Ascorbic acid as a key signaling molecule integrating the activities of 2-oxoglutarate-dependent dioxygenases: shifting the paradigm

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Abstract

The integration of different environmental cues and the harmonization of proper responses require molecular communication at different levels. Enzymes belonging to the large superfamily of 2-oxoglutarate-dependent dioxygenases (2-ODDs) use for their catalytic activity different cofactors, including molecular oxygen, 2-oxoglutarate, and ascorbic acid (ASC). In plants, 2-ODDs are involved in the synthesis of hydroxyproline (largely present in cell wall proteins), gibberellins, ethylene, zeaxanthin, and many more relevant molecules. In addition, 2-ODDs operating in the epigenetic control of gene expression by regulating cytosine and histone methylation have been recently identified in both animals and plants. It is here hypothesized that each of the co-substrates required for the complex catalytic mechanism of 2-ODDs provides specific molecular information. Such information is integrated by 2-ODDs, which in turn regulate the synthesis of information-bearing metabolites (e.g. hormones), and control epigenetic processes. The evolutionary success of the catalytic mechanism shared by 2-ODDs is possibly related to the parallel activation of different biosynthetic pathways.

Introduction

The development of living organisms is regulated by both internal (genetic) and environmental cues. An astonishing amount of information must be connected and interrelated to ensure proper developmental responses under the most diverse conditions. Understanding how a plurality of molecular signals is translated into developmental choices is a major challenge in today’s biological investigations.

Disentangling the complex networks of multi-layered molecular signals underlying each response, and the coordination of such responses at the pre-and post-transcriptional, translational and post-translational level, may seem at first sight an impossible mission. Notably, the complexity of signaling modules reflects the mechanisms of molecular evolution: an interesting simulation of the evolving of a simple three-component signaling module showed that signaling pathways tend to increase in complexity far above the minimum size necessary to fulfill their function (Soroya and Bonhoeffer 2006). A key to finding one’s way in this signaling jungle is interpreting available data with an integrative approach, i.e. identifying ways in which signaling molecules deriving from different aspects of cell metabolism are integrated. If molecules tell stories (De Tullio and Asard, 2012), we need to learn the syntax of this molecular language, which sometimes can only be understood going beyond the generally held views we are currently exposed to.

It is long known that 2-oxoglutarate-dependent dioxygenases (2-ODDs) require, in addition to their substrate(s), the presence of an unusual number of cofactors to perform their catalytic activity (Fowler and Facchini, 2014). The possibility that such complex catalytic mechanism is used to integrate different molecular signals has been suggested (Salminen et al., 2015; Kundu, 2015), but not thoroughly explored so far. In particular, the role of ascorbate (ASC) in the reaction mechanism of 2-ODDs, and its role as a key component of the possible function of 2-ODDs in the integration of molecular signals has been overlooked. Different lines of evidence supporting this view will be discussed in the following sections.

Molecular evolution and the role of cofactors

In spite of several studies, the mechanisms of molecular (and namely of protein) evolution remain only partly understood (Suarez-Diaz, 2016). Caetano-Anolles et al. (2012), by analyzing in an evolutionary perspective the domains of the 54 most ancient protein fold families, identified “a clear progression of cofactor recruitment” in the timeline. This interesting observation suggests that cofactors are involved in the evolution of protein shape and function. Analysis of the cofactors used by a class of enzymes can provide relevant information that goes beyond the strict function of each enzyme and of the products deriving from the reaction they catalyze. Jeong et al. (2000) demonstrated that single molecules are not randomly connected in metabolic networks, and the average number of connections increases with the increase in organism complexity and the number of available metabolites (Fig. 1). Such increase in connectivity implies that some molecules (namely those involved in many different reactions) actually operate some form of integration. By extending this concept, the association of enzyme-catalyzed reaction can be viewed as a possible way of coordinating different and apparently independent pathways. The availability of all required substrates and cofactors makes a reaction possible. When one of them is lacking, the reaction is impaired. If we assume that each cofactor is an indicator (meter) of something occurring in the cell, the plurality of cofactors required by some enzymes underlies the necessity of collecting information to operate informed molecular choices.

2-Oxoglutarate-dependent dioxygenases: a superfamily at work

Many different reactions (including epoxidation, desaturation, hydroxylation, and ring opening) are catalyzed by enzymes belonging to the 2-ODD superfamily (Farrow and Facchini 2014). With the notable exception of the ethylene-forming enzyme ACC oxidase (see below), 2-ODDs require for their catalytic activity molecular oxygen, 2-oxoglutarate, iron (Fe^{2+}) and ASC. A very large number of genes coding for 2-ODDs was identified in plant genomes. Interestingly, the number of putative 2-ODD-coding genes apparently increases along with plant evolution: Kawai et al. (2014) retrieved 41 sequences related to 2-ODDs in the green algae *Chlamydomonas reinhardtii;* 66 in the moss *P. patens;* 74 in the lycophyte *Selaginella mollendorfii*. A marked increase in 2-ODD sequences occurred in seed plants, with 142 sequences identified in the gymnosperm *Picea abies*, 130 and 114 in the model angiosperms *Arabidopsis thaliana* and *Oryza sativa*, respectively. Three main classes of 2-ODDs have been identified on the basis of protein sequence similarity (Kawai et al. 2014): the DOXA class includes homologs of the bacterial DNA repair protein AlkB; prolyl hydroxylases and related enzymes catalyzing post-translational hydroxylation of proline and lysine are the core of the DOXB class; the third class (DOXC) includes enzymes involved in hormone (e.g. gibberellin, ethylene, auxin) synthesis/catabolism and other specialized pathways for the synthesis of secondary metabolites. In seed plants a marked increase is observed mainly in the number of 2-ODDs belonging to the DOXC class, whereas the number of DOXA and DOXB sequences is not much different among the different plant genomes analyzed (Kawai et al., 2014). It should be considered that no biochemical information is available for most of the putative 2-ODD sequences retrieved by sequence comparison, whereas only few 2-ODD proteins and their catalytic activity have been fully characterized so far. An exhaustive list of 2-ODDs goes beyond the scope of this article (more details can be found in the excellent reviews by Farrow and Facchini, 2014; White and Flashman 2016, Nadi et al. 2018; see also Table1), but reporting here a few examples can be useful to highlight the outstanding importance of this superfamily.

