

## 3.1. The members of the ALDH family 3 and the corresponding Cys mutants

ALDH3H1 contains three Cys residues located at positions 45, 247 and 253 of the amino acid sequence. Single mutants were generated, in which Cys residues were substituted (Figure 7). Cys-45 was substituted by Ser or Ala residue, whereas Cys-247 and Cys-253 were substituted by Ser. Substitution of Cys-253 by Ser in the ALDH3H1 protein caused a total loss of the dehydrogenase activity, but maintained the esterase activity.

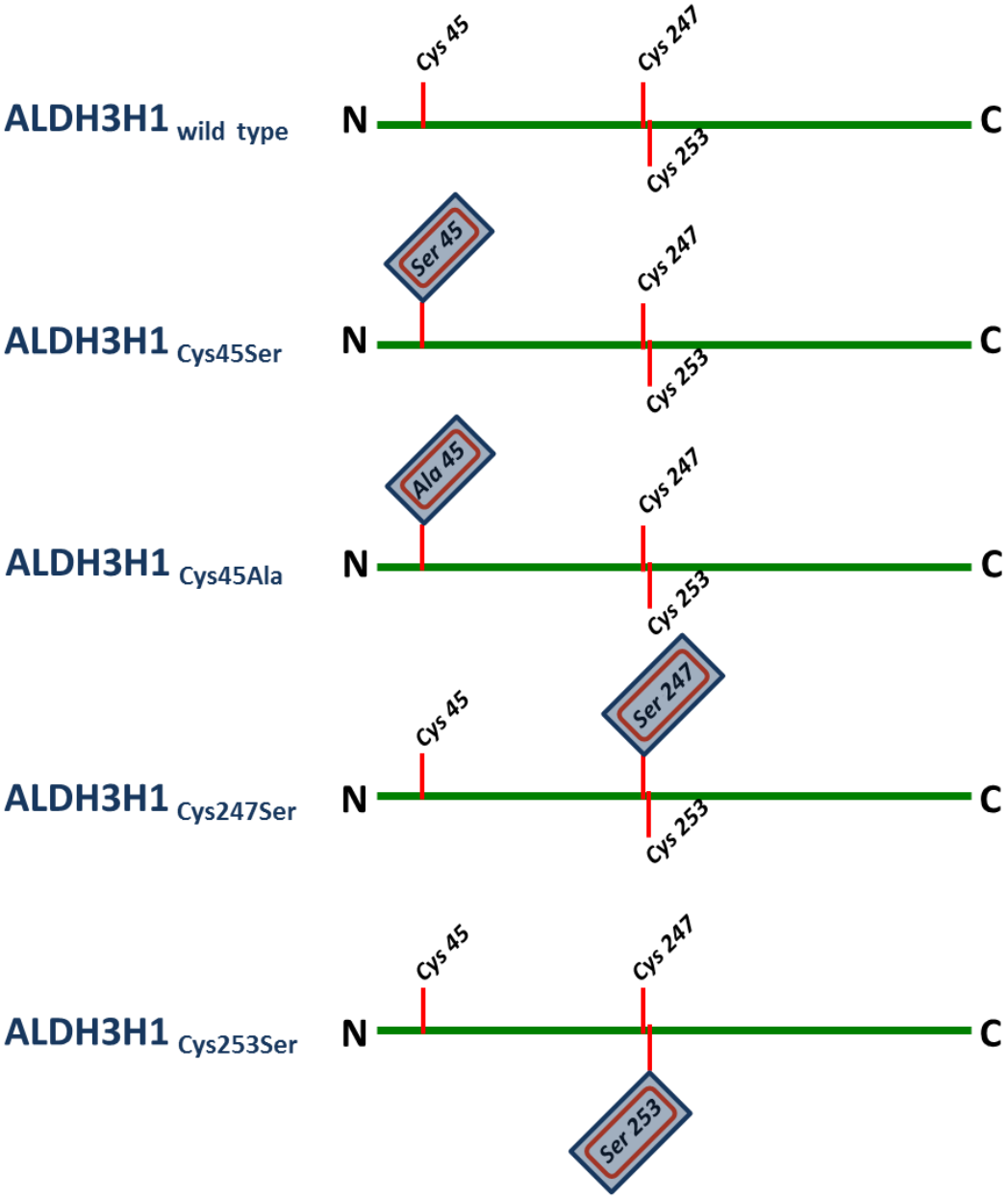



**Figure 7: Cysteine mutants generated in the recombinant ALDH3H1 protein.**

The positions of the substituted amino acid residues are indicated in the boxes. The Cys residue located at position 45 was substituted by Ser or Ala and two mutant enzymes for this Cys were generated.

ALDH3I1 contains nine Cys residues located at positions 14, 42, 46, 55, 114, 142, 286, 310 and 316 of the amino acid sequence. ALDH3I1 contains an N-terminal plastid targeting sequence and four Cys residues (14, 42, 46 and 55) are located in the chloroplast signal peptide. The recombinant protein lacks most of the plastid targeting sequence. Single mutants were generated, in which Cys residues were substituted (Figure 8).

Cys-114 was substituted by Ser or Ala or Val residues, whereas Cys-142, Cys-286, Cys-310 and Cys-316 were substituted by Ser. Substitution of Cys-316 by Ser in the ALDH3I1 protein caused a total loss of the dehydrogenase activity, but maintained the esterase activity.

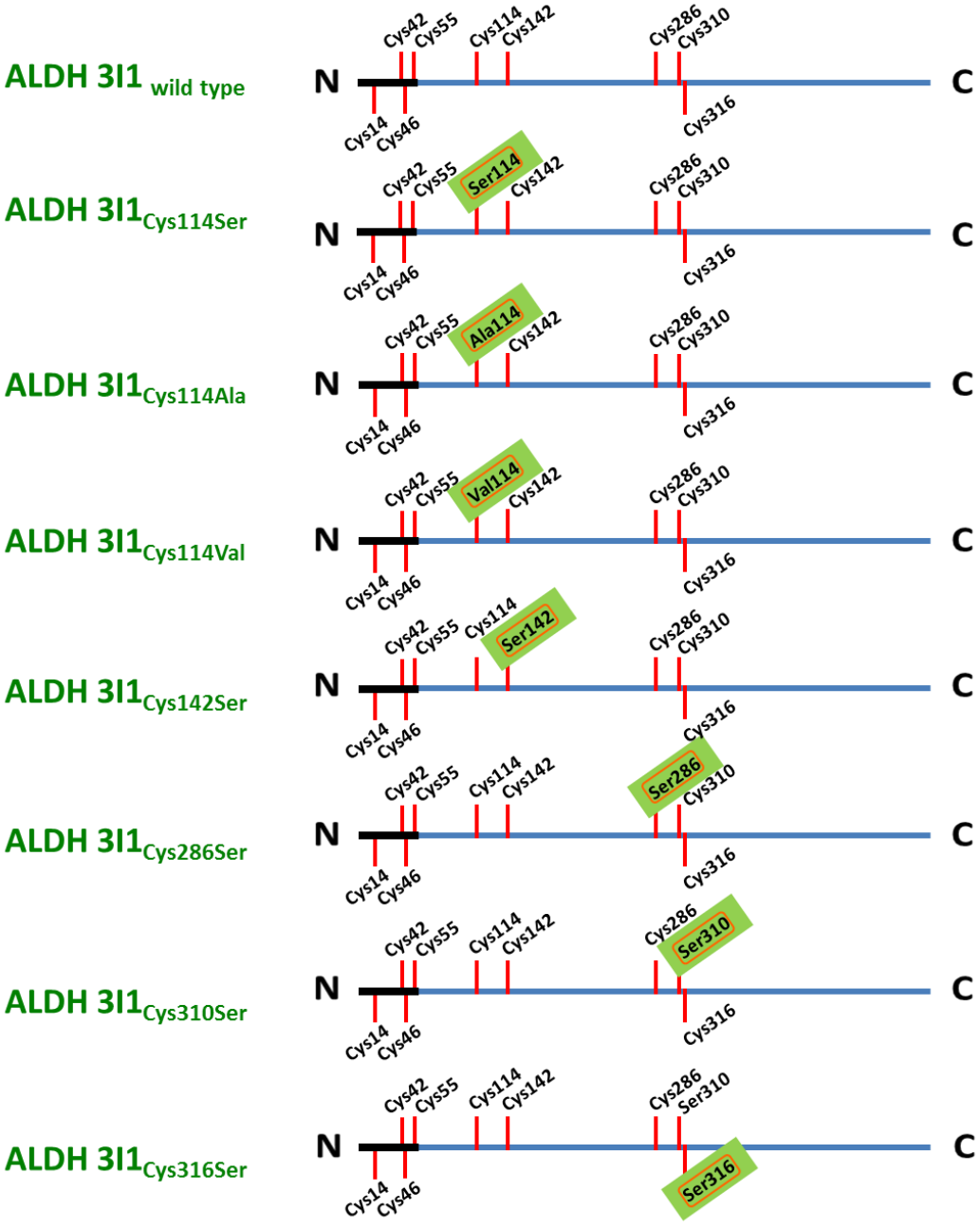



**Figure 8: Cysteine mutants generated in the recombinant ALDH3I1 protein.**

The positions of the substituted amino acid residues are indicated in the boxes. The Cys residue located at position 114 was substituted by Ser, Ala or Val and three mutant enzymes for this Cys were generated. The plastid targeting sequence is indicated in black.

## 3.2. Purification of recombinant ALDH family 3 proteins and the corresponding Cys mutants

An *EcoRI/EcoRI* DNA fragment (1410 bp; 470 amino acids; nucleotides 197-1607) was subcloned into the *EcoRI/EcoRI* sites of the pET-28a expression vector (Novagen) to express the recombinant protein ALDH3H1 (Stiti *et al.*, 2011). The ALDH3H1 fragment was amplified by PCR from a cDNA clone (GenBank®; AY072122) with the following primers:

Forward primer, position 183-204: 5'-CTGCGAAGAAGGAATTCGGATC-3'

Reverse primer, position 1620-1596: 5'-AGAAGGACTTTGAATTCATCGAAT-3'

The expression vector comprises a sequence corresponding to a 6His-tag which was fused to the ALDH3H1 gene sequence at the 5'-end. The induction by IPTG of the BL21 *E. coli* strain bearing this vector resulted in the expression of a recombinant ALDH3H1 enzyme with a 6xHis-tag at the N-terminal end. The recombinant protein has 528 amino acids, and the predicted molecular weight is 56 kDa (Stiti *et al.*, 2011).

An *EcoRI/XhoI* DNA fragment (1470 bp; 490 amino acids; nucleotides 195-1665) was subcloned into *EcoRI/XhoI* sites of the pET-28a expression vector (Novagen) to express the recombinant protein ALDH3I1 (Stiti *et al.*, 2011). The ALDH3I1 fragment was amplified by PCR from a cDNA clone (GenBank®; AJ306961) with the following primers:

Forward primer, position 178-200: 5'-CCTTATCGGTTGGAATTC-3'

Reverse primer, position 1681-1659: 5'-CTTTAGAGAACTCGAGGAAAGCC-3'

The expression vector comprises a sequence corresponding to a 6His-tag which was fused to the ALDH3I1 gene sequence at the 3' and 5'-ends. The induction by IPTG of the BL21 *E. coli* strain bearing this vector resulted in the expression of a recombinant ALDH3I1

enzyme with a 6xHis-tag at the C- and N-terminal ends. The recombinant protein has 534 amino acids, and the predicted molecular weight is 58 kDa (Stiti *et al.*, 2011).

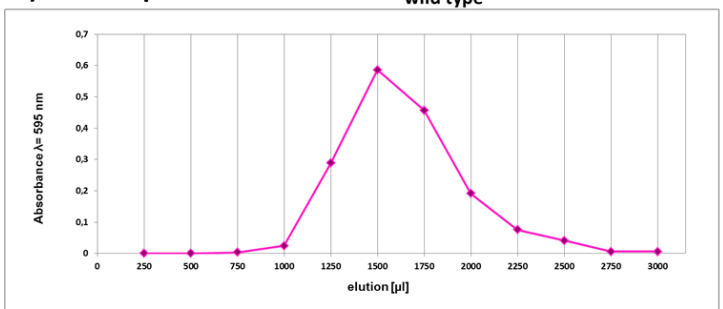
To investigate the role of the cysteine residues, cysteines were individually mutated to serine or alanine. The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate cysteine mutants. Primers carrying the desired mutations were designed. The following mutants were produced for ALDH3H1: Cys45Ser, Cys45Ala, Cys247Ser and Cys253Ser. The following mutants were produced for ALDH3I1: Cys114Ser, Cys114Ala, Cys114Val, Cys142Ser, Cys286Ser, Cys310Ser and Cys316Ser.

All proteins were purified from bacterial pellets using metal ion affinity chromatography on His-tag binding columns under native conditions. The protein concentrations in the eluted fractions were determined using the Bradford assay. The elution profile for each enzyme is illustrated in Figure 9 and Figure 10.

Protein mutants ALDH3H1$_{Cys45Ser}$ and ALDH3H1$_{Cys45Ala}$ have also a peak in fraction 6 like the wild-type, but the protein content is five times lower compared to wild type. For mutants ALDH3H1$_{Cys247Ser}$ and ALDH3H1$_{Cys253Ser}$ the protein content in the peak fraction is higher compared to the wild type. Cysteine mutants of ALDH3I1 contain similar amounts of proteins in the peak fractions compared to the wild type.

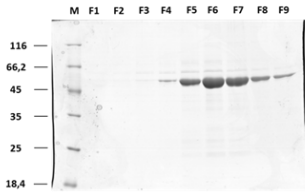
All purified enzymes shared the same pattern of the elution profile and the purity of the eluted fractions is similar (Figures 9 and 10).

**A) Elution profile for ALDH3H1 wild type**

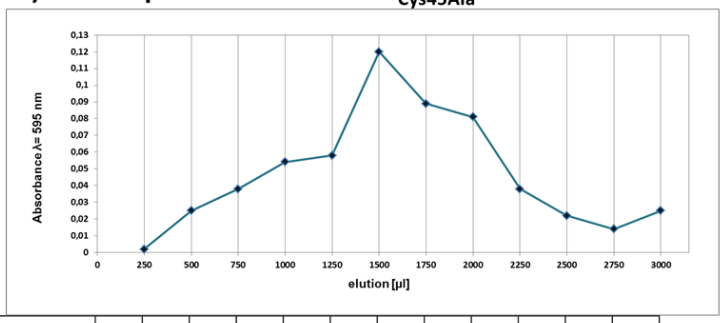



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0 | 0 | 0,003 | 0,024 | 0,288 | 0,586 | 0,457 | 0,191 | 0,075 | 0,041 | 0,006 | 0,006 |
| Protein concentration [µg/µl] | 0 | 0 | 0,0050 | 0,0397 | 0,4760 | 0,9686 | 0,7554 | 0,3157 | 0,1240 | 0,0678 | 0,0099 | 0,0099 |

**SDS-PAGE gel of the ALDH3H1 wild type**

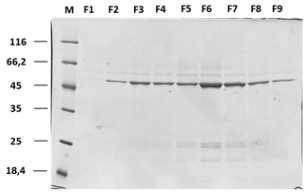


**B) Elution profile for ALDH3H1$_{Cys45Ala}$**

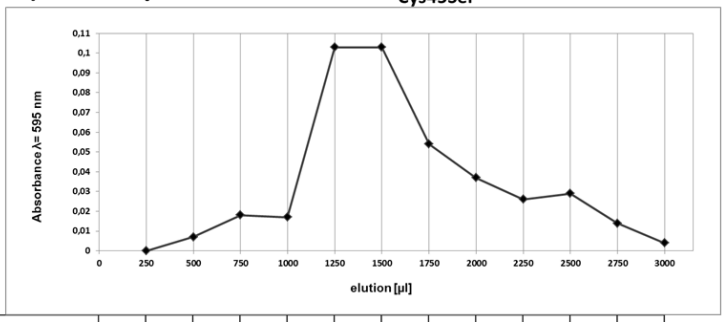



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,002 | 0,025 | 0,038 | 0,054 | 0,058 | 0,12 | 0,089 | 0,081 | 0,038 | 0,022 | 0,014 | 0,025 |
| Protein concentration [µg/µl] | 0,0033 | 0,0413 | 0,0628 | 0,0893 | 0,0959 | 0,1983 | 0,1471 | 0,1339 | 0,0628 | 0,0364 | 0,0231 | 0,0413 |

**SDS-PAGE gel of the ALDH3H1$_{Cys45Ala}$**

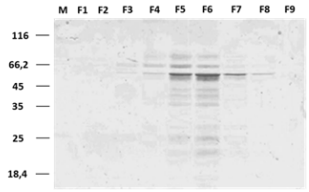



**C) Elution profile for ALDH3H1$_{Cys45Ser}$**

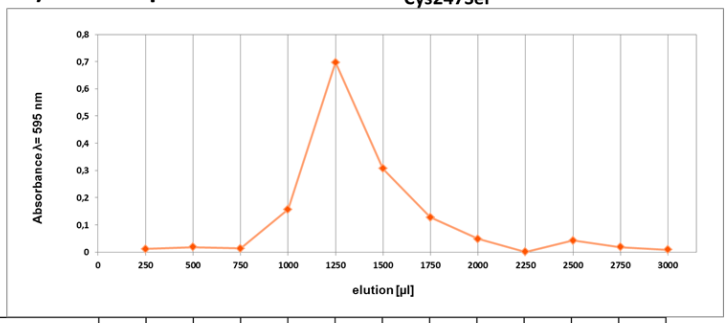



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0 | 0,007 | 0,018 | 0,017 | 0,103 | 0,103 | 0,054 | 0,037 | 0,026 | 0,029 | 0,014 | 0,004 |
| Protein concentration [µg/µl] | 0 | 0,0116 | 0,0298 | 0,0281 | 0,1702 | 0,1702 | 0,0893 | 0,0612 | 0,0430 | 0,0479 | 0,0231 | 0,0066 |

**SDS-PAGE gel of the ALDH3H1$_{Cys45Ser}$**

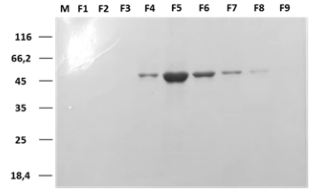



**D) Elution profile for ALDH3H1$_{Cys247Ser}$**

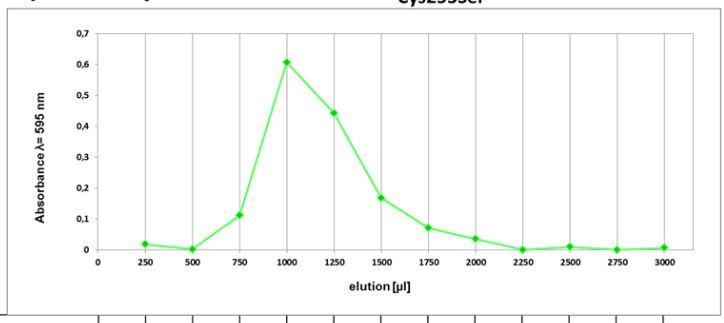



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,012 | 0,018 | 0,014 | 0,157 | 0,697 | 0,307 | 0,128 | 0,048 | 0,001 | 0,043 | 0,018 | 0,009 |
| Protein concentration [µg/µl] | 0,0198 | 0,0298 | 0,0231 | 0,2595 | 1,1521 | 0,5074 | 0,2116 | 0,0793 | 0,0017 | 0,0711 | 0,0298 | 0,0149 |

**SDS-PAGE gel of the ALDH3H1$_{Cys247Ser}$**



**E) Elution profile for ALDH3H1$_{Cys253Ser}$**




| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,018 | 0,002 | 0,111 | 0,607 | 0,442 | 0,168 | 0,071 | 0,035 | 0 | 0,01 | 0 | 0,006 |
| Protein concentration [µg/µl] | 0,0298 | 0,0033 | 0,1835 | 1,0033 | 0,7306 | 0,2777 | 0,1174 | 0,0579 | 0,0000 | 0,0165 | 0,0000 | 0,0099 |

**SDS-PAGE gel of the ALDH3H1$_{Cys253Ser}$**

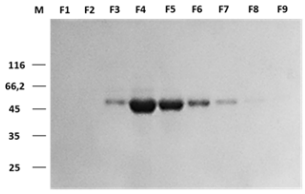
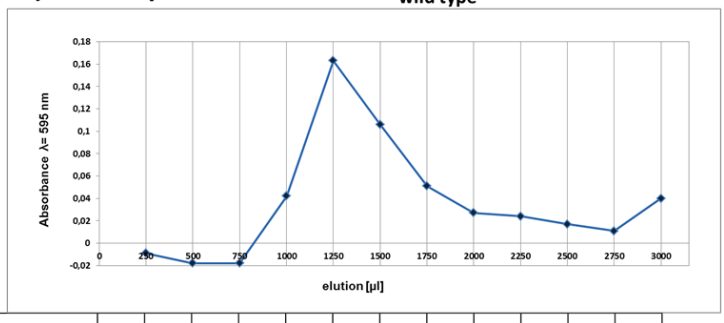



**Figure 9: Protein elution profiles and SDS-PAGE gels of the recombinant ALDH3H1 wild-type enzyme and the corresponding Cys mutants after purification by affinity chromatography on Ni-NTA column.**

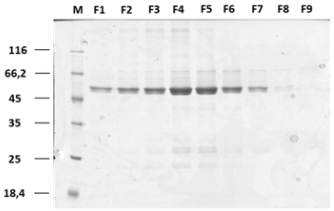
Recombinant ALDH3H1 mutants were produced in *E. coli* and purified as described under 2.6.4. The fractions containing 250 µl of the eluate were collected and the absorbance at 595 nm was measured for the each fraction. Subsequently the protein concentration of each fraction was determined. The values for the absorbance and the protein concentrations are presented in the tables below the elution profiles. The fractions containing the highest protein concentration are shown on SDS gels. Elution profiles of ALDH3H1 wild type (A), ALDH3H1$_{Cys45Ala}$ (B), ALDH3H1 $_{Cys45Ser}$ (C), ALDH3H1$_{Cys247Ser}$ (D) and ALDH3H1$_{Cys253Ser}$ (E).

