Alternative respiratory pathways in plants:

Established and proposed roles of xanthine oxidoreductase in oxidative and reductive pathways in plants

Estibaliz Urarte[1], Raquel Esteban[1], José Fernando Morán[1] Florian Bittner[2*]

[1]Institute of Agrobiotechnology, IdAB-CSIC-UPNA-Government of Navarre, E-31192 Pamplona, Spain

[2]Department of Plant Biology, Braunschweig University of Technology, 38106 Braunschweig, Germany

*Corresponding author: f.bittner@tu-braunschweig.de

Running title: ***Xanthine oxidoreductase in plants***

Abbreviations

ABA, abscisic acid

AO, aldehyde oxidase

GOGAT cycle, Glutamine oxoglutarate aminotransferase cycle

H_2O_2, hydrogen peroxide

NO, nitric oxide

NR, nitrate reductase

$O_2^{\cdot -}$, superoxide radical

$ONOO^-$, peroxynitrite

SOD, superoxide dismutase

RNS, reactive nitrogen species

ROS, reactive oxygen species

SHAM, salicylhydroxamic acid

XDH, xanthine dehydrogenase

XO, xanthine oxidase

XOR, xanthine oxidoreductase

Index

Abstract

Xanthine oxidoreductase (XOR) is among the most-intensively studied enzymes known to participate in the consumption of oxygen in cells. However, it attracted the attention of researchers due its participation in free radical production *in vivo*, mainly through the production of superoxide radicals. In plants, XOR is a key enzyme in purine degradation where it catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. Both reactions are accompanied by electron transfer to either NAD^+ with simultaneous formation of NADH or to molecular oxygen, which results in formation of superoxides. Characterization of plant XOR mutants and isolated XOR proteins from various plant species provided evidence that the enzyme plays significant roles in plant growth, leaf senescence, fruit size, synthesis of nitrogen storage compounds, and plant-pathogen interactions. Moreover, the ability of XOR to carry out redox reactions as NADH oxidase and to produce reactive oxygen species and nitric oxide, together with a possible complementary role in abscisic acid synthesis have raised further attention on the importance of this enzyme. Based on these established and proposed functions, XOR is discussed as regulator of different processes of interest in plant biology and agriculture.

I. Introduction

Despite the current description of more than 50 molybdoenzymes in living organisms (Mo-enzymes; Bittner and Mendel, 2010), only five of them are found in plants: nitrate reductase (NR), aldehyde oxidase (AO), sulfite oxidase (SO), mitochondrial amidoxime reducing component (mARC) and xanthine oxidoreductase (XOR). This review will focus on the latter, a FAD-, molybdenum-, iron- and sulphur-containing hydroxylase fundamental in nucleic acid metabolism.

Xanthine oxidoreductase (XOR, EC 1.17.1.4., formerly EC 1.1.1.204) is an oxygen consuming enzyme which catalyzes the conversion of the purines hypoxanthine and xanthine into uric acid, with the concomitant formation of either NADH or superoxide radical (Bittner and Mendel, 2010). The monomer can be subdivided into three distinct domains: a N-terminal domain binding two iron-sulfur clusters of the [2Fe-2S]-type, a domain harboring a FAD-binding site, and a C-terminal domain required for Moco-binding and dimerization. Currently, the crystal structure of plant XOR is not available. However, due to its similarity with bovine, human and rat XOR, the structure of plant XOR may be assumed based on these models. XOR is a ubiquitous enzyme among the kingdom *Plantae* and for the last three decades it has been deeply studied by different approaches. At early stages, studies with inhibitors like allopurinol provided a useful tool, followed in the last years by *Arabidopsis* knockout plants (Watanabe et al., 2010).

The study on XOR gave origin to the idea of the free radical chemistry in living organisms, mostly based on work by Fridovich and Handler on O_2 consumption by XOR (1958). 10 years later McCord and Fridovich (1968) envisaged that a free radical product derived from oxygen may be produced in biological systems during the reaction of XOR with O_2. The importance of this enzyme lies thus on the fact that it participates in the regulation of reactive oxygen species (ROS) production (Montalbini, 1992a). This ROS production in plants by XOR might have outstanding physiological consequences in plant signalling, in the processes of host-pathogen relationships (Moltalbini, 1992a,b), during abiotic stresses (Zdunek-Zastocka and Lips, 2003), or for natural plant senescence (Hesberg, et al. 2004). By a common regulatory sulfuration step, the ratio of inactive and active XOR and AO enzymes can be changed rapidly in order to increase the amount of certain important plants hormones such as abscisic acid (ABA) as was shown in drought and salt stressed *Arabidopsis thaliana* (Xiong et al. 2001). Under physiological aspects, this sulfuration step provides an efficient way of regulating the amount of active XOR and AO within the cell and thus, to adopt it to the physiological demands of the plant (Mendel, 2011).

This chapter reviews recent insights into the study of the enzyme XOR in plants, with a specific focus on the established and possible physiological substrates, inhibitors and feasible function in plants.

II. History and evolution of XOR

Due to its high abundance in milk and the simplicity of purification procedures mammalian XOR is among the most-intensively studied enzymes and was originally referred to as the "Schardinger enzyme" that catalyzes the reduction of methylene blue when formaldehyde served as substrate (Schardinger, 1902). Nearly two decades later, extracts of yeast and animal tissues were demonstrated to harbor certain substances that are likewise involved in the reduction of methylene blue in milk (Hopkins, 1921), and only one year later these substances have been identified as hypoxanthine and xanthine, which lead to the renaming of the enyzme into xanthine oxidase (XO) (Morgan et al., 1922; Booth, 1935 and references therein). Later, the term XO became representative for a specific condition of the enzyme (along with xanthine dehydrogenase, XDH), and the term xanthine oxidoreductase (XOR) was introduced as a more general and ubiquitous name for this enzyme that is found in all kingdoms of life. In particular mammalian XOR attracted, and still attracts, many researchers due to its implications in ischemia/reperfusion injury and reactive oxygen species (ROS) generation, but also because of its possible involvement in nitric oxide (NO) and peroxynitrite formation, and because of XOR-related diseases such as xanthinuria and hyperuricemia/gout (reviewed by Harrison, 2002; Agarwal et al., 2011). Moreover, mammalian XOR was proven to have another distinct function that is associated with the formation of milk-fat droplets but totally unrelated to enzymatic activity of XOR (Vorbach et al., 2002). However, XOR is generally recognized as a key enzyme in purine degradation where it catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. In primates and many other animals, the latter reaction represents the last step in this pathway, thus demanding excretion of poorly soluble uric acid with the urine to prevent severe consequences due to crystal formation in blood, serum, tissues and muscles. In plants however, uric acid is likewise the product of XOR catalysis but far away from being the final product of purine degradation (Fig. 1).

Typesetter for Figure 1

Instead, uric acid is further decomposed to allantoin and allantoate, two important nitrogen storage and transport compounds at least in tropical legumes (Smiths and Atkins, 2002). Subsequently, allantoate is converted to glyoxylate via three enzymatic steps, which include the formation of ureidoglycine and ureidoglycolate and the release of carbon dioxide and ammonia (Werner and Witte, 2011; Werner et al., 2013, and references therein). It is assumed that the latter compounds have the potential of being reused in photosynthesis or reassimilated by the photorespiratory glycolate and GOGAT cycles. Remarkably, the XOR substrate xanthine is the first common intermediate in the degradation of all purine nucleotides, thus representing an important bottleneck in this pathway and making XOR a key enzyme in purine catabolism.

In prokaryotes, XOR has been found exclusively in the XDH form and enables bacteria such as *Comamonas acidovorans* to grow on minimal medium supplemented with hypoxanthine or xanthine as the sole source of carbon and nitrogen (Xiang and Edmonson., 1996; Ivanov et al., 2004). In contrast, other bacteria like *Escherichia coli* turned out to be unable to live on purines as sole carbon or nitrogen source (Xi et al., 2000), indicating that XOR in these bacteria is involved in purine salvage rather than being part of a complete purine catabolic pathway. The *C. acidovorans* enzyme consists of two 58 kDa α subunits, which each bind two non-identical [2Fe-2S] iron-sulfur clusters, and two 87 kDa β subunits each binding one flavin adenine dinucleotide (FAD) and one molybdenum cofactor (Moco) in its dinucleotide form. The fully constituted and active enzyme thus is an $(\alpha\beta)_2$ heterodimer with a molecular mass of about 290 kDa indistinguishable from the mass of the eukaryotic XOR enzymes. While several other bacteria such as *Rhodobacter capsulatus* (Leimkühler et al., 1998) basically harbor the same type of XOR with identical subunit composition, XOR enzymes from other prokaryotic species such as *Pseudomonas putida*, *Veillonella atypica* and *Eubacterium barkeri* have been demonstrated to consist of only one or even three subunits, which associate to functionally active enzymes with molecular masses of up to 550 kDa (Woolfolk, 1985; Hettrich and Lingens, 1991; Gremer et al., 1996; Schräder et al., 1999). Differences between these enzymes do also concern variations of the bound redox groups, the composition of redox groups and their oligomerization states, but also the ligands bound to the molybdenum centres as well as the preferred co-substrate, which in some cases is NAD^+ and in others is $NADP^+$. Interestingly, in some bacteria the oxidation of hypoxanthine to xanthine and of xanthine to uric acid involves two enzymes, with purine hydroxylase catalyzing the first reaction and XOR catalyzing the latter (Self, 2002). Yet, among the diverse bacterial XOR forms, the one found in *R. capsulatus* appeared to be more similar to

its eukaryotic counterparts than to other prokaryotic XOR enzymes with respect to molecular mass, domain composition, bound redox groups, catalytical properties and 3D structure (Leimkühler et al., 1998; Truglio et al., 2002), suggesting that eukaryotic XOR has evolved from an ancient bacterial XOR form as found in *R. capsulatus*.

Besides prokaryotes and eukaryotes, XOR homologues can be found also in archaea, with their physiological roles being largely unraveled in most cases. It is noteworthy that XOR is not present in all species but absent in those, which are specialized in a way that the function of XOR is not required (some bacteria and most archaea) or which have adopted to parasitic living styles that either enable the respective organism to acquire all necessary purine intermediates from their host or that have established alternative pathways. In eukaryotes, the loss of XOR genes seems to be accompanied always by the complete loss of molybdenum metabolism as is found in some yeasts like *Saccharomyces cerevisiae* and *Schizosaccharomyces pomb*e but not in other yeasts such as *Pichia* (*Komagataella*) *pastoris*. Remarkably, the loss of XOR and molybdenum metabolism in eukaryotes appears to be limited to unicellar organisms, whereas multicellular eukaryotes generally appear to have an essential demand for molybdenum metabolism including XOR, which might be ascribed to the specialization of cells and tissues and the need for well-orchestrated pathways.

III. Distribution of XOR among the kingdom *Plantae* and subcellular localization

XOR occurs ubiquitously within the kingdom *Plantae* and it has been isolated from all organs of plants: root nodules (Tripplett et al., 1982; Boland et al., 1983; Nguyen et al., 1986), roots (Barabás et al., 2000), leaves (Nguyen and Feierabend, 1978; Montalbini, 2000; Vitória and Mazzafera, 1999), seedlings and whole plants (Suzuki and Takahachi 1975; Tajima and Yamamoto, 1975; Kumar and Taneja, 1977; Sauer et al., 2002), and fruits (Vitória and Mazzafera, 1999; Taylor and Cowan, 2001). Indeed, XOR activity has been reported in a great variety of plant species, including soybean, pea, wheat, maize, tea, lentil, tomato, coffee, alfalfa, avocado, tobacco and *Arabidopsis* (i.e. Moltalbini, 1998; Sauer et al., 2002; Nakagawa et al., 2007; Corpas et al., 2009; Shakirov et al., 2012). Despite this widespread distribution and the reasonable number of studies of XOR in plants, the exact subcellular localization has been discussed rather controversially. Initially, XOR was found associated to microbodies (Nguyen, 1986), glyoxysomes and peroxisomes (Sandalio et al., 1988; Del Río et al., 1989), but later immunolabeling techniques revealed that XOR was present in the cytosol of infected and uninfected cells of legume root nodules, and that this feature was related to the process of ureide biosynthesis (Datta et al., 1991). Parallel and later studies supported the

hypothesis of XOR being involved in stress response, and other locations within the plant cell were explored. In this respect, XOR was once more localized in peroxisomes, which are organelles with a potent oxidative metabolism that are additionally well known as ROS and reactive nitrogen species (RNS) generators (Corpas et al., 2008 and 2009). In pea leaf peroxisomes, XOR was found to act with both oxidizing substrates, NAD^+ and O_2, where the activity with the latter was surprisingly two-fold more pronounced, as indicated by enzymatic assays (Corpas et al., 2008). However, this recent article localizes XOR also in the cytosol, suggesting that XOR has several localizations within the plant cell.

IV. Structure of XOR and its redox centres

Unfortunately, no crystal structure of a plant XOR is available until now and a detailed description with regard to structure-function relationships has thus been precluded. Nevertheless, based on the high sequence similarity, the identical domain composition, and nearly identical substrate preferences, the well known structures of bovine (Enroth et al., 2000), human (Yamaguchi et al., 2007) and rat XOR (Nishino et al., 2005) might well serve as model structures for plant and other eukaryotic XOR enzymes.