Prolyl hydroxylases in animals and plants

The hydroxylation of proline residues within polypeptide chains is crucial for the functioning of extracellular proteins in animals (collagen) and plants (cell wall extensin and arabinogalactan proteins). Prolyl-4-hydroxylases (P4H), probably the most studied and best characterized 2-ODDs, have been initially identified for their role in collagen hydroxylation (Myllyharju 2003). Early studies by Alton Meister (Stone and Meister, 1962) highlighted the connection between ASC deficiency (scurvy) and collagen-related disorders caused by defects in blood vessels, bone, and cartilage: all structures characterized by abundant collagen. The discovery of the function of P4H in collagen hydroxylation was a fundamental step in the development of molecular medicine, as it provided the first molecular explanation of the symptomatology of scurvy. The pioneer work by Paul Bolwell highlighted the involvement of plant P4H in cell wall re-arrangement in response to fungal elicitors (Bolwell et al. 1985; Wojtazsek et al. 1999). Several decades after the identification of collagen P4H, a different class of specialized P4H was discovered in animal cells, targeting specific proline residues in the hypoxia-induced transcription factor HIF (Semenza, 2002). If the full set of typical 2-ODD cofactors is available, two proline residues in the HIF sequence are hydroxylated, leading the transcription factor to proteolysis via ubiquitination; in the absence of one or more cofactors, no hydroxylation occurs and HIF moves to the nucleus for transcriptional regulation. The mechanism of oxygen sensing mediated by HIF has several relevant implications in physiology and medicine (Pugh and Ratcliffe 2017), and the 2019 Nobel prize was awarded to the scientists responsible for this outstanding discovery. Panos Kalaitzis and his coworkers (Vlad et al. 2007a, 2007b) provided indirect evidence that a similar hydroxylating mechanism for oxygen sensing occurs in *Arabidopsis* as well.

2-ODDs involved in plant hormone metabolism and in sensing/signaling

Going through the list of plant 2-ODDs, the massive presence of hormone-related enzymes catches the eye (Table 2A) . Different 2-ODDs catalyze key steps in the synthesis or the degradation (or both) of virtually all hormones: abscisic acid (ABA), ethylene, various gibberellins, oxylipins (which include jasmonates), auxin, and strigolactones. The ethylene-forming enzyme ACCO (1-aminocyclopopane-1-carboxylic acid oxidase) makes an interesting exception to the typical cofactor requirement of 2-ODDs (Fig.2), in that ASC is used as a substrate instead of 2-oxoglutarate (Dilley et al. 2013). Recently, the expression of the tomato dioxygenase-coding gene *SlF3HL* has been correlated with chilling tolerance and lower levels of reactive oxygen species (ROS), possibly in connection with jasmonate metabolism (Hu et al. 2019).

The presence of 2-ODDs potentially involved in sensing and signaling mechanisms (Table 2B) makes another interesting point. In addition to the possible plant counterpart of the prolyl hydroxylase/HIF-mediated oxygen sensing mentioned above (Vlad et al. 2007a, 2007b) an intriguing oxygen-sensing mechanism involving the 2-ODD cysteine oxidase has been initially observed in plants (Weits et al. 2014; White et al. 2018), and only later found in animal cells (Masson et al. 2019). Five Plant Cysteine Oxidase (PCO 1-5) have been identified and characterized in *Arabidopsis thaliana* (White et al. 2018). In this case the oxygen-sensing mechanism is based upon the hydroxylation of a terminal cysteine residue in transcription factors of the *Arabidopsis* ERF group VII (Fig. 3). This hydroxylation, in normoxic conditions, targets the transcription factor to degradation, whereas the unhydroxylated transcription factor activates an orchestrated hypoxic response. Recent data also show that oxygen availability is not the only trigger of this mechanism, which is responsive to different forms of abiotic stress (Vicente et al. 2017). Kinetic analysis of PCO1-5 showed that ASC (1 mM) significantly increased the specific activity of PCO2 and, to lesser extent, PCO5, whereas the remaining PCO are apparently not affected by ASC availability, at least in the condition tested (White et al. 2018).

Additional interesting 2-ODDs apparently act in the regulation of plant responses to biotic stress (Zeilmaker et al. 2015). In this case, DMR6 and DOL1 operate as repressors of plant immunity.

Two 2-ODDs are clearly involved in epigenetic mechanisms regulating gene expression. A group of histone demethylases sharing the Jumonji C (JmjC) signature catalyze the removal of methyl groups from lysine residues of histone proteins (Tsukuda et al., 2006; Lu et al. 2008). The histone demethylation activity of JUMONJI proteins was recently correlated to salt and drought stress resistance in rice plants (Song et al. 2018; Chowrasia et al. 2018). An additional 2-ODD-dependent epigenetic mechanism is the first step in the demethylation of cytosine operated by Ten-Eleven-Translocation (TET) enzymes (Ito et al. 2011, Blashke et al. 2013). The first intermediate in TET-catalyzed cytosine demethylation is hydroxymethylcytosine. Although the presence of TET activity in plants has not been ascertained so far, the presence of this intermediate was detected in rice plants (Wang et al. 2015). Detailed biochemical analysis confirmed that the activity of TET enzymes is ASC-dependent (Minor et al. 2013), supporting the hypothesis of a general role of ASC in the epigenetic control of gene expression (Camarena and Wang, 2016).

The potential informational role of the molecules involved in the catalytic mechanism of 2-ODDs

As mentioned above, in spite of the large diversity in the “main” substrate modified by 2-ODDs, especially in the DOXC class (Kawai et al. 2014, Farrow and Facchini 2014), 2-ODDs share for their catalytic activity molecular oxygen, 2-oxoglutarate, iron (Fe2+), and ASC (but 2-oxoglutarate is not used by ACCO, Fig. 2). In an evolutionary perspective, we observe that the same mechanism has been conserved and adapted to the needs of a huge number of different metabolic pathways. The spread of 2-ODDs could be simply explained in terms of catalytic efficiency of the mechanism. However, there is mounting evidence that each of the four cofactors could bear relevant metabolic information, that is integrated at the enzyme level (Salminen et al. 2015, Kundu 2015a, Kundu 2015b). Some points in support of this view are discussed below.

Molecular oxygen is the main player in the reactions catalyzed by 2-ODDs, but also by enzymes of the even larger family of cytochrome P450 (Mitchell and Weng 2019). In aerobic organisms, all cellular activities must be harmonized with oxygen availability, therefore oxygen obviously bears information of general interest that can be used for tuning cell metabolism.

Araujo et al. (2015) reported several interesting considerations on the possible role of 2-oxoglutarate as a connector between different biosynthetic pathways. Antisense inhibition of the gene coding for the TCA cycle enzyme 2-oxoglutarate dehydrogenase resulted in early senescence, possibly correlated with altered gibberellin biosynthesis, and altered biosynthesis of secondary metabolites.

The iron-dependency of 2-ODDs has been considered part of an iron-sensing mechanism in plants (Farrow and Facchini 2014). Iron is used in many ways in the cell, and has pivotal importance in photosynthesis (Briat et al. 2007, Kobayashi 2019). The relationship between iron and 2-ODD activity in connection with oxygen and within a signaling perspective has been recently discussed (Vigani and Murgia 2018).