**A) Elution profile for ALDH3I1 wild type**

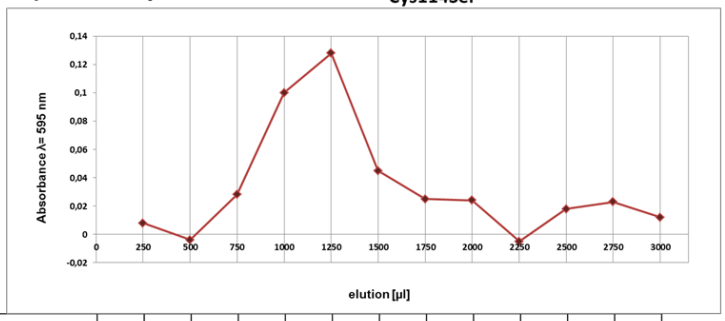



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | -0,009 | -0,018 | -0,018 | 0,042 | 0,163 | 0,106 | 0,051 | 0,027 | 0,024 | 0,017 | 0,011 | 0,04 |
| Protein concentration [µg/µl] | 0 | 0 | 0 | 0,0694 | 0,2694 | 0,1752 | 0,0843 | 0,0446 | 0,0397 | 0,0281 | 0,0182 | 0,0661 |

**SDS-PAGE gel of the ALDH3I1 wild type**

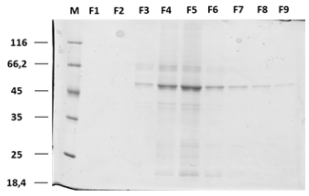


**B) Elution profile for ALDH3I1$_{Cys114Ser}$**

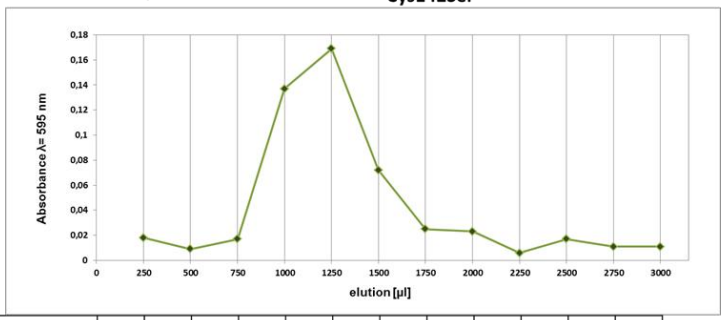



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,008 | -0,004 | 0,028 | 0,1 | 0,128 | 0,045 | 0,025 | 0,024 | -0,005 | 0,018 | 0,023 | 0,012 |
| Protein concentration [µg/µl] | 0,0132 | 0 | 0,0463 | 0,1653 | 0,2116 | 0,0744 | 0,0413 | 0,0397 | -0,0083 | 0,0298 | 0,0380 | 0,0198 |

**SDS-PAGE gel of the ALDH3I1 $_{Cys114Ser}$**

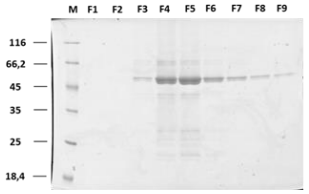



**C) Elution profile for ALDH3I1$_{Cys142Ser}$**

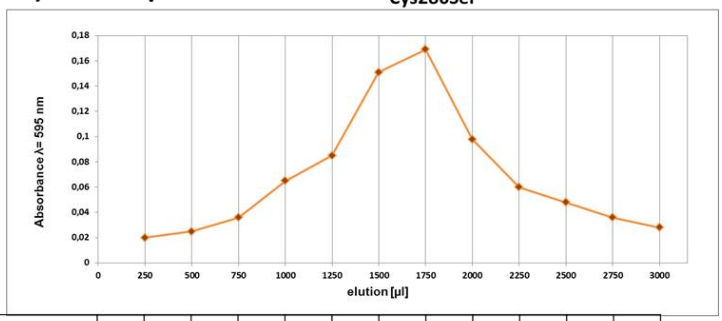



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,018 | 0,009 | 0,017 | 0,137 | 0,169 | 0,072 | 0,025 | 0,023 | 0,006 | 0,017 | 0,011 | 0,011 |
| Protein concentration [µg/µl] | 0,0298 | 0,0149 | 0,0281 | 0,2264 | 0,2793 | 0,1190 | 0,0413 | 0,0380 | 0,0099 | 0,0281 | 0,0182 | 0,0182 |

**SDS-PAGE gel of the ALDH3I1 $_{Cys142Ser}$**

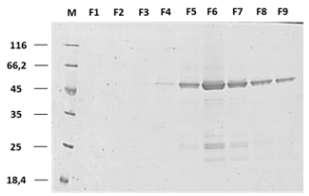



**D) Elution profile for ALDH3I1$_{Cys286Ser}$**

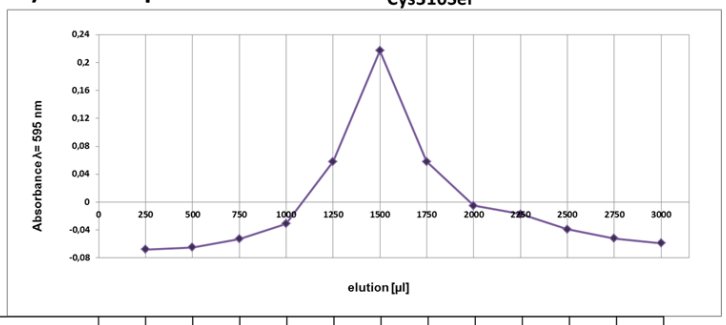



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,02 | 0,025 | 0,036 | 0,065 | 0,085 | 0,151 | 0,169 | 0,098 | 0,06 | 0,048 | 0,036 | 0,028 |
| Protein concentration [µg/µl] | 0,0331 | 0,0413 | 0,0595 | 0,1074 | 0,1405 | 0,2496 | 0,2793 | 0,1620 | 0,0992 | 0,0793 | 0,0595 | 0,0463 |

**SDS-PAGE gel of the ALDH3I1 $_{Cys286Ser}$**

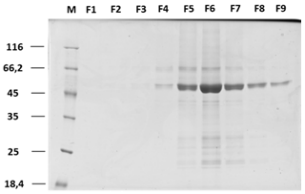


**E) Elution profile for ALDH3I1$_{Cys310Ser}$**

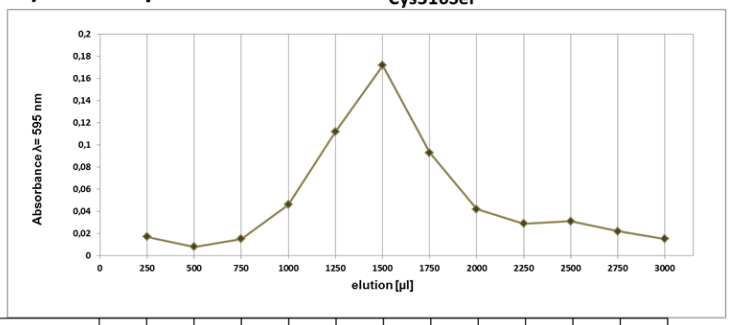



| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | -0,068 | -0,065 | -0,053 | -0,031 | 0,058 | 0,217 | 0,058 | -0,005 | -0,017 | -0,039 | -0,052 | -0,059 |
| Protein concentration [µg/µl] | 0 | 0 | 0 | 0 | 0,0959 | 0,3587 | 0,0959 | 0 | 0 | 0 | 0 | 0 |

**SDS-PAGE gel of the ALDH3I1 $_{Cys310Ser}$**




**F) Elution profile for ALDH3I1$_{Cys316Ser}$**




| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,017 | 0,008 | 0,015 | 0,046 | 0,112 | 0,172 | 0,093 | 0,042 | 0,029 | 0,031 | 0,022 | 0,015 |
| Protein concentration [µg/µl] | 0,0281 | 0,0132 | 0,0248 | 0,0760 | 0,1851 | 0,2843 | 0,1537 | 0,0694 | 0,0479 | 0,0512 | 0,0364 | 0,0248 |

**SDS-PAGE gel of the ALDH3I1 $_{Cys316Ser}$**

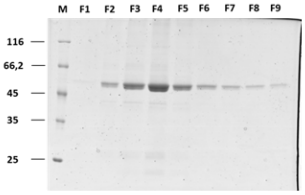



**Figure 10: Protein elution profiles and SDS-PAGE gels of the recombinant ALDH3I1 wild-type enzyme and the corresponding Cys mutants after the purification by affinity chromatography on Ni-NTA column.**

Recombinant ALDHI1 mutants were produced in *E. coli* and purified as described under 2.6.4. The fractions containing 250 µl of the eluate were collected and the absorbance at 595 nm was measured for the each fraction. Subsequently the protein concentration of each fraction was determined. The values for the absorbance and the protein concentrations are presented in tables below the elution profiles. The fractions containing the highest protein concentration are shown on SDS gels. Elution profiles of ALDH3I1 wild type (A), ALDH3I1$_{Cys114Ser}$ (B), ALDH3I1 $_{Cys142Ser}$ (C), ALDH3I1$_{Cys286Ser}$ (D), ALDH3I1$_{Cys310Ser}$ (E) and ALDH3I1$_{Cys316Ser}$ (F).

# 3.3. The effect of nitric oxide donors on enzyme activity of members of the ALDH family 3

The members of the ALDH family 3 (ALDH3H1 and ALDH3I1) are responsible for the oxidation of aldehydes to the corresponding carboxylic acids according to the reaction shown in Figure 11. The activity of aldehyde dehydrogenases may be modulated by several molecules. The inhibitors decrease the enzyme activity, whereas the activators increase the enzyme activity. In this work the effect of nitric oxide donors on dehydrogenase activity is investigated.

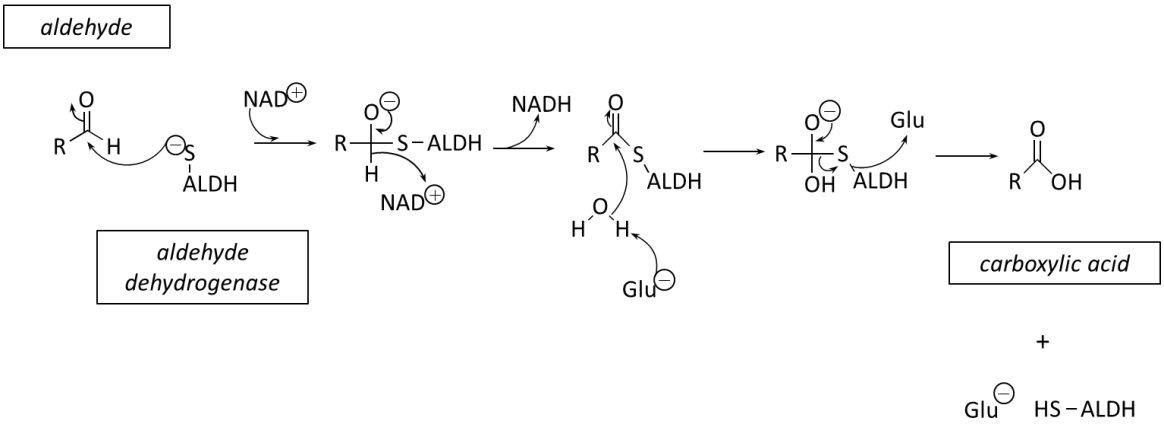



**Figure 11: The enzymatic conversion of aldehyde to carboxylic acid.**

The active site of ALDH has a conserved cysteine and glutamate residue. In the first step the cofactor ($NAD^+$ or $NADP^+$) binds to the coenzyme binding site. The thiol group from a cysteine in the active site makes a nucleophilic attack on the carbonyl carbon on the aldehyde. The hydrogen is released as a hydride and attacks $NAD(P)^+$ to form NAD(P)H. In the next step an isomorphic change takes place in the active site of the enzyme and NAD(P)H is removed. In the final step the water molecule can access the substrate. The water is primed by a glutamate in the active site. Due to the nucleophilic attack of the water, sulphur is removed as a leaving group and the carboxylic acid is formed. Ref. Liu *et al.*, (1997).

### 3.3.1. Determination of the optimum concentration of nitrosative agents for the *S*-nitrosylation process

The purified enzymes ALDH3H1 and ALDH3I1 were incubated with different concentrations of the nitric oxide donors: *S*-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) for 2.5 hours. To study the effect of GSNO and SNP on ALDH3H1 and ALDH3I1 by GSNO and SNP, enzymatic activity was monitored spectrophotometrically (Figure 12). The enzymatic activity of the ALDH3H1 and ALDH3I1 was only partially inhibited with the highest concentration of GSNO, but never completely.

Incubation of the ALDH3H1 wild type enzyme with the different concentrations (from 50 µM to 2 mM) of GSNO showed that the concentration of 250 µM reduced the enzyme activity to 50%. The exposure to the lowest concentration (50 µM) of GSNO led to the reduction of the ALDH activity to 58%, whereas the exposure to the highest concentration (2 mM) of GSNO led to the reduction of the ALDH activity to 26%.

Incubation of the ALDH3I1 wild type enzyme with the different concentrations (from 50 µM to 2 mM) of GSNO showed that 500 µM GSNO reduced the enzyme activity to 50%. The exposure to the lowest concentration (50 µM) of GSNO led to the reduction of the ALDH activity to 73%, whereas the exposure to the highest concentration (2 mM) of GSNO led to the reduction of the ALDH activity to 39%.

The same range of GSNO concentrations has a stronger inhibition effect on the enzymatic activity of the ALDH3H1 wild type enzyme than on the enzymatic activity of the ALDH3I1 wild type enzyme. For both enzymes the rate of inactivation increased with higher GSNO concentrations.

Incubation of the ALDH3H1 wild type enzyme with the different concentrations (from 50 µM to 10 mM) of SNP showed that already the lowest concentration (50 µM) reduced the ALDH activity to 50%. The exposure to the highest concentration (10 mM) of SNP led to the reduction of the ALDH activity to 52%.

Incubation of the ALDH3I1 wild type enzyme with the different concentrations (from 50 µM to 10 mM) of SNP showed that concentrations of SNP used in the experiment did not reduce the ALDH activity to 50%. The exposure to the lowest concentration (50 µM)

of SNP led to the reduction of the ALDH activity to 80%, whereas the exposure to the highest concentration (10 mM) of SNP led to the reduction of the ALDH activity to 68%.

The same range of SNP concentrations has the stronger inhibition effect on the enzymatic activity of the ALDH3H1 wild type than on the enzymatic activity of the ALDH3I1 wild type

For both enzymes the rate of inactivation remained unchanged with the higher SNP concentrations.

Based on the results obtained GSNO showed a stronger inhibition of ALDH activity compared to SNP, even if higher SNP concentrations were used. It suggests that GSNO is a better NO donor than SNP.

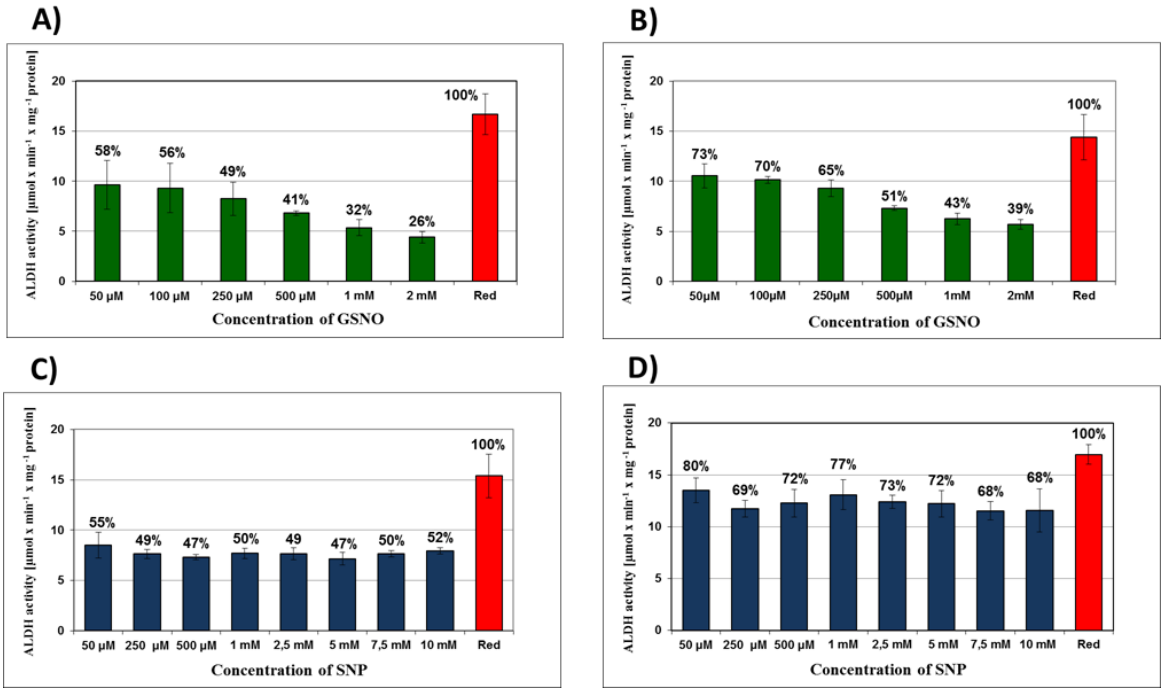



**Figure 12: The effect of GSNO and SNP concentrations on the dehydrogenase activity of the ALDH3H1 and ALDH3I1 wild-type enzymes.**

ALDH activities of reduced ALDH3H1 and ALDH3I1 enzymes (red columns) and ALDH activities of treated ALDH3H1 and ALDH3I1 enzymes for 2.5 hours at room temperature in the dark with the indicated concentrations of either GSNO (50 µM-2 mM) (green columns) or SNP (50 µM-10 mM) (dark blue columns). The dehydrogenase activity of the recombinant ALDH3H1 was inhibited by GSNO (A) or SNP (C), the dehydrogenase activity of the recombinant ALDH3I1 was inhibited by GSNO (B) or SNP (D). The initial dehydrogenase activity of the reduced form was taken as 100%. Values represent the means ± SEM of three independent determinations.

## 3.3.2. Determination of the optimum incubation time for nitric oxide donors

The effect of the incubation time with NO donors on enzyme activity was determined. ALDH3H1 and ALDH3I1 enzymes were incubated in the presence of 500 µM GSNO or 500 µM SNP for various times from 0 to 240 min in the dark after which dehydrogenase activity was measured by monitoring the absorbance at 340 nm. The results, presented in Figure 13, showed the change of the enzymatic activity. Only 30% of dehydrogenase activity of ALDH3H1 remained upon 4 h of incubation with GSNO, whereas 60% of the dehydrogenase activity remained in the case of ALDH3I1. However, in the presence of SNP 40% of dehydrogenase activity was conserved for ALDH3H1 after 4 h of incubation versus 80% for ALDH3I1.

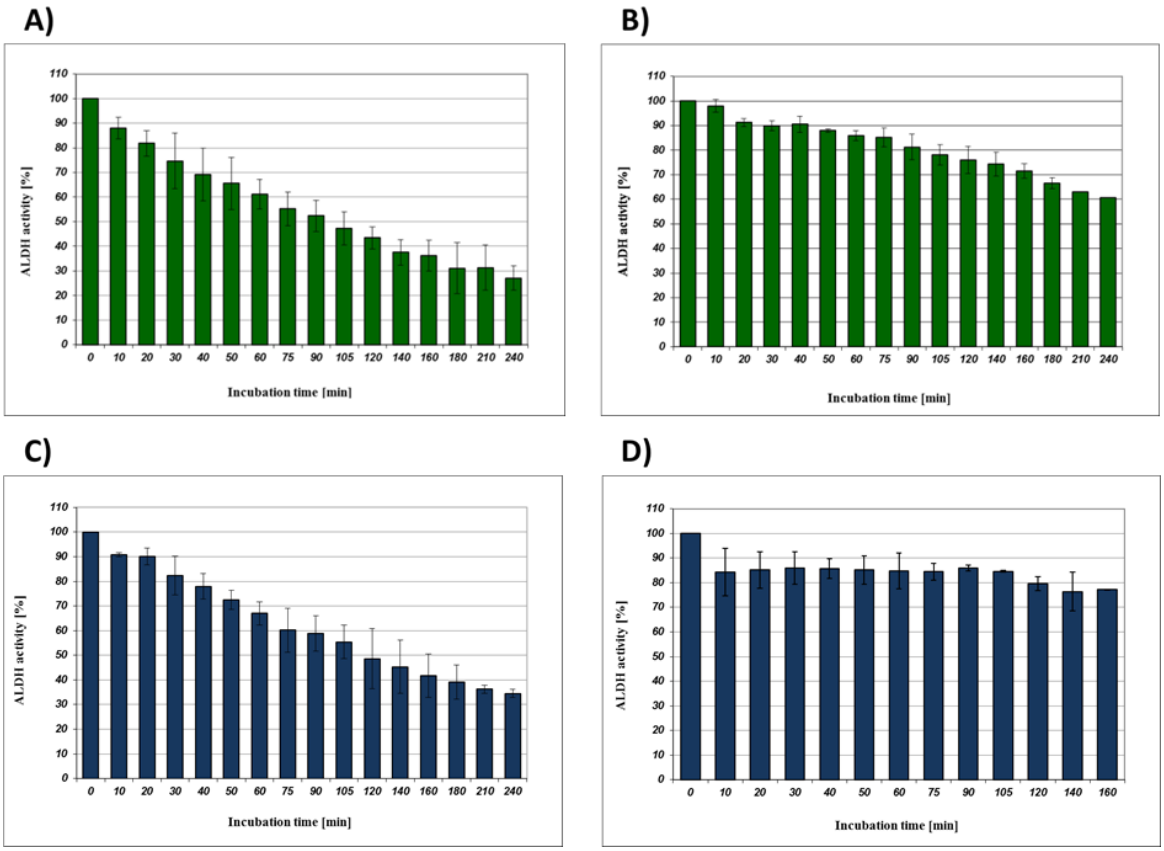



**Figure 13: The effect of the incubation time on the dehydrogenase activity in the presence of NO donors**

The purified, recombinant enzymes ALDH3H1 and ALDH3I1 were incubated in the presence of either 500 µM GSNO (green columns) or 500 µM SNP (dark blue columns) for 240 min at room temperature in the dark. After the indicated incubations times, the dehydrogenase activities were determined by monitoring the absorbance at 340 nm and presented as ALDH activity [%]. The activities of untreated enzymes at 0 min were set to 100%. The inhibition of the dehydrogenase activity of ALDH3H1 by GSNO (A) or SNP (C); the inhibition of the dehydrogenase activity of ALDH3I1 by GSNO (B) or SNP (D). Values represent the means ± SEM of three independent determinations.

### 3.3.2.1. The effect of the incubation time on enzyme activity

It was further investigated whether loss of dehydrogenase activity in response to GSNO or SNP is affected by incubation time with the NO donors. ALDH3H1 and ALDH3I1 enzymes were incubated in the presence of 500 µM GSNO or 500 µM SNP for various times from 0 to 240 min in the dark after which the dehydrogenase activity was measured by monitoring the absorbance at 340 nm. Simultaneously ALDH3H1 and ALDH3I1 enzymes were incubated in the absence of the NO donors for various times from 0 to 240 min at room temperature in the dark and the dehydrogenase activity was measured by monitoring the absorbance at 340 nm. The results, presented in Figure 14, showed that dehydrogenase activity of ALDH3H1 and ALDH3I1 was only reduced in the presence of GSNO. In the absence of NO donors the dehydrogenase activity of the reduced form of ALDH3H1 and ALDH3I1 did not vary considerably. It suggests that there are no additional factors (temperature, light) which influence the loss of activity rather than the nitrosylation triggered by NO donors. The enzyme stability was not affected during experiments at room temperature.