All eukaryotic XOR enzymes are homodimeric proteins of 290-300 kDa with each monomer of approximately 145 kDa being constituted of three distinct domains (Fig. 2A). The N-terminal domain of 20 kDa is responsible for binding of two non-identical iron-sulfur clusters of the [2Fe-2S] type and is followed by a larger middle domain of 40 kDa that binds FAD. The C-terminal domain of 85 kDa exhibits two functions, one of which is the binding of one Moco while the other is to mediate the dimerization of two identical, monomeric XOR polypeptides (Fig. 2B). Iron-sulfur clusters, FAD and Moco are all prosthetic groups that are permanently bound by the enzyme and participate in catalysis and electron transfer. As is known for other molybdenum-dependent enzymes, Moco has a very particular role in forming an essential part of the active site of XOR where substrates are bound and converted and where the respective product is released. Notably, in the crystal structures of mammalian XOR proteins both molybdenum centres are about 50 Å apart, indicating that the two XOR monomers act independently from each other.

Typesetter for Figure 2

Once a reducing substrate such as xanthine or hypoxanthine is bound in the active site of XOR, the oxidative hydroxylation of these substrates takes place at the molybdenum centre, whereby the molybdenum becomes protonated and reduced from MoVI to MoIV in the reductive half-reaction of the catalytic sequence (Fig. 3). Electrons thus liberated from the

substrate are passed on via the two iron-sulfur clusters to the FAD site, where they are finally removed either by NAD^+ or O_2 in the oxidative half-reaction of the catalytic sequence. In this reaction, a particular glutamate residue strictly conserved among all XOR and AO proteins (e.g. Glu1261 in bovine XOR, Glu1297 in *Arabidopsis* AtXDH1) is assumed to function as an active site base required for the activation of substrates (Hille et al., 2011). While the ultimate source of the oxygen atom incorporated by XOR into the hydroxyl group of the respective product has been identified as water (Murray et al., 1966), it has been demonstrated that a hydroxyl ligand of the molybdenum centre (Mo-OH) represents the catalytically labile oxygen within the protein (Hille and Sprecher, 1987), which is supported by the crystal structure of bovine XOR in which the Mo-OH group points toward the substrate binding site (Enroth et al., 2000). Accordingly, substrates are coordinated to the catalytically introduced hydroxyl group of the molybdenum atom in a Mo-O-substrate fashion, thereby converting the respective substrate into hydroxylated product (e.g. hypoxanthine into xanthine or xanthine into uric acid, respectively). The product is finally released from the enzyme upon displacement by solvent-derived hydroxide, with electron transfer from the molybdenum centre to the iron-sulfur clusters and FAD.

Typesetter for Figure 3

V. Established and possible physiological substrates of plant XOR

For all XOR proteins, irrespective of their origin, hypoxanthine and xanthine are the preferred substrates (Fig. 4A), which points out the involvement of XOR enzymes in purine catabolism (see above). Besides these purine substrates however, XOR enzymes catalyze the conversion of many other substrates, with some of them having medicinal or pharmaceutical relevance, some others being discussed to be of physiological importance, and those, whose importance for the respective organism is questionable. In case of plant XOR, one class of alternative substrates is represented by aldehydes, whose conversion is likely to be facilitated in a fashion basically identical to the oxidation of hypoxanthine and xanthine, respectively (Coelho et al., 2012). It is thus not surprising that aldehyde oxidase (AO) proteins, which share a high degree of sequence similarity with XOR and which present identical domain structures and prosthetic group composition, have been identified to derive from XOR by gene duplication and subsequent neo-functionalization before the origin of multicellularity (Rodríguez-Trelles et al., 2003). In this process, the substrate-binding site of AO has been modified in a way that the ability to bind purine substrates got lost while the preference for aldehyde substrates was significantly enhanced. In addition, the FAD-binding site underwent certain modifications in

which NAD^+ was precluded and O_2 was favoured as final electron acceptor. Even though AO is now specialized for oxidation of aldehydes, it appears reasonable to assume that XOR contributes to a certain extent to the oxidation of certain aldehydes *in vivo*. In fact, among the various aldehyde substrates tested for plant XOR the ABA precursor abscisic aldehyde was shown to be oxidized with greatest efficiency of about 30% relative to the native substrates xanthine/hypoxanthine (Hesberg et al., 2004), thus allowing to speculate that XOR has a minor function in ABA biosynthesis *in vivo*, e.g. under conditions of decreased AO activity. Albeit with lower efficiency, other compunds such as indole-3-carbaldehyde, 1-naphthaldehyde, heptaldehyde, acetaldehyde, benzaldehyde, hydroxylamine and salicylhydroxamic acid (SHAM) were likewise oxidized by plant XOR proteins to the respective carboxylic acids (Montalbini, 1998; Montalbini, 2000; Sauer et al., 2002; Hesberg et al., 2004; Rümer et al., 2009), thereby proving the broad substrate specificity of plant XOR for cyclic, *N*-heterocyclic and non-cyclic aldehydes (Fig. 5).

Typesetter for Figure 4

A typical feature of molybdenum-containing enzymes seems to be the capacity to catalyze the one-electron reduction of nitrite to nitric oxide (NO) under low-oxygen conditions as has been shown for plant nitrate reductase (summarized by Meyer et al., 2005; Gupta et al., 2011a), the human mitochondrial reducing components (Sparacino-Watkins et al., 2014), rat AO (Li et al., 2008 and 2009), and also mammalian XOR (summarized by Cantu-Medellin and Kelley, 2013). Importantly, in contrast to the oxidation of xanthine/hypoxanthine and aldehydes electrons are consumed rather than produced upon nitrite reduction, meaning that the molybdenum centre of the enzyme needs to be reduced before nitrite can be converted into NO (Fig. 4C+D). For rat XOR, an interesting observation concerns the capability of the enzyme to reduce nitrite to NO under both anaerobic and aerobic conditions, with the NO formation rate under aerobic conditions and with NADH as reducing substrate reaching about 70% of the anaerobic rate (Li et al., 2008). Though not in the context of NO production, also plant XOR has been demonstrated to react with NADH (Yesbergenova et al., 2005; Zarepour et al., 2010) and it can thus be speculated that similar to mammalian XOR, plant XOR is able to catalyze the NADH-dependent reduction of nitrite in the presence of oxygen (Fig. 4D). Moreover, as NO is involved in the regulation of many important processes in plants including seed germination, root growth, respiration, stomatal closure, as well as in a variety of response reactions to abiotic and biotic stresses (Gupta et al., 2011a and references therein), XOR-derived NO might well be of physiological relevance, in particular due to the abundance of XOR in nearly all plant tissues and at all developmental stages.

Typesetter for Figure 5

Another possible physiological substrate of XOR is represented by NADH, which has been demonstrated to be oxidized not only by mammalian (Harrison, 2002; Maia et al., 2007) but also by *Arabidopsis* and tomato XOR (Yesbergenova et al., 2005; Zarepour et al., 2010). The reaction takes place exclusively at the FAD domain of the enzyme and is believed not to involve the other redox centres Moco and iron-sulfur clusters (Fig. 4B). Once NADH is oxidized and its electrons are transferred to FAD, the reduced flavin tends to transfer its electrons to O_2 with concomitant formation of superoxide anions ($O_2^{\cdot -}$). Interestingly, the rate of NADH oxidation by the XOR enzyme AtXDH1 from *Arabidopsis* is extremely high as compared to the NADH oxidation rates of mammalian XOR enzymes, suggesting that XOR-dependent NADH oxidation might be an important source of $O_2^{\cdot -}$ in plant cells (Zarepour et al., 2010). In combination with the use of NAD^+ as preferred electron acceptor, the intrinsic NADH oxidase activity of plant XOR might also be involved in the regulation of the cellular NAD^+/NADH ratio. In this respect, it was demonstrated that NAD^+ and NADH indeed compete for the same binding site at the FAD domain of plant XOR, with NAD^+ being prefered over NADH when simultaneously offered to AtXDH1. This indicates that, as long as the respective components are available, the physiological activity with xanthine/hypoxanthine as substrates and NAD^+ as electron acceptor dominates over the alternative NADH oxidase activity of XOR.

VI. Inhibitors of XOR

Within the last years research on the possible physiological roles of XOR in plants has concentrated on the analysis of T-DNA insertion lines and RNAi knockdown plants from *Arabidopsis* (Nakagawa et al., 2007; Brychkova et al., 2008; Watanabe et al., 2010). However, before such mutants became available other approaches aimed to modulate XOR activity by use of appropriate and more or less specific inhibitors mostly in other plant species. As such inhibitors provide powerful tools, in particular when lacking a XOR mutant in a non-model species, some of these inhibitors are briefly presented and discussed in the following section with respect to their suitability:

The presently best established and most-specific inhibitor of XOR enzymes from all organisms including plants is allopurinol. This inhibitor has been established in the therapy of patients suffering from hyperuricemia/gout, which is characterized by abnormally high levels of uric acid in the blood, serum, muscles and urine, often leading to urate crystals in these

tissues. In those cases, where hyperuricemia and the elevation of uric acid levels is due to an extremely high XOR activity allopurinol is used as a substrate analogue that competes with hypoxanthine/xanthine for the substrate-binding site in XOR. Once allopurinol is bound in the active site it is oxidized to oxypurinol (also referred to as alloxanthine), which remains covalently bound to the reduced molybdenum atom in the active site of XOR (Okamoto and Nishino, 2008). In this way, oxypurinol serves as a suicide inhibitor of XOR, acting in a mechanism-based fashion. Treatment of plants, plant extracts and purified plant XOR enzymes with allopurinol has been proven to be very efficient and has thus been reported frequently and for diverse plant species including *Arabidopsis* (Hesberg et al., 2004, Yesbergenova et al., 2005; Nakagawa et al., 2007; Brychkova et al., 2008; Zarepour et al., 2010), avocado (Taylor and Cowan, 2004), cowpea (Woo et al., 1980; Triplett, 1986; Atkins et al., 1988), fava bean (Montalbini, 2000), french bean (Quiles et al., 2009), lima bean (Triplett, 1986), soybean (Fujihara and Yamaguchi, 1978; Triplett et al., 1980; Boland and Schubert, 1982), lentil (Montalbini, 2000), pea (Sauer et al., 2002), and wheat (Montalbini, 1998). Since during allopurinol treatment side effects have never been reported, allopurinol can indeed be considered the most-suitable XOR inhibitor in plants. The relatively new inhibitors Febuxostat and Y-700 likewise act near to the active site of XOR, but in contrast to allopurinol/oxypurinol these compounds serve as structure-based inhibitors by interacting with several amino acids in the active site tunnel (Okamoto and Nishino, 2008), thereby precluding hypoxanthine and xanthine from the substrate-binding site. Although the use of these inhibitors in plants has not yet been reported, Febuxostat and Y-700 are likely to act as efficient and specific in inhibition of plant XOR as allopurinol.

Unlike allopurinol, cyanide treatment of XOR in plant extracts is much less specific, even though XOR activity is very efficiently blocked. The reason for this is the interaction of cyanide with a variety of micro- and macromolecules, including proteins. However, purified XOR (and also XOR in plant extracts) can be very effectively and reversibly inactivated by cyanide, which under oxidative conditions removes the terminal, or so-called "cyanolyzable" sulfur ligand of the molybdenum atom in the active site of XOR (Wahl and Rajagopalan, 1982; Schwartz et al., 1997; Wollers et al., 2008). By anaerobic treatment with sulfide and reducing agents such as dithionite the sulfur can be re-introduced, whereby the enzyme's activity is largely restored. *In vivo*, the absence of the terminal/cyanolyzable sulfur in XOR is caused by a deficiency in the Moco sulfurase gene (*ABA3* in *Arabidopsis*, *Flacca* in tomato, *ABA1* in tobacco), which has been demonstrated to be essentially required for generating and providing the sulfur ligand of the Moco in both XOR and AO (Schwartz et al., 1997; Bittner

et al., 2001; Sagi et al., 2002; Wollers et al., 2008). Like XOR, AO therefore is likewise susceptible to cyanide treatment and subsequent re-sulfuration.

Another inhibitor that affects the activity of XOR is the molybdate (MoO_4^{2-}) analogue tungstate (WO_4^{2-}) (Bentley et al., 1981; Arst et al., 1982; Kawada et al., 1982; Schieber and Edmondson, 1993; Nielsen et al., 1996). While molybdate is the natural form of molybdenum required for synthesis of Moco in eukaryotic and most prokaryotic molybdenum-dependent enzymes, tungstate is used instead of molybdate by a number of enzymes in archaea and certain bacteria (Grimaldi et al., 2013). Due to their similar size, charge and physicochemical properties, tungstate can substitute molybdate, which usually results in inactivation of the respective Mo-enzyme, with the strength of inactivation depending on various parameters such as concentration, affected enzyme, and plant species. As tungstate affects not only XOR but all molybdenum-dependent enzymes and also many physiological processes such as seedling growth, biomass, cell cycle, components of the cytoskeleton, and gene regulation (Adamakis et al., 2012), tungstate generally appears to be a relative inappropriate inhibitor for studying XOR-related functions in plants and its use in plants is thus documented by only very few reports.

In contrast to all the afore-mentioned inhibitors, diphenyleneiodonium (DPI) does not attack the active site or the molybdenum centre but rather the flavin moiety in the FAD domain, where it forms a covalent complex with the FAD itself (O'Donnell et al., 1994). However, DPI is far from being a specific inhibitor of XOR enzymes but rather interacts with basically any type of enzyme that has a reduced FAD or FMN molecule bound. DPI is thus a very potent inhibitor of cellular $O_2^{\cdot -}$ generating systems, which often carry flavins as redox groups and catalyze one-electron transfer reactions, as is the case for several enzymes of the respiratory chain and the photosystems, NAD(P)H oxidases and XOR. Nevertheless, DPI is a very powerful tool to study the contribution of the flavin cofactor to a specific sub-activity of a flavin-dependent enzyme such as XOR (Yesbergenova et al., 2005; Zarepour et al., 2010), but its use is largely limited to purified enzymes.