Last but not least, the role of ASC in the reactions catalyzed by 2-ODDs deserves proper discussion. Early studies claimed that ASC is only required in the reaction to keep iron in the reduced state (De Jong et al. 1982, De Jong and Kemp 1984, Myllyla et al. 1984). If so, any reductant should be as effective as ASC in promoting 2-ODD activity. However, this is contradicted by the interesting data reported by Flashman et al. (2010), showing that dithiothreitol (which is a non-physiological reductant) is less effective than ASC, and glutathione is almost ineffective in promoting the hydroxylation of proline residues, although on the basis of their standard reduction potential they both should be able of reducing Fe^{3+} to Fe^{2+} more efficiently than ASC (Flashman et al. 2010). An increasing deal of papers were published in the last 5-6 years, highlighting the role of 2-ODDs in many aspects of animal and plant biology, but many of them, and especially those using a bioinformatic approach (Kundu 2015a, 2015b) do not take into consideration ASC in this context.

ASC is widely known as an antioxidant molecule, mainly involved in the scavenging of reactive oxygen species (ROS). However, it is increasingly clear that this is probably just the tip of the iceberg. The ROS/antioxidant duality as a competition between dangerous and positive forces, respectively, represents an oversimplification of the complex relationship between them (Noctor and Foyer 2005). It should be considered that ROS are generated by stress conditions, but they are also purportedly formed by specialized enzymes belonging to the class of NADH oxidases (Marino et al. 2012). Moreover, as brilliantly and concisely claimed by Ron Mittler, “ROS are good” because they are required for signaling and for basic cellular functions (Mittler 2017). On the other hand, the presence of the enzyme ASC oxidase, which apparently wastes ASC without any clear advantage (Balestrini et al. 2012) can only be explained in the context of a signaling-related function (De Tullio et al. 2013). In this framework, ASC peculiar reactivity with ROS, rather than a simple scavenging system, could be seen as an extremely efficient way to gain information about the cellular redox status in response to environmental cues, since it is clear that virtually any stress condition results in ROS production and consequent lower ASC availability. In turn, low ASC could impact on the function of some 2-ODDs, resulting in the activation of stress responses. This “double life” of vitamin C as a stress sensor and a signal transducer (De Tullio 2012) could accommodate much of the available information on the peculiar reactivity of this molecule.

Ascorbate deficiency and altered functioning of 2-ODDs

We (humans) and a few animal species are unable to synthesize ASC because of the accumulation of mutations in the L-gulonolactone oxidase gene, which in synthesizing animals encodes for the protein catalyzing the last step in ASC biosynthesis (Nishikimi and Yagi, 1991). Being the natural equivalent of knockout mutants, we can easily observe in humans and other non-synthesizing organisms the effects of the lack of dietary ASC, which results in the deadly syndrome known as scurvy caused by the progressive inactivation of many 2-ODDs (De Tullio 2004).

“Ascorbic” literally means “against scurvy”, the name deriving from the discovery of ASC as the anti-scurvy factor Vitamin C. No “scurvy” plant species or mutant has ever been observed, suggesting that plants totally devoid of ASC are not viable. All low-ASC single mutants isolated so far have “leaky” mutations, which result in lowering ASC content, but not in totally blocking its biosynthesis. Notably, seeds of a *vtc2* double mutant of *Arabidopsis thaliana* unable to perform L-galactose phosphorylase activity (a key step in ASC biosynthesis) can still germinate, possibly due to the presence in mature dry seeds of a small but significant amount of dehydroascorbic acid, but seedlings stop growing and cotyledons rapidly bleach in the absence of ASC supplementation (Dowdle et al. 2007).

In ASC-synthesizing organisms, including plants, the possible influence of ASC deficiency on the activity of 2-ODDs can be indirectly inferred by analyzing the phenotype of ASC-deficient mutants. Reduced growth and early senescence are features shared by many low-ASC mutants/antisensed plants (Keller et al. 1999, Veljovic-Jovanovic et al. 2001, Barth et al. 2004, Colville and Smirnoff 2008). In our lab, wild-type plants and two antisense potato (*Solanum tuberosum*) lines cultured *in vitro* (kindly provided by the Kossmann group, Keller et al. 1999), showed an interesting correlation between ASC content and shoot length (Fig.4). Shoots of the antisense line with lowest ASC content were also thicker than WT. Shoot cross sections showed in the WT a well-ordered collenchyma, that was dramatically disassembled in the low-ASC line (Fig. 4), with large cells and intercellular spaces. Using an anti-extensin primary antibody and FITC-tagged secondary antibodies, the fluorescence pattern in ASC-deficient potato plants evidenced cell wall disassembly.

Somehow even more interesting is the unexpected correlation between low ASC content and increased pathogen resistance (Pastori et al. 2003, Barth et al. 2004, Pavet et al. 2005, Mukherjee et al. 2010). The basal expression of pathogenesis-related (PR) proteins is higher in ASC-deficient plants (Colville and

Smirnoff 2008, Mukherjee et al. 2010) and, most interestingly, a GFP-tagged PR protein signal was constitutively found in the nucleus in the ASC deficient *vtc1* mutant of *Arabidopsis* (Pavet et al. 2005). If we look at the above-reported information on the effects of ASC deficiency in plants, and compare it to what we know about 2-ODD involvement in the regulation of stress responses (discussed in the previous paragraphs) the similarity is quite remarkable. Both the HIF1alpha and PCO signaling modules, by acting on stress responsive genes, set the basal level of stress alert in relation to the availability of the signaling molecules participating to the 2-ODD reaction. Strikingly, plants with low ASC availability show increased resistance to biotic stress, which is surprising and counterintuitive, if we rely on the widely held assumption of a generally beneficial role of ASC in stressful conditions. It is tempting to speculate that low ASC is involved in the regulation of the basal level of plant stress responses, possibly via a still uncharacterized 2-ODD-mediated mechanism. Keeping the basal level of stress response high is also likely to result in grow defects and early senescence. The observed alterations in the cell wall organization of *S. tuberosum* cells in the ASC-deficient plants, with the apparent disassembly of the hydroxyproline-containing protein extensin, also suggest that the hydroxylation of proline residues is reduced. Eventually, the increase in the size of intercellular spaces could be interpreted as a possible hypoxic-like response caused by ASC deficiency.