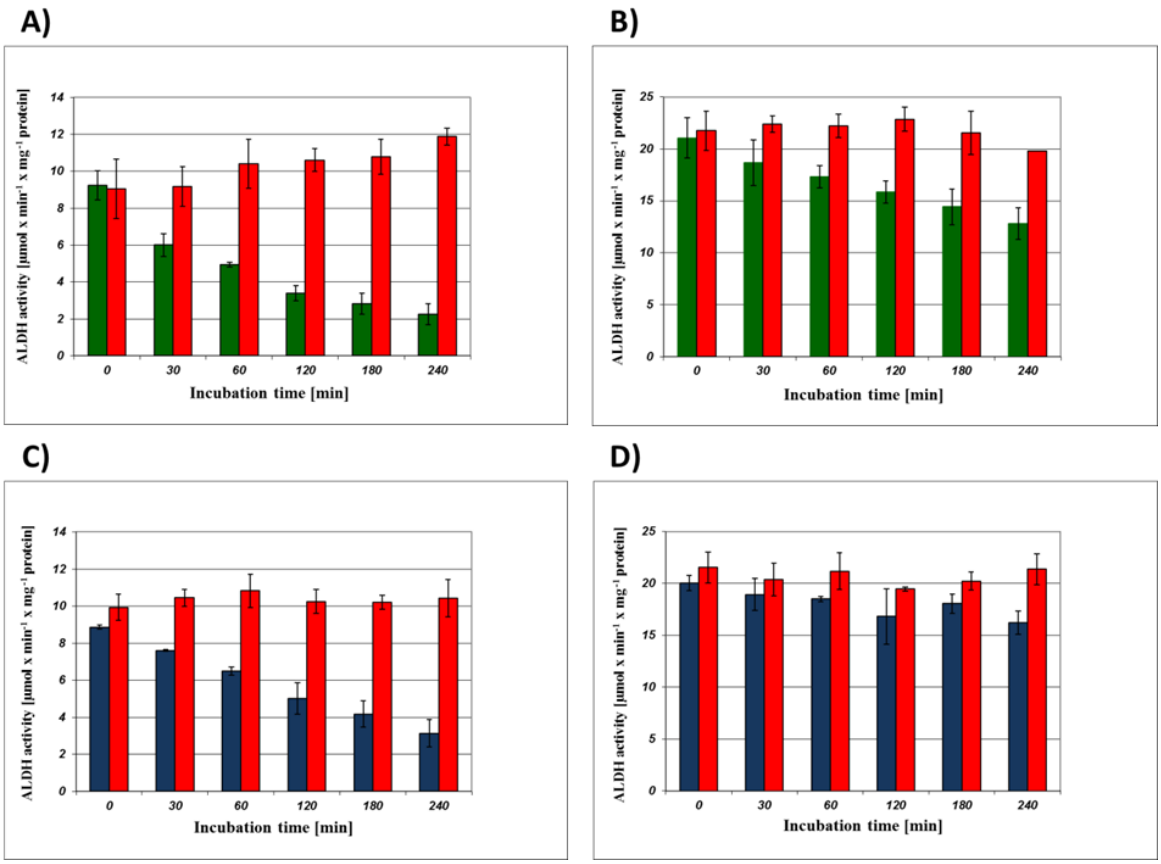



**Figure 14: The effect of the incubation time on the dehydrogenase activity in the presence or absence of NO donors**

The purified, recombinant enzymes ALDH3H1 and ALDH3I1 were incubated in the absence (red columns) or presence of 500 µM GSNO (green columns) or 500 µM SNP (dark blue columns) for 240 min at room temperature in the dark. After the indicated incubation times, the dehydrogenase activities were determined by monitoring the absorbance at 340 nm. The activity of untreated enzymes at 0 min set to 100%. The inhibition of the dehydrogenase activity of ALDH3H1 in the absence and presence of GSNO (A) or SNP (C); the inhibition of the dehydrogenase activity of ALDH3I1 in the absence and presence of GSNO (B) or SNP (D). Values represent the means ± SEM of three independent determinations.

The strongest inhibition of the dehydrogenase activity was achieved during the first 30 min for ALDH3H1 (decrease of about 35%) and ALDH3I1 (decrease of about 12%) when the enzymes were treated with 500 µM GSNO. The strongest inhibition of the dehydrogenase activity was achieved after 120 min for ALDH3H1 (decrease of about 17%) and ALDH3I1 (decrease of about 10%) when the enzymes were treated with 500 µM SNP.

## 3.3.3. Analysis of the reversibility of *S*-nitrosylation by application of reducing agents

### 3.3.3.1. Determination of the optimum concentration of reducing agents

A series of experiments was performed to analyse whether the *S*-nitrosylation can be reversible. ALDHs were treated with different concentrations of reducing reagents dithiothreitol (DTT) and glutathione (GSH) after *in vitro S*-nitrosylation. After treatment with 500 µM GSNO or 500 µM SNP the ALDH3H1 or ALDH3I1 wild-type enzymes were incubated with variable concentrations (from 50 µM to 20 mM) of DTT or GSH in the dark for 1.5 hour. To study the reversibility of the *S*-nitrosylation and its impact on ALDH activity, the enzymatic activities of tested samples were measured and compared to the control (reduced enzyme, non-nitrosylated).

The results, presented in Figure 15, show that the treatment of ALDH3H1 wild-type enzyme with 500 µM GSNO resulted in a decrease of 80% of the enzyme activity compared to the control. *S*-nitrosylated ALDH3H1 wild-type enzyme retained 20% of the initial enzyme activity. Re-reduction using DTT at concentrations of 50 µM to 1 mM led to a recovery of the ALDH activity to 30%. A significant recovery was achieved when the concentration of DTT was raised to 2.5 mM (73%). 10 mM DTT led to an activity which is comparable to what was observed for the non-nitrosylated enzyme.

The treatment of ALDH3H1wild-type enzyme with 500 µM GSNO resulted in a decrease of 60% of the enzyme activity compared to the control. *S*-nitrosylated ALDH3H1 wild-type enzyme retained 40% of the initial enzyme activity. Re-reduction using GSH at concentrations of 50 µM to 5 mM led to a recovery of the ALDH activity to 60%. 10 mM DTT led to a recovery of the ALDH activity to 70%.

The treatment of ALDH3H1 wild-type enzyme with 500 µM SNP resulted in a decrease of 70% of the enzyme activity compared to the control. *S*-nitrosylated ALDH3H1 wild-type enzyme retained 30% of the initial enzyme activity. Re-reduction using DTT at concentrations of 50 µM to 1 mM led to a recovery of the ALDH activity to 40%. At concentrations of 2.5 mM to 10 mM of DTT the ALDH activity was recovered to 50%.

The treatment of ALDH3H1 wild-type enzyme with 500 µM SNP resulted in a decrease of 90% of the enzyme activity compared to the control. *S*-nitrosylated ALDH3H1

wild-type enzyme retained 10% of the initial enzyme activity. Re-reduction using DTT at concentrations of 50 µM to 2.5 mM led to a recovery of the ALDH activity to 20%. At concentrations of 5 mM to 20 mM of DTT the ALDH activity was recovered to 40%.

The best recovery (80%) of the ALDH activity was achieved for ALDH3H1 wild-type enzyme with high concentrations of DTT after the treatment with GSNO, whereas the same concentrations of GSH led to a recovery of the ALDH activity to 70%. A stronger recovery was achieved with using DTT after the treatment with GSNO than after the treatment with SNP.

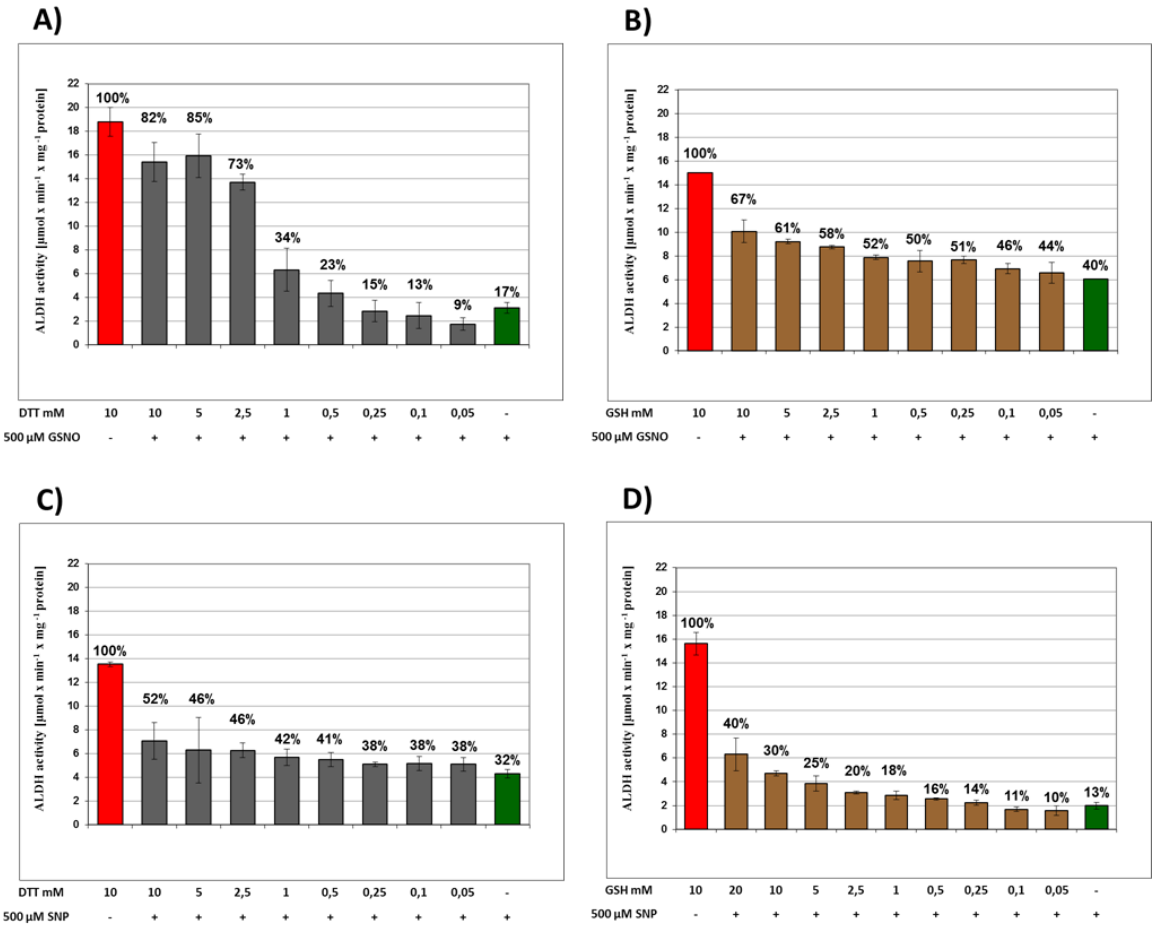



**Figure 15: The effect of DTT and GSH concentrations on the reversibility of the *S*-nitrosylation.**

The purified, recombinant enzyme ALDH3H1 was incubated with either 500 µM GSNO or 500 µM SNP (green columns) for 2.5 h at room temperature in the dark. ALDH activities of the reduced ALDH3H1 are presented as red columns. For restoring the dehydrogenase activity, the indicated increasing concentrations of either DTT (grey columns) or GSH (light brown) were added to the inhibited enzymes and incubated for 1.5 h at room temperature in the dark. The enzyme activity was determined by monitoring the absorbance at 340 nm. The initial dehydrogenase activity of the reduced form was taken as 100%. Re-reduction of the dehydrogenase activity of ALDH3H1 with DTT after an inactivation with GSNO (A) and SNP (C), re-reduction of the dehydrogenase activity of ALDH3H1 with GSH after an inactivation with GSNO (B) and SNP (D). Values represent the means ± SEM of three independent determinations.

### 3.3.3.2. Recovery ratio of enzyme activity of ALDH3H1 and ALDH3I1 and cysteine mutants after *S*-nitrosylation

To determine the function of Cys substitution, the impact of *S*-nitrosylation on the dehydrogenase activity of Cys mutants: ALDH3H1$_{Cys45Ser}$, ALDH3H1$_{Cys45Ala}$ and ALDH3H1$_{Cys247Ser}$ were compared with the values of the dehydrogenase activity obtained for ALDH3H1 wild-type enzyme. The results, presented in Figure 16, show that the treatment with 500 µM GSNO strongly inhibited the dehydrogenase activity of ALDH3H1 wild-type enzyme and the Cys mutants compared to the corresponding untreated samples. The dehydrogenase activity of the mutated enzymes ALDH3H1$_{Cys45Ser}$ and ALDH3H1$_{Cys247Ser}$ was reduced up to 44% and 43%, respectively. The dehydrogenase of ALDH3H1$_{Cys45Ala}$ was stronger inhibited (9%) compared to the dehydrogenase activity of the wild-type enzyme (17%). Re-reduction using DTT showed a significant recovery of the ALDH activity for ALDH3H1 wild-type enzyme (82%), ALDH3H1$_{Cys45Ser}$ (90%), ALDH3H1$_{Cys45Ala}$ (57%) and ALDH3H1$_{Cys247Ser}$ (92%). Re-reduction using GSH led only to a recovery of the ALDH activity for ALDH3H1 wild-type enzyme (82%), but did not lead to a recovery of the Cys mutants.

The treatment with 500 µM SNP inhibited the dehydrogenase activity of ALDH3H1 wild-type enzyme and the Cys mutants compared to their corresponding untreated samples. The dehydrogenase activity of ALDH3H1 wild-type enzyme was reduced to 34%, whereas the dehydrogenase activity of ALDH3H1$_{Cys45Ala}$ was reduced to 63%. The enzyme activity of the mutants ALDH3H1$_{Cys45Ser}$ and ALDH3H1$_{Cys247Ser}$ was unchanged. Re-reduction using DTT showed only a slight recovery of the dehydrogenase activity for ALDH3H1 wild-type enzyme (44%) and ALDH3H1$_{Cys45Ala}$ (84%). Re-reduction using GSH did not yield a recovery of the dehydrogenase activity neither for ALDH3H1 wild-type enzyme nor for the Cys mutants.

The dehydrogenase activity of ALDH3H1 wild-type enzyme and ALDH3H1$_{Cys45Ala}$ was reduced by both NO donors: GSNO and SNP.

For ALDH3H1 wild-type and the corresponding Cys mutants GSNO was a stronger *S*-nitrosylating reagent compared to SNP.

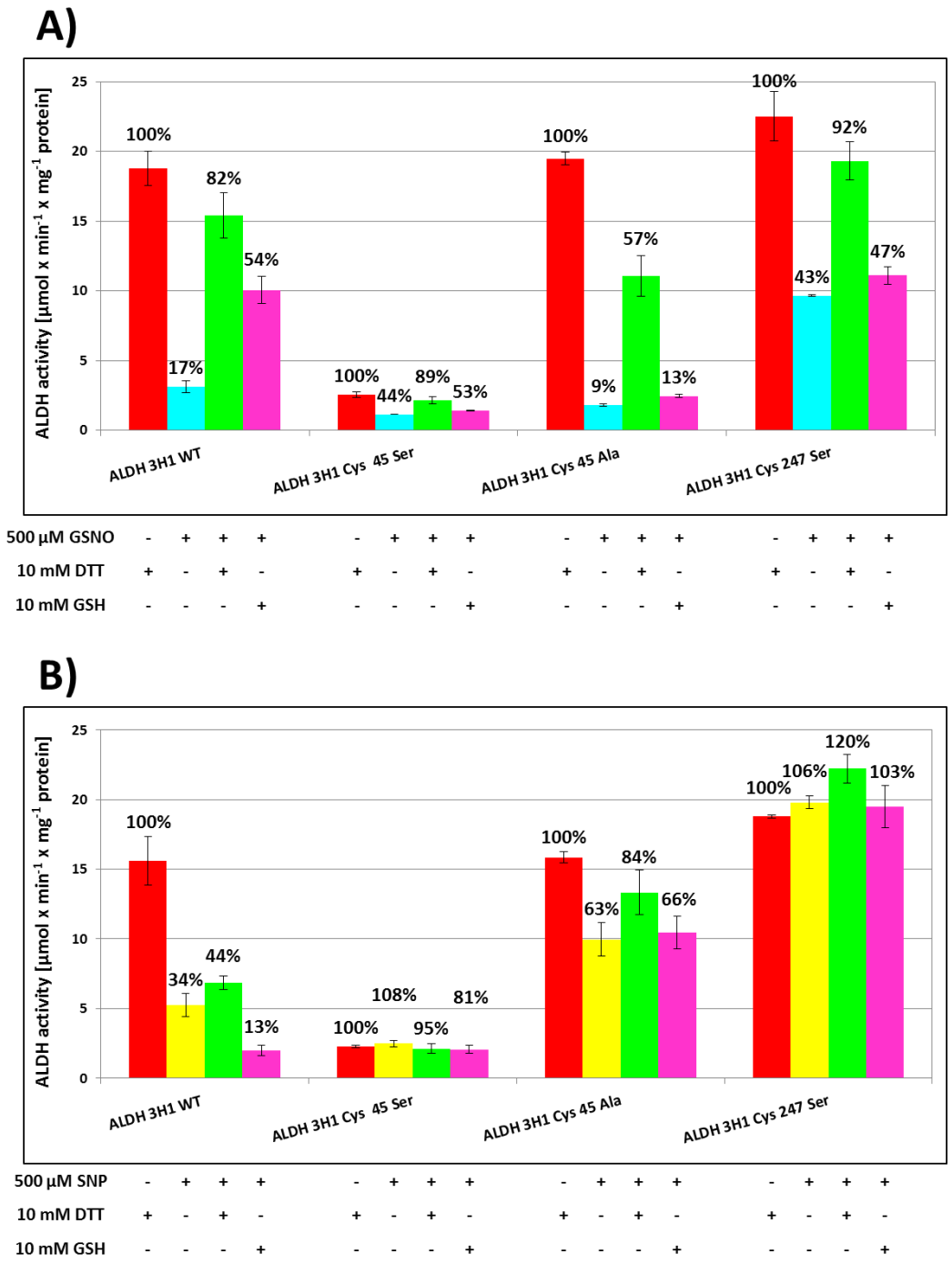



**Figure 16: The effect of *S*-nitrosylation on the dehydrogenase activity of ALDH3H1 wild-type enzyme and the corresponding Cys mutants.**

The purified, recombinant enzyme ALDH3H1 and the corresponding Cys mutants were incubated in the presence of either 500 µM GSNO (light blue columns) or 500 µM SNP (yellow columns) for 2.5 h at room temperature in the dark and then enzyme activity was measured. ALDH activities of the reduced forms of ALDH3H1 enzymes are presented as red columns. For restoring the dehydrogenase activity of ALDH3H1 and the corresponding Cys mutants, 10 mM DTT (light green columns) or 10 mM (pink columns) were added to the inhibited enzymes and incubated for 1.5 h at room temperature in the dark. The enzyme activity was determined by monitoring the absorbance at 340 nm. The activity of the reduced form was taken as 100%. The inactivation by GSNO and recovery of the dehydrogenase activity for ALDH3H1 and the corresponding Cys mutants (A), the inactivation by SNP and recovery of the dehydrogenase activity for ALDH3H1 and the corresponding Cys mutants (B). Values represent the means ± SEM of three independent determinations.

The results, presented in Figure 17, show that the treatment with 500 µM GSNO inhibited the dehydrogenase activity of ALDH3I1 wild-type enzyme and the Cys mutants compared to the corresponding untreated samples. The dehydrogenase activity of the mutated enzymes ALDH3I1$_{Cys114Ser}$ and ALDH3I1$_{Cys286Ser}$ was reduced to 63% and 69%, respectively. This shows the similarity to the inhibition of the dehydrogenase activity for ALDH3I1 wild-type enzyme (68%). The dehydrogenase activity achieved of the following Cys mutants ALDH3I1$_{Cys142Ser}$ (31%) and ALDH3I1$_{Cys310Ser}$ (56%) was much stronger inhibited than ALDH3I1 wild-type enzyme. The dehydrogenase activity of ALDH3I1$_{Cys114Ala}$ was reduced to 79%. Re-reduction using DTT led to a recovery of the dehydrogenase activity for the following enzymes: ALDH3I1 wild-type enzyme (79%), ALDH3I1$_{Cys114Ala}$ (91%), ALDH3I1$_{Cys142Ser}$ (45%) and ALDH3I1$_{Cys310Ser}$ (63%). The dehydrogenase activity of ALDH3I1$_{Cys286Ser}$ was not recovered with DTT. Re-reduction using GSH did not lead to a recovery of the dehydrogenase activity neither for ALDH3I1 wild-type enzyme nor for the Cys mutants.

The treatment with 500 µM SNP inhibited very slightly the dehydrogenase activity of ALDH3I1 wild-type enzyme and the Cys mutants compared to the corresponding untreated samples. The dehydrogenase activity of the mutated enzymes ALDH3I1$_{Cys114Ala}$, ALDH3I1$_{Cys286Ser}$ and ALDH3I1$_{Cys310Ser}$ was reduced to 92%, 93% and 92% respectively. This shows the similarity to the inhibition of the dehydrogenase activity for ALDH3I1 wild-type enzyme (90%). The dehydrogenase activity of the Cys mutants ALDH3I1$_{Cys114Ser}$ (83%) and ALDH3I1$_{Cys142Ser}$ (74%) was much stronger inhibited than the ALDH3I1 wild-type enzyme. Re-reduction using DTT showed a slight recovery of the dehydrogenase activity only for ALDH3I1$_{Cys142Ser}$ (90%). Also re-reduction using GSH showed a slight recovery of the dehydrogenase activity only for ALDH3I1$_{Cys142Ser}$ (93%) and additionally for ALDH3I1$_{Cys286Ser}$ (103%).

