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BELGRADE SERBIA 15-18 JUNE 2021

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Urate oxidase is modulated by NO-derived post-translational modifications during the ripening of sweet pepper fruit

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Abstract

In plants, urate oxidase (UO; EC 1.7.3.3), also called uricase, is an important enzyme involved in the nitrogen cycle through the ureides' metabolism. This enzyme catalyzes the oxidation of urate to allantoin and is localized in unspecialized peroxisomes, where it plays an important role in nodules from leguminous species. In addition, UO has also been detected in other plant organs, such as leaves from several species, although at a very low level. Thus far there are no reports on either the presence of urate oxidase in fleshy fruits or how this enzyme is modulated by nitric oxide (NO) and other reactive nitrogen species (RNS) in higher plants.

In this work, comparative and complementary RNA-seq transcriptomic, gene expression through RT-PCR, and iTRAQ proteomic analyses of green and red sweet pepper (*Capsicum annuum* L.) fruits were carried out. XOD activity in peroxisomes isolated from pepper fruits, and the presence of uric acid and allantoin by HPLC-MS in this reproductive organ was also studied. The results show that UO from pepper fruits contains 307 amino acids, with a molecular weight of 34.82 kDa and pI 6.31. Although XOD activity remains unchanged, the gene expression is up regulated during ripening, a physiological process where NO and RNS play relevant roles. Besides, it was found by in vitro assays that UO is modulated by nitration and S-nitrosation events. By coupling a biotin-switch method and mass spectrometry, a cysteine susceptible to be S-nitrosated was identified in the enzyme purified from the yeast *Candida utilis*. Overall, the results suggest that UO could be involved in the ripening process of pepper fruits, where the enzyme might regulate the levels of both, uric acid and peroxynitrite (ONOO-), two molecules that interact and scavenge one to another.

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Material and methods

California-type pepper

Plant material and RNA extraction. California-type pepper (Capsicum annuum L., cv. Melchor) fruits, were collected from plants grown in plastic-covered greenhouse (Syngenta Seeds, Ltd., El Ejido, Almería, Spain). Fruits were harvested at two different developmental stages: green immature and red ripe. Total RNA was isolated from pepper fruits using a two-step method based on Trizol® Reagent (Gibco BRL) and the RNAeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions.

Library preparation and RNA-sequencing. Libraries were prepared using an optimized Illumina protocol and were sequenced on an Illumina NextSeq550 platform using 2 x 75 bp paired-end reads. Reads were pre-processed to remove low-quality sequences. Clean reads were used to perform the *de novo* transcriptome assembly. Bowtie2 was used to realign the reads and Samtools to quantify known transcripts. Differential expression analyses were carried out using DEgenes-Hunter.

Semiquantitative RT-PCR. Two micrograms of total RNA was used to produce cDNA by reverse transcription. Amplification by PCR was performed as follows: 1 µl of each cDNA (30 ng) was added to 0.250 mM dNTPs, 2.5 mM MgCl2, 1 × PCR buffer, 0.5 U of Biotools High Retrotranscriptase (Biotools B&M Labs S.A., Madrid, Spain) and 0.5 µM of specific primer in a final volume of 20 µl. *Actin* was used as internal control.

Isobaric tags for relative and absolute quantitation (iTRAQ ®). After reduction and alkylation, protein samples were combined with trypsin at a final trypsin:protein ratio of 1:10 and digested overnight at 37 ºC. Tryptic peptides were dried by vacuum centrifugation, reconstituted in labeling buffer (70% ethanol/25 mM TEAB) and labeled with iTRAQ reagents, according to the manufacturer's protocol (ABSciex, Framingham, MA, USA). Protein identification and quantification was performed by LC-MS/MS analysis using a nanoLC Ultra 1D plus/Triple TOF 5600 analyzer (ABSciex, Framingham, MA, USA).

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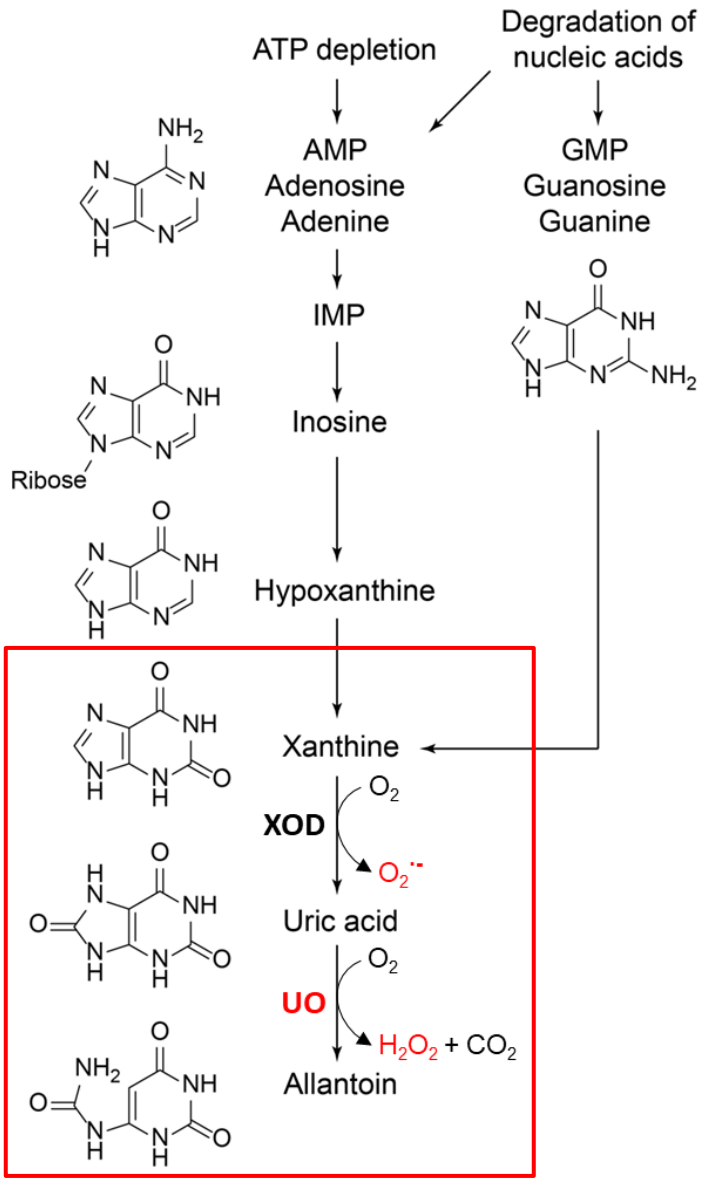
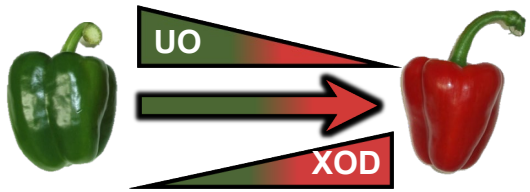
Results

Metabolism of ureides