Modulation of the parallel activation of 2-ODDs

Given the high number of 2-ODDs potentially working in the same cellular environment, it is unlikely that they all could be activated by the same conditions. Indeed, whenever the kinetic properties of 2-ODD proteins have been considered, distinct 2-ODDs performing the same catalytic activity show different affinity for their cofactors. This is the case, for example with PCO 1-5 cysteine oxidases. As mentioned above, only two PCOs (namely PCO2 and PCO5) are ASC-dependent (and to different degrees) for their catalytic activity (White et al. 2018). This finding, rather than limiting the importance of ASC in the reaction and making it a “facultative” cofactor, further strengthens the hypothesis that the plurality of cofactors is required for the fine-tuning of the responses. If all 2-ODDs had exactly the same qualitative and quantitative cofactor requirement, they would be activated at the same time to the same extent, and this is definitely not the best way to face a plurality of different (stress) conditions. A balance in cofactor availability, and the different requirements of single 2-ODDs potentially makes the many different 2-ODD-dependent pathways adjustable.

Conclusions: the paradigm shift

Thomas Kuhn has been probably the most influential philosopher of science of the last century. In his seminal work “The structure of scientific revolutions” (Kuhn 1962) he described the events leading to a change of perspective from a mainstream view (“normal science”) to a different level of awareness (the so called Kuhn cycle), which in turn becomes the new standard of normal science. His concept of “paradigm shift” has been popularized and introduced in economics and other fields of human activity. A paradigm shift is probably required to move away from the simplistic view of a function of ascorbic acid just confined to his antioxidant role (Loiacono and De Tullio 2012) and consider the much more realistic perspective of a key role in signaling.

References

Alder A, Jamil M, Marzorati M, Bruno M, Vermathen M, Bigler P, Ghisla S, Bouwmeester H, Beyer P, Al-Babili S (2012) The path from beta-carotene to carlactone, a strigolactone-like plant hormone. Science, 335: 1348-1351

Araújo WL, Martins AO, Fernie AR, Tohge T (2014) 2-Oxoglutarate: linking TCA cycle function with amino acid, glucosinolate, flavonoid, alkaloid, and gibberellin biosynthesis. *Front Plant Sci*. 5:552. doi:10.3389/fpls.2014.00552

Balestrini R, Ott T, Güther M, Bonfante P, Udvardi MK, De Tullio MC (2012) Ascorbate oxidase: the unexpected involvement of a 'wasteful enzyme' in the symbioses with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi. Plant Physiol Biochem. 59:71-9. doi: 10.1016/j.plaphy.2012.07.006

Barth C, Moeder W, Klessig DF, Conklin PL. (2004) The timing of senescence and response to pathogens is altered in the ascorbate-deficient Arabidopsis mutant vitamin c-1. Plant Physiol. 134:1784-1792.

Blaschke K, Ebata KT, Karimi MM, Zepeda-Martínez JA, Goyal P, Mahapatra S et al. Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. *Nature* (2013) **500**:222-226. doi: 10.1038/nature12362

Bolwell GP, Robbins MP, Dixon RA (1985) Metabolic changes in elicitor-treated bean cells. Enzymic responses associated with rapid changes in cell wall components Eur J Biochem 148:571-578 doi:10.1111/j.1432-1033.1985.tb08878.x

Briat J-F, Curie C, Gaymard F (2007) Iron utilization and metabolism in plants.Curr Opin Plant Biol 10:276-282 doi:10.1016/j.pbi.2007.04.003

Caarls L, Elberse J, Awwanah M, Ludwig NR, de Vries M, Zeilmaker T, Van den Ackerveken G. (2017). *Arabidopsis* JASMONATE-INDUCED OXYGENASES down-regulate plant immunity by hydroxylation and inactivation of the hormone jasmonic acid. Proc Natl Acad Sci USA 114: 6388–6393. doi:10.1073/pnas.1701101114

Caetano-Anollés G, Kim KM, Caetano-Anollés D. The phylogenomic roots of modern biochemistry: origins of proteins, cofactors and protein biosynthesis. *J Mol Evol* (2012) **74**:1–34. doi 10.1007/s00239-011-9480-1

Camarena V, Wang G. (2016). The epigenetic role of vitamin C in health and disease. *Cellular and molecular life sciences : CMLS*, *73*(8), 1645–1658. doi:10.1007/s00018-016-2145-x

Chowrasia S, Panda AK, Rawal HC, Kaur H, Mondal TK (2018) Identification of JumonjiC domain containing gene family among the *Oryza* species and their expression analysis in FL478, a salt tolerant rice genotype. Plant Physiol Biochem 130: 43-53. doi10.1016/j.plaphy.2018.06.031

De Jong L, Albracht SP, Kemp, A. (1982) Prolyl 4-hydroxylase activity in relation to the oxidation state of enzyme-bound iron. The role of ascorbate in peptidyl proline hydroxylation. Biochim Biophys Acta 704: 326-332

De Jong, L. and Kemp, A. (1984) Stoicheiometry and kinetics of the prolyl 4- hydroxylase partial reaction. Biochim. Biophys. Acta 787, 105-111

De Tullio MC (2004) How does ascorbic acid prevent scurvy? A survey of the nonantioxidant functions of vitamin C. In Vitamin C: functions and biochemistry in animals and plants. Asard H, May JM, Smirnoff N eds. BIOS scientific publishers, London and New York.

De Tullio MC (2010) Antioxidants and redox regulation: changing notions in a changing world. Plant Physiol Biochem 48:289–291. doi 10.1016/j.plaphy.2010.02.011

De Tullio MC (2012) Beyond the antioxidant: the double life of vitamin C. Subcell Biochem 56:49-65. doi: 10.1007/978-94-007-2199-9_4.

De Tullio MC, Asard, H (2012) Molecules tell stories: reactive nitrogen, oxygen, carbonyl and sulfur species take center stage. Plant Physiol Biochem 59: 1-2 doi10.1016/j.plaphy.2012.07.025

De Tullio, M. C., Guether, M., & Balestrini, R. (2013). Ascorbate oxidase is the potential conductor of a symphony of signaling pathways. Plant signaling & behavior, 8, e23213. doi:10.4161/psb.23213

Dilley DR, Wang Z, Kadirjan-Kalbach DK, Ververidis F, Beaudry R, Padmanabhan K (2013) 1-Aminocyclopropane-1-carboxylic acid oxidase reaction mechanism and putative post-translational activities of the ACCO protein. AoB Plants 5: plt031. doi: 10.1093/aobpla/plt031

Dowdle J, Ishikawa T, Gatzek S, Rolinski S, Smirnoff N. (2007) Two genes in Arabidopsis thaliana encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. Plant J. 52:673-89. 10.1111/j.1365-313X.2007.03266.x

Farrow SC, Facchini PJ (2014) Functional diversity of 2-oxoglutarate/Fe(II)-dependent dioxygenases in plant metabolism. Front Plant Sci 10.3389/fpls.2014.00524

Flashman E, Davies SL, Yeho KK, Schofield CJ (2010) Investigating the dependence of the hypoxia-inducible factor hydroxylases (factor Inhibiting HIF and prolyl hydroxylase domain 2) on ascorbate and other reducing agents. Biochem J 427: 135-142 doi.org/10.1042/BJ20091609

Foyer CH, Noctor G. (2005) Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Env 28:1056–1071.