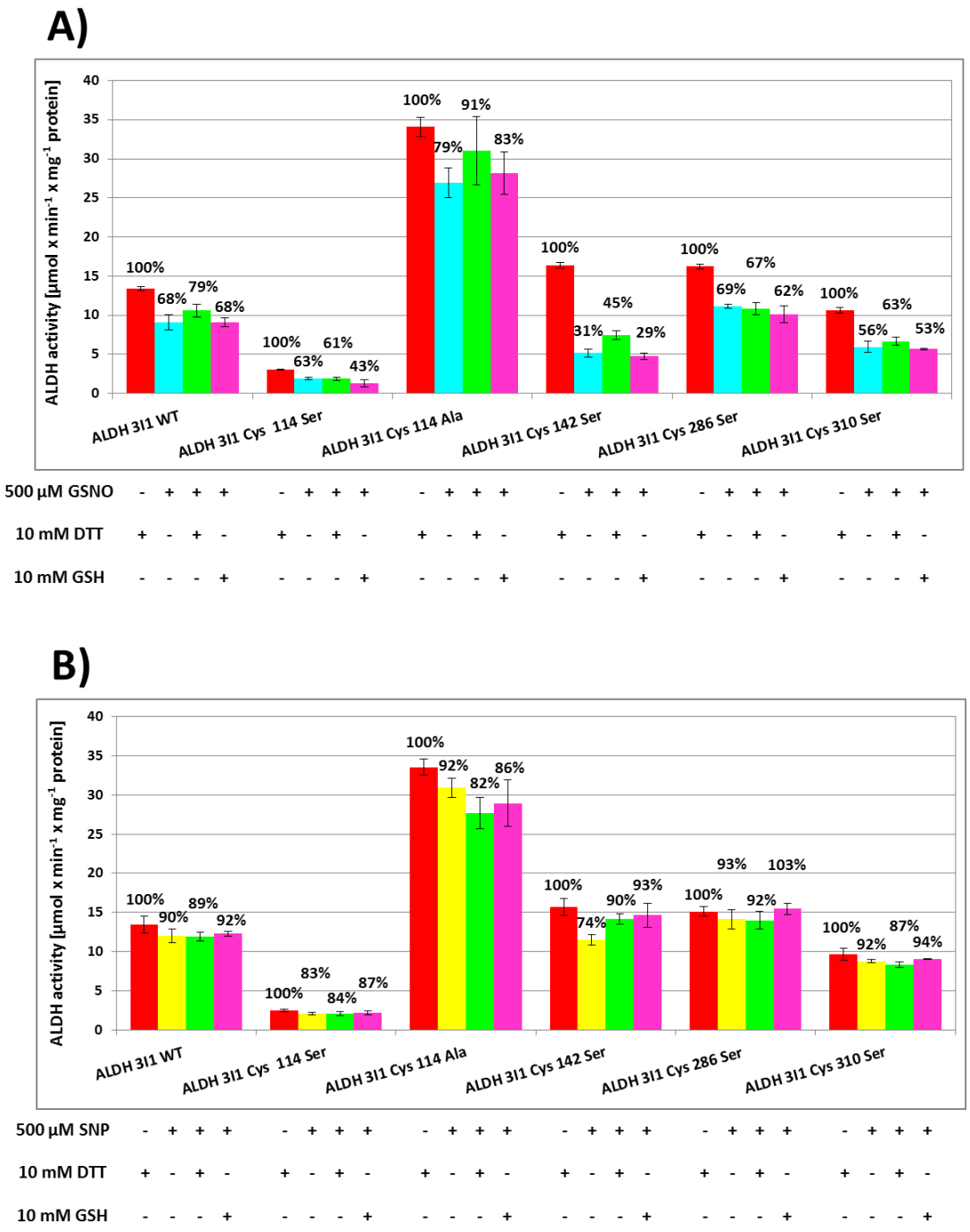



**Figure 17: The effect of *S*-nitrosylation on the dehydrogenase activity of ALDH3I1 wild-type enzyme and the corresponding Cys mutants.**

The purified, recombinant enzyme ALDH3I1 and the corresponding Cys mutants were incubated in the presence of either 500 µM GSNO (light blue columns) or 500 µM SNP (yellow columns) for 2.5 h at room temperature in the dark and then enzyme activity was measured. ALDH activities of the reduced forms of ALDH3I1 enzymes are presented as red columns. For restoring the dehydrogenase activity of ALDH3I1 and the Cys mutants, 10 mM DTT (light green columns) or 10 mM (pink columns) were added to the inhibited enzymes and incubated for 1.5 h at room temperature in the dark. The enzyme activity was determined by monitoring the absorbance at 340 nm. The activity of the reduced form was taken as 100%. The inactivation by GSNO and recovery of the dehydrogenase for ALDH3I1 and Cys mutants (A), the inactivation by SNP and recovery of the dehydrogenase activity for ALDH3I1 and Cys mutants (B). Values represent the means ± SEM of three independent determinations.

# 3.4. Comparison of *S*-nitrosylation patterns and kinetic parameters for ALDH3H1 and ALDH3I1 and the corresponding Cys mutants

## 3.4.1. Esterase activity

ALDH3H1 and ALDH3I1 from *A. thaliana* are capable of hydrolysing *p*-nitrophenyl acetate (*p*-NPA), a standard substrate for esterase. The reaction catalysed by aldehyde dehydrogenase is presented in Figure 18.

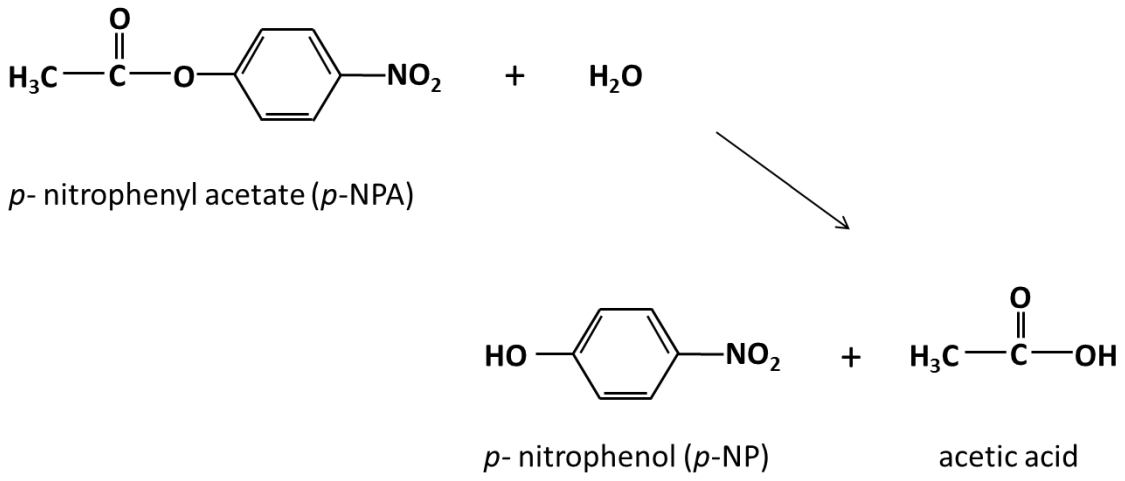



**Figure 18: The hydrolysis of *p*-nitrophenyl acetate (*p*-NPA).**

During the reaction yellow coloured product *p*-nitrophenol (*p*-NP) is released, and the absorbance may be monitored at 400 nm.

The purified, recombinant ALDH3H1 and ALDH3I1 wild-type enzymes and the corresponding Cys mutants were incubated with the oxidizing (50 µM $CuCl_2$) and with the nitrosylating (500 µM GSNO) reagents. To study the effect of $CuCl_2$ and GSNO, the esterase activity of ALDH3H1 and ALDH3I1 and the corresponding Cys mutants was monitored spectrophotometrically. The results are shown in Figures 19 and 20.

The treatment of ALDH3H1 wild-type enzyme and the Cys mutants with 500 µM GSNO showed a slight decrease of the esterase activity compared to the esterase activity of the corresponding untreated samples. The esterase activity of the mutated enzymes $ALDH3H1_{Cys247Ser}$ and $ALDH3H1_{Cys253Ser}$ was reduced to 83% and 81%, respectively which is similar to the inhibition of the esterase activity for ALDH3H1 wild-type enzyme

(88%). A stronger inhibition of the esterase activity was obtained for the mutants ALDH3H1$_{Cys45Ser}$ (70%) and ALDH3H1$_{Cys45Ala}$ (70%) compared to the esterase activity of ALDH3H1 wild-type enzyme. The treatment of ALDH3H1 wild-type enzyme and the Cys mutants with 50 µM $CuCl_2$ showed a slight decrease of the esterase activity compared to the esterase activity of the corresponding untreated samples. The esterase activity of the wild-type enzyme was reduced to 87%. The stronger inhibition of the esterase activity was achieved for the mutants ALDH3H1$_{Cys45Ala}$ (75%), ALDH3H1$_{Cys247Ser}$ (78%) ALDH3H1$_{Cys253Ser}$ (61%) and compared to the esterase activity of ALDH3H1 wild-type enzyme. The esterase activity of the ALDH3H1$_{Cys45Ser}$ showed the strongest inhibition and was reduced to 46%. Re-reduction using DTT did not lead to a recovery of the esterase activity for ALDH3H1 wild-type enzyme either after treatment with GSNO or after treatment with $CuCl_2$. Re-reduction using DTT showed a recovery of the esterase activity for ALDH3H1$_{Cys45Ser}$ (71%) only after treatment with $CuCl_2$.

Esterase activity of ALDH3H1 wild-type enzyme was not affected after treatment with GSNO or $CuCl_2$. Since the measured activity of ALDH3H1 wild-type enzyme did not vary significantly after treatment with GSNO or $CuCl_2$ substitution of Cys-45 by Ser or Ala caused a significant increase of esterase activity compared to ALDH3H1 wild-type enzyme. The esterase activity increased nearly five-fold upon substitution of Cys-45 by Ser and two-fold following the substitution by Ala. Esterase activity of ALDH3H1$_{Cys45Ser}$ and ALDH3H1$_{Cys45Ala}$ is partially reduced by treatment with GSNO or $CuCl_2$, but can be restored by incubation with the reducing reagent DTT. However, *S*-nitrosylation and oxidation have no effect on esterase activity of the mutated enzymes as also observed for the ALDH3H1 wild-type enzyme.

Previous experiments showed that the dehydrogenase activity of ALDH3H1$_{Cys253Ser}$ was abolished, but it was demonstrated that this enzyme has esterase activity (Stiti *et al.*, 2011). These results suggest that Cys-253 is responsible only for dehydrogenase activity and not for the esterase activity. ALDH3H1 might have probably a second active centre which is responsible for the esterase activity.

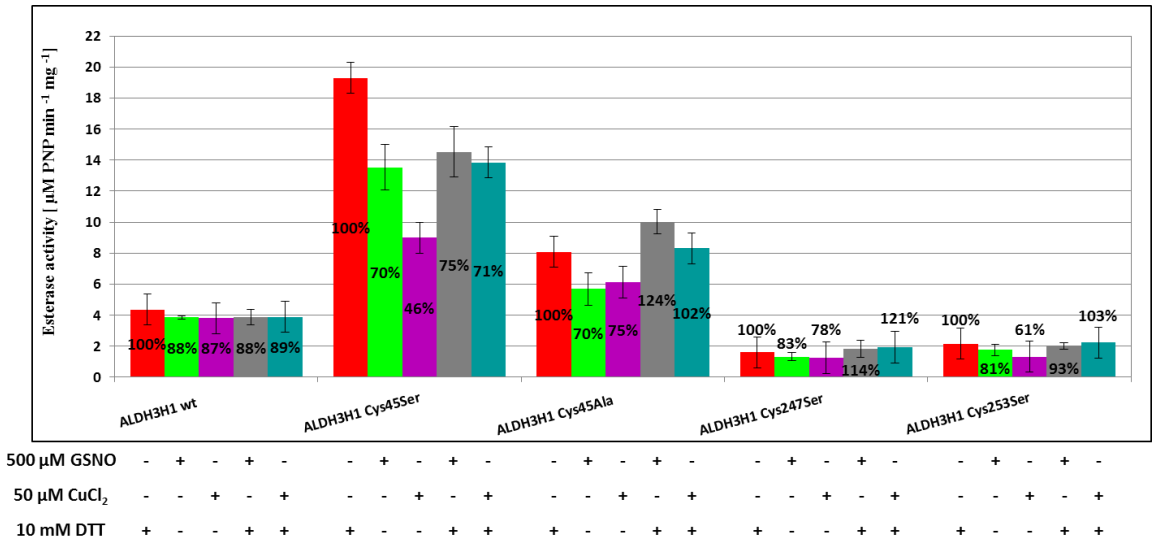



**Figure 19: Esterase activity of *S*-nitrosylated ALDH3H1 wild-type enzyme and the corresponding Cys mutants.**

The purified, recombinant enzyme ALDH3H1 and the corresponding Cys mutants were incubated in the presence of 500 µM GSNO (light green column) for 2.5 h in the dark or 50 µM $CuCl_2$ (purple column) for 1 h at room temperature and then enzyme activity was measured. The esterase activities of the reduced ALDH3H1 enzymes are presented as red columns. For restoring the esterase activity of ALDH3H1 and the Cys mutants, 10 mM DTT was added to the *S*-nitrosylated enzymes (grey columns) and to the oxidized enzymes (turquoise columns), then incubated for 1.5 h at room temperature in the dark. The esterase activity was determined by monitoring the absorbance at 400 nm. The activity of the reduced form was taken as 100 %. Values represent the means ± SEM of three independent determinations.

The treatment of ALDH3I1 wild-type enzyme and the Cys mutants with 500 µM GSNO showed a significant decrease of the esterase activity compared to the esterase activity of the corresponding untreated samples. The Cys mutants, except for $ALDH3I1_{Cys310Ser}$ (78%), showed a stronger inhibition of the esterase activity compared to the esterase activity of the wild-type enzyme (59%). The esterase activity of *S*-nitrosylated $ALDH3I1_{Cys114Ala}$ decreased two-fold (31%) and the esterase activity of *S*-nitrosylated $ALDH3I1_{Cys286Ser}$ decreased three-fold (22%) compared to the esterase activity of *S*-nitrosylated ALDH3I1 wild-type enzyme.

The treatment of ALDH3I1 wild-type enzyme and the Cys mutants with 50 µM $CuCl_2$ showed a significant decrease of the esterase activity compared to the esterase activity of the corresponding untreated samples. The esterase activity of ALDH3I1 wild-type enzyme was reduced to 39%. The esterase activity of the mutated enzymes $ALDH3I1_{Cys114Ala}$, $ALDH3I1_{Cys114Ser}$, $ALDH3I1_{Cys114Val}$, $ALDH3I1_{Cys142Ser}$ and $ALDH3I1_{Cys316Ser}$ was reduced between 35% and 45%. The esterase activity of $ALDH3I1_{Cys286Ser}$ was reduced to 27%, whereas the esterase activity of $ALDH3I1_{Cys310Ser}$

was reduced to 74%. ALDH3I1$_{Cys286Ser}$ showed a stronger inhibition of the esterase activity after the treatment with GSNO as well as after the treatment with $CuCl_2$ than ALDH3I1 wild-type enzyme. ALDH3I1$_{Cys310Ser}$ showed the smallest inhibition of the esterase activity after the treatment with GSNO as well as after the treatment with $CuCl_2$ compared to ALDH3I1 wild-type enzyme. Re-reduction using DTT led to a significant recovery of the esterase activity of ALDH3I1 wild-type enzyme and the Cys mutants after the treatment with GSNO as well as after the treatment with $CuCl_2$. The strongest recovery of the esterase activity showed ALDH3I1$_{Cys114Val}$ (120%) and ALDH3I1$_{Cys310Ser}$ (145%) after *S*-nitrosylation compared to ALDH3I1 wild-type enzyme (73%). The following enzymes ALDH3I1$_{Cys114Val}$ and ALDH3I1$_{Cys310Ser}$ showed also the strongest recovery of the esterase activity after oxidation 101% and 119%, respectively compared to the esterase activity for ALDH3I1 wild-type enzyme (83%). ALDH3I1 wild-type enzyme and the corresponding Cys mutants were more strongly inhibited by GSNO and $CuCl_2$ than the ALDH3H1 wild-type enzyme and the corresponding Cys mutants. Also re-reduction using DTT led to a recovery of the esterase activity for ALDH3I1 wild-type enzyme and the Cys mutants, that was not observed for ALDH3H1 wild-type enzyme and the Cys mutants.

The results, presented in Figure 20, show that the Cys mutants of ALDH3I1 have an esterase activity comparable to ALDH3I1 wild-type enzyme. Almost all of these enzymes are inhibited by GSNO. Only esterase activity of ALDH3I1$_{Cys310Ser}$ was not significantly affected by the treatment with GSNO and $CuCl_2$. Esterase activity of the ALDH3I1 wild-type enzyme and the Cys mutants can be recovered by DTT after the treatment with GSNO and $CuCl_2$. The cysteine residue located at position 114 (Cys-114) in ALDH3I1 was substituted by three other amino acid residues: Ala, Ser and Val. Esterase activity of ALDH3I1$_{Cys114Ala}$ is more strongly inhibited by GSNO than $CuCl_2$.

Previous experiments showed that the dehydrogenase activity of ALDH3I1$_{Cys316Ser}$ was abolished, but it was demonstrated that this enzyme has esterase activity which is even modified under oxidizing and nitrosating conditions (Stiti *et al.,* 2011). These results suggest that Cys-316 is responsible only for dehydrogenase activity and not for the esterase activity. Probably ALDH3I1 might have a second active centre which is responsible for the esterase activity.

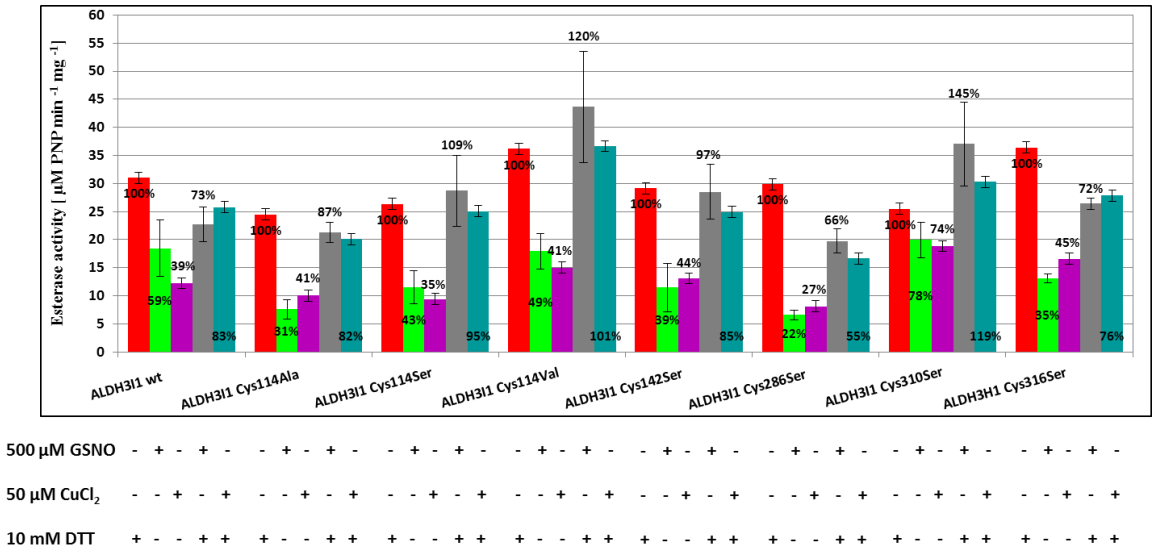



**Figure 20: Esterase activity of *S*-nitrosylated ALDH3I1 wild-type enzyme and the corresponding Cys mutants.**

The purified, recombinant enzyme ALDH3I1 and the corresponding Cys mutants were incubated in the presence of 500 µM GSNO (light green column) for 2.5 h in the dark or 50 µM $CuCl_2$ (purple column) for 1 h at room temperature and then enzyme activity was measured. The esterase activities of the reduced ALDH3I1 enzymes are presented as red columns. For restoring the esterase activity of ALDH3I1 and the corresponding Cys mutants, 10 mM DTT was added to the *S*-nitrosylated enzymes (grey columns) and to the oxidized enzymes (turquoise columns), then incubated for 1.5 h at room temperature in the dark. The esterase activity was determined by monitoring the absorbance at 400 nm. The activity of the reduced form was taken as 100 %. Values represent the means ± SEM of three independent determinations.

The enzymatic activities (dehydrogenase activity and esterase activity) of ALDH3H1 and ALDH3I1 wild-type enzymes and the corresponding Cys mutants are inhibited by nitric oxide donors (Table 7 and Table 8, respectively).

**Table 7: Dehydrogenase and esterase activities of the ALDH3H1 wild-type enzyme and the corresponding Cys mutants modified by *S*-nitrosylation and oxidation**

| | Dehydrogenase activity [%] | | | | | | | Esterase activity [%] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Name of enzyme | GSNO | GSNO + DTT | GSNO + GSH | SNP | SNP + DTT | SNP + GSH | Name of enzyme | GSNO | GSNO + DTT | $CuCl_2$ | $CuCl_2$ + DTT |
| ALDH3H1 $_{wild\ type}$ | 17 | 82 | 54 | 34 | 44 | 13 | ALDH3H1 $_{wild\ type}$ | 88 | 88 | 87 | 89 |
| ALDH3H1 $_{Cys45Ser}$ | 44 | 89 | 53 | 108 | 95 | 81 | ALDH3H1 $_{Cys45Ser}$ | 70 | 75 | 46 | 71 |
| ALDH3H1 $_{Cys45Ala}$ | 9 | 57 | 13 | 63 | 84 | 66 | ALDH3H1 $_{Cys45Ala}$ | 70 | 124 | 75 | 102 |
| ALDH3H1 $_{Cys247Ser}$ | 43 | 92 | 47 | 106 | 120 | 103 | ALDH3H1 $_{Cys247Ser}$ | 83 | 114 | 78 | 121 |
| ALDH3H1 $_{Cys253Ser}$ | no dehydrogenase activity | | | | | | ALDH3H1 $_{Cys253Ser}$ | 81 | 93 | 61 | 103 |

**Table 8: Dehydrogenase and esterase activities of the ALDH3I1 wild-type enzyme and the corresponding Cys mutants modified by *S*-nitrosylation and oxidation**

| | Dehydrogenase activity [%] | | | | | | | Esterase activity [%] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Name of enzyme | GSNO | GSNO + DTT | GSNO + GSH | SNP | SNP + DTT | SNP + GSH | Name of enzyme | GSNO | GSNO + DTT | $CuCl_2$ | $CuCl_2$ + DTT |
| ALDH3I1 $_{wild\ type}$ | 68 | 79 | 68 | 90 | 89 | 92 | ALDH3I1 $_{wild\ type}$ | 59 | 73 | 39 | 83 |
| ALDH3I1 $_{Cys114Ser}$ | 63 | 61 | 43 | 83 | 84 | 87 | ALDH3I1 $_{Cys114Ser}$ | 43 | 109 | 35 | 95 |
| ALDH3I1 $_{Cys114Ala}$ | 79 | 91 | 83 | 92 | 82 | 86 | ALDH3I1 $_{Cys114Ala}$ | 31 | 87 | 41 | 82 |
| ALDH3I1 $_{Cys114Val}$ | not tested | | | | | | ALDH3I1 $_{Cys114Val}$ | 49 | 120 | 41 | 101 |
| ALDH3I1 $_{Cys142Ser}$ | 31 | 45 | 29 | 74 | 90 | 93 | ALDH3I1 $_{Cys142Ser}$ | 39 | 97 | 44 | 85 |
| ALDH3I1 $_{Cys286Ser}$ | 69 | 67 | 62 | 93 | 92 | 103 | ALDH3I1 $_{Cys286Ser}$ | 22 | 66 | 27 | 55 |
| ALDH3I1 $_{Cys310Ser}$ | 56 | 63 | 53 | 92 | 87 | 94 | ALDH3I1 $_{Cys310Ser}$ | 78 | 145 | 74 | 119 |
| ALDH3I1 $_{Cys316Ser}$ | no dehydrogenase activity | | | | | | ALDH3I1 $_{Cys316Ser}$ | 35 | 72 | 45 | 76 |

## 3.4.2. Ellman's test: Quantification of free thiol groups

To check if the decrease of dehydrogenase activity of ALDH3H1 and ALDH3I1 under the nitrosylating conditions is related to the loss of sulfhydryl groups, the enzymatic assay was performed during *S*-nitrosylation (mediated by treatment with GSNO or SNP) free thiol groups were quantified. The results, presented in Figure 21, show that loss of dehydrogenase activity is correlated with the decrease of the number of sulfhydryl groups. It confirms that *S*-nitrosylation is the process that targets all the cysteines of the enzyme and has consequently an impact on the ALDH activity.