VII. Interconversion of XOR into xanthine dehydrogenase and xanthine oxidase forms

An often-discussed issue concerns the discrimination of eukaryotic XOR enzymes into xanthine dehydrogenase (XDH) forms and xanthine oxidase (XO) forms. Basically, all XOR enzymes are initially produced in the XDH form and the term XDH is thus likewise adequate for XOR enzymes. Yet, mammalian XOR can be converted from its initial XDH form into the

XO form either reversibly by disulfide bond formation between the sulfhydryl groups of two conserved cysteine residues (Cys535 and Cys992 in rat XOR; Nishino and Nishino, 1997; Nishino et al., 2005) or irreversibly by limited proteolysis, the latter resulting in cleavage between the three functional domains of XOR with maintenance of catalytic activity (Amaya et al., 1990) (Fig. 6). Under physiological conditions, the XDH form appears to dominate with 80% over the XO form with 20% in tissues (Della Corte et al., 1969; Stirpe et al., 2002), while the XO form clearly predominates in milk, most likely due to a specific function of XO in the formation of milk-fat droplets (Jeong et al., 2013). Both, reversible and irreversible interconverion result in conformational changes near to the NAD^+ binding site at the FAD domain, thereby preventing access of NAD^+ to the FAD cofactor (Nishino et al., 2005). The interconversion between XDH and XO therefore affects the preference for the oxidizing substrates NAD^+ and O_2: whereas XDH possesses high reactivity toward NAD^+ and low reactivity toward O_2 as electron acceptor, XO uses O_2 very efficiently and has negligible reactivity toward NAD^+ (Hille and Nishino, 1995). In contrast to mammalian XOR proteins, other animal XOR enzymes such as those from chicken and insects do not possess the conserved cysteine couple required for reversible conversion into XO and are exclusively found in the XDH form (Nishino and Nishino, 1989; Komoto et al., 1999). It should thus be considered that interconversion between XDH and XO might be a regulatory element in mammals required to separate and control the specific functions of XOR in tissues and milk. In fact, also all plant XOR proteins analyzed so far from nodules of bean (Boland, 1981), pea seedlings (Sauer et al., 2002), wheat leaves (Montalbini, 1998), *Arabidopsis* (Hesberg et al., 2004), and leaves of various legumes (Montalbini, 2000) gave no indications for a possible interconversion into XO and it therefore appears likely that plant XOR enzymes are present exclusively in the XDH form. This is supported by the fact that the two conserved cysteine residues required for reversible conversion of XDH into XO by disulfide bond formation are absent in all accessible sequences of plant XOR proteins. Furthermore, irreversible proteolytic cleavage of plant XOR into several distinct domains that maintain catalytic activity as known from the mammalian enzymes has not been reported until now. Although not absolutely conclusive, these observations indicate that plant XOR proteins exist exclusively in the XDH form and are not, neither reversibly nor irreversibly, converted into XO. It should be noted in this respect that some research aimed to discriminate between XDH and XO forms in plant cell extracts. In all cases, this discrimination was based on the misleading assumption that XDH exclusively transfers its electrons to NAD^+ whereas XO exclusively transfers its electrons to O_2. However, due to the fact that XDH is capable of transferring its

hypoxanthine/xanthine-derived electrons alternatively also to O_2 such discrimination between XDH and XO forms is presently impossible.

Typesetter for Figure 6

VIII. Plant XOR: an important source of ROS and NO in plants

In 1968 McCord and Fridovich turned evident that a basal production of free radicals like $O_2^{\cdot-}$ may take place in biological systems ~~and~~ originated from XOR catalysis (McCord and Fridovich, 1969; Fridovich, 1998). Since then, ROS and RNS became acknowledged as a group of highly reactive molecules playing key roles in cellular signalling. In the past decades, their involvement in numerous plant physiological processes has been ascertained. For instance, $O_2^{\cdot-}$ appears to be principally involved in programmed cell death (PCD) associated to hypersensitive response (HR) (Doke and Ohashi, 1988; Jabs et al., 1996). Hydrogen peroxide (H_2O_2) is also an active participant of the HR (Desikan et al., 1998; Yoda et al., 2003), further mediating stomatal closure (Pei et al., 2000), mechanical wounding response (Orozco-Cárdenas et al., 2001) and plant-pathogen interaction (Mittler et al., 1999). In the last 20 years NO has come to light as an essential second messenger in a wide range of phenomena such as seed germination, root growth, leaf expansion, senescence and stress response (Delledonne et al., 2001; Besson-Bard et al., 2008; Wang et al., 2010; Gupta et al., 2011a,b). Other nitrogen oxidized species, represented by nitrite (NO_2^-), nitrate (NO_3^-) or peroxynitrite ($ONOO^-$), have also been described to act as signals in various responses within plant cells but further research is required in order to clarify these issues (Scheible et al., 1997; Zhang and Forde, 2000; Serrano et al., 2012; Konishi and Yanagisawa, 2013). It has been ascertained that the production of these molecules may be derived from NO via different reactions, so it is considered that their participation in diverse plant signalling pathways is fundamental (Dordas et al., 2003; Begara-Morales et al., 2013).

In mammals, XOR is able to reduce O_2 with the subsequent generation of $O_2^{\cdot-}$, and it may also reduce NO_2^- to NO. In principal, both species can rapidly react, which results in $ONOO^-$ formation (Harrison, 2002). In plants, in addition to purine catabolism, XOR is often related to plant-pathogen interaction phenomena, PCD and natural senescence (Bittner and Mendel, 2010), with these reactions representing a spotlight of $O_2^{\cdot-}$ production within the cells. There are early descriptions of $O_2^{\cdot-}$ production by XOR in peroxisomes of watermelon and pea (Sandalio et al., 1988; Del Río et al., 1989). Later on, *Arabidopsis* recombinant AtXDH1 was described as a $O_2^{\cdot-}$ producer *in vitro*, transferring around 20% of the electrons from xanthine to O_2 (Hesberg et al., 2004). Enzymatic AtXDH1 activity rises at senescence *in planta* giving

one more hint of $O_2^{\cdot-}$ involvement in this physiological process. Tomato XOR was also found to produce $O_2^{\cdot-}$ and it was observed that both tomato and *Arabidopsis* XOR from leaves were capable of producing $O_2^{\cdot-}$ with either xanthine/hypoxanthine or NADH as reducing substrate (Yesvergenova et al., 2005). Subsequently, $O_2^{\cdot-}$ production by AtXDH1 was characterized and it was established that NADH-dependent $O_2^{\cdot-}$ generation was pH-dependent, suggesting a certain regulation of the process (Zarepour et al., 2010). AtXDH1 exhibited the highest rates of NADH oxidase activity among all enzymes studied so far and, besides, this reaction was not dependent of the sulfuration state of the Moco centre, which enables the plant to control $O_2^{\cdot-}$ contents and maintain a constitutive cellular pool of this molecule (Zarepour et al., 2010).

Superoxide dismutases (SODs) are powerful antioxidant metalloenzymes whose primary activity is the dismutation of $O_2^{\cdot-}$ resulting in H_2O_2 and molecular oxygen (Fridovich, 1995). As it was mentioned before, an excess of H_2O_2 within the cell is often related with HR and PCD, as well as with nodule formation and nodule/leaf senescence (Levine et al., 1994; Escuredo et al., 1996; Evans et al., 1999; Grant and Loake, 2000; Santos et al., 2001; Alesandrini et al., 2003). In this context, and taking into account that plant XOR is involved in many of these processes, its implication in H_2O_2 production could be explained, although none of the plant XOR enzymes analysed to date ~~where~~ shown to directly produce this ROS (Fig. 7). Rubio et al. (2004) proved that some of the H_2O_2 in legume root nodules is produced through two consecutive reactions involving an NAD(P)H oxidase-like $O_2^{\cdot-}$-generating enzyme and a CuZnSOD, and complementary scientific data gave light to the possibility that XOR may also be involved in cytosolic H_2O_2 generation (Datta et al., 1991; Zarepour et al., 2010).

In the late 80s NO was revealed as the endothelium-derived relaxing factor (EDRF), responsible for the relaxation of the smooth muscle (Ignarro et al., 1987). In consequence, there was a clear paradigm shift regarding this molecule, drastically changing the vision of NO by the scientific community. The most important NO source in metazoans is the enzyme nitric oxide synthase (NOS), a hemeprotein that transfers 5 electrons and oxidizes L-arginine to NO and citrulline, using NADPH and O_2 as co-substrates (Halliwell and Gutteridge, 2007). A decade ago, XOR was also discovered as a NO-generating enzyme in animal systems, especially under anaerobiosis (i.e. ischemia) based on its capacity to reduce NO_2^- in the presence of either xanthine or NADH (Godber et al., 2000; Harrison, 2002). Concerning oxidative pathways of NO production in plants, the enzymatic reaction analogous to those in animal cells involving NOS has been greatly discussed, including the retraction of several papers which had previously affirmed the existence of a mammalian-like NOS (Travis, 2004;

Crawford et al., 2006; Zemojtel et al., 2006). Reductive NO formation from NO_2^- has long been studied in plant systems and cytosolic nitrate reductase (cNR) is currently considered the major source for NO in plants (Planchet and Kaiser, 2006). Knowing that plants are able to grow and complete their life cycle using ammonium as the sole nitrogen source (Ariz et al., 2011), the existence of oxidative reactions for NO production in plants is essential. XOR was initially proposed as a reductive NO source, being the catalyst of NO_2^- reduction to NO in analogy to animals, but definitive evidence is still lacking (Del Río et al., 2004). Concerning this issue, there is some evidence of XOR involvement in oxidative NO production from hydroxylamines in plant cells (Rümer et al., 2009) but there is little knowledge about this issue.

IX. Implication of plant XOR in abiotic stress and plant-microbe interactions

i) Abiotic stresses and senescence

Plant growth is ultimately modulated by the environment with the drawback of suffering stress and affection of the metabolic functions under extreme conditions, such as extreme temperatures, drought or salinity. Under these circumstances, XOR together with other molybdo-enzymes (AO, NR) may constitute one of the earliest metabolic adjustments towards abiotic stress in plants. However, the involvement of XOR in the response mechanisms of plants to stress and plant-microbe signalling remains unknown; hence, these are reviewed and discussed herein (Fig. 7).

The involvement of XOR in salt tolerance is controversial. Shakirov et al. (2012) described that the different activities of XOR isoforms increased with salinity. XOR activity also increased in leaves of wheat genotypes with differing salt tolerance. However, root XOR activity decreased with increasing concentrations of NaCl in the salt tolerant genotype and was enhanced in the salt-sensitive genotype (Xu et al., 2012). On the other hand, the expression of the XOR gene *AtXDH1* in *Arabidopsis thaliana* was induced by salt stress; however, XOR activity was not affected (Hesberg et al. 2004). The different implications of XOR activity in different species and different organs under salinity stress suggest that XOR is subject of various regulatory mechanisms (Xu et al., 2012). When salinity is combined with ammonium (NH_4^+) nutrition, XOR activity was enhanced in maize nodal roots (Barabás et al., 2000) and ryegrass, where it correlated with a higher content of ureides in plant tissues (Sagi et al., 1998). In pea roots however, salinity did not significantly affect XOR activity, and there was even a sharp depression of XOR activity in leaves (Zdunek-Zastocka and Lips, 2003).

The changes of root and leaf XOR activity in pea plants seem to correlate with ureide biosynthesis that is induced by NH_4^+, the product of N fixation, and rather not by salinity.

Under drought stress, Yesbergenova et al. (2005) demonstrated that XOR was induced by water deprivation but also by the main hormone that regulates the response to drought, ABA. It is suggested that XOR, besides the main producer AO, takes part in the synthesis of this phytohormone, as it was demonstrated by Sagi et al. (1999) in Moco-deficient tomato mutants lacking AO and XOR activities, and hence exhibiting a reduced capacity to produce ABA. Besides, it has been reported that XOR responds to drought with transcriptional up-regulation and with a concomitant increase of the enzymatic activity (Hesberg et al., 2004). This is in agreement with Watanabe et al. (2010), who demonstrated that XOR-suppressed lines of *Arabidopsis* having a deprived purine metabolism were more susceptible to drought-induced oxidative damage. These results indicate that XOR has critical roles in maintaining plant growth and development under both normal and stressful growth conditions.

In leaf peroxisomes cadmium treatment reduced XOR activity (Romero-Puertas et al., 1999; Corpas et al., 2008), whereas cadmium stress enhanced XOR activities in roots of *Phragmites australis* (Jiang and Wang, 2007). These results suggest the possible implication of XOR in plant response mechanisms to abiotic stress by heavy metals, and it is discussed that in peroxisomes the ratio of NAD^+ and O_2 as oxidizing substrates for XOR is regulated in a way which is dependent on the physiological stage and stress conditions (Corpas et al., 2008).

Another example for the possible implication of XOR in the response to abiotic stresses is the enhanced chlorophyll degradation in leaves in the absence of XOR, which might result from ROS production during dark stress (Guo and Crawford, 2005; Brychkova et al., 2008). Apart from environmental stresses, it has also been proposed that XOR contributes to plant hormone homeostasis and to the control of fruit size in avocado (Cowan et al., 2001).