Hu T, Wang Y, Wang Q, Dang N, Wang L, Liu C, Zhu J, Zhan X (2019) The tomato 2-oxoglutarate-dependent dioxygenase gene *SlF3HL* is critical for chilling stress tolerance. Hortic Res 6: 45 doi:10.1038/s41438-019-0127-5

Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science. 2011;333:1300–1303.

Ji, L., Jordan, W. T., Shi, X., Hu, L., He, C., & Schmitz, R. J. (2018). TET-mediated epimutagenesis of the Arabidopsis thaliana methylome. *Nature communications*, *9*(1), 895. doi:10.1038/s41467-018-03289-7

Jeong H, Tombor B, Albert R, Zoltvai ZN, Barabasi A-L. (2000) The large-scale organization of metabolic networks. Natur*e* 407:651-654

Kawai Y, One E, Mizutani M (2014) Evolution and diversity of the 2-oxoglutarate-dependent dioxygenase superfamily of plants. Plant J. 78:328-343 doi10.1111/tpj.12479

Keller R, Renz FS, Kossmann J (1999) Antisense inhibition of the GDP-mannose pyrophosphorylase reduces the ascorbate content in transgenic plants leading to developmental changes during senescence. Plant J.19:131-141.

Kobayashi T (2019) Understanding the complexity of iron sensing and signaling cascades in plants Plant Cell Physiol 60:1440–1446 doi:10.1093/pcp/pcz038

Kuhn T (1962) The structure of scientific revolutions. University of Chicago Press, Chicago

Kundu S (2015a) Co-operative intermolecular kinetics of 2-oxoglutarate dependent dioxygenases may be essential for system-level regulation of plant cell physiology. Front Plant Sci 6: doi:10.3389/fpls.2015.00489

Kundu S (2015b) Unity in diversity, a systems approach to regulating plant cell physiology by 2-oxoglutarate-dependent dioxygenases. Front Plant Sci 6:98 doi:10.3389/fpls.2015.00489

Loiacono FV, De Tullio MC (2012) Why we should stop inferring simple correlations between antioxidants and plant stress resistance: towards the antioxidomic era. OMICS 16:160-167. doi: 10.1089/omi.2011.0099

Lu F, Li G, Cui X, Liu C, Wang X-J, Cao X (2008) Comparative analysis of JmjC domain-containing proteins reveals the potential histone demethylases in *Arabidopsis* and rice. J Integrative Plant Biol 50:886-896.

Marino D, Dunand C, Puppo A, Pauly N (2012) A burst of plant NADPH oxidases. Trends Plant Sci 17:9-15. doi: 10.1016/j.tplants.2011.10.001

Masson N, Keeley TP, Giuntoli B, White MD, Lavilla Puerta M, Perata P, Hopkinson RJ, Flashman E, Licausi F, Ratcliffe PJ (2019) Conserved N-terminal cysteine dioxygenases transduce responses to hypoxia in animals and plants. Science 365: 65-69 10.1126/science.aaw0112

Mittler R (2017) ROS are good. Trends Plant Sci. 22:11-19. doi:10.1016/j.tplants.2016.08.002.

Myllyharju J (2003) Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. Matrix Biology 22: 15-24

Myllyla, R., Majamaa, K., Gunzler, V., Hanauske-Abel, H. M. and Kivirikko, K. I. (1984) Ascorbate Is Consumed Stoichiometrically in the Uncoupled Reactions Catalyzed by Prolyl 4-Hydroxylase and Lysyl Hydroxylase. J. Biol. Chem. 259, 5403-5405

Minor EA, Court BL, Young JE, Wang G. (2013) Ascorbate induces Ten-Eleven Translocation (Tet) methylcytosine dioxygenase-mediated generation of 5-hydroxymethylcytosine. J Biol Chem 288:13669-13674. doi: 10.1074/jbc.C113.464800

Mitchell EJ, Weng JK (2019) Unleashing the synthetic power of plant oxygenases: from mechanism to application. Plant Physiol 179: 813–829 doi:10.1104/pp.18.01223

Mukherjee M, Larrimore KE, Ahmed NJ, Bedick TS, Barghouthi NT, Traw MB, Barth C. (2010) Ascorbic acid deficiency in *Arabidopsis* induces constitutive priming that is dependent on hydrogen peroxide, salicylic acid, and the NPR1 gene. Mol Plant Microbe Interact. 23:340-51. doi: 10.1094/MPMI-23-3-0340.

Nadi R, Mateo-Bonmatì E, Juan-Vicente L, Micol JL (2018) The 2OGD Superfamily: Emerging Functions in Plant Epigenetics and Hormone Metabolism. Molecular Plant 11: 1222–1224 doi:10.1016/j.molp.2018.09.002

Nishikimi M, Yagi K (1991) Molecular basis for the deficiency in humans of gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis. Am J Clin Nutr. 1991 Dec;54(6 Suppl):1203S-1208S. doi: 10.1093/ajcn/54.6.1203s.

Noctor G, Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* (1998) **49**:249-279.

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH (2003) Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. Plant Cell 15:939-51.

Pavet V, Olmos E, Kiddle G, Mowla S, Kumar S, Antoniw J, Alvarez ME, Foyer CH. (2005) Ascorbic acid deficiency activates cell death and disease resistance responses in Arabidopsis. Plant Physiol 139:1291-1303

Porta H, M. Rocha-Sosa (2002) Plant lipoxygenases. Physiological and molecular features Plant Physiol 130:15-21

Pugh CW, Ratcliffe PJ (2017) New horizons in hypoxia signaling pathways. Exp Cell Res 356:116-121 doi10.1016/j.yexcr.2017.03.008

Salminen A., Kauppinen A, Kaarniranta K (2015) 2-Oxoglutarate-dependent dioxygenases are sensors of energy metabolism, oxygen availability, and iron homeostasis: potential role in the regulation of aging process. Cell Mol Life Sci 72:3897-3914 doi:10.1007/s00018-015-1978-z

H. Schmidt, R. Kurtzer, W. Eisenreich, W. Schwab (2006) The carotenase AtCCD1 from *Arabidopsis thaliana* is a dioxygenase. J Biol Chem 281: 9845-9851

Semenza G (2002) Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology 19: 176-182 doi:10.1152/physiol.00001.2004

Song T, Zhang Q, Wang H, Han J, Xu Z, Yan S, Zhu Z (2018) *OsJMJ703*, a rice histone demethylase gene, plays key roles in plant development and responds to drought stress. Plant Physiol Biochem 132:183-188. doi10.1016/j.plaphy.2018.09.007

Soyer O, Bonhoeffer S (2006) Evolution of complexity in signaling pathways. Proceed Natl Acad Sci USA 103:16337-16342 doi10.1073pnas.0604449103/

Stone N, Meister A (1962) Function of ascorbic acid in the conversion of proline to collagen hydroxyproline. Nature 194:555-557. doi10.1038/194555a0

Suarez-Diaz E (2016) Molecular evolution in historical perspective. J Mol Evol 83: 2014-213 doi10.1007/s00239-016-9772-6

Tsukada Y, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, Zhang Y (2006) Histone demethylation by a family of JmjC domain-containing proteins. Nature 439: 811-816.