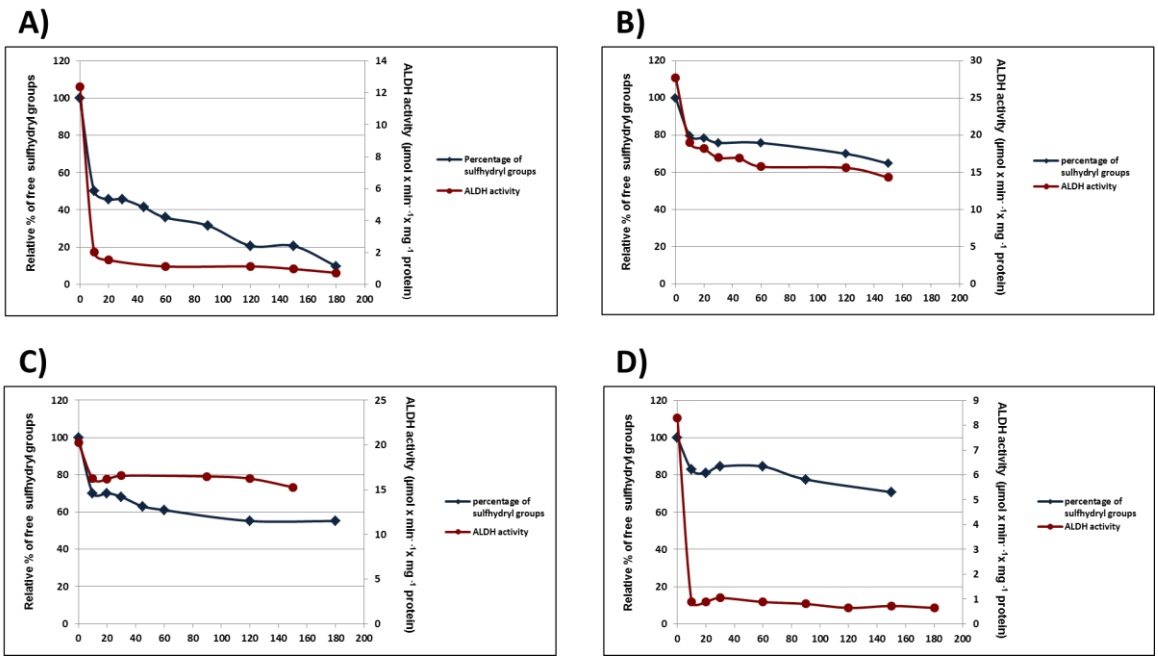



**Figure 21: Loss of the sulfhydryl groups during *S*-nitrosylation of ALDH3H1 and ALDH3I1 wild-type enzymes.**

ALDH proteins were incubated with either 500 µM GSNO or 500 µM SNP up to 180 min. The dehydrogenase activity (red line) was determined and the relative percentage of the sulfhydryl groups (blue line) was measured. The dehydrogenase inhibition during *S*-nitrosylation is correlated with loss of the sulfhydryl groups. ALDH3H1 *S*-nitrosylated with GSNO (A) and SNP (B), ALDH3I1 *S*-nitrosylated with GSNO (C) and SNP (D). Values represent the means ± SEM of three independent determinations.

# 3.5. Detection of *S*-nitrosylated aldehyde dehydrogenases using the biotin switch method

To detect *S*-nitrosylated aldehyde dehydrogenase ALDH3H1 the biotin switch technique (BST) was used (Jaffrey *et al.*, 2001). In this technique *S*-nitrosylated cysteines are converted to biotinylated cysteines, and then biotinylated proteins are subsequently detected by immunoblotting.

## 3.5.1. Detection of *S*-nitrosylated recombinant ALDH proteins

Freshly purified ALDH3H1 and ALDH3I1 recombinant proteins were incubated with 500 µM GSNO for 2.5 hours. Excess of GSNO was removed by acetone precipitation (ALDH3H1). During the precipitation a significant loss of protein occurred and the sample was not completely free of GSNO and blocking reagent. For removal of GSNO after treatment with ALDH3I1 TCA precipitation was used. This method was more

effective, because only small losses of protein occurred and sharper protein bands in the separating gel after SDS-PAGE electrophoresis were observed. *S*-nitrosylated ALDH3H1 and untreated ALDH3H1 were blocked by *S*-methyl methanethiosulfonate (MMTS). This reagent was not effective to block free thiols. The reversible thiol blocking reagent MMTS used in the blocking step for ALDH3H1 was replaced by an irreversible thiol reactive reagent N-ethylmaleimide (NEM). The blocking reagents were removed and the *S*-nitrosylated proteins and untreated proteins were incubated with N-[6-(Biotinamido)hexyl]-3´-(2´-pyridyldithio)propionamide (biotin-HPDP) in the presence or absence of 1 mM sodium ascorbate for 1 h.

### 3.5.1.1. Detection of *S*-nitrosylated recombinant ALDH3H1 wild-type protein

The immunoblot, presented in Figure 22, shows a strong signal of biotinylation in the sample containing the ALDH3H protein, which was incubated with GSNO. There is no significant difference in the signal between line 2 and 4, which suggests that the presence of sodium ascorbate did not enhance the biotinylation process. For further experiments the sodium ascorbate was not used in the biotinylation step.

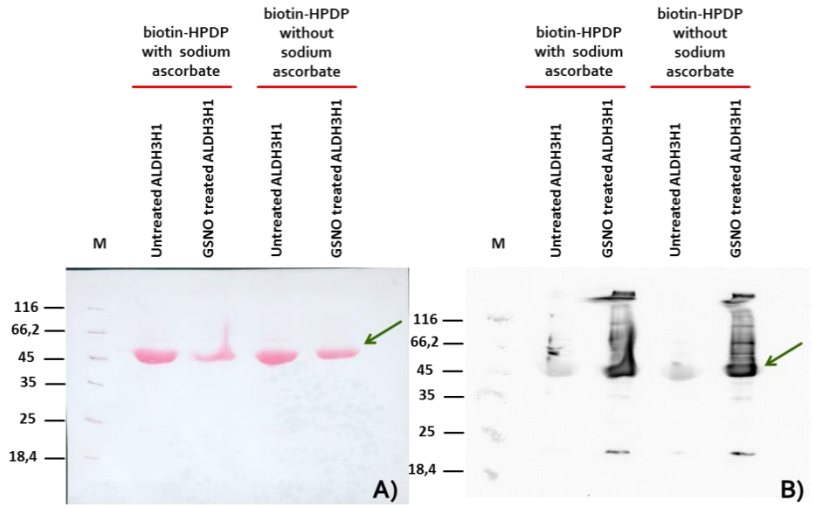



**Figure 22: Detection of *S*-nitrosylated recombinant ALDH3H1 protein.**

Equal amounts (5 µg) of protein were treated with 500 µM GSNO and labelled with biotin-HPDP in the absence or presence of sodium ascorbate using the biotin switch method. As control, untreated recombinant ALDH3H1 labelled with biotin-HPDP in the absence or presence of the sodium ascorbate using the biotin switch method. Proteins were separated by SDS-PAGE, blotted on a nitrocellulose membrane and immunologically detected with anti-biotin antibody. Panel A shows the samples incubated with Ponceau S. Panel B shows the samples incubated with anti-biotin antibody. Molecular mass markers (kDa) are shown on the left. The arrow indicates the band containing ALDH3H1 protein.

### 3.5.1.2. Detection of S-nitrosylated recombinant ALDH3I1 wild-type protein

The immunoblot, presented in Figure 23, shows a clear signal of biotinylation in the sample of the ALDH3I1 recombinant protein, which was previously incubated with GSNO.

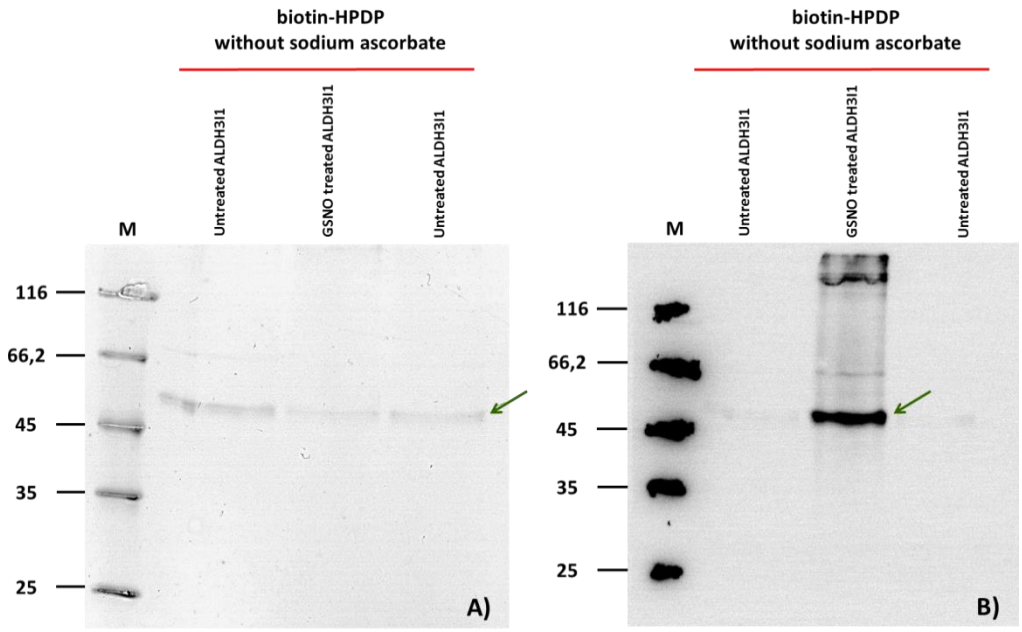



**Figure 23: Detection of *S*-nitrosylated recombinant ALDH3I1 protein.**

Equal amounts (7 µg) of protein were treated with 500 µM GSNO and labelled with biotin-HPDP in the absence of the sodium ascorbate using the biotin switch method. As control, untreated recombinant ALDH3H1 labelled with biotin-HPDP in the absence of the sodium ascorbate using the biotin switch method. Proteins were separated by SDS-PAGE, blotted on a nitrocellulose membrane and immunologically detected with anti-biotin antibody. Panel A shows the samples incubated with Ponceau S. Panel B shows the samples incubated with anti-biotin antibody. Molecular mass markers (kDa) are shown on the left. The arrow indicates the band containing ALDH3I1 protein.

The results obtained suggest that precipitation by TCA and NEM as blocking reagent gives the best detection of *S*-nitrosylated ALDH3I1 compared to the detection of *S*-nitrosylated ALDH3H1, where acetone precipitation and MMTS were used.

## 3.5.2. Change of migration of *S*-nitrosylated ALDH3H1 protein after separation in two-dimensional gel electrophoresis

To evaluate and confirm the *S*-nitrosylation of ALDH3H1, two-dimensional gel electrophoresis (2D) was performed. ALDH3H1 was treated with GSNO and then labelled with biotin-HPDP. By separating the biotinylated protein according to pI, it is possible to compare the position of *S*-nitrosylated ALDH3H1 with untreated ALDH3H1. The attachment of biotin-HPDP to the protein causes a shift of ALDH3H1 towards the basic region of the gel (Figure 24, Panel B). Treated and untreated samples of ALDH3H1 were separated by 2D SDS-PAGE electrophoresis. The separated proteins were transferred onto the nitrocellulose membrane and incubated with anti-biotin antibody. As a control biotinylated ovalbumin was loaded in the combination with the protein sample. Biotinylated ovalbumin can be recognized by anti-biotin antibody.

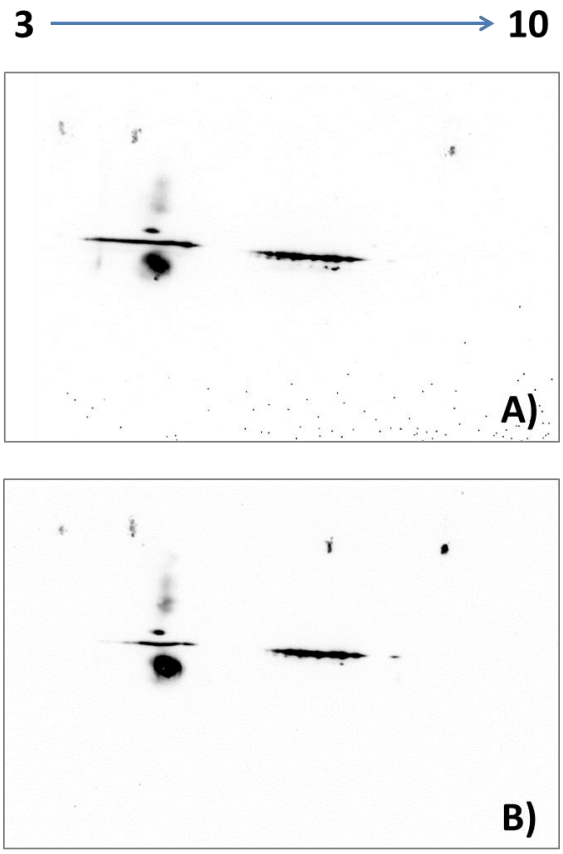



**Figure 24: Two-dimensional separation of *S*-nitrosylated and untreated ALDH3H1 wild-type protein.**

The purified recombinant protein ALDH3H1 was separated by 2D SDS-PAGE. Panel A shows untreated ALDH3H1 and panel B shows GSNO-treated ALDH3H1. The gels were transferred on a nitrocellulose membrane and immunologically detected with anti-biotin antibody. The band corresponding to ALDH3H1 protein is indicated by an arrow. Biotinylated ovalbumin was used as reference protein.

## 3.5.3. Detection of *S*-nitrosylated ALDH3H1 protein from *A. thaliana* root culture

Two-weeks-old seedlings of *A. thaliana* were transferred to Erlenmeyer flasks containing liquid MS media for growing root cultures. The flasks were wrapped with aluminum foil paper and incubated for three weeks in a shaker at 120 rpm. The flasks containing root cultures were divided into two equal sets. The root cultures of the first set were exposed to nitrosative stress by the application of 100 µM GSNO for 4 hours. The root cultures of the second set were untreated and served as the controls for the *S*-nitrosylation process. In the next step roots from both sets were air-dried and frozen in liquid nitrogen. Total proteins of non-treated and GSNO-treated root cultures were extracted. The proteins extracted from control plants were incubated with 100 mM DTT to remove biotinylation occurring *in vivo*. The proteins in the control sample and *S*-nitrosylated sample were blocked using NEM. The excess of the blocking reagent was removed by TCA precipitation and samples were incubated with biotin-HPDP for 1h.

The immunoblot, presented in Figure 25, shows the root proteins which are *S*-nitrosylated. Among these proteins one band corresponds to the size of ALDH3H1.

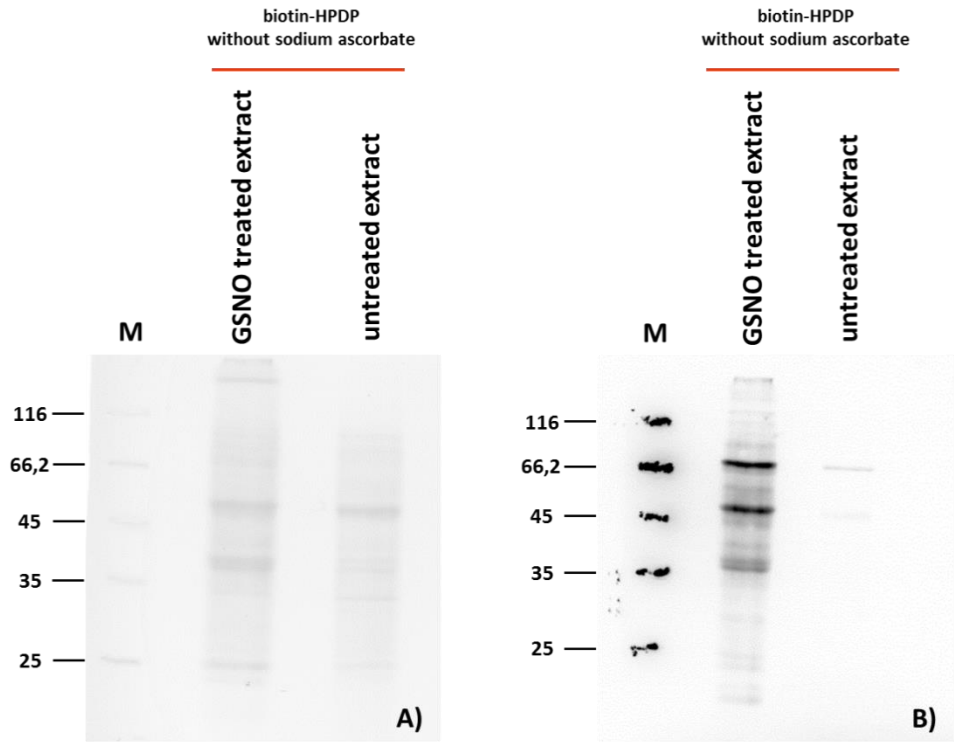



**Figure 25: Detection of *S*-nitrosylated ALDH3H1 enzyme from *A. thaliana* root cultures.**

The root culture was treated with 500 µM GSNO and total proteins were extracted. Proteins extracted from untreated root culture were treated with 100 mM DTT. All protein extracts were further labelled with biotin-HPDP using the biotin switch method. Protein samples were separated by SDS-PAGE electrophoresis and blotted on the nitrocellulose membrane and then immunologically detected with anti-biotin antibody. Panel A shows the samples incubated with Ponceau S. Panel B shows the samples incubated with anti-biotin antibody. Molecular mass markers (kDa) are shown on the left.

# 3.6. Kinetic characterization of the ALDH7B4 protein

The previous research showed that ALDH7B4 is a stress-responsive protein (Kotchoni *et al.*, 2006; Missihoun *et al.*, 2014). The posttranslational modifications as *S*-nitrosylation may modify the enzyme activity of ALDH7B4 and at the same time participate in the plant responses to drought stress. The objective of this study was to investigate the effect of *S*-nitrosylation on the activity of the ALDH7B4 enzyme and compare the outcome with the results obtained for ALDH3H1 and ALDH3I1 enzymes.

## 3.6.1. Generation of a construct encoding ALDH7B4 protein and over-expression of recombinant ALDH7B4 enzyme.

The amino acid sequence of ALDH3H1 contains three Cys residues, whereas the amino acid sequence of ALDH3I1 includes nine Cys residues. The predicted amino acid sequence of the recombinant ALDH7B4 protein contains eleven Cys residues. Due to the different number of cysteine residues occurring in aldehyde dehydrogenases ALDH3I1, ALDH3H1 and ALDH7B4, the patterns of *S*-nitrosylation and the effects of *S*-nitrosylation on the enzyme activity may vary. In order to compare the *S*-nitrosylation patterns of these three enzymes, the construct encoding ALDH7B4 protein was generated.

An *NcoI/HindIII* fragment (1541 bp; 542 amino acids; nucleotides 5070-6711) was subcloned into *NcoI/HindIII* sites of the pET-28a expression vector (Novagen) to express the recombinant protein ALDH7B4. The ALDH7B4 fragment was amplified by PCR with the following primers:

| | |
|---|---|
| Forward primer: | 5'- CTGAGTGAGATTGGGCTGAC -3' |
| Reverse primer: | 5'- GCTACTAGCTTGGTCATTGCG -3' |

The expression vector comprises a sequence corresponding to 6His-tag which was fused to ALDH7B4 gene sequence at the 5'-end. The induction by IPTG of the BL21 *E. coli* strain bearing this vector resulted in the expression of a recombinant ALDH7B4 protein with a 6xHis-tag at the N-terminal end. The recombinant protein has 542 amino acids, and the predicted molecular weight is 57 kDa.

To establish optimal conditions for expression of ALDH7B4, the bacterial culture was induced at 26ºC by adding IPTG to a final concentration of 0.5 mM. As shown in Figure 26, the expression of the recombinant protein ALDH7B4 was monitored every 2 hours and compared to the expression of the vector pET-28a. The best induction of soluble protein was obtained after 8 hours of incubation.

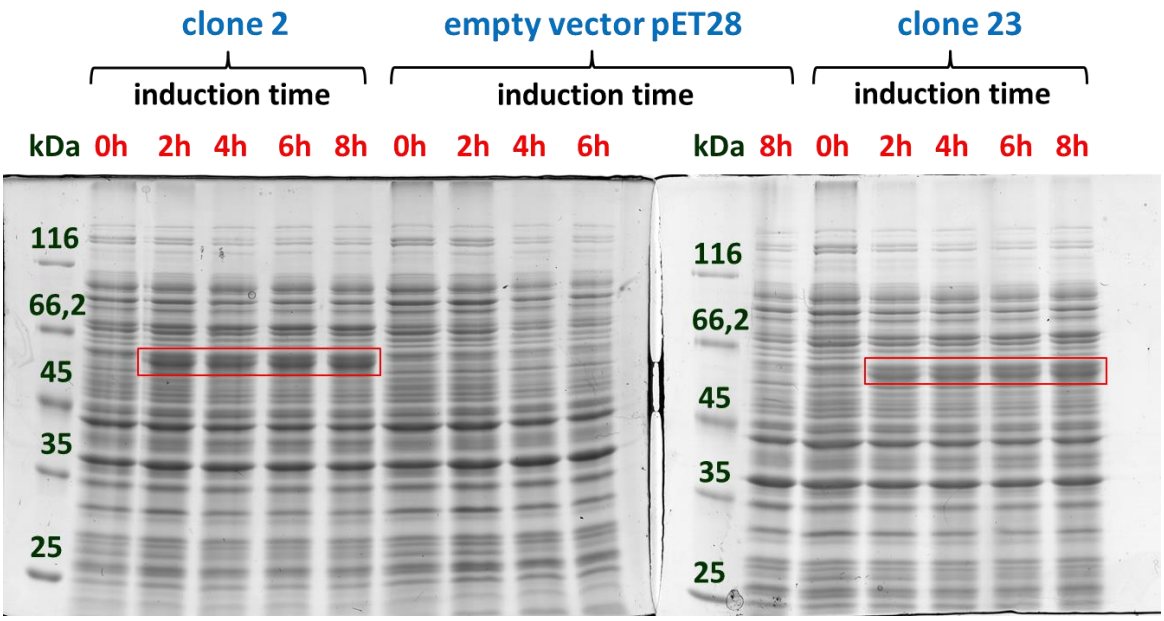



**Figure 26: Induction and expression of the histidine-tagged ALDH7B4 protein.**

Bacterial cultures of two positive clones (2 and 23) containing ALDH7B4 fragment and the empty vector pET-28a were induced for 8 hours with 1 mM IPTG. The samples of the liquid bacterial culture were collected every 2 hours and the cells were harvested. The induced protein has a mass of about 57 kDa and is indicated in the red frame.