Senescence is a natural phenomenon occurring in plant tissues, which is known to induce oxidative processes, including lipid peroxidation and protein carbonylation among others. Although the precise role of XOR in senescence remains unclear, the fact that this enzyme is a natural producer of $O_2^{\cdot-}$ (Hesberg et al., 2004; Yesvergenova et al., 2005) gives an idea on the kind of involvement XOR may have in cell ageing phenomena. Furthermore, there is evidence of an augmentation of enzymatic levels both in mammalian heart (Willems et al., 2003) and plant XOR in ageing and senescence procedures. In pea leaves, natural senescence showed an increase in peroxisomal XOR activity, along with the enzymatic activities of uricase (UO) and superoxide dismutase (SOD) (Pastori and Del Río, 1997). These facts indicate that purine catabolism may be taking place in this organelle during leaf senescence.

In *Arabidopsis*, mRNA levels of *AXDH1* notably increased in aging (6-week-old) and senescent (8-week-old) leaves, but enzymatic activity only rose significantly in senescent tissues (Hesberg et al., 2004). Further studies revealed that *Arabidopsis* XOR gene silencing lines showed an accelerated senescence phenotype caused by a decrease of XOR levels, while wild-type plants presented an age-dependent increase of XOR levels (Nakagawa et al., 2007). Additionally, research with *AtXDH1* mutants confirmed that XOR regulates ureide levels during nutrient mobilization, the latter being a process highly associated with senescence (Brychkova et al., 2008). Finally, senescence of *Phalaenopsis* flower was likewise correlated with an increase of XOR activity (Tewari et al., 2009). In view of the controversial results, Zarepour et al., (2010) proposed that in *Arabidopsis thaliana* XOR is involved in a general and constitutive function during purine degradation but probably not at the level of stress adaptation.

ii) XOR and plant-microbe interactions

Plants identify and defend against many invading pathogens by inducing the HR, which leads to localized PCD at the site of infection, confining the disease and preventing it from spreading through the whole plant. The HR is characterized by triggering oxidative burst including production of ROS like $O_2^{\cdot -}$ and H_2O_2. As mentioned previously, plant XOR is often related to pathogenic defence and HR, as these processes require ROS formation to be triggered (Montalbini, 1995; Montalbini and Della Torre, 1996). In this respect, it was proven that allopurinol treatment decreased both ureide levels and rust infection in wheat leaves (Montalbini, 1992a), the HR induced by the incompatible interaction between bean and *Uromyces phaseoli* (Montalbini, 1992b), and additionally the HR triggered by tobacco necrosis virus in tobacco leaves (Montalbini and Della Torre, 1996). Moreover, it was observed that XOR activity increased with the infection of wheat leaves by *Puccinia recondita* (Montalbini, 1995). A recent work pointed out that XOR activity and H_2O_2 contents rapidly increased in leaves of a rust-sensitive wheat, whereas in the resistant variety such changes were not observable (Sarsenbaev et al., 2013). All these facts suggest that XOR is a key enzyme in the plant-pathogen interaction process.

It is well known that XOR is one of the key enzymes during purine metabolism in the nitrogen-fixing nodules of tropical legumes, where it has a key role in ureide biosynthesis and major nitrogen storage and transport (Smith and Atkins, 2002). In fact, inhibition of nodule functioning has been observed when plants were supplied with allopurinol (Atkins et al., 1988). As mentioned earlier, allopurinol is a competitive inhibitor of XOR which impairs

hypoxanthine/xanthine oxidation to uric acid, thus preventing ureide synthesis in the nodule and resulting in nitrogen starvation, chlorosis and reduced growth (Atkins et al., 1988). No connection between XOR and root nodulation has been established so far, although it is widely known that in legumes ROS levels increase during the rhizobial infection (Santos et al., 2001; Rubio et al., 2004). In this regard, the elimination of $O_2^{\cdot -}$ by SOD during the legume-Rhizobium symbiosis is known to be essential for the establishment of symbiosis and nodule development (Santos et al., 2000). SOD from either plant legume or bacterials cells localized in the nucleus of the plant nodule cells evidenced that the elimination of $O_2^{\cdot -}$ exerted a positive signaling during the symbiosis initial stages (Rubio et al., 2009; Asensio et al., 2011). All these facts suggest that XOR isoforms may have a role in this process.

iii) Does XOR have a relevant function in plant stress and plant-microbe interactions?

Although it has been broadly suggested that XOR may have a function in plant stress, there is still no overwhelming evidence to assure that this enzyme is responsible for the resistance to environmental stresses. It is proposed that the role of XOR during stress might be related to the need for a more efficient use of available carbon skeletons, which are required to synthesize organic nitrogen compounds with a low C/N ratio for their transport through the xylem to the shoot (Sagi et al., 1998). However, as described in this review, the implication of XOR under stress conditions seems to be quite complex. In all these stresses, it remains unclear whether the enzymatic activity is physiologically relevant for plant protection from stressful conditions. The question whether XOR is involved in plant injury (due to ROS production and consequent damage of plant tissues) or whether it is protective (due to the production of ureides to alleviate ROS) is still not clarified. Further studies are needed in order to fill the knowledge gap in the involvement of XOR in plant adaptation to environmental stresses.

Typesetter for Figure 7

Concluding remarks

In the last 50 years, the study of XOR has led to a detailed view of this enzyme and to a better understanding of its biochemical features and physiological significance in plant metabolism. Important aspects have been elucidated or are in the process, such as its key role in ureide biosynthesis, its subcellular localization and its capacity of ROS production under certain circumstances. However, questions dealing with the possible involvement of XOR in the response to stress conditions and with XOR as a tangible NO generator within plant cells remain unclear. The advanced study of these reactions appears fundamental and future research should focus on the exact reactions that ascribe XOR a function in stress response and production of oxidized nitrogen species, including NO, under NH_4^+ nutrition.

Acknowledgements and funding

The authors acknowledge the support of the research grants AGL2010-16167 to J.F.M. from the Spanish Ministry of Science and Innovation and Bi 1075/5-1 to F.B. by the Deutsche Forschungsgemeinschaft. R.E. received a JAE-Doctor grant from the Spanish Research Council (CSIC). We are greatly thankful to all the researchers who worked and have been involved in the XOR enzyme study, for advancing knowledge in this field.

References

Adamakis, I.D.S., Panteris, E., Eleftheriou, E.P. (2012) Tungsten toxicity in plants. *Plants*, **1**, 82–99.

Agarwal, A., Banerjee, A., Banerjee, U.C. (2011) Xanthine oxidoreductase: a journey from purine metabolism to cardiovascular excitation-contraction coupling. *Critical Reviews on Biotechnology*, **31**, 264–280.

Alesandrini, F., Mathis, R., Van de Sype, G. (2003) Possible roles for a cysteine protease and hydrogen peroxide in soybean nodule development and senescence. *New Phytologist*. **158**, 131–138.

Amaya, Y., Yamazaki, K., Sato, M. *et al*. (1990) Proteolytic conversion of xanthine dehydrogenase from the NAD-dependent type to the O_2-dependent type. Amino acid sequence of rat liver xanthine dehydrogenase and identification of the cleavage sites of the enzyme protein during irreversible conversion by trypsin. *Journal of Biological Chem*istry, **265**, 14170–14175.

Ariz, I., Artola, E., Cabrera Asensio, A. *et al*. (2011) High irradiance increases NH_4^+ tolerance in *Pisum sativum*: Higher carbon and energy availability improve ion balance but not N assimilation. *Journal of Plant Physiology*, **168**, 1009–1015.

Arst, H.N. Jr., Tollervey, D.W., Sealy-Lewis, H.M. (1982) A possible regulatory gene for the molybdenum-containing cofactor in *Aspergillus nidulans*. *Journal of General Microbiology*, **128**, 1083–1093.

Asensio, A.C., Marino, D., James, E.K., Ariz, I., Arrese-Igor, C., Aparicio-Tejo, P.M., Arredondo-Peter, R., Moran, J.F. (2011) Expression and localization of a Rhizobium-derived cambialistic superoxide dismutase in pea (Pisum sativum) nodules subjected to oxidative stress. *Molecular Plant Microbe Interaction* **24**, 1247-1257.

Atkins, C.A., Sanford, P.J., Storer P.J., Pate, J.S. (1988) Inhibition of nodule functioning in cowpea by a xanthine oxidoreductase inhibitor, allopurinol. *Plant Physiol*ogy, **88**, 1229–1234.

Barabás, N.K., Omarov, R.T., Erdei, L., Lips, S.H. (2000) Distribution of the Mo-enzymes aldehyde oxidase, xanthine dehydrogenase and nitrate reductase in maize (*Zea mays* L.) nodal roots as affected by nitrogen and salinity. *Plant Science*, **15**, 49–58.

Begara-Morales, J.C., Chaki, M., Sánchez-Calvo, B. *et al.* (2013) Protein tyrosine nitration in pea roots during development and senescence. *Journal of Experimental Botany*, **64**, 1121–1134.

Bentley M.M., Williamson, J.H., Oliver, M.J. (1981) The effects of molybdate, tungstate and lxd on aldehyde oxidase and xanthine dehydrogenase in *Drosophila melanogaster*. *Canadian Journal of Genetics and Cytol*ogy, **23**, 597–609.

Besson-Bard, A., Pugin, A., Wendehenne, D. (2008) New insights into nitric oxide signalling in plants. *Annual Review of Plant Biology*, **59**, 21–39.

Bittner, F., and Mendel, R.R. (2010) Cell biology of molybdenum. *Plant Cell Monographs*, **17**, 119–143.

Bittner, F., Oreb, M., Mendel, R.R. (2001) ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in *Arabidopsis thaliana*. *Journal of Biological Chemistry*, **276**, 40381–40384.

Boland, M.J. (1981) NAD^+: xanthine dehydrogenase from nodules of navy beans: partial purification and properties. *Biochemistry International*, **2**, 567-574.

Boland, M.J., Blevins, D.G., Randall, D.D. (1983) Soybean nodule xanthine dehydrogenase: a kinetic study. *Archives of Biochemistry and Biophysics*, **222**, 435–441.

Boland, M.J., and Schubert, K.R. (1982) Purine biosynthesis and catabolism in soybean root

nodules: incorporation of 14C from 14CO2 into xanthine. *Archives of Biochemistry and Biophysics*, **213**, 486–491.

Booth, V.H. (1935) The identity of xanthine oxidase and the Schardinger enzyme. *The Biochemical Journal*, **29**, 1732–1748.

Brychkova, G., Alikulov, Z., Fluhr, R., Sagi, M. (2008) A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the AtXDH1 *Arabidopsis* mutant. *The Plant Journal*, **54**, 496–509.

Cantu-Medellin, N., and Kelley, E.E. (2013) Xanthine oxidoreductase-catalyzed reduction of nitrite to nitric oxide: insights regarding where, when and how. *Nitric Oxide*, **34**, 19–26.

Coelho, C, Mahro, M., Trincão, J. *et al*. (2012) The first mammalian aldehyde oxidase crystal structure: insights into substrate specificity. *Journal of Biological Chemistry*, **287**, 40690–40702.

Corpas, F.J., Hayashi, M., Mano, S. et al. (2009) Peroxisomes are required for in vivo nitric oxide accumulation in the cytosol following salinity stress of arabidopsis plants. *Plant Physiology*, **151**, 2083–2094.

Corpas, F.J., Palma, J.M., Sandalio, L.M. *et al.* (2008) Peroxisomal xanthine oxidoreductase: characterization of the enzyme from pea (*Pisum sativum* L.) leaves. *Journal of Plant Physiology*, **165**, 1319–1330.

Cowan, A.K., Cripps, R.F., Richings, E.W., Taylor, N.J. (2001) Fruit size: towards an understanding of the metabolic control of fruit growth using avocado as a model system. *Physiologia Plantarum*, **111**, 127–136.

Crawford, N.M., Galli, M., Tischner, R. *et al*. (2006) Response to Zemojtel *et al*: Plant nitric oxide synthase: back to square one. *Trends in Plant Science*, **11**, 526.

Datta, D.B., Triplett, E.W., Newcomb, E.H. (1991) Localization of xanthine dehydrogenase in cowpea root nodules: implications for the interaction between cellular compartments during ureide biogenesis. *Proceedings of the National Academy of Science USA*, **88**, 4700–4702.

Della Corte, E., Gozzetti, G., Novello, F., Stirpe, F. (1969) Properties of the xanthine oxidase from human liver. *Biochimica et Biophysica Acta*, **191**, 164–166.

Delledonne, M., Zeier, J., Marocco, A., Lamb, C. (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceedings of the National Academy of Science USA*, **98**, 13454–13459.

Del Río, L.A., Fernández, V.M., Rupérez, F.L. *et al*. (1989). NADH induces the generation of superoxide radicals in leaf peroxisomes. *Plant Physiology*, **89**, 728–731.

Del Río, L.A., Corpas, F.J., Barroso, J.B. (2004) Nitric oxide and nitric oxide synthase activity in plants. *Phytochemystry*, **65**, 783–792.

Desikan, R, Burnett, E.C., Hancock, J.T. *et al*., (1998) Harpin and hydrogen peroxide induce the expression of a homologue of gp91-phox in *Arabidopsis thaliana* suspension cultures. *Journal of Experimental Botany*, **49**, 1767–1771.