Veljovic-Jovanovic SD, Pignocchi C, Noctor G, Foyer CH. (2001) Low ascorbic acid in the *vtc-1* mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system. Plant Physiol. 127:426-435.

Vicente J, Mendiondo GM, Movahedi M, Peirats-Llobet M, Juan YT, Shen YY, Dambire C, Smart K, Rodriguez PL, Charng YY, Gray JE, Holdsworth MJ (2017) The Cys-Arg/N-End Rule Pathway Is a General Sensor of Abiotic Stress in Flowering Plants. *Curr Biol*. 2017 Oct 23;27(20):3183-3190.e4. doi: 10.1016/j.cub.2017.09.006.

Vigani G, Murgia I (2018) Iron-requiring enzymes in the spotlight of oxygen. Trends Plant Sci 23:874-882 doi:10.1016/j.tplants.2018.07.005

Vlad F, Spano T, Vlad D, Daher FB, Ouelhadj A, Kalaitzis P (2007a) *Arabidopsis* prolyl 4-hydroxylases are differentially expressed in response to hypoxia, anoxia and mechanical wounding. Physiol Plant 130:471-483 doi:10.1111/j.1399-3054.2007.00915.x

Vlad F, Spano T, Vlad D, Daher FB, Ouelhadj A, Fragkostefanakis S, Kalaitzis P (2007b) Involvement of *Arabidopsis* prolyl 4 hydroxylases in hypoxia, anoxia and mechanical wounding. Plant signaling and behavior *2*:368–369. doi:10.4161/psb.2.5.4462

Wang X, Song S, Wu Y, Li Y, Chen T, Huang Z, Liu S, Dunwell TL, Pfeifer GP, Dunwell JM, Wamaedeesa R, Ullah I, Wang Y, Hu S (2015) Genome-wide mapping of 5-hydroxymethylcytosine in three rice cultivars reveals its preferential localization in transcriptionally silent transposable element genes. J Exp Bot 66: 6651–6663. doi: 10.1093/jxb/erv372

Weits DA, Giuntoli B, Kosmacz M, Parlanti S, Hubberten HM, Riegler H, Hoefgen H, Perata P, van Dongen JP, Licausi F (2014) Plant cysteine oxidases control the oxygen-dependent branch of the N-end-rule pathway. Nat Commun 5:3425

White MD, Flashman E (2016) Catalytic strategies of the non-heme iron dependent oxygenases and their roles in plant biology. Current Opinion in Chemical Biology 31:126-135 doi:10.1016/j.cbpa.2016.02.017

White MD, Kamps JJAG, East S, Taylor Kearney LJ, Flashman E (2018) The plant cysteine oxidases from Arabidopsis thaliana are kinetically tailored to act as oxygen sensors. J Biol Chem 293: 11786–11795 10.1074/jbc.RA118.003496

Wojtaszek P, Smith GC, Bolwell GP (1999) Ultrastructural localisation and further biochemical characterisation of prolyl 4-hydroxylase from *Phaseolus vulgaris*: comparative analysis. Eur J Biochem 43:463-477 doi10.1016/S1357-2725(98)00126-5

S. Yamaguchi (2008) Gibberellin metabolism and its regulation Annu Rev Plant Biol 59: 225-251

Young JI, Zuchner S, Wang G (2015) Regulation of the epigenome by Vitamin C. Annu Rev Nutr 35: 545-564. doi10.1146/annurev-nutr-071714-034228

Zeilmaker T, Ludwig NR, Elberse J, Seidl MF, Berke L, Van Doorn A, Schuurink RC, Snel B, Van den Ackerveken G (2015) DOWNY MILDEW RESISTANT 6 and DMR6-LIKE OXYGENASE 1 are partially redundant but distinct suppressors of immunity in Arabidopsis. Plant J 81: 210–222 doi:10.1111/tpj.12719

Zechmann B, Stumpe M, Mauch F. Immunocytochemical determination of the subcellular distribution of ascorbate in plants. *Planta* (2011) **233**:1-12. doi: 10.1007/s00425-010-1275-x

Zhang J, Peer WA (2017) Auxin homeostasis: the DAO of catabolism. J Exp Bot. 68:3145-3154. doi: 10.1093/jxb/erx221.