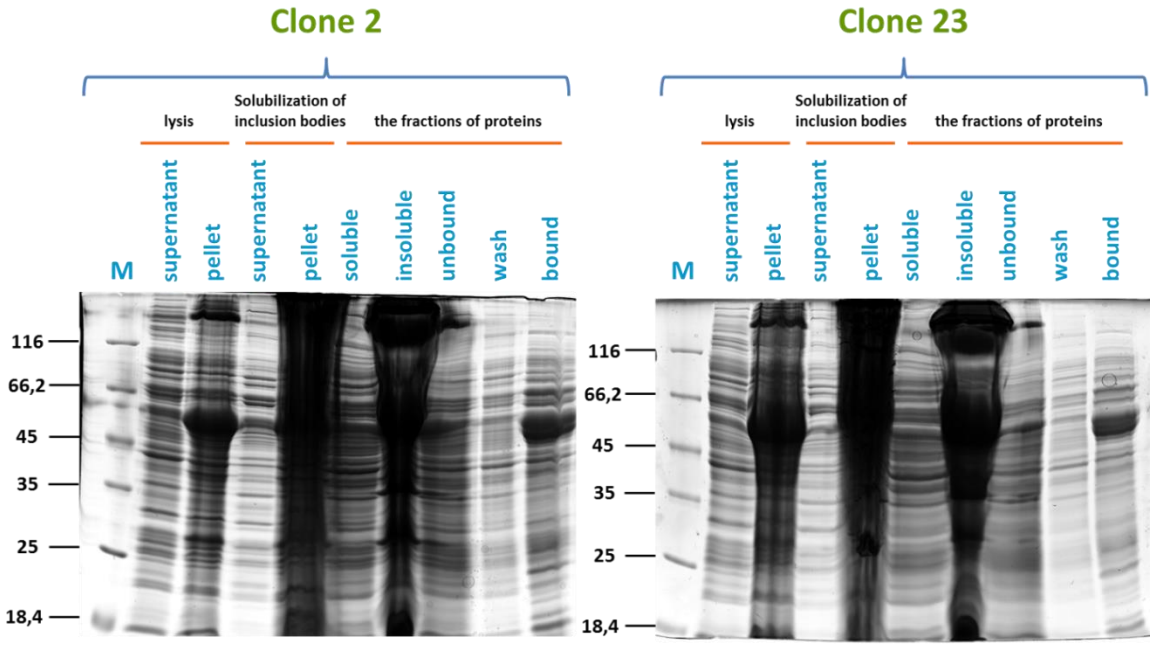



**Figure 27: Purification of the histidine-tagged ALDH7B4 protein from inclusion bodies.**

Bacterial culture of clone 2 and 23 were induced for 8 hours with 1 mM IPTG and further the cells were harvested. The pellet containing ALDH7B4 protein was lysed using lysozyme and subsequently the lysate was solubilized using deoxycholic acid. The samples collected in each step of the procedure were loaded on the gels and separated using SDS-PAGE electrophoresis.

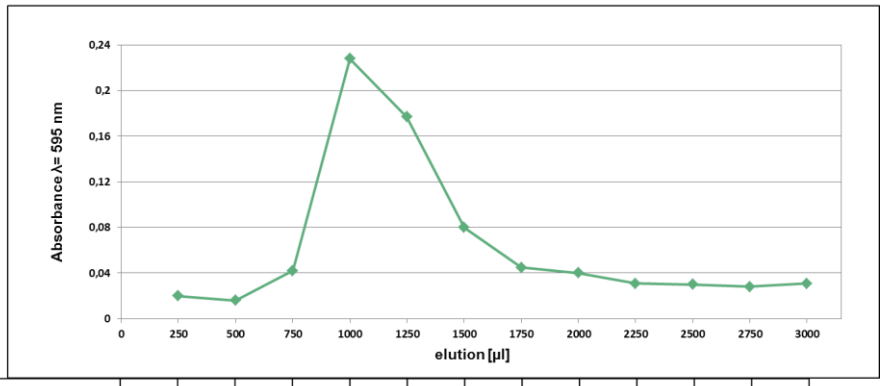
## 3.6.2. Purification of His-tagged ALDH7B4 protein by nickel affinity chromatography

The recombinant protein ALDH7B4 was purified from bacterial pellets using metal ion affinity chromatography on His-tag binding columns under native conditions. The proteins were eluted from the column using high concentration (250 mM) of imidazole. The concentration of the proteins in eluted fractions was determined using the Bradford assay. The elution profiles for each ALDH7B4 protein obtained from two clones of *E. coli* BL 21 are illustrated in Figure 28. The results, presented in Figure 29, show that the protein ALDH7B4 is present mainly in inclusion bodies. The soluble protein occurs only in small amounts in the supernatant fraction.

**A) Elution profile for ALDH7B4 clone 2**


| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,03 | 0,03 | 0,02 | 0,142 | 0,151 | 0,079 | 0,06 | 0,049 | 0,037 | 0,02 | 0,025 | 0,025 |
| Protein concentration [µg/µl] | 0,050 | 0,050 | 0,033 | 0,235 | 0,250 | 0,131 | 0,099 | 0,081 | 0,061 | 0,033 | 0,041 | 0,041 |

**B) Elution profile for ALDH7B4 clone 23**




| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OD595 | 0,02 | 0,016 | 0,042 | 0,228 | 0,177 | 0,08 | 0,045 | 0,04 | 0,031 | 0,03 | 0,028 | 0,031 |
| Protein concentration [µg/µl] | 0,033 | 0,026 | 0,069 | 0,377 | 0,293 | 0,132 | 0,074 | 0,066 | 0,051 | 0,050 | 0,046 | 0,051 |

**Figure 28: Protein elution profile of the recombinant ALDH7B4 wild-type enzyme after the purification by the affinity chromatography on Ni-NTA column.**

The fractions containing 250 µl of the eluate were collected and the absorbance at 595 nm was measured for the each fraction. Subsequently the protein concentration of the each fraction was determined. The values for the absorbance and the protein concentration are presented in the table below the elution profile. Elution profile of ALDH7B4 clone 2 (A) and ALDH7B4 clone 23 (B).

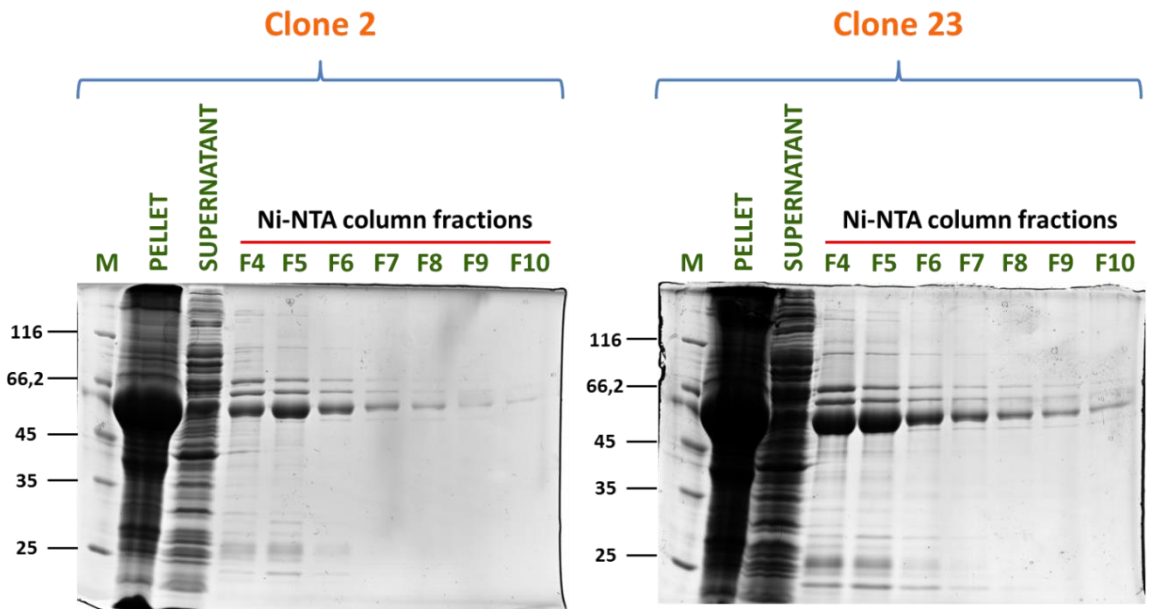



**Figure 29: Isolation and purification of the histidine-tagged ALDH7B4 protein.**

Bacterial cultures (clone 2 and 23) were induced for 8 hours with 1 mM IPTG and then the cells were harvested. The ALH7B4 protein was pre-fractionated by incubation with lysozyme. The cell suspension was sonicated and centrifuged. The proteins in the supernatant were purified by immobilized metal affinity chromatography (IMAC) using a Ni-NTA column.

# 4. Discussion

Nitric oxide (NO) is known as a small radical with diverse signalling functions. Over the last few years several studies have provided evidence that nitric oxide (NO) and NO-related molecules, such as *S*-nitrosothiols, *S*-nitrosoglutathione (GSNO) and peroxynitrite ($ONOO^-$) play an important role in regulation of enzyme activity as well as in signalling in animals and plants under physiological and stress conditions. Nitric oxide can react with thiol groups derived from protein and non-protein molecules, and also with other free radicals (Stamler *et al.,* 1992). Nitric oxide reacts with Cys residues and forms one or several reversible or irreversible modification. Among all Cys modifications, *S*-nitrosylation as covalent incorporation of a nitrogen monoxide group into the thiol chain of cysteine, is considered to be functionally important posttranslational modification mediated by NO (Hess *et al.,* 2005) and has been extensively studied. It has been shown that *S*-nitrosylation affects not only the protein activity (either by activation or inhibition) but also leads to changes in protein-protein interactions and/or subcellular localization (Benhar and Stamler 2005).

In the literature, a large number of examples of protein *S*-nitrosylation in animal systems and functional consequences of this posttranslational modification has been reported (Stamler *et al.,* 2001). A comparative study reveals common targets for *S*-nitrosylation between animals and plants. Until now, little is known about the extent and physiological function of *S*-nitrosylated proteins in plants. The results of microarray analysis have shown that expression of about 2% of the transcripts in the *Arabidopsis* genome is NO-responsive. Analysis of SNP-treated seedlings showed 342 up and 80 down-regulated genes (Parani *et al.,* 2004). In recent years, few proteomic studies have been performed to identify targets for protein *S*-nitrosylation in plants. In *A. thaliana* 63 proteins have been identified from GSNO-treated cell-culture and 52 proteins have been identified from NO-treated leaves (Lindermayr *et al.,* 2005), furthermore 16 proteins have been found to be differentially *S*-nitrosylated in *A. thaliana* plants under a hypersensitive response (Romero-Puertas *et al.,* 2008) and 42 proteins have been found in *A. thaliana* plantlets (Puyaubert *et al.,* 2014). The other proteomic approaches led to the identification of *S*-nitrosylated proteins in the Crassulacean Acid Metabolism (CAM) plants *Kalanchoe pinnata* (Abat *et al.,* 2008), *Brassica juncea* (Abat and Deswal 2009) and *Citrus*

*aurantium* L. (Tanou *et al.,* 2009). The identification of the candidates was the starting point for the functional and biochemical characterization of *S*-nitrosylation in plants.

In plants, the knowledge about the role of the *S*-nitrosylation is rather limited and restricted to results obtained for a few proteins. Experimental evidence of regulation by *S*-nitrosylation has been described for non-symbiotic haemoglobin (AHb1) (Perazzolli *et al.,* 2004), metacaspase 9 (AtMC9) (Belenghi *et al.,* 2006), *S*-adenosyl-methionine synthetase 1 (SAMS1) (Lindermayr *et al.,* 2006), peroxiredoxin II E (PrxIIE) (Romero-Puertas *et al.,* 2007), glyceraldehyde-3-phosphate (GAPDH) (Holtgrefe *et al.,* 2008; Wawer *et al.,* 2010; Vescovi *et al.,* 2013; Zaffagnini *et al.,* 2013) salicylic acid-binding protein 3 (AtSABP3) (Wang *et al.,* 2009), TGA1 transcription factor (Lindermayr *et al.,* 2010) and *S*-nitrosoglutathione reductase (GSNOR) (Frungillo *et al.,* 2014).

In a proteomic study of *A. thaliana* cell cultures aldehyde dehydrogenase has been considered as a putative target of *S*-nitrosylation (Lindermayr *et al.,* 2005). This is confirmed by a recent study in which several aldehyde dehydrogenases (ALDH3B4, ALDH5F1, ALDH7B4, ALDH10A9 and ALDH11A3) were found among endogenously *S*-nitrosylated proteins in *A. thaliana* (Hu *et al.,* 2015).

Since the inactivation of ALDH3H1 and ALDH3I1 activities by oxidation has been demonstrated (Stiti *et al.,* 2011), this study was focused on the effect of *S*-nitrosylation on stress-responsive aldehyde dehydrogenases ALDH3H1, ALDH3I1 and ALDH7B4 from *A. thaliana.* The first objective of this study was the detection of *S*-nitrosylated aldehyde dehydrogenases *in vitro* and *in vivo* using the biotin switch method. The second objective was the investigation of nitric oxide donors on dehydrogenase and esterase activity of aldehyde dehydrogenases. The third objective was to explore whether *S*-nitrosylation is reversible. The fourth objective was the identification of cysteine residues involved in *S*-nitrosylation.

# 4.1. *S*-nitrosylation of aldehyde dehydrogenases ALD3H1 and ALDH3I1 from *A. thaliana* depends on the type of NO donor used

## 4.1.1. The effect of NO donors *S*-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) on dehydrogenase activity of purified aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1 from *A. thaliana*

Here evidence from *in vitro* studies for the *S*-nitrosylation of the aldehyde dehydrogenase enzymes ALDH3H1 and ALDH3I1 from *A. thaliana* was provided. It have been identified the aldehyde dehydrogenase enzymes ALDH3H1 and ALDH3I1 from *A. thaliana* as targets for protein *S*-nitrosylation using the biotin switch method (Jaffrey *et al.*, 2001). The results of studies from *A. thaliana* root culture showed that ALDH3H1 is *S*-nitrosylated *in vivo*. These findings led to evaluate the effect of NO on ALDH activities.

Incubation of purified ALDH isozymes ALDH3H1 and ALDH3I1 with each nitric oxide donor *S*- nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) resulted in a loss of enzymatic activity. The findings presented in this work showed that the inactivation of dehydrogenase activity for both enzymes ALDH3H1 and ALDH3I1 became stronger with increasing GSNO concentrations and longer incubation times, whereas inactivation of dehydrogenase activity for both enzymes ALDH3H1 and ALDH3I1 were not affected by increasing SNP concentrations. The inhibition of the dehydrogenase activity of the purified ALDH3H1 enzyme with SNP was dependent on the duration of the incubation time, whereas the inhibition of the dehydrogenase activity of the ALDH3I1 enzyme with SNP did not depend on the duration of the incubation time. Based on the results obtained in this study, it was concluded that the duration of the incubation time and the concentrations of NO donors have differential influence on the *S*-nitrosylation of ALDH3H1 and ALDH3I1 enzymes. The difference might result from the distinct properties of NO donors and number of Cys residues present in the ALDH3H1 and ALDH3I1 enzymes. The data presented in section 3.3.1. (Fig. 12) showed that dehydrogenase activity of aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana* is inactivated by both NO donors GSNO and SNP, while GSNO is a stronger

*S*-nitrosylating agent than SNP. This result is in agreement with a study in *A. thaliana* cell suspension cultures where it has been demonstrated that the *S*-nitrosothiol content was very low (2 µM/mg protein) in SNP-treated extracts, whereas the *S*-nitrosothiol content in GSNO-treated extracts was higher and reached values up to 40 µM/mg protein (Lindermayr *et al.,* 2005). It was demonstrated in the same study that the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in crude extracts was inactivated by GSNO and SNP. The GAPDH activity was reduced to 90% with 1 mM GSNO, whereas the same concentration of SNP inhibited the GAPDH activity to only 50% (Lindermayr *et al.,* 2005). Similar findings have been reported in the literature. Different results for GSNO and SNP were obtained in wheat leaves. Contrary to the results obtained here, it was reported that the nitrate reductase activity was inactivated by SNP at all incubation times (3, 6, 9 and 21 hours) and the reduction in nitrate reductase activity was concentrations dependent, whereas the same GSNO concentrations and incubation times (3 and 21 hours) had no significant effects on the nitrate reductase activity (Rosales *et al.,* 2011). In the work presented here it was shown that NO donors GSNO and SNP inactivated the dehydrogenase activity of purified aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana*. Contrary to these results, it was shown that lipoxygenase (LOX) activity in *Pelargonium peltatum* L. leaves was enhanced in response to 100 µM SNP and almost a 30% increase of enzyme activity was found after 24 h of incubation, whereas the same GSNO concentration had no effect on lipoxygenase activity (Arasimowicz-Jelonek *et al.,* 2011). A similar observation was made for the enzyme activity of glutathione peroxidase from *P. peltatum* L. leaves in response to GSNO and SNP (Arasimowicz-Jelonek *et al.,* 2011). The authors showed that *S*-nitrosylation resulted in an increase of the lipoxygenase (LOX) and the glutathione peroxidase (GPX) activities in *P. peltatum* L. leaves.

In the present work it was shown that the type of NO donor used in the experiments influenced the *S*-nitrosylation pattern of aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1. This could be explained by the different chemical properties of the NO donors GSNO and SNP and the interaction with the cysteine thiol residue. The nitric oxide group in SNP has a triple-bond character, whereas the nitric oxide group in GSNO exhibits significant double-bond character, which allows GSNO to exist as two isomers: *syn* and *anti*. It has been shown by Bartberger *et al*. (2000; 2001) that the "*syn*" conformation has the dihedral angle close 0º and is preferred in a primary *S*-nitrosothiols,

whereas the "*anti*" conformation has the dihedral angle of about 180º and is preferred in tertiary *S*-nitrosothiols. Previous studies have demonstrated that specific NO redox forms in which NO is released, namely $NO^+$ for SNP and $NO^\bullet$ for GSNO, have diverse metabolic effects (Wink *et al.,* 1996; Kim and Ponka 1999, 2000; Cairo and Pietrangelo 2000; Wardrop *et al.,* 2000; Murgia *et al.,* 2004; Ederli *et al.,* 2008). Nitrosonium cation ($NO^+$) released from SNP directly attacks the thiol group at neutral pH, but it is unstable in water and it is immediately hydrolysed to nitrite ($NO_2$) (Kettenhofen *et al.,* 2007), which can further react with $NO^\bullet$ to generate $N_2O_3$ (Goldstein *et al.,* 1996). It has been demonstrated by Wang *et al*. (2005) that NO radical ($NO^\bullet$) released from SNAP and GSNO does not react directly with Cys thiols, but requires the conversion to higher oxidized nitrogen species such as $N_2O_3$. As previously reported the efficiency of NO release both in solution and leaf tissue of *Nicotiana tabacum* L. varies among different NO donors (Ederli *et al.,* 2008). The difference in the effect of NO donors on various cellular functions is derived from the varying amounts of NO released from particular NO donors under specific conditions (Wink *et al.,* 1996). Floryszak-Wieczorek *et al*. (2006) have analysed the mechanism and kinetics of NO emission from the following NO donors GSNO, SNP and SNAP. The authors found that not only the rate of NO generation but also the half-life of donors in solutions were different for all tested compounds. For instance, the half-life of GSNO in solution is 7 h and is shorter than the half-life of SNP (12 h) (Floryszak-Wieczorek *et al.,* 2006). Additionally, Heikal *et al*. (2009) demonstrated that several physicochemical factors, including light, pH and temperature have an impact on GSNO decomposition and an increase in the values of these parameters result in a decrease in the stability of the GSNO molecule. In the studies presented here, all experiments were carried out at the same temperature (25ºC) avoiding exposure to light, but the pH values of a solution (100 mM sodium pyrophosphate) used for incubation with GSNO and SNP varied depending on the aldehyde dehydrogenase isozymes. The pH-optima for ALDH3H1 and ALDH3I1 were determined and were 8.0 and 9.0, respectively (Stiti *et al.,* 2011). The results obtained in section 3.3.1. (Fig.12. A, B) showed that the exposure of ALDH3H1 to the highest concentration (2 mM) of GSNO led to the reduction of dehydrogenase activity to 26%, whereas the same concentration of GSNO resulted in the reduction of ALDH3I1 activity to 39%. Based on these observations the difference in the inactivation of dehydrogenase activity of ALDH3H1 and ALDHI1 isozymes may relate to the GSNO decomposition at different pH values. Several factors have to be taken into account to explain why dehydrogenase activity of

both enzymes is inhibited in different ways by NO donors. In the studies presented here it was found that *S*-nitrosylation is a time-dependent process, especially for aldehyde dehydrogenase ALDH3H1 (Section 3.3.2., Fig. 13. and Section 3.3.2.1., Fig. 14). It is suggested that the amount of NO released is related to the incubation time of NO donors with the enzyme. Similarly, it has been reported that a two-fold higher concentration of NO was released from 1 mM GSNO than from 1 mM SNP under continuous illumination during 24 h (Semchuk *et al.,* 2011). SNP solution is extremely photosensitive (Zhelayaskov and Godwin 1999) and its decomposition is accelerated by oxygen (Feelisch, 1991; Wang *et al.,* 2002). It has been reported that the degradation of SNP in aqueous solution is caused by the exposure to white or blue light (Arnold *et al.,* 1984; Feelisch 1998). For instance, Floryszak-Wieczorek *et al.* (2006) demonstrated that light and incubation time modified the content of NO released from GSNO and SNP. The authors found that a temporary lack of light inhibited NO release from SNP, both in solution and SNP-treated leaf tissue, whereas release of NO was observed in the dark in the case of GSNO. Seabra *et al*. (2004) suggested that release of NO from GSNO might be due to the auto decomposition promoted by room temperature. It was also found that the maximum NO concentration (6 µM) in SNP solution was recorded after 2 h under continuous light and after this time the content of NO decreased gradually. Conversely, the maximum NO concentration (12 µM) in GSNO solution was recorded after 5 h under continuous light. In the experiments presented in this work the aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1 were incubated with NO donors for 2.5 h in the dark. The time of incubation (2.5 h) was perhaps insufficient to release the complete amount of NO from GSNO, especially in darkness where GSNO decomposition is weaker than under continuous light. This may explain why the dehydrogenase activity of ALDH3H1 and ALDH3I1 was not completely inhibited after 4 h of incubation (Fig. 13 A, B). Both enzymes ALDH3H1 and ALDH3I1 maintained about 30% of enzymatic activity. On the other hand, 2.5 h of incubation was sufficient time to release the maximum amount of NO from SNP, but under continuous light as was reported by Seabra *et al*. (2004). In the case of ALDH3H1 and ALDH3I1 enzymes from *A. thaliana*, darkness may have limited the decomposition of SNP. The experiments were carried out in darkness at room temperature; therefore the content of NO release from SNP under this condition might be lower than from GSNO and it might provide an explanation why the inactivation of dehydrogenase activity of ALDH3H1 and ALDH3I1 was stronger in the case of GSNO treatment than in the case of SNP treatment. Based on the observations that the

decomposition of nitric oxide donors is a light dependent reaction, it would be worth to investigate in further studies the effect of light on *S*-nitrosylation of aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1 from *A. thaliana*, maintaining all other parameters unchanged.