Doke, N., and Ohashi, Y. (1988) Involvement of an O_2^--generating system in the induction of necrotic lesions on tobacco leaves infected with tobacco mosaic virus. *Physiological and Molecular Plant Pathology*, **32**, 163–175.

Dordas, C., Rivoal, J., Hill, R.D. (2003) Plant haemoglobins, nitric oxide and hypoxic stress. *Annals of Botany,* **91**,173–178.

Enroth, C., Eger, B.T., Okamoto, K. *et al*. (2000) Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: structure-based mechanism of conversion. *Proceedings of the National Academy of Science USA*, **97**, 10723–10728.

Escuredo, P.R., Frank, R. Minchin F.R. *et al*. (1996) lnvolvement of activated oxygen in nitrate-induced senescence of pea root nodules. *Plant Physiology*, **11**, 1187–1195.

Evans, P.J., Gallesi, D., Mathieu, C. *et al*. (1999) Oxidative stress occurs during soybean nodule senescence. *Planta*, **208**, 73–79.

Fridovich, I. (1995) Superoxide radical and superoxide dismutases. *Annual Review of Biochemistry*, **64**, 97–112.

Fridovich, I. (1998) The trail to superoxide dismutase. *Protein Science*, **7**, 2688–2690.

Fridovich I., and Handler, P. (1958) Xanthine oxidase. III. Sulfite oxidation as an ultra sensitive assay. *Journal of Biological Chemistry*, **233**, 1578–1580.

Fujihara, S., and Yamaguchi, M. (1978) Effects of allopurinol [4-hydroxypyrazolo(3,4-d)pyrimidine] on the metabolism of allantoin in soybean plants. *Plant Physiological*, **62**, 134–138.

Godber, B.L.J., Doel, J.J., Sapkota, G.P. *et al.* (2000) Reduction of nitrite to nitric oxide catalysed by xanthine oxidoreductase. *Journal of Biological Chemistry*, **275**, 7757–7763.

Grant, J.J., and Loake, G.J. (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiology*, **124**, 21–29.

Gremer, L., and Meyer, O. (1996) Characterization of xanthine dehydrogenase from the anaerobic bacterium *Veillonella atypica* and identification of a molybdopterin-cytosine-dinucleotidecontaining molybdenum cofactor. *European Journal of Biochemistry*, **238**, 862–866.

Grimaldi, S., Schoepp-Cothenet, B., Ceccaldi, P. *et al*. (2013) The prokaryotic Mo/W-bisPGD enzymes family: a catalytic workhorse in bioenergetic. *Biochimica et Biophysica Acta*, **1827**, 1048–1085.

Gupta, K.J., Alisdair, R.F., Kaiser, W.M., van Dongen, J. (2011a) On the origins of nitric oxide. *Trends in Plant Science*, **16**, 160–168.

Gupta, K.J., Hincha, D.K., Mur, L.A.J. (2011b) NO way to treat a cold. *New Phytologist*, **189**, 360–363.

Guo, F.Q., and Crawford, N.M. (2005) Arabidopsis nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. *The Plant Cell*, **17**, 3436–3450.

Halliwell, B., and Gutteridge, J.M.C. (eds) (2007). *Free radicals in biology and medicine*, Clarendon Press, Oxford.

Harrison, R. (2002) Structure and function of xanthine oxidoreductase: where are we now?. *Free Radical Biology and Medicine*, **33**, 774–797.

Hesberg, C., Hänsch, R., Mendel, R.R., Bittner, F. (2004) Tandem orientation of duplicated xanthine dehydrogenase genes from *Arabidopsis thaliana*. *Journal of Biological Chemistry*, **279**, 13547–13554.

Hettrich, D., and Lingens, F. (1991) Microbial metabolism of quinoline and related compounds. VIII. Xanthine dehydrogenase from a quinoline utilizing *Pseudomonas putida* strain. *Biological Chemistry Hoppe-Seyler*, **372**, 203–211.

Hille, R., and Nishino, T. (1995) Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase. *The FASEB Journal*, **9**, 995–1003.

Hille, R., Nishino, T., Bittner, F. (2011) Molybdenum enzymes in higher organisms. *Coordination Chemistry Reviews*, **255**, 1179–1205.

Hille, R., and Sprecher, H. (1987) On the mechanism of action of xanthine oxidase. Evidence in support of an oxo transfer mechanism in the molybdenum-containing hydroxylases. *Journal of Biological Chemistry*, **262**, 10914–10917.

Hopkins, F.G. (1921) On an autoxidisable constituent of the cell. *The Biochemical Journal*, **15**, 286–305.

Jeong, J., Lisinski, I., Kadegowda, A.K. *et al*. (2013) A test of current models for the mechanism of milk-lipid droplet secretion. *Traffic*, **14**, 974–986.

Jiang, X., and Wang, C. (2007) Cadmium distribution and its effects on molybdate-containing hydroxylases in *Phragmites australis*. *Aquatic Botany*, **86**, 353–360.

Ignarro, L.J., Buga, G.M., Wood, K.S. *et al*. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Science USA*, **84**, 9265–9269.

Ivanov, N.V., Trani, M., Edmondson, D.E. (2004) High-level expression and characterization of a highly functional *Comamonas acidovorans* xanthine dehydrogenase in *Pseudomonas aeruginosa*. *Protein Expression and Purification*, **37**, 72–82.

Jabs, T., Dietrich, R.A., Dangl, J.L. (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science*, **273**, 1853–1856.

Jeong, J., Lisinski, I., Kadegowda, A.K., *et al*. (2013) A test of current models for the mechanism of milk-lipid droplet secretion. *Traffic*, **14**, 974–986.

Kawada, J., Shirakawa, Y., Yoshimura, Y., Nishida, M. (1982) Thyroid xanthine oxidase and its role in thyroid iodine metabolism in the rat: difference between effects of allopurinol and tungstate. *Journal of Endocrinology*, **95**, 117–241.

Komoto, N., Yukuhiro, K., Tamura, T. (1999) Structure and expression of tandemly duplicated xanthine dehydrogenase genes of the silkworm (*Bombyx mori*). *Insect Molecular Biology*, **8**, 73–83.

Konishi, M., and Yanagisawa, S. (2013) Arabidopsis NIN-like transcription factors have a central role in nitrate signalling. *Nature Communications*, **4**, 1617.

Kumar, R., and Taneja, V.A. (1977) Xanthine oxidase in lentil (*Lens esculenta*) seedlings, *Biochimica et Biophysica Acta*, **485**, 489–491.

Leimkühler, S., Kern, M., Solomon, P.Z. *et al*. (1998) Xanthine dehydrogenase from the phototrophic purple bacterium *Rhodobacter capsulatus* is more similar to its eukaryotic counterparts than to prokaryotic molybdenum enzymes. *Molecular Microbiology*, **27**, 853–869.

Levine, A., Tenhaken, R., Dixon, R., Lamb, C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, **79**, 583–593.

Li, H., Cui, H., Kundu, T.K. *et al*. (2008) Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. *Journal of Biological Chemistry*, **283**, 17855–17863.

Li, H., Kundu, T.K., Zweier, J.L. (2009) Characterization of the magnitude and mechanism of aldehyde oxidase-mediated nitric oxide production from nitrite. *Journal of Biological Chemistry*, **284**, 33850–33858.

Maia, L., Duarte, R.O., Ponces-Freire, A. *et al*. (2007) NADH oxidase activity of rat and human liver xanthine oxidoreductase: potential role in superoxide production. *Journal of*

Biological Inorganic Chemistry, **12**, 777–787.

Mendel, R.R. (2011) Cell biology of molybdenum in plants. *Plant Cell Reports*, **30**, 1787–1797.

McCord, J.M., and Fridovich, I. (1968) The reduction of cytochrome *c* by milk xanthine oxidase. *Journal of Biological Chemistry*, **243**, 5753–5760.

McCord, J.M., and Fridovich, I. (1969) An enzymic function for erythrocuprein (Hemocuprein) *Journal of Biological Chemistry*, 244:6049-6055.

Meyer, C., Lea, U.S., Provan, F. *et al*. (2005) Is nitrate reductase a major player in the plant NO (nitric oxide) game? *Photosynthesis Research*, **83**, 181–189.

Mittler, R., Herr, E.H., Orvar, B.L. *et al.* (1999) Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyperresponsive to pathogen infection. *Proceedings of the National Academy of Science USA*. **96,** 14165–14170.

Montalbini, P. (1992a) Inhibition of hypersensitive response by allopurinol applied to the host in the incompatible relationship between *Phaseolus vulgaris* and *Uromyces phaseoli*. *Journal of Phytopathology*, **134**, 218–228.

Montalbini, P. (1992b) Ureides and enzymes of ureide synthesis in wheat seeds and leaves and effect of allopurinol on *Puccinia recondita* f. sp. *tritici* infection. *Plant Science*, **87**, 225–231.

Montalbini, P. (1995) Effect of rust infection on purine catabolism enzyme levels in wheat leaves. *Physiological and Molecular Plant Pathology*, **46**, 275–292.

Montalbini, P., and Della Torre, G. (1996) Evidence of a two-fold mechanism responsible for the inhibition by allopurinol of the hypersensitive response induced in tobacco by tobacco necrosis virus. *Physiological and Molecular Plant Pathology*, **48**, 273–287.

Montalbini, P. (1998) Purification and some properties of xanthine dehydrogenase from wheat leaves. *Plant Science*, **134**, 89–102.

Montalbini, P. (2000) Xanthine dehydrogenase from leaves of leguminous plants: purification, characterization and properties of the enzyme. *Journal of Plant Physiology*, **156**, 3–16.

Morgan, E.J., Stewart, C.P., Hopkins, F.G. (1922) On the anaerobic and aerobic oxidation of xanthine and hypoxanthine by tissues and by milk. *Proceedings of the Royal Society of London Series B*, **94**, 109–131.

Murray, K.N., Watson, J.G., Chaykin, S. (1966) Catalysis of the direct transfer of oxygen from nicotinamide N-oxide to xanthine by xanthine oxidase. *Journal of Biological Chemistry*, **241**, 4798–4801.

Nakagawa, A., Sakamoto, S., Takahashi, M. *et al*. (2007) The RNAi-mediated silencing of xanthine dehydrogenase impairs growth and fertility and accelerates leaf senescence in transgenic *Arabidopsis* plants. *Plant and Cell Physiology*, 48, 1484–1495.

Nguyen, J. (1986) Plant xanthine dehydrogenase: its distribution, properties and function. *Physiologie Végétale*, **24**, 263–281.

Nguyen, J., Machal, L., Vidal, J. *et al*. (1986) Immunochemical studies on xanthine dehydrogenase of soybean root nodules: ontogenic changes in the level of enzyme and immunocytochemical localization. *Planta*, 167, 190–195.

Nguyen, J., and Feierabend, J. (1978) Some properties and sub cellular localization of xanthine dehydrogenase in pea leaves. *Plant Science Letters*, **13**, 125–132.

Nielsen, V.G., Tan, S., Weinbroum, A. et al. (1996) Lung injury after hepatoenteric ischemia-reperfusion: role of xanthine oxidase. *American Journal of Respiratory and Critical Care Medicine*, 154, 1364–1369.

Nishino, T., and Nishino, T. (1989) The nicotinamide adenine dinucleotide-binding site of chicken liver xanthine dehydrogenase. Evidence for alteration of the redox potential of the flavin by NAD binding or modification of the NAD-binding site and isolation of a modified peptide. *Journal of Biological Chemistry*, **264**, 5468–5473.

Nishino, T., and Nishino, T. (1997) The conversion from the dehydrogenase type to the oxidase type of rat liver xanthine dehydrogenase by modification of cysteine residues with fluorodinitrobenzene. *Journal of Biological Chemistry*, **272**, 29859–29864.

Nishino T., Okamoto K., Kawaguchi Y. *et al*. (2005) Mechanism of the conversion of xanthine dehydrogenase to xanthine oxidase: identification of the two cysteine disulfide bonds and crystal structure of a non-convertible rat liver xanthine dehydrogenase mutant. *Journal of Biological Chemistry*, **280**, 24888–24894.

O'Donnell, V.B., Smith, G.C.M., Jones, O.T.G. (1994) Involvement of phenyl radicals in lodonium compound inhibition of flavoenzymes. *Molecular Pharmaceutics*, **46**, 778–785.

Okamoto, K., Nishino, T. (2008) Crystal structures of mammalian xanthine oxidoreductase bound with various inhibitors: allopurinol, febuxostat, and FYX-051. *Journal of Nippon Medical School*, **75**, 2–3.

Orozco-Cárdenas, M.L., Narváez-Vásquez, J., Ryan, C.A. (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *The Plant Cell,* **13**, 179–191.

Pastori, G.M., and Del Río, L.A. (1997) Natural senescence of pea leaves: an activated oxygen-mediated function of peroxisomes. *Plant Physiol*ogy, **113**, 411–418.

Planchet, E., and Kaiser, W.M. (2006) Nitric oxide production in plants: Facts and fictions. *Plant Signaling & Behaviour*, **1**, 46–51.

Pei, Z.M., Mutrata, Y., Benning, G. *et al.* (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cell. *Nature*, **406**, 731–734.

Quiles, F.A., Raso, M.J., Pineda, M., Piedras, P. (2009) Ureide metabolism during seedling development in French bean (*Phaseolus vulgaris*). *Physiologia Plantarum*, **135**, 19–28.