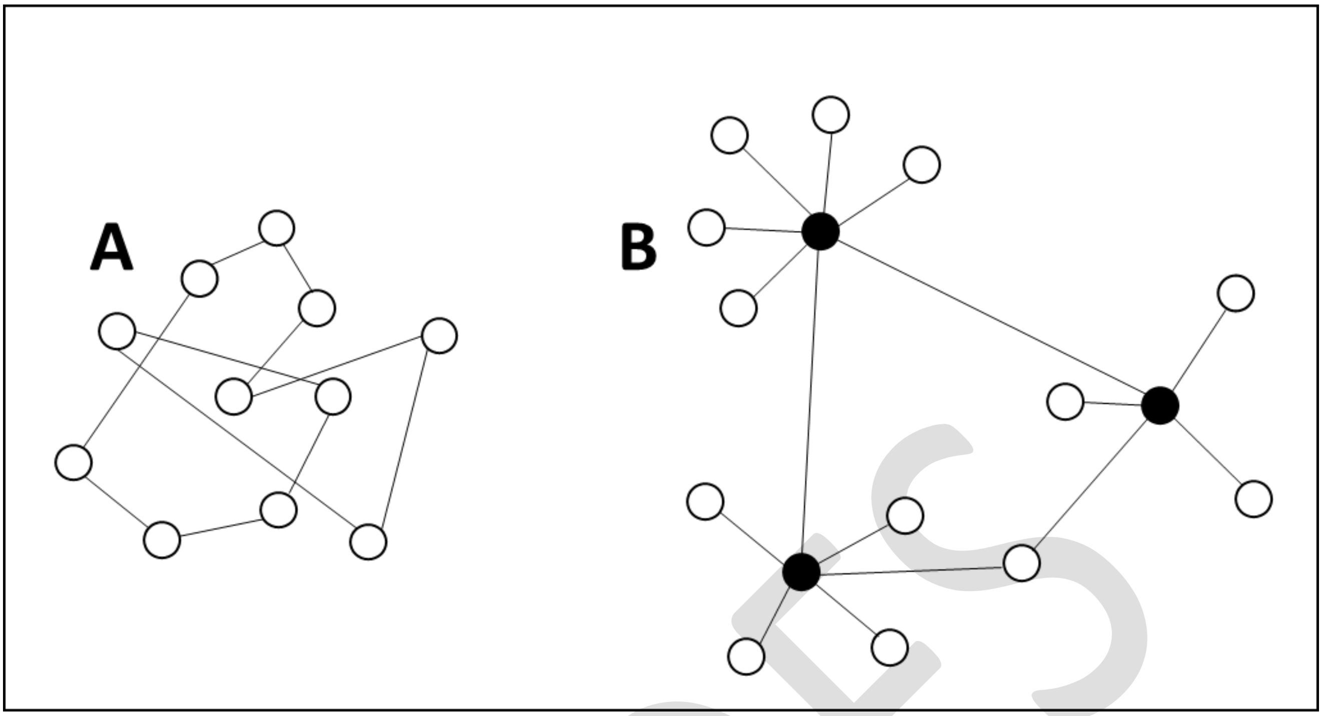

Fig.1 Two alternative models of interaction within a metabolic network. A: each node is connected randomly within a statistically homogeneous network (Erdos-Rényi model). B: scale-free model with hubs (black circles) having a large number of links. (Redrawn from Jeong et al. 2000).

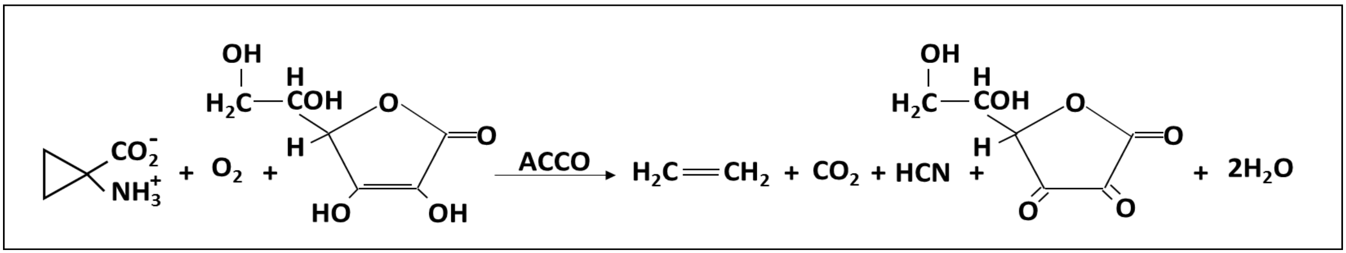

Fig.2 The reaction catalyzed by ACCO (aminocyclopropane carboxylic acid oxidase) for ethylene biosynthesis. In the reaction, ascorbic acid is oxidized to dehydroascorbic acid.

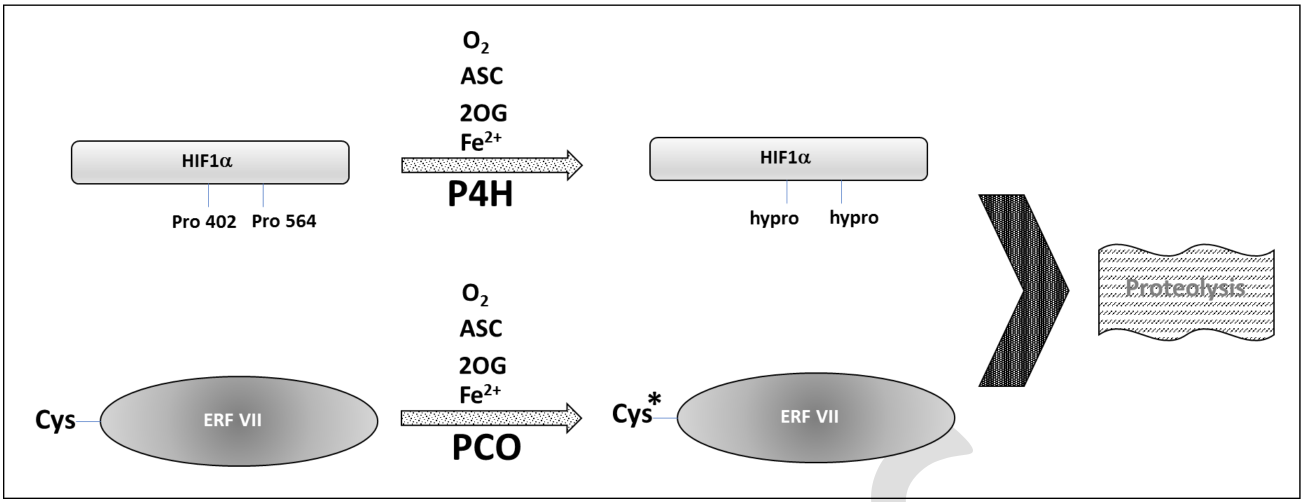

Fig.3 The similarities between the two dioxygenase-dependent oxygen-sensing mechanisms described in the text. Hydroxylation, catalyzed by prolyl hydroxylase (P4H), of two proline residues in the HIF transcription factor targets the protein to degradation. Similarly, the oxidized terminal cysteine (Cys*) resulting from the hydroxylation catalyzed by Plant Cysteine Oxidase (PCO) targets the *Arabidopsis* transcription factors of the ERF VII group to proteolysis. In the absence of the hydroxylation reaction, transcription factors move to the nucleus and operate transcriptional activation of an array of stress-responsive genes.