SNP contains five tightly bound cyanide ligands and one linear nitric oxide ligand, which are attached to an iron atom. The reduction and decomposition of SNP is accompanied by the release of cyanide and this leads to pronounced cellular toxicity (Arnold *et al.,* 1984; Bates *et al.,* 1991). It has been demonstrated by Han *et al*. (2004) that cyanide released from SNP decreased the formation of *S*-nitrosylated haemoglobin (SNO-Hb) by 30-40%. Possibly the *S*-nitrosylation of recombinant aldehyde dehydrogenase enzymes ALDH3H1 and ALDH3I1 from *A. thaliana* by the SNP donor might be slightly attenuated by the presence of cyanide ions in the protein sample.

All observations suggest that the dehydrogenase activity of the recombinant aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana* is modulated by the nitric oxide donors GSNO and SNP in two different ways. Diverse inhibition pattern of the dehydrogenase activity of ALDH3H1 and ALDH3I1 resulted from redox forms of nitric oxide released from GSNO and SNP and the physicochemical factors, including light, temperature, pH and incubation time, which have an effect on the *S*-nitrosylation process. It is proposed that the inactivation of dehydrogenase activity of recombinant ALDH3H1 and ALDH3I1 by nitric oxide donors might be a starting point to investigate the mechanism and physiological function of the *S*-nitrosylation process in *A. thaliana* plants. Further studies on the *S*-nitrosylation in *A. thaliana* plants over-expressing aldehyde dehydrogenases ADH3H1 and ALDH3I1 might provide data to investigate the role of the *S*-nitrosylated aldehyde dehydrogenases under non-stress conditions and in response to drought stress.

## 4.1.2. The effect of NO donors *S*-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) on esterase activity of purified aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1 from *A. thaliana*. Comparison of the esterase activity modulated by *S*-nitrosylation and oxidation

Several aldehyde dehydrogenases have the ability to catalyse the hydrolysis of *p*-nitrophenyl acetate (*p*-NPA), a specific substrate for esterases (Park *et al.,* 1961; Feldman *et al.,* 1972; Sidhu *et al.,* 1975; Mukerjee and Pietruszko 1992). The previous study showed that aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I from *A. thaliana* have also an esterase activity apart from the dehydrogenase activity (Pandey 2013). Based on these observations, the effect of GSNO on the esterase activity of the aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1was investigated. According to the results obtained for ALDH3H1 wild-type enzyme and ALDH3I1 wild-type enzyme (section 3.4.1. Fig. 19 and Fig. 20), it was found that the esterase activity of ALDH3H1 wild-type enzyme and ALDH3I1 wild-type enzyme were inactivated in response to 500 µM GSNO treatment. Similarly, it was reported by Beretta *et al*. (2008) that the hydrolysis of *p*-nitrophenyl acetate catalysed by human mitochondrial aldehyde dehydrogenase (ALDH2) was inhibited by nitroglycerin (GTN), which was classified as an organic NO donor. The inactivation of the esterase activity of ALDH2 by GTN was time dependent, the loss of esterase activity was faster with increasing GTN concentrations and slower with increasing substrate concentrations. The inhibition of esterase activity of ALDH2 by GTN was strongly enhanced in the presence of $NAD^+$ (Beretta *et al.,* 2008). Based on the results presented in this study, the inhibition of esterase activity by GSNO was stronger for ALDH3I1 wild-type enzyme than for ALDH3H1 wild-type enzyme.

It was further investigated the effect of oxidation on the esterase activity of ALDH3H1 and ALDH3I1 wild-type enzymes. In a previous study, the decrease of the dehydrogenase activity of ALDH3H1 and ALDH3I1 wild-type enzymes resulted from cysteine oxidation as shown by Stiti *et al*. (2011). According to the results obtained here, the esterase activity of the aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1 was inhibited under oxidizing conditions. The inhibition of esterase activity by $CuCl_2$ was stronger for ALDH3I1 wild-type enzyme than for ALDH3H1 wild-type enzyme. The comparison of the esterase activity of ALDH3H1 and ALDH3I1 wild-type enzyme modified by

*S*-nitrosylation and oxidation led to the conclusion that both cysteine posttranslational modifications (*S*-nitrosylation and oxidation) have different effects on the esterase activity of aldehyde dehydrogenase enzymes. In the case of ALDH3H1 the rate of inhibition (about 88%) was similar in response to GSNO and $CuCl_2$, whereas in the case of ALDH3I1 enzyme we found that the esterase activity was more strongly inhibited by $CuCl_2$ than by GSNO.

The findings demonstrated that the esterase activity of the recombinant aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1 from *A. thaliana*, similarly to the dehydrogenase activity, was inhibited in response to *S*-nitrosylation and oxidation.

## 4.2. The number of cysteines/and their localization influence the *S*-nitrosylation of aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana*

It has been reported in many studies that the *S*-nitrosylation of cysteine residues is a very specific process and it is determined by several factors, including the number of cysteine residues (Lander *et al.,* 1997; Gow and Stamler 1998; Sun*et al.,* 2001; Faccenda and Mutus 2011), the presence of the residues surrounding the reactive thiol of cysteine (acid, basic and hydrophobic amino acids) (Stamler *et al.,* 1997; Nedospasov *et al.,* 2000; Kovacs and Lindermayr 2013), protein-protein interactions (O'Brian and Chu 2005), the local pH, and the presence of ions ($Mg^{2+}$ and $Ca^{2+}$) (Hess *et al.,* 2001). Although the knowledge about the factors which control thiol accessibility and reactivity is increasing, up to now it is still difficult to determine the rules which could explain which cysteine would be modified by NO donors in which manner. Several algorithms have been developed to predict the *S*-nitrosylated sites in proteins (Chaki *et al.,* 2014).

## 4.2.1. Identification of cysteine residues involved in *S*-nitrosylation of aldehyde dehydrogenase ALDH3H1 wild-type enzyme

To investigate the role of an individual cysteine residue in the aldehyde dehydrogenase ALDH3H1 enzyme from *A. thaliana* in the *S*-nitrosylation, cysteine residues were individually replaced by serine or alanine using site-directed mutagenesis. The previous studies of Stiti *et al*. (2011) showed that Cys-45 in the ALDH3H1 enzyme is redox-responsive residue and is involved in the intermolecular disulphide bond formation when exposed to oxidizing conditions. According to these findings, it was proposed that Cys-45 might also be the target of posttranslational modification induced by NO donors. This prediction was confirmed by the results presented in this work since the inhibitory effect of GSNO was reduced when Cys-45 was substituted by a serine residue. Additionally, the data obtained from *S*-nitrosylation of mutated enzymes in this work showed that Cys-247 in ALDH3H1 is a second residue responsible for NO-induced inhibition of ALDH3H1 enzyme. The substitution of Cys-247 of ALDH3H1 enzyme by serine reduced the inhibitory effect of GSNO. Unfortunately, it was not possible to identify the *S*-nitrosylated cysteine residues in ALDH3H1 enzyme using mass spectrometry (MS). Although mass spectrometry allows the identification of cysteine residues modified by NO (Torta *et al.,* 2008), the labile and dynamic nature of the *S*-nitrosothiols is a major limitation for direct analysis of *S*-nitrosylated cysteines. It was suggested previously that the exposure of *S*-nitrosylated ALDH3H1 enzyme to the ionization might cause the decomposition of *S*-nitrosothiols. Although the nitrosocysteines present in the ALDH3H1 enzyme were converted to stable biotinylated cysteines, it was still impossible to identify the cysteine residues involved in *S*-nitrosylation.

Based on the results obtained in this study and the findings of Stiti *et al*. (2011), it was shown that the conversion of Cys-253 of ALDH3H1 enzyme to a serine residue caused a total loss of the dehydrogenase activity. Therefore, it was impossible to determine the contribution of Cys-253 to *S*-nitrosylation of the ALDH3H1 enzyme. Cys-253 is a conserved amino acid critical for the catalytic activity of ALDH3H1 enzyme (Stiti *et al.,* 2011). According to the results obtained in these studies, it was suggested that different Cys residues of ALDH3H1 enzyme are responsible for the dehydrogenase and the esterase activities. It has been reported by several investigators that the conversion of aldehydes to corresponding carboxylic acids and the hydrolysis of (*p*-NPA) occurred at

separate active sites (Blackwell *et al.,* 1983), whereas others suggested that these two reactions occurred at the same site (Kitson 1986). Here it was shown that *S*-nitrosylation of ALDH3H1 by GSNO caused the differences in the inhibition of the dehydrogenase activity and the esterase activity. This supports the assumption that the aldehyde dehydrogenase ALDH3H1 enzyme has a second active site responsible for the esterase activity (Pandey 2013). Contrary to the results obtained in this work, when the active site cysteine of glyceraldehyde-3-phosphate dehydrogenase from *E. coli* was substituted by an alanine residue (Corbier *et al.,* 1992) and the mutated enzyme retained some dehydrogenase activity, although it completely lost the esterase activity.

## 4.2.2. Identification of cysteine residues involved in *S*-nitrosylation of aldehyde dehydrogenase ALDH3I1 wild-type enzyme

To investigate the role of an individual cysteine residue present in the aldehyde dehydrogenase ALDH3I1 wild-type from *A. thaliana* in the *S*-nitrosylation, the cysteine residues in ALDH3I1 enzyme were individually replaced by serine, alanine or valine. Previous studies of Stiti *et al*. (2011) showed that Cys-114 in ALDH3I1 enzyme is a redox-responsive residue. Cys-114 seems to be involved in the intermolecular disulphide bond formation when exposed to oxidizing conditions. According to these findings, Cys-114 might be also a target of posttranslational modification induced by NO donors. The results presented here confirmed that Cys-114 in ALDH3I1 enzyme is responsible for NO-induced inhibition of the ALDH3I1 enzyme. The substitution of Cys-114 of ALDH3HI1 enzyme by an alanine residue reduced the inhibitory effect of GSNO. It has been taken into consideration that Cys-286 might also participate in *S*-nitrosylation. The substitution of Cys-286 in ALDH3I1 enzyme by a serine residue maintained the inhibitory effect of GSNO comparable to ALDH3I1 wild-type enzyme and therefore it was concluded that the contribution of Cys-114 in *S*-nitrosylation of ALDH3I1 enzyme is bigger than Cys-286.

Here it was demonstrated that the conversion of Cys-316 of the ALDH3I1 enzyme to a serine residue caused a total loss of the dehydrogenase activity, but maintained the esterase activity. Therefore, it was impossible to determine the contribution of Cys-316 to *S*-nitrosylation of the ALDH3I1 enzyme. Cys-316 is a conserved amino acid critical for the catalytic activity of ALDH3I1 enzyme (Stiti *et al.,* 2011). According to the

observations concluded from this work, it is suggested that different Cys residues of the ALDH3I1 enzyme are responsible for the dehydrogenase and the esterase activities. It was demonstrated that *S*-nitrosylation of ALDH3I1 by GSNO caused the differences in the inhibition of the dehydrogenase activity and the esterase activity. It confirmed the assumption that the aldehyde dehydrogenase ALDH3I1 enzyme has a second active site responsible for the esterase activity (Pandey 2013).

Taken all results together, it is suggested that Cys-45 in ALDH3H1 and Cys-114 in ALDH3I1 are involved in *S*-nitrosylation. It is speculated that Cys-45 and Cys-114 occur as thiolate and have therefore strong nucleophilic properties. This might explain why both cysteine residues (Cys-45 in ALDH3H1 and Cys-114 in ALDH3I1) are the main targets of posttranslational modifications, including oxidative formation of disulphide bonds (Stiti *et al.,* 2011) and *S*-nitrosylation.

## 4.3. The importance of reversible *S*-nitrosylation for the enzyme activities of aldehyde dehydrogenase isozymes ALDH3H1 and ALDH3I1 from *A. thaliana*

*S*-nitrosylation plays an important role as signalling mechanism due to its reversibility (Benhar *et al.,* 2009). The reversion of *S*-nitrosylation is named denitrosylation. It has been reported that non-enzymatic and enzymatic ways might promote denitrosylation of target proteins and regulate this Cys modification (Tada *et al.,* 2008; Benhar *et al.,* 2008; Benhar *et al.,* 2009; Astier *et al.,* 2011).

*S*-nitrosothiols are unstable in a reducing environment due to reversible oxidative modification (Sen 1998). The NO removal is caused by exposure to heat, light (Singh *et al.,* 1996b), and reducing agents, including glutathione, ascorbate, sodium dodecyl sulphate, nucleophilic compounds and transition metals (Stubauer *et al.,* 1999). In biological systems half-lives of protein *S*-nitrosothiols are very short and range from seconds to minutes (Singh *et al.,* 1996; Kashiba-Iwatsuki *et al.,* 1997). The levels of some protein *S*-nitrosothiols in cells are regulated by the intracellular concentration of glutathione (GSH) (Clementi *et al.,* 1998). For instance, in plants the concentration of glutathione reaches 2-3 mM (Ball *et al.,* 2004), whereas in animal cells the concentration of glutathione ranges from 0.5 up to 10 mM (Kosower and Kosower 1978; Mayer *et al.,* 1995).

Based on the results presented in section 3.3.3.1.; Fig. 15 and section 3.3.3.2.; Fig. 17, the inhibition of the dehydrogenase activity of the aldehyde dehydrogenases ALDH3H1 and ALDH3I1 enzymes from *A. thaliana* by GSNO and SNP was restored by the reducing reagents DTT and GSH. The rate of the restored dehydrogenase activity increased with the increase of DTT and GSH concentrations. Zaffagnini *et al*. (2013) showed that the dehydrogenase activity of the recombinant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *A. thaliana* was inhibited by GSNO and the inactivation of the dehydrogenase activity was restored by GSH, but not by cytosolic thioredoxins. Holtgrefe *et al*. (2008) demonstrated that the inactivation of the recombinant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoforms GapC1 and GapC2 from *A. thaliana* by GSNO was restored by the treatment with DTT. The findings obtained for glyceraldehyde-3-phosphate dehydrogenase from *A. thaliana* by two independent research groups showed the similarities to the results obtained in our study. It was concluded that the inhibition of the dehydrogenase activity of the recombinant ALDH3H1 wild-type enzyme was largely reversed by DTT, whereas GSH was less efficient. Similarly, the inhibition of yeast ALDH by NO was more strongly reversed by DTT than by ß-mercaptoethanol (ß-ME) and GSH (DeMaster *et al.*, 1997). The dehydrogenase activity of the recombinant ALDH3H1 and ALDH3I1 wild-type enzymes was restored by DTT and GSH after GSNO-inactivation. Also SNP-inactivation of the dehydrogenase activity of the recombinant ALDH3H1 and ALDH3I1 enzymes was restored by DTT and GSH. Similarly, Vaidyanathan *et al*. (1993) reported that the dehydrogenase activity of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was inactivated in a concentration- and time-dependent manner in the presence of sodium nitroprusside and the inhibition was restored by 10 mM DTT and 10 mM GSH. Nearly half of the initial activity was restored by the treatment with GSH and DTT. The complete inactivation of the human mitochondrial dehydrogenase activity by nitroglycerin (GTN) was very modestly (≤ 16%) restored by DTT (Beretta *et al.*, 2008). The results obtained by Beretta *et al*. (2008) provide evidence that the reversibility of *S*-nitrosylation might depend on the type of reducing agent, but also on the NO donor used for *S*-nitrosylation. The results obtained in this work showed that neither DTT nor GSH was able to completely restore the dehydrogenase activity of the recombinant ALDH3H1 and ALDH3I1 wild-type enzymes. It was concluded that DTT and GSH partially could react with the *S*-nitrosylated ALDH 3H1 and ALDH3I1 enzymes. It is speculated that the remaining in the protein samples GSNO and SNP molecules could decrease the reducing properties of

DTT and GSH. On the one hand, the alternative solution would be the removal of the remaining GSNO and SNP from *S*-nitrosylated protein sample by precipitation method, but on the other hand this could increase the risk of the *S*-nitrosothiols decomposition. Therefore, the precipitation method could introduce a false-positive result into the reversibility of *S*-nitrosylation by DTT and GSH.

The inhibition of the esterase activity of the recombinant aldehyde dehydrogenase ALDH3I1 enzyme from *A. thaliana* by GSNO was restored by the 10 mM DTT (Fig. 20), whereas the slightly inactivated esterase activity of the aldehyde dehydrogenase ALDH3H1 enzyme maintained unchanged in the presence of 10 mM DTT (Fig.19). The esterase activity of the aldehyde dehydrogenase ALDH3I1 enzyme is restored by 10 mM DTT after oxidation in the presence of 50 µM $CuCl_2$, whereas the slightly inactivated esterase activity of aldehyde dehydrogenase ALDH3H1 enzyme maintained unchanged in the presence of 10 mM DTT. The esterase activity of the aldehyde dehydrogenase ALDH3I1 enzyme was not completely restored either after *S*-nitrosylation or after oxidation. Little is known about posttranslational modification (*S*-nitrosylation, oxidation) of the esterase activity of aldehyde dehydrogenases. It was demonstrated that nitric oxide donor GSNO and oxidizing reagent $CuCl_2$ modulate the esterase activity of the aldehyde dehydrogenase ALDH3I1 enzyme by the *S*-nitrosylation and oxidation of cysteine residues, respectively. Additionally, the findings presented in this study showed that the inactivated esterase activity of the aldehyde dehydrogenase ALDH3I1 enzyme resulted from *S*-nitrosylation and oxidation was restored in the presence of 10 mM dithiothreitol (DTT).

Some data have demonstrated that small-molecules such as *S*-nitrosoglutathione are rapidly denitrosylated (Sing *et al.*, 1996; Kashiba-Iwatsuki *et al*., 1997), whereas in most cases protein *S*-nitrosothiols are less susceptible to denitrosylation by glutathione. This can be due to conformational changes that occur after *S*-nitrosylation in these proteins. Paige *et al*. (2008) proposed that the protein structure may be the limitation of accessibility of the *S*-nitrosothiol to the solvent or may reduce the *S*-nitrosothiol reactivity. These factors decrease the possibility of *S*-nitrosothiol to be reduced (Paige *et al.*, 2008). Based on these observations, Paige *et al*. (2008) suggested that *S*-nitrosothiols in proteins can be classified in two different types: stable and unstable. The *S*-nitrosothiols in a cytosolic brain lysate that can only be reduced by 0 to 20% in the presence of GSH are considered to be stable. These *S*-nitrosothiols are not prone to

denitrosylation in contrast to unstable *S*-nitrosothiols which are susceptible to denitrosylation. The unstable *S*-nitrosothiols showed a reduction from 80 to 100% of *S*-nitrosylation in the presence of GSH (Paige *et al.*, 2008). These two types of *S*-nitrosothiols may have a different impact on protein function and simultaneously play different roles in NO signalling. Several studies emphasize the effect of the *cis/trans* conformation of the *S*-nitrosothiols on the stability (Stamler and Toone 2002; Weichsel *et al.*, 2007). Several studies suggest that the regulation of *S*-nitrosylation as well as denitrosylation depend on NO-derived species, reducing agents and redox-related enzymes (Astier *et al.*, 2011).

The results indicated that there are differences in the ability to restore the dehydrogenase and the esterase activity of the aldehyde dehydrogenase enzymes ALD3H1 and ALDH3I1. Taking into consideration that the reduction of *S*-nitrosylation depends on the type and the concentrations of reducing compounds, accessibility of protein *S*-nitrosothiols and their structural conformations, it was concluded that these factors affected the susceptibility of the ALDH3H1 and ALDH3I1 enzymes to denitrosylation. To explain the differences between GSH and DTT to restore the enzymatic (dehydrogenase and esterase) activities of the ALDH3H1 and ALDH3I1 enzymes from *A. thaliana*, the biochemical properties of both reducing compounds were compared. Dithiothreitol (DTT) is a strong reducing agent known also as Cleland's reagent (Cleland 1964). It contains two sulfhydryl groups (-SH) and at pH 7 at room temperature possesses a redox potential of around -0.33 volts. Although DTT has a small tendency to be oxidized by air, it plays an important role in maintaining thiols in a reduced form and reducing disulphides quantitatively. The reducing power of DTT is limited to pH values above 7. Due to the $pK_a$ of thiol groups occurring in DTT is 9.2 and 10.1, dithiothreitol occurs as a negatively charged thiolate ($RS^-$). In the protonated form of the thiol, DTT does not have the possibility to reduce disulphides. Additionally, dithiothreitol may be also used as an oxidizing reagent. Glutathione (GSH) is a tripeptide which plays an important role as an endogenous antioxidant in all organisms. It contains one thiol group (-SH). GSH is also responsible for the reduction of disulphide bonds. The redox potential at pH 7 at room temperature for GSH is -0.24 volts. Glutathione exists in two different redox forms: reduced (GSH) and oxidized (GSSG). The oxidized form of dithiothreitol (GSSG) is responsible for the protein glutathionylation. It has been demonstrated that *S*-nitrosylation may serve as an intermediate for *S*-glutathionylation

due to the reaction between *S*-nitrosothiols and reduced form of glutathione (GSH) (1) (Martínez-Ruiz and Lamas 2007).

(1) RS−N=O + GSH → RS−SG + HNO

The transnitrosylation of glutathione (GSH) by *S*-nitrosylated proteins has been shown in several studies (Meyer *et al.,* 1994; Arnelle and Stamler 1995; Tsikas *et al.,* 1999). As the result of the reaction between *S*-nitrosylated protein and the reduced form of glutathione (GSH), the native reduced protein and *S*-nitrosoglutathione are formed (2).

(2) $R_1$− SNO + GSH → $R_1$−SH + GSNO

In biological systems, *S*-nitrosoglutathione is reduced to GSSG via *S*-nitrosoglutathione reductase (GSNOR) (Letterier *et al.,* 2011). *In vitro* the excess of newly generated GSNO may cause the re-nitrosylation of ALDH, limiting the possibility of GSH to restore the enzymatic activity.

Based on the results, it is concluded that DTT has stronger reducing properties than GSH. It has been suggested that there are several factors which affect the capability of GSH and DTT to restore the dehydrogenase and the esterase activity of the aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana*. One of these factors is the size of reducing compounds. The molecular weight of DTT is 154.2 g/mol, whereas for GSH this value is 307.3 g/mol. The smaller size of DTT can facilitate the accessibility to *S*-nitrosylated cysteines, particularly it has been shown that the attachment of NO to thiols can cause conformational changes in the protein structures (Mou *et al.,* 2003; Wang *et al.,* 2006; Tada *et al.,* 2008) and limit the possibility to denitrosylate modified cysteine residues. Moreover, the value of the redox potential of DTT is lower than that of GSH under the same conditions (pH 7; 25ºC), which indicates that DTT has more free electrons than GSH and therefore it has also stronger reducing properties. The comparison of DTT and GSH structures showed that DTT contains one thiol group more than GSH and the amount of thiol groups might also influence the reducing ability of these reducing compounds. The reductive mechanism of protein denitrosylation depends on the number of electrons. In pH above 7, two sulfhydryl groups of DTT might be negatively charged, forming two thiolate groups ($RS^-$). During the denitrosylation process both thiolate groups are able to remove NO from two cysteine residues, whereas GSH might remove NO from only one cysteine residue. *S*-nitrosylation and denitrosylation of the aldehyde

dehydrogenase ALDH3H1 and ALDH3I1 enzymes from *A. thaliana* may change the functions of these proteins.