Rodríguez-Trelles, F, Tarrío, R, Ayala, FJ. (2003) Convergent neofunctionalization by positive Darwinian selection after ancient recurrent duplications of the xanthine dehydrogenase gene. *Proceedings of the National Academy of Science USA*. **100**, 13413–13417.

Romero-Puertas, M.C., McCarthy, I., Sandalio, L.M. *et al.* (1999) Cadmium toxicity and oxidative metabolism of pea leaf peroxisomes. *Free Radical Research*, **31**, S25–31.

Rubio, M.C., James, E.K., Clemente, M.R. *et al*. (2004) Localization of superoxide dismutases and hydrogen peroxide in legume root nodules. *Molecular Plant-Microbe Interactions*, **17**, 1294–1305.

Rubio, M. C., Becana, M., Kanematsu, S. *et al.* (2009) Immunolocalization of antioxidant enzymes in high-pressure frozen root and stem nodules of *Sesbania rostrata*. *New Phytologist* **183**, 395-407.

Rümer, S., Gupta, K.J., Kaiser, W.M. (2009) Plant cells oxidize hydroxylamines to NO. *Journal of Experimental Botany*, **60**, 2065–2072.

Sagi, M., Fluhr, R., Lips, S.H. (1999) Aldehyde oxidase and xanthine dehydrogenase in a *flacca* tomato mutant with deficient abscisic acid and wilty phenotype. *Plant Physiology*, **120**, 571–578.

Sagi, M., Omarov, R.T., Lips, S.H. (1998) The Mo-hydroxylases xanthine dehydrogenase and aldehyde oxidase in ryegrass as affected by nitrogen and salinity. *Plant Science,* **135**, 125–135.

Sagi, M., Scazzocchio, C., Fluhr, R. (2002) The absence of molybdenum cofactor sulfuration is the primary cause of the *flacca* phenotype in tomato plants. *Plant Journal*, **31**, 305–317.

Sandalio, L.M., Fernández, V.M., Rupérez, F.L., Del Río, L.A. (1988) Superoxide free radicals are produced in glyoxysomes. *Plant Physiology*, **87**, 1–4.

Santos, R., Herouart, D., Puppo, A., Touati, D. (2000) Critical protective role of bacterial superoxide dismutase in *Rhizobium*-legume symbiosis. *Molecular Microbiology* **38**, 750-759.

Santos, R., Hérouart, D., Sigaud, S. *et al*. (2001) Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. *Molecular Plant-Microbe Interactions*. **14**, 86–89.

Sarsenbaev, K.N., Abiyev, S.A., Kozhamzharova, L.S. *et al*. (2013) Effect of leaf rust on the molybdenum-containing enzymes activity in spring wheat varieties differing in resistance to infection. *World Applied Sciences Journal,* **25**, 1308–1313.

Sauer, P., Frébortová, J., Sebela, M. *et al*. (2002) Xanthine dehydrogenase of pea seedlings: a member of the plant molybdenum oxidoreductase family. *Plant Physiology and Biochemistry*, **40**, 393–400.

Schardinger, F. (1902) Über das Verhalten der Kuhmilch gegen Methylenblau und seine Verwendung zur Unterscheidung von ungekochter und gekochter Milch. *Zeitschrift für Untersuchung der Nahrungs- und Genussmittel* **5**, 1113–1121.

Scheible, W.-R., González-Fontes, A., Lauerer, M. *et al*. (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *The Plant Cell*, **9**, 783–798.

Schieber, A., and Edmondson, D.E. (1993) Studies on the induction and phosphorylation of xanthine dehydrogenase in cultured chick embryo hepatocytes. *European Journal of Biochemistry*, **215,** 307–314.

Schräder, T., Rienhöfer, A., Andreesen, J.R. (1999) Selenium-containing xanthine dehydrogenase from *Eubacterium barkeri*. *European Journal of Biochemistry*, **264**, 862–871.

Schwartz, S.H., Léon-Kloosterziel, K.M., Koornneef, M., Zeevaart, J.A. (1997) Biochemical characterization of the aba2 and aba3 mutants in *Arabidopsis thaliana*. *Plant Physiology*, **114**, 161–166.

Self, W.T. (2002) Regulation of purine hydroxylase and xanthine dehydrogenase from Clostridium purinolyticum in response to purines, selenium, and molybdenum. *Journal of Bacteriology*, **184**, 2039–2044.

Serrano, I., Romero-Puertas, M.C., Rodríguez Serrano, M. *et al*. (2012) Role of peroxynitrite in programmed cell death induced in self-incompatible pollen. *Plant Signaling & Behaviour*, **7**, 779–781.

Shakirov, Z.S., Khakimov, S.A., Shomurodov, K.F. (2012) Effect of salinity and drought on symbiotical and biochemical properties of *Onobrychis* and alfalfa. *Agricultural Sciences*, **3**, 444–454.

Smith, P.M.C., and Atkins C.A. (2002) Purine biosynthesis. Big in cell division, even bigger in nitrogen assimilation. *Plant Physiology*, **128**, 793–802.

Sparacino-Watkins, C.E., Tejero, J., Sun, B. *et al*. (2014) Nitrite reductase and NO synthase activity of the mitochondrial molybdopterin enzymes mARC1 and mARC2. *Journal of Biological Chemistry*, doi:10.1074/jbc.M114.555177.

Stirpe, F., Ravaioli, M., Battelli, M.G. *et al*. (2002) Xanthine oxidoreductase activity in human liver disease. *The American Journal of Gastroenterology*, **97**, 2079–2085.

The Biochemical Journal, **146**, 79–85.

Tajima, S., and Yamamoto, Y. (1975) Enzymes of purine catabolism in soybean plants. *Plant and Cell Physiology*, **16**, 271–282.

Taylor, N., and Cowan, K. (2001) Plant hormone homeostasis and the control of avocado fruit size. *Plant Growth Regulation*, **35**, 247–255.

Taylor, N.J., and Cowan, A.K. (2004) Xanthine dehydrogenase and aldehyde oxidase impact plant hormone homeostasis and affect fruit size in 'Hass' avocado. *Journal of Plant Research*, **117**, 121–130.

Tewari, R.K., Kumar, P., Kim, S. et al. (2009) Nitric oxide retards xanthine oxidase-mediated superoxide anion generation in *Phalaenopsis* flower: an implication of NO in the senescence and oxidative stress regulation. *Plant Cell Reports*, **28**, 267–279.

Travis, J. (2004) NO-making enzyme no more: Cell, PNAS papers retracted. *Science*, **306**, 960.

Triplett, E.W. (1986) Two indirect methods for detecting ureide synthesis by nodulated legumes. *Plant Physiology*, **81**, 566–571.

Triplett, E.W., Blevins, D.G., Randall, D.D. (1980) Allantoic acid synthesis in soybean root nodule cytosol via xanthine dehydrogenase. *Plant Physiology*, **65**, 1203–1206.

Triplett, E.W., Blevins, D.G., Randall, D.D. (1982) Purification and properties of soybean nodule xanthine dehydrogenase. *Archives of Biochemistry and Biophysics*, **219**, 39–46.

Truglio, J.J., Theis, K., Leimkühler, S. *et al*. (2002) Crystal structures of the active and alloxanthine-inhibited forms of xanthine dehydrogenase from *Rhodobacter capsulatus*. *Structure*, **10**, 115–125.

Vitória, A.P., and Mazzafera, P. (1999) Xanthine degradation and related enzyme activities in leaves and fruits of two coffea species differing in caffeine catabolism. *Journal of Agricultural and Food Chemistry*, **47**, 1851–1855.

Wang, B.L., Tang, X.Y., Cheng, L.Y. *et al*. (2010) Nitric oxide is involved in phosphorus deficiency-induced cluster-root development and citrate exudation in white lupin. *New Phytologist*, **187**, 1112–1123.

Vorbach, C., Scriven, A., Capecchi, M.R. (2002) The housekeeping gene xanthine oxidoreductase is necessary for milk fat droplet enveloping and secretion: gene sharing in the lactating mammary gland. *Genes & Development*, **16**, 3223–3235.

Wahl, R.C., and Rajagopalan, K.V. (1982) Evidence for the inorganic nature of the cyanolyzable sulfur of molybdenum hydroxylases. *Journal of Biological Chemistry*, **257**, 1354–1359.

Watanabe, S., Nakagawa, A., Izumi, S. *et al*. (2010) RNA interference-mediated suppression of xanthine dehydrogenase reveals the role of purine metabolism in drought tolerance in *Arabidopsis*. *FEBS Letters*, **584**, 1181–1186.

Werner, A.K., Medina-Escobar, N., Zulawski M. *et al*. (2013) The ureide-degrading reactions of purine ring catabolism employ three amidohydrolases and one aminohydrolase in *Arabidopsis*, soybean, and rice. *Plant Physiology*, **163**, 672–681.

Werner, A.K., and Witte, C.P. (2011) The biochemistry of nitrogen mobilization: purine ring catabolism. *Trends in Plant Science*, **16**, 381–387.

Willems, L., Garnham, B., Headrick, J.P. (2003) Aging-related changes in myocardial purine metabolism and ischemic tolerance. *Experimental Gerontology*, **38**, 1169–1177.

Wollers, S., Heidenreich, T., Zarepour, M. *et al*. (2008) Binding of sulfurated molybdenum cofactor to the C-terminal domain of ABA3 from *Arabidopsis thaliana* provides insight into the mechanism of molybdenum cofactor sulfuration. *Journal of Biological Chemistry*, **283**, 9642–9650.

Woo, K.C., Atkins, C.A., Pate, J.S. (1980) Biosynthesis of ureides from purines in a cell-free system from nodule extracts of cowpea [*Vigna unguiculata* (L) Walp.]. *Plant Physiology*, **66**, 735–739.

Woolfolk, CA. (1985). Purification and properties of a novel ferricyanide-linked xanthine dehydrogenase from *Pseudomonas putida* 40. *Journal of Bacteriology*, **163**, 600–609.

Xi, H., Schneider, B.L., Reitzer, L. (2000) Purine catabolism in *Escherichia coli* and function of xanthine dehydrogenase in purine salvage. *Journal of Bacteriology*. **182**, 5332–5341.

Xiang, Q, and Edmondson, D.E. (1996) Purification and characterization of a prokaryotic

xanthine dehydrogenase from *Comamonas acidovorans*. *Biochemistry*, **35**, 5441–5450.

Xiong, L., Gong, Z., Rock, C.D. *et al*. (2001) Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in *Arabidopsis*. *Developmental Cell*, **1**, 771–781.

Xu, H., Zhai, J., Liu, Y. *et al.* (2012) The response of Mo-hydroxylases and abscisic acid to salinity in wheat genotypes with differing salt tolerances. *Acta Physiologiae Plantarum*, **34**, 1767–1778.

Yamaguchi, Y., Matsumura, T., Ichida, K. *et al*. (2007) Human xanthine oxidase changes its substrate specificity to aldehyde oxidase type upon mutation of amino acid residues in the active site: roles of active site residues in binding and activation of purine substrate. *Journal of Biochemistry*, **141**, 513–524.

Yesbergenova, Z., Yang, G., Oron, E. *et al*. (2005) The plant Mo-hydroxylases aldehyde oxidase and xanthine dehydrogenase have distinct reactive oxygen species signatures and are induced by drought and abscisic acid. *The Plant Journal*, **42**, 862–876.

Yoda, H., Yamaguchi, Y., Sano, H. (2003) Induction of hypersensitive cell death by hydrogen peroxide produced through polyamine degradation in tobacco plants. *Plant Physiology*, **132**, 1973–1981.

Zarepour, M., Kaspari, K., Stagge, S. *et al*. (2010) Xanthine dehydrogenase AtXDH1 from *Arabidopsis thaliana* is a potent producer of superoxide anions via its NADH oxidase activity. *Plant Molecular Biology*, **72**, 301–310.

Zdunek-Zastocka, E., and Lips, H.S. (2003) Is xanthine dehydrogenase involved in response of pea plants (*Pisum sativum* L.) to salinity or ammonium treatment? *Acta Physiologiae Plantarum*, **25**, 395–401.

Zemojtel, T., Fröhlich, A., Palmieri, M.C. *et al.* (2006) Plant nitric oxide synthase: a never-ending story? *Trends in Plant Science*, **11**, 524–525.

Zhang, H., and Forde, B.G. (2000) Regulation of *Arabidopsis* root development by nitrate availability. *Journal of Experimental Botany*, **51**, 51–59.

Zrenner, R., Stitt, M., Sonnewald, U., Boldt, R. (2006) Pyrimidine and purine biosynthesis and degradation in plants. *Annual Review of Plant Biology*, **57**, 805–836.

Figure captions

Fig. 1: General scheme of purine degradation in higher plants. The purine degradation pathway is depicted starting with adenosine monophosphate (AMP) at the crosspoint between purine *de novo* synthesis and catabolism. Only the relevant intermediates are shown according to Zrenner et al. (2006), Werner and Witte (2011) and Werner et al. (2013), presumed back-reactions are not considered. Abbreviation are: inosine monophosphate (IMP), guanosine monophosphate (GMP), and xanthosine monophosphate (XMP).

Fig. 2: Schematic presentation of the structure of xanthine oxidoreductase. (**A**) Linear presentation of the domain arrangement in one XOR polypeptide. Each domain is separated from the others by flexible hinge regions. (**B**) Simplified scheme of the 3-dimensional structure of eukaryotic XOR according to Enroth et al. (2000). The two monomers of the XOR homodimer are shown in green and blue, respectively, with the dimerization interface being located within the large Moco domain. In contrast to what the linear structure suggests, electron transfer in the fully folded enzyme is directed from Moco via the two [2Fe-2S] clusters to the FAD, which is enabled by folding of the N-terminal Fe-S cluster domain between the Moco- and FAD-binding domains.