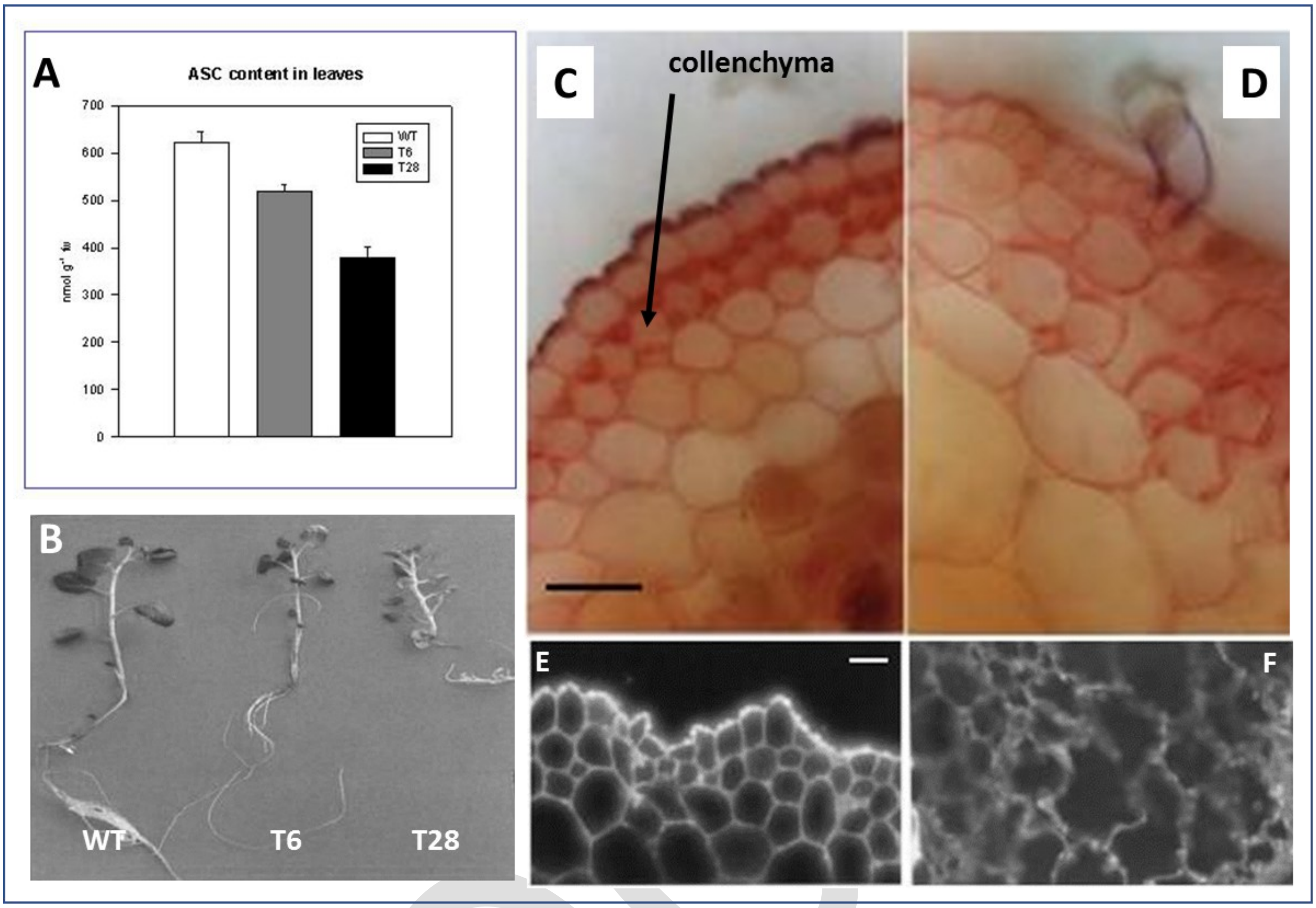

Fig. 4 The phenotype of ASC-deficient antisense *S. tuberosum* plants. WT and the two transgenic lines (T6 and T28) with reduced GDP-mannose pyrophosphorylase activity. A: ascorbic acid content measured according to Zhang and Kirkham (1996). B: phenotype of the plants after 3 weeks of culture in MS medium with 1% agar (12h photoperiod, PFD 400 micromoles m^{-2} sec^{-1}). C, D: Cross sections of the stems (third internode) of WT and T28 plants, respectively, stained with Congo Red and Methyl Green. Collenchyma cells are indicated by the arrow. Scale bar: 20 μm. E,F: Immunolocalization of extensin in WT and T28 cells, respectively. Anti-extensin antibodies were a kind gift of Mike Brownleader, University of London. Experiments performed by P. Russo and MC De Tullio using transgenic plants kindly provided by Jens Kossmann (Keller et al. 1999).

Table 1. Enzymes, which use Ascorbic acid as a cofactor (from http://www.ebi.ac.uk/thornton-srv/databases/CoFactor/enzymes.php?cid=12&pidArray=Array&oneDigitRefs=Array)

Enzyme name	**E.C. number**
violaxanthin de-epoxidase	1.10.99.3
L-ascorbate peroxidase	1.11.1.11
Gamma-butyrobetaine dioxygenase	1.14.11.1
Pyrimidine-deoxynucleoside 1'-dioxygenase	1.14.11.10
Hyoscyamine (6S)-dioxygenase	1.14.11.11
6-beta-hydroxyhyoscyamine epoxidase	1.14.11.14
Gibberellin 3-beta-dioxygenase	1.14.11.15
Taurine dioxygenase	1.14.11.17
Phytanoyl-CoA dioxygenase	1.14.11.18
Leucocyanidin oxygenase	1.14.11.19
Procollagen-proline dioxygenase	1.14.11.2
Deacetoxyvindoline 4-hydroxylase	1.14.11.20
Flavone synthase	1.14.11.22
Pyrimidine-deoxynucleoside 2'-dioxygenase	1.14.11.3
Procollagen-lysine 5-dioxygenase	1.14.11.4
Thymine dioxygenase	1.14.11.6
Procollagen-proline 3-dioxygenase	1.14.11.7
Trimethyllysine dioxygenase	1.14.11.8
Flavanone 3-dioxygenase	1.14.11.9
2-acetolactate mutase	5.4.99.3

Table 2. List of 2-oxoglutarate-dependent dioxygenases involved in plant hormone metabolism (A) and in signaling (B)

A	Hormone-related	**Name**	**Hormone**	**Reference**
		9′-Cis-epoxycarotenoid dioxygenase (NCED)	ABA	Schmidt et al. 2006
		1-Aminocyclopropane-1-carboxylate oxidase (ACCO)	Ethylene	Dilley et al. 2013
		Gibberellin GA$_3$ and GA$_{20}$ oxidase	GA	Yamaguchi 2008
		Carotenoid cleavage dioxygenase 7 (CCD7) and 8 (CCD8)	Strigolactones	Alder et al. 2012
		9-Lipoxygenase (9LOX) 13-lipoxygenase (13LOX)	Jasmonates	Porta & Rocha-Sosa 2002
		Dioxygenase for auxin oxidation (DAO)	IAA catabolism	Zhang & Peer 2017
B	Sensing/signaling	**Name**	**Function**	**Reference**
		Feruloyl CoA hydroxylase (F6′H1)	Iron deficiency	Vigani & Murgia 2018
		Plant cysteine oxidase 1-5 (PCO1-5)	O$_2$ sensing	Weits et al. 2014
		Histone arginine demethylases Jumonji	Epigenetic	Chen et al 2011
		TET methylcytosine dioxygenase	Epigenetic	Ito et al. 2011
		DOWNY MILDEW REPRESSOR (DMR6- DLO1)	Plant immunity	Zeilmaker et al. 2015