## 4.4. Detection of the *S*-nitrosylated aldehyde dehydrogenases using the biotin switch technique (BST)

Detection and quantification of protein *S*-nitrosothiols is a challenge due to the low levels of *S*-nitrosothiols and the labile and dynamic nature of the S-NO bond (Kashiba-Iwatsuki *et al.,* 1997; Bartberger *et al.,* 2001; Jaffrey 2005; Lo Conte 2012). Previous methods, including spectrophotometry, fluorometry and chemiluminescence, enabled to measure the total content of *S*-nitrosothiols at the whole-cell level (Rassaf *et al.,* 2002;Yang *et al.,* 2003; Giustarini *et al.,* 2004; Giustarini *et al.,* 2007; Kettenhofen *et al.,* 2008), but did not identify an individual *S*-nitrosylated protein in cells or tissues. The biotin switch technique developed by Jafferey *et al*. (2001) enabled the identification of *S*-nitrosylated proteins from nearly any source including purified proteins (Belenghi *et al.,* 2007), cultured cells (Lindermayr *et al.,* 2005; Maldonado-Alconada *et al.,* 2011), isolated organelles (Palmieri *et al.,* 2010; Ortega-Galisteo *et al.,* 2012). The previous techniques such as chemiluminescence were based on the detection and the quantification of the liberated NO. In contrast to the methods based on the NO liberation, the biotin switch technique is based on targeting the sulphur atom of *S*-nitrosothiol (Forrester *et al.,* 2009b). Furthermore, in the biotin switch method unstable *S*-nitrosylated Cys groups are converted into stable biotinylated Cys groups (Jaffrey *et al.,* 2001) and facilitate the indirect detection of *S*-nitrosylated proteins.

Although the biotin switch method is the most widely applied method, it is still not the perfect technique; because it consists of three steps (blocking of free thiols, the reduction of *S*-nitrosothiol groups to thiol groups by ascorbate and labelling of the nascent thiols with biotin-HPDP) and each step contains potential sources of errors (Kettenhofen *et al.,* 2007; Forrester *et al.,* 2009b). There are two main limitations of the biotin switch method: firstly, the efficiency and sensitivity of this technique depends on blocking of free thiols. Secondly, the specificity of the ascorbate to reduce *S*-nitrosothiols is a critical factor and affects the detection of *S*-nitrosylated proteins (Wang and Xian 2011).

## 4.4.1. Detection of the *S*-nitrosylated aldehyde dehydrogenase ALDH3H1 enzyme using the biotin switch technique (BST)

In the study presented here it has been demonstrated that the purified aldehyde dehydrogenase ALDH3H1 enzyme from *A. thaliana* was *S*-nitrosylated and the results obtained in this work confirmed that the biotin switch technique (BST) was useful for the detection of *S*-nitrosylated ALDH3H1 *in vitro* by GSNO (Section 3.5.1.; Fig. 22).

It has been shown that there is no significant difference in the biotinylation step in the presence and in the absence of ascorbate, thus it was suggested that the reduction of *S*-nitrosothiols by ascorbate did not enhance the labelling of thiols by biotin-HPDP. Similarly to the findings presented here the reductive effect of ascorbate in the biotin switch method has been questioned in several studies. For instance, it has been reported that the reduction of *S*-nitrosothiols by ascorbate depends on the substrate. Some *S*-nitrosothiols were not reduced by ascorbate efficiently (Zhang *et al.,* 2005; Forrester *et al.,* 2007; Kettenhofen *et al.,* 2007; Wang *et al.,* 2008), whereas in other cases ascorbate enabled the reduction of not only *S*-nitrosothiols, but also disulphides (Landino *et al.,* 2006; Huang and Chen 2006; Giustarini *et al.,* 2008). Simultaneous reduction of disulphides is a source of false-positive signals in the biotin switch method. In connection with reduction of disulphide by ascorbate, it has been proposed to substitute ascorbate by sinapinic acid, a non-disulphide reducing reagent (Kallakunta *et al.,* 2010). Another possibility for errors is the ascorbate can artificially increase the unspecific binding of biotin-HPDP to proteins and generate false-positive signals (Huang and Chen 2006).

The reason that we detected no difference in *S*-nitrosylation between the treated sample and the non-treated sample with ascorbate might result from the low ascorbate concentration (1 mM) and insufficient incubation time (1 h). In the study presented here, the standard conditions (ascorbate concentration and incubation time) as in the original method were used (Jaffrey *et al.,* 2001). On the one hand it has been questioned whether the application of the higher ascorbate concentrations and increased incubation time might enhance the reduction of *S*-nitrosothiols in the biotin switch technique. Holmes and Williams (2000) showed that the reaction of ascorbate with protein *S*-nitrosothiols is slow and pH-dependent. It has been demonstrated by Zhang *et al*. (2005) that higher concentrations of ascorbate (30 mM) and longer incubation time (3h) significantly enhanced the detection of *S*-nitrosothiols using the biotin switch method. On the other

hand, it has been reported that a long, high-dose treatment with ascorbate reduced some disulphides and generated false-positive signals (Landino *et al.,* 2006). Similar observations were reported by Huang and Chen (2006). The authors showed that both ascorbate and heat treatment accelerated the generation of the false-positive signals during the increased time of biotinylation.

Taken all parts together, the results obtained in this study showed that omitting ascorbate in the step before labelling with biotin-HPDP maintained the signal derived from biotinylation and avoided an increase of false-positive signals.

## 4.4.2. Detection of the *S*-nitrosylated aldehyde dehydrogenase ALDH3H1 enzyme from *A. thaliana* root culture using the biotin switch technique (BST)

The data presented here showed that several proteins are *S*-nitrosylated *in vivo* by GSNO in *A. thaliana* root culture. Among the *S*-nitrosylated proteins, one protein band on the nitrocellulose membrane which corresponds to the size of ALDH3H1 enzyme was identified. Based on the previous results obtained in section 3.5.1., the ascorbate for the reduction of *S*-nitrosothiols was not applied to avoid the reduction of disulphides present not only in *S*-nitrosylated proteins but also in non-modified proteins. It has been taken into account that false positive signals might be obtained by endogenously *S*-nitrosylated and biotinylated proteins.

The molecular weight of aldehyde dehydrogenase ALDH3H1 protein is 56 kDa. Similarly, the molecular weight of the large subunit in ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the protein abundantly occurring in plants, is 55 kDa. Lindermayr *et al*. (2005) identified the large subunit of RuBisCO among the *S*-nitrosylated proteins from *A. thaliana* cell cultures and leaves, whereas Abat and Deswal (2009) identified the large subunit of RuBisCO among the *S*-nitrosylated proteins from GSNO-treated crude extracts of *Brassica juncea*. Although the content of RuBisCO in root culture is much lower than in leaves, we cannot exclude the possibility that the signal derived from *in vivo S*-nitrosylation of the aldehyde dehydrogenase ALDH3H1 enzyme in *A. thaliana* root culture might be enhanced due to the presence of the *S*-nitrosylated large subunit of RuBisCO. Further analyses are required to confirm that the aldehyde dehydrogenase ALDH3H1 is *S*-nitrosylated by GSNO under *in vivo* conditions.

It has been proposed to investigate the *S*-nitrosylation in *A. thaliana* plants over-expressing ALDH3H1 protein to increase the specificity of the ALDH3H1 *S*-nitrosylation. Additionally, it is suggested to remove the RuBisCO protein from *S*-nitrosylated root extracts before the application of biotin switch method to avoid false-positive signals.

## 4.4.3. Detection of the *S*-nitrosylated aldehyde dehydrogenase ALDH3I1 enzyme using the biotin switch technique (BST)

The data presented here demonstrated that the purified aldehyde dehydrogenase ALDH3I1 enzyme from *A. thaliana* was *S*-nitrosylated and the findings obtained in this work confirmed that the biotin switch technique was useful for the detection of *S*-nitrosylated ALDH3I1 after treatment with GSNO, however two modifications were introduced: firstly, the blocking reagent *S*-methyl methanoethiosulfonate (MMTS), used in the detection of the *S*-nitrosylated aldehyde dehydrogenase ALDH3H1 protein by the biotin switch method, was substituted with N-ethylmaleimide (NEM). The application of MMTS in the blocking step was not efficient for aldehyde dehydrogenase ALDH3I1 enzyme even with an increased incubation time (1.5 h) and caused the appearance of the false-positive signal in non-treated sample after labelling with biotin-HPDP. It has been supposed that an incomplete blocking of free thiols in treated (*S*-nitrosylated) samples might enhance the signal derived from *S*-nitrosothiols. It has been observed that MMTS possesses diverse efficiencies of blocking free thiols in the aldehyde dehydrogenase ALDH3H1 and ALDH3I1 enzymes from *A. thaliana*. This difference resulted from the distinct number of Cys residues present in ALDH3H1 and ALDH3I1 enzymes. Furthermore, it has been taken into consideration that not only the number of Cys residues, but also the localization of these amino acids and the three-dimensional (3D) protein structure of aldehyde dehydrogenase ALDH3I1 might influence the access of MMTS to buried cysteines. It has been demonstrated by Kohr *et al*. (2011) that NEM was especially useful for blocking the peptides containing more than one Cys residue. Additionally, the difference in the blocking of free thiols by MMTS and NEM might be due to the fact that MMTS is a reversible thiol blocking reagent, whereas NEM is an irreversible thiol blocking reagent (Huang and Chen 2010). MMTS forms a disulphide bond with protein sulphur residues and might be more susceptible to the reduction, whereas NEM forms a more stable thioether (Kettenhofen *et al.,* 2007). Thus the

application of MMTS in the blocking step might be inefficient due to the reversibility. Incomplete blocking of thiols will result in false-positive identification of protein *S*-nitrosothiols. The blocking step of free thiols is critical, because the efficiency of this step determines the specificity and sensitivity of the biotin switch method. The application of NEM caused the complete blocking of free thiols in non-treated sample, thus we did not observe the false-positive signals in non-treated sample (Section 3.5.1., Fig. 23).

Secondly, ethanol precipitation method, used in the detection of the recombinant aldehyde dehydrogenase ALDH3H1 by the biotin switch method, was substituted by trichloroacetic acid (TCA) precipitation method. In this case sharper protein bands were obtained in the separating gel after SDS-PAGE electrophoresis and immunodetection.

Taken all parts together, it was confirmed that the biotin switch method is a useful tool for detection of the *S*-nitrosylated aldehyde dehydrogenases, but the particular steps, previously optimized by Jaffrey *et al*. (2001), could not be used for both proteins. The data obtained in this work demonstrated that the detection of aldehyde dehydrogenase ALDH3H1 enzyme was successful, whereas in the case of aldehyde dehydrogenase ALDH3I1 enzyme the introduction of modifications was necessary.

## 4.4. Conclusions and future perspectives

The findings demonstrated in this study contribute to understanding the mechanism and regulation of plant aldehyde dehydrogenases non-enzymatic modification mediated by nitric oxide and identifying of cysteine residues involved in this process. It has been shown, similar to mammalian dehydrogenases, that the dehydrogenase activity of stress-responsive aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana* is affected by *S*-nitrosylation. It has been suggested that the data obtained in this work might provide the basis to investigate the role of ALDHs *S*-nitrosylation in plants under physiological and drought stress. The results presented here showed that the plant aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana* might be inactivated through *S*-nitrosylation. *S*-nitrosylation diminishes the dehydrogenase activity of the ALDH3H1 and ALDH3I1 enzymes *in vitro*. Additional study is required to optimize the conditions of the enzymatic assay for ALDH7B4 enzyme and investigate the effect of nitric oxide donors on the dehydrogenase activity of the ALDH7B4 enzyme. It is speculated whether the reduced activity of ALDH enzymes mediated by *S*-nitrosylation *in vivo* would be expected to lead to accumulation of aldehydes, thus would cause an increase of reactive oxygen species (ROS). However, it is taken into consideration whether the presence of free thiols, like reduced glutathione (GSH) and cysteine, as well as the enzymes mediating Cys denitrosylation, including thioredoxin and glutathione reductase might regenerate *in vivo* the inactivated dehydrogenase activity of ALDH3H1 and ALDH3I1 from *A. thaliana.* It has been proposed the further studies using the tandem affinity purification (TAP) might be a useful tool for protein-protein interactions analysis. Application of this technique might provide the protein partners which interact with aldehyde dehydrogenases and modulate its enzymatic activity through denitrosylation. The identification of protein partners might provide the basis for the investigation of a possible protective mechanism against *S*-nitrosothiol formation and subsequent activity loss. Since it has been shown that glutathione reductase is critical for maintaining nitric oxide homeostasis, it would be useful to further investigate the effect of *S*-nitrosoglutathione on aldehyde dehydrogenases using the mutants of *S*-nitrosoglutathione reductase from *A. thaliana*.

The data obtained from *A. thaliana* root cultures showed that *S*-nitrosoglutathione as a main physiologically active nitric oxide donor has the ability to modify aldehyde

dehydrogenase under *in vivo* conditions. To fully confirm this finding, further studies in *A. thaliana* plants over-expressing aldehyde dehydrogenases ALDH3H1 and ALDH3I1 enzymes are required.

It has been shown that the biotin switch method enabled the detection of *S*-nitrosylated aldehyde dehydrogenases *in vitro* as well as *in vivo*. Taking into consideration that the results obtained in this work provides the methods for an over-expression and purification of recombinant ALDH7B4 enzyme, it has been suggested that biotin switch method might be also useful for detection of *S*-nitrosylated ALDH7B4, as was shown in case of ALDH3H1 and ALDH3I1 enzymes. However, the further studies using ALDH7B4 enzyme are required to prove this hypothesis, since the results obtained in this work did not provide the evidence for *S*-nitrosylation of ALDH7B4 due to difficulties concerning optimizing enzymatic assay.

Owing to the fact that aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana* have the second enzymatic activity which is affected by *S*-nitrosylation and oxidation and the lack of the evidence which could explain the function of esterase activity in *A. thaliana* plants, further research is required to investigate the role of dual function of aldehyde dehydrogenases under *in vivo* conditions. Considering the presence of two separate distinct active sites in aldehyde dehydrogenases, the promiscuous function of esterase activity would be expected.

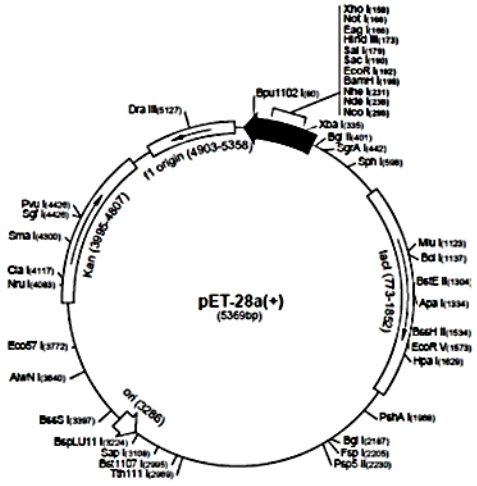
The identification of the target cysteine residues involved in *S*-nitrosylation of aldehyde dehydrogenases provide additional information about the putative function of the *S*-nitrosylation and together with the results derived from the identification of cysteine residues involved in oxidation and dimer formation provide the basis for the regulation of enzyme activity under nitrosative and oxidative conditions generated by environmental stress such as drought.

# 5. Summary

*S*-nitrosylation is a posttranslational modification that is known to affect many biochemical processes in plants. In the current study the ability of NO donors to *S*-nitrosylate aldehyde dehydrogenases ALDH3H1 and ALDH3I1 from *A. thaliana* was investigated. It was shown that ALDH3H1 is strongly nitrosylated by GSNO than ALDH3I1 showing the differences in nitrosylating capacity between the two enzymes. The experiments proved that oxidative posttranslational modification modulates the dehydrogenase activity in both enzymes in differential ways. It has been demonstrated that the effect of NO donors is related to the physicochemical properties of *S*-nitrosoglutathione and sodium nitroprusside. The following conclusions were made from this study. (i) Prolonged incubation time and high dose of NO donors (GSNO and SNP) regulate the inactivation and the rate of dehydrogenase activity. (ii) The decrease of dehydrogenase activity during *S*-nitrosylation of cysteine residues is related to the loss of sulfhydryl groups.

It is also clearly shown that the reducing reagents DTT and GSH have the differential abilities to restore the GSNO and SNP-mediated inhibition of the dehydrogenase activity in both the enzymes. The effect of the reducing agents on dehydrogenase activity was monitored and it was observed that DTT possessed stronger reducing properties than GSH. The concentration of reducing reagents (DTT and GST) regulated the ability to restore the inactivation of the dehydrogenase activity. Additionally, the dual enzyme activity (dehydrogenase and esterase activities) of aldehyde dehydrogenases ALDH3H1 and ALDH3I1 was effectively demonstrated. Further, the effect of GSNO and $CuCl_2$ on the esterase activity was investigated and the results indicated that the reactions of dehydrogenation and hydrolysis are probably catalysed at two separate active sites of the protein. Using the non-direct method for detection of *S*-nitrosylated aldehyde dehydrogenases it is proved that this application can be successfully used in the case of recombinant, purified enzymes ALDH3H1 and ALDH3I1 as well as for ALDH3H1enzyme *S*-nitrosylated under *in vivo* conditions. Altogether it can be concluded that ALDH enzymes used in the current study can undergo nitrosylation and apart from having dehydrogenase activity they also possess esterase activity.

# 6. Appendix




| pET-28a(+) sequence landmarks | |
|---|---|
| T7 promoter | 370-386 |
| T7 transcription start | 369 |
| His•Tag coding sequence | 270-287 |
| T7•Tag coding sequence | 207-239 |
| Multiple cloning sites | |
| (*BamH* I - *Xho* I) | 158-203 |
| His•Tag coding sequence | 140-157 |
| T7 terminator | 26-72 |
| *lacI* coding sequence | 773-1852 |
| pBR322 origin | 3286 |
| Kan coding sequence | 3995-4807 |
| f1 origin | 4903-5358 |

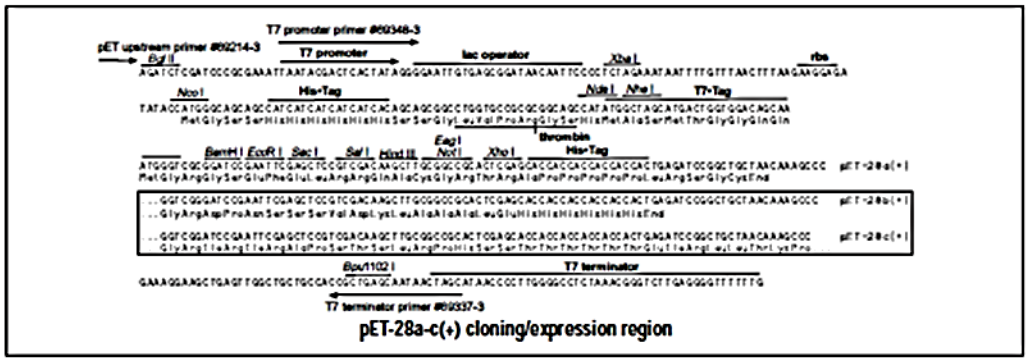



**Figure 30: The map and the sequence of the pET-28a vector**

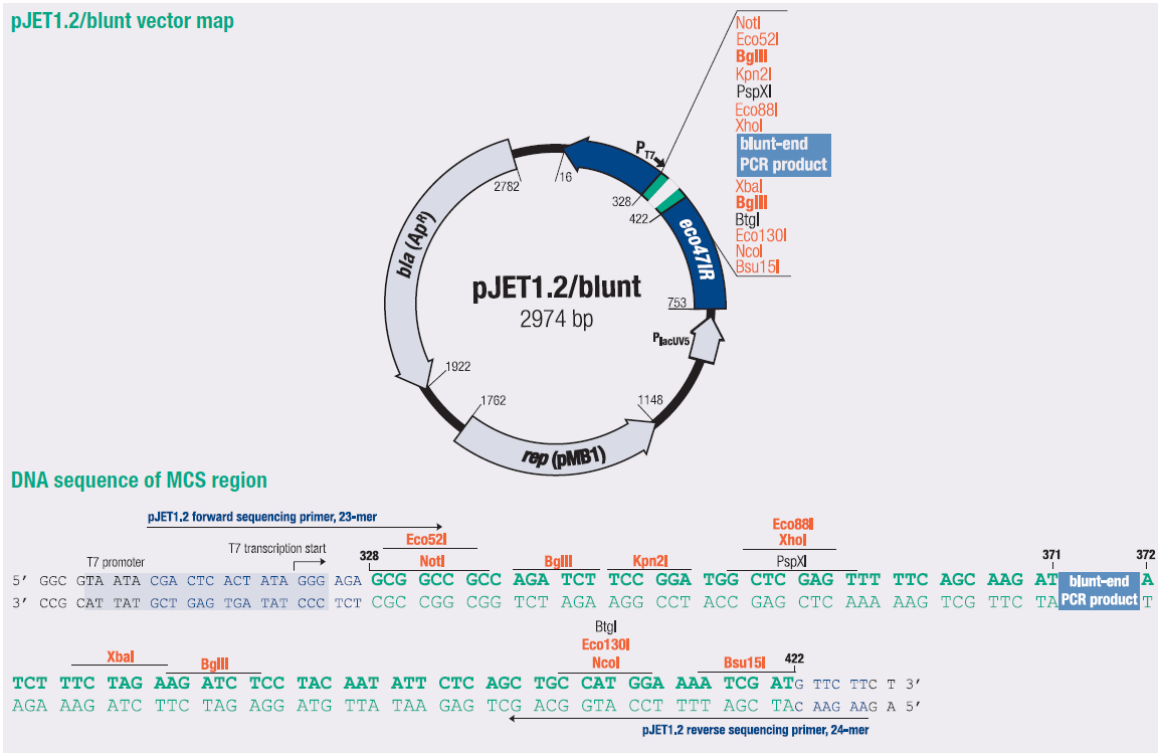



**Figure 31: The map and the sequence of the pJET1.2/blunt vector**

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# 8. Acknowledgements

First and foremost, I would like to sincerely thank my supervisor Professor Dr. Dorothea Bartels for giving me the great opportunity to work on this project and carry out my Ph.D. thesis in her laboratory. I would like to express my appreciation for your patience, guidance, support and valuable comments and advices during my research work.

My very special thanks go out to Dr. Naïm Stiti for support and guidance throughout my work. I deeply appreciate your continued encouragement. Without giving me constant motivation, the fulfillment of this project would not have been possible.

I would like thank Dr. Dinakar Challabathula for answering my countless questions as well as for your daily and spontaneous help in many occasions and creative discussions.

I would also like to thank the current and former members of Prof. D. Bartels's lab for an excellent working environment and technical support.

Chciałabym serdecznie podziękować moim wspaniałym rodzicom za życie, którym mnie obdarowali, za ich miłość i troskę, trud jaki włożyli w moje wychowanie, a także za wsparcie zarówno duchowe jak i finansowe. Dziekuje im, że zawsze we mnie wierzyli, nawet w natrudniejszych chwilach.