Fig. 3: General reaction mechanism of xanthine oxidoreductase with xanthine as substrate. Hydroxylation of xanthine is likely to be initiated by (1) proton abstraction of the hydroxyl group of the molybdenum centre (Mo-OH) supported by a strictly conserved and catalytically active glutamate residue in the active site of XOR enzymes. (2) By base-assisted nucleophilic attack of the formed Mo-O^- on the C8 position of xanthine and by concomitant hydride transfer to the Mo=S group a $Mo^{IV}O(SH)$-O-substrate intermediate is generated, which (3) breaks down by electron transfer to the other redox centres (Fe-S and FAD) and deprotonation, with the occurence of a Mo^V species. Alternatively, the intermediate can be broken down by hydroxide displacement of the product from the molybdenum centre and subsequent transfer of electrons to the other redox centres (according to Hille et al., 2011).

Fig. 4: The diverse sub-activities of plant xanthine oxidoreductase. (**A**) Major activity of XOR with xanthine as reducing substrate, NAD^+ as the preferred oxidizing substrate and O_2 as alternative oxidizing substrate, which are reduced to NADH or superoxide anions (O_2^-), respectively. This activity involves all four redox groups. In place of xanthine, various other

compounds such as hypoxanthine and aldehydes can likewise serve as reducing substrate. (**B**) NADH oxidase activity of XOR, which takes place solely at the FAD domain and involves oxidation of NADH with concomitant reduction of O_2 to O_2^-. (**C**) Nitrite reductase activity of XOR as exhibited by the substrate(xanthine)-reduced enzyme. Electrons derived from xanthine are not passed to other redox centres but to nitrite (NO_2^-), which is thereby reduced to nitric oxide ($^{\bullet}NO$). This activity is strictly dependent on low-oxygen conditions. (**D**) Nitrite reductase activity of XOR after reduction of the enzyme by NADH. This activity involves all four redox-active groups and resembles the inverse reaction of (A) with regard to electron flow. It is assumed that this activity also occurs in the presence of O_2. For clarity, only one monomer of the otherwise homodimeric enzyme is presented.

Fig. 5. Established and possible physiological substrates of plant xanthine oxidoreductase. Xanthine and hypoxanthine are the preferred substrates for XOR, which points out the involvement of XOR enzymes in purine catabolism. Albeit with lower efficiency, other compounds such as uric acid, abscisic aldehyde, 1-naphthaldehyde, heptaldehyde, acetaldehyde, hydroxylamine and SHAM were likewise oxidized by plant XOR proteins. For more detailed information see section V.

Fig. 6: Schematic presentation of interconversion of mammalian xanthine oxidoreductase into dehydrogenase and oxidase forms. Mammalian XOR is converted from the initially existing dehydrogenase form (middle) into the oxidase form either irreversibly by limited proteolysis within the hinge regions between the domains (right) or reversibly by oxidation of conserved cysteine thiols (left). For clarity, only one monomer of the otherwise homodimeric enzyme is presented.

Fig. 7: Schematic view of the implication of plant xanthine oxidoreductase in abiotic stress and plant-microbe interactions. Plant XOR is implicated in the ROS generation in plant tissues under abiotic or biotic stresses. These ROS are involved in oxidative stress, stress signaling and senescence. At the same time, XOR produces ureides that alleviate ROS. XOR levels/activities increase under some of stresses, e.g. ammonium, drought, plant-microbe interactions (indicated by the green dashed arrows), whereas in other cases, a reduction of XOR levels/activities has been demonstrated (indicated by the red dashed arrows). Besides, XOR is also involved in fruit development.

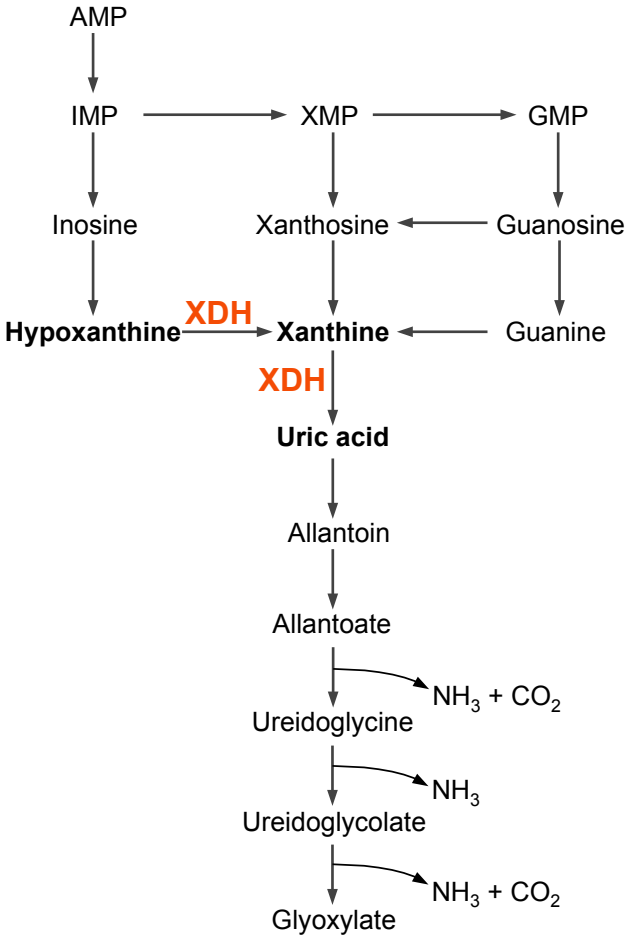

Fig. 1: General scheme of purine degradation in higher plants. The purine degradation pathway is depicted starting with adenosine monophosphate (AMP) at the crosspoint between purine *de novo* synthesis and catabolism. Only the relevant intermediates are shown according to Zrenner et al. (2006), Werner and Witte (2011) and Werner et al. (2013), presumed back-reactions are not considered. Abbreviations are: inosine monophosphate (IMP), guanosine monophosphate (GMP), and xanthosine monophosphate (XMP).

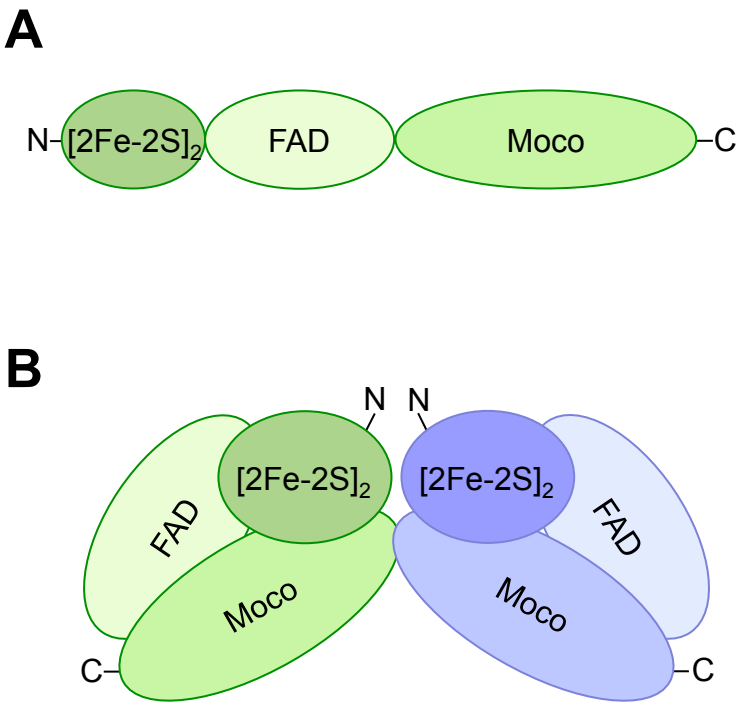

Fig. 2: Schematic presentation of the structure of xanthine oxidoreductase. (**A**) Linear presentation of the domain arrangement in one XOR polypeptide. Each domain is separated from the others by flexible hinge regions. (**B**) Simplified scheme of the 3-dimensional structure of eukaryotic XOR according to Enroth et al. (2000). The two monomers of the XOR homodimer are shown in green and blue, respectively, with the dimerization interface being located within the large Moco domain. In contrast to what the linear structure suggests, electron transfer in the fully folded enzyme is directed from Moco via the two [2Fe-2S] clusters to the FAD, which is enabled by folding of the N-terminal iron-sulfur cluster domain between the Moco- and FAD-binding domains.

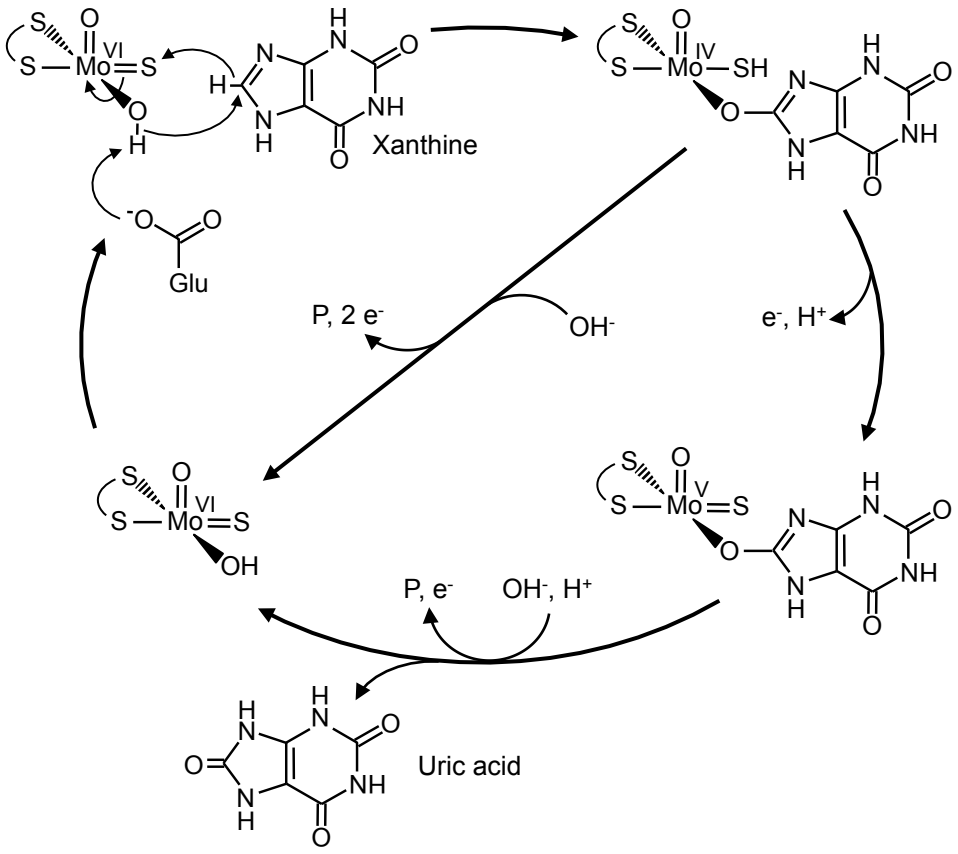

Fig. 3: General reaction mechanism of xanthine oxidoreductase with xanthine as substrate. Hydroxylation of xanthine is likely to be initiated by (1) proton abstraction of the hydroxyl group of the molybdenum center (Mo-OH) supported by a strictly conserved and catalytically active glutamate residue in the active site of XOR enzymes. (2) By base-assisted nucleophilic attack of the formed Mo-O^- on the C8 position of xanthine and by concomitant hydride transfer to the Mo=S group a $Mo^{IV}O(SH)$-O-substrate intermediate is generated, which (3) breaks down by electron transfer to the other redox centers (Fe-S and FAD) and deprotonation, with the occurence of a Mo^V species. Alternatively, the intermediate can be broken down by hydroxide displacement of the product from the molybdenum center and subsequent transfer of electrons to the other redox centers (according to Hille et al., 2011).

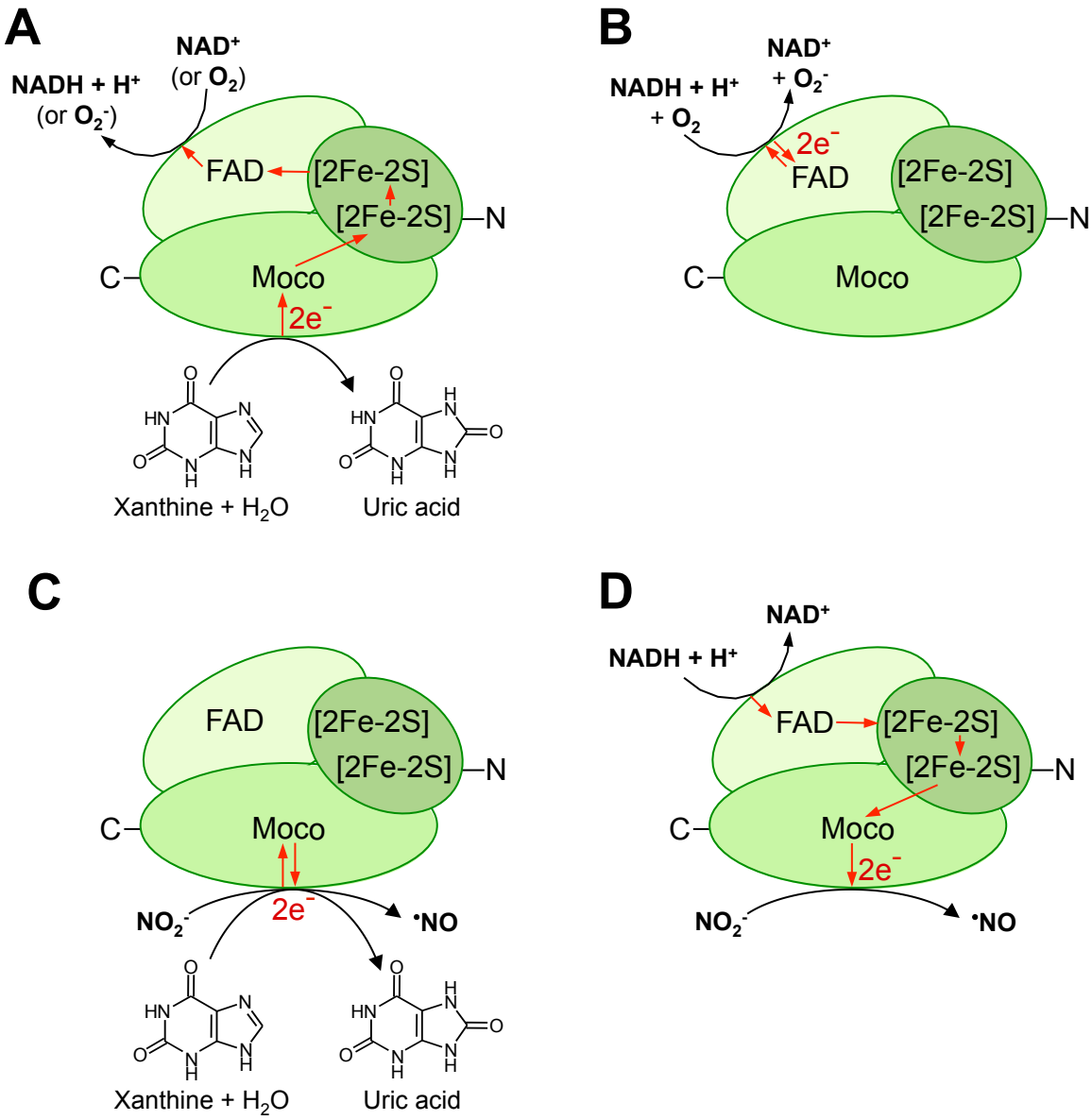

Fig. 4: The diverse sub-activities of plant xanthine oxidoreductase. (**A**) Major activity of XOR with xanthine as reducing substrate, NAD^+ as the preferred oxidizing substrate and O_2 as alternative oxidizing substrate, which are reduced to NADH or superoxide anions (O_2^-), respectively. This activity involves all four redox groups. In place of xanthine, various other compounds such as hypoxanthine and aldehydes can likewise serve as reducing substrate. (**B**) NADH oxidase activity of XOR, which takes place solely at the FAD domain and involves oxidation of NADH with concomitant reduction of O_2 to O_2^-. (**C**) Nitrite reductase activity of XOR as exhibited by the substrate(xanthine)-reduced enzyme. Electrons derived from xanthine are not passed to other redox centers but to nitrite (NO_2^-), which is thereby reduced to nitric oxide ($^\bullet NO$). This activity is strictly dependent on low-oxygen conditions. (**D**) Nitrite reductase activity of XOR after reduction of the enzyme by NADH. This activity involves all four redox-active groups and resembles the inverse reaction of (A) with regard to electron flow. It is assumed that this activity also occurs in the presence of O_2.

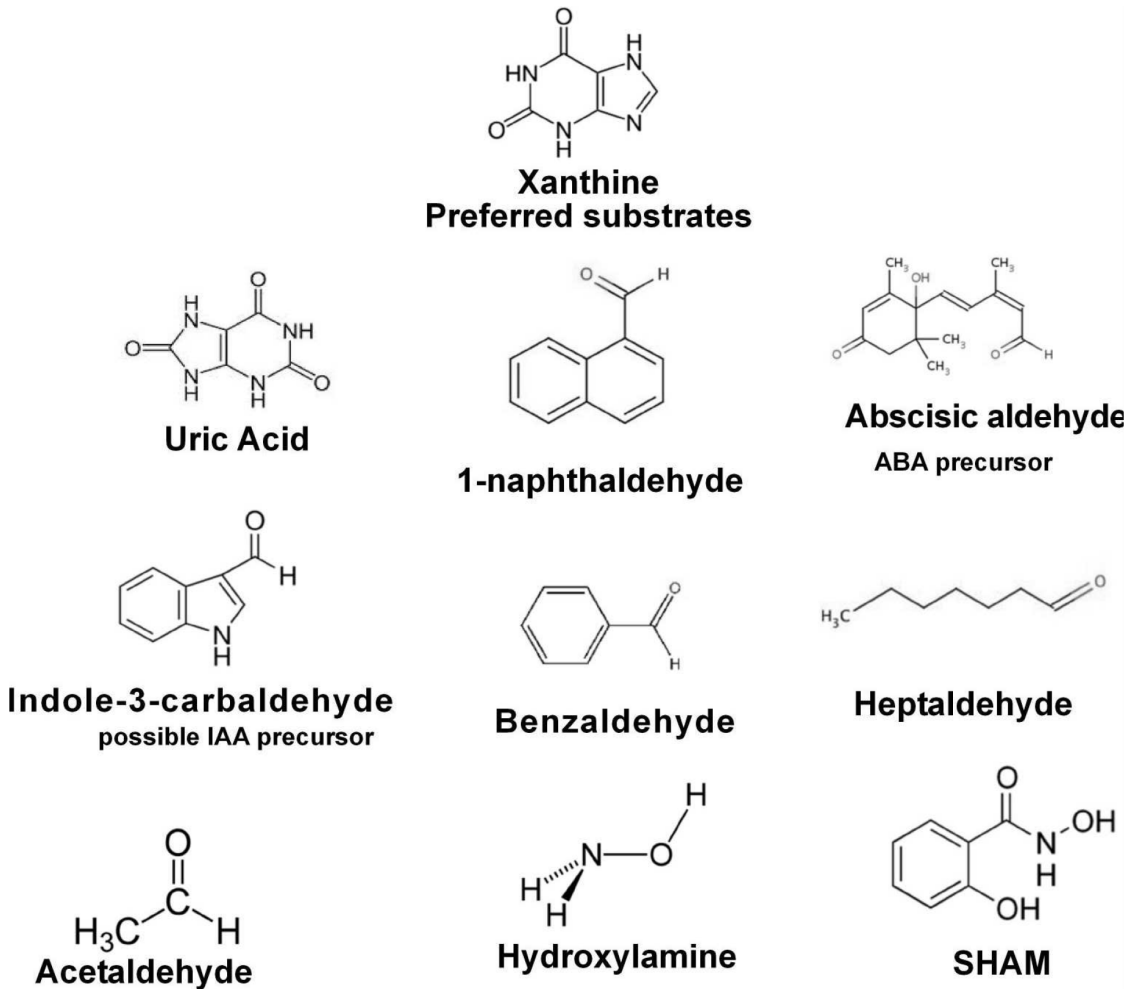

Fig. 5. Established and possible physiological substrates of plant xanthine oxidoreductase. Xanthine and hypoxanthine are the preferred substrates for XOR, which points out the involvement of XOR enzymes in purine catabolism. Albeit with lower efficiency, other compounds such as uric acid, abscisic aldehyde, 1-naphthaldehyde, heptaldehyde, acetaldehyde, hydroxylamine and SHAM were likewise oxidized by plant XOR proteins. For more detailed information see section V.

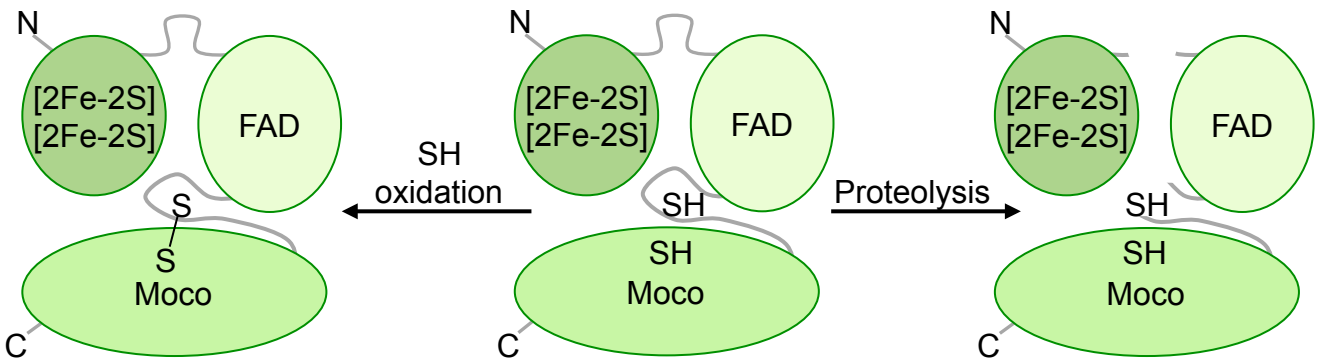

Fig. 6: Schematic presentation of interconversion of mammalian xanthine oxidoreductase into dehydrogenase and oxidase forms. Mammalian XOR is converted from the initially existing dehydrogenase form (middle) into the oxidase form either irreversibly by limited proteolysis within the hinge regions between the domains (right) or reversibly by oxidation of conserved cysteine thiols (left). For clarity, only one monomer of the otherwise homodimeric enzyme is presented.

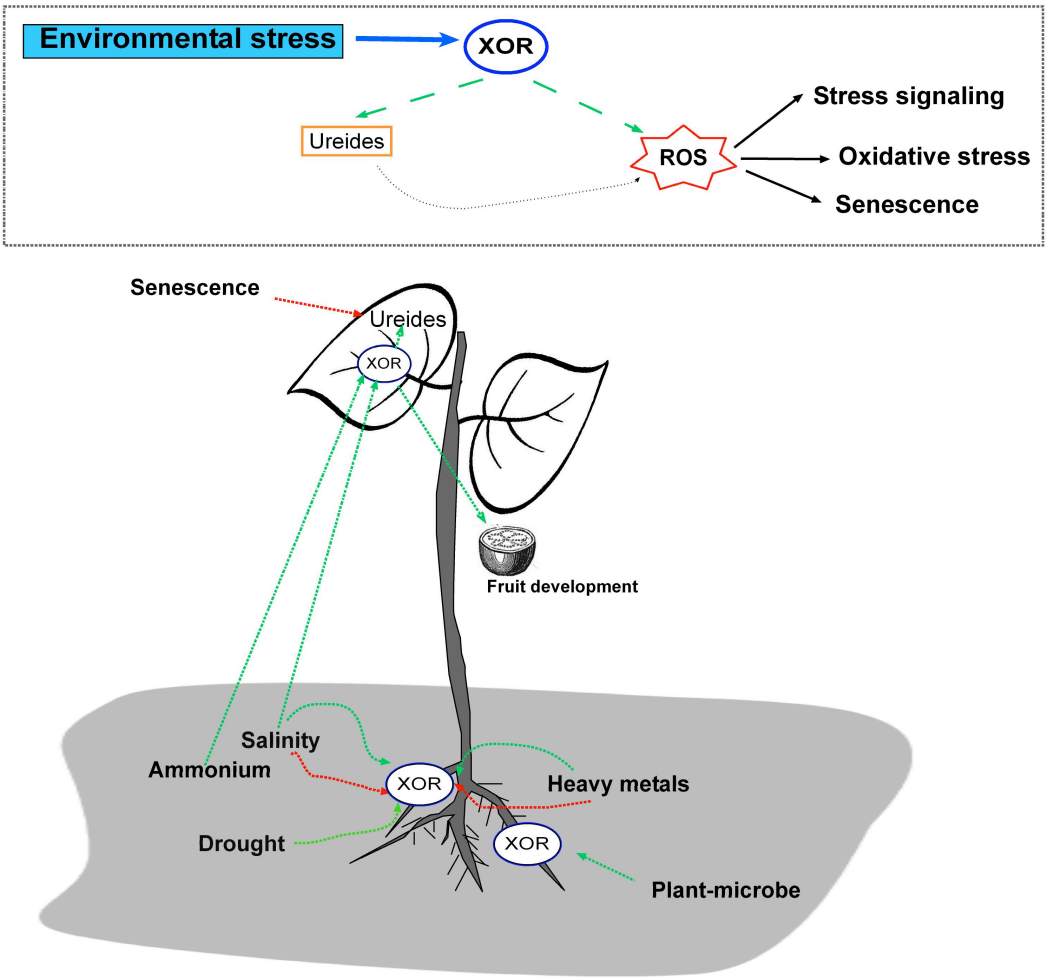

Fig. 7: Schematic view of the implication of plant xanthine oxidoreductase in abiotic stress and plant-microbe interactions. Plant XOR is implicated in the ROS generation in plant tissues under abiotic or biotic stresses. These ROS are involved in oxidative stress, stress signaling and senescence. At the same time, XOR produces ureides that alleviate ROS. XOR levels/activities increase under some of stresses, e.g. ammonium, drought, plant-microbe interactions (indicated by the green dashed arrows), whereas in other cases, a reduction of XOR levels/ activities has been demonstrated (indicated by the red dashed arrows). Besides, XOR is also involved in fruit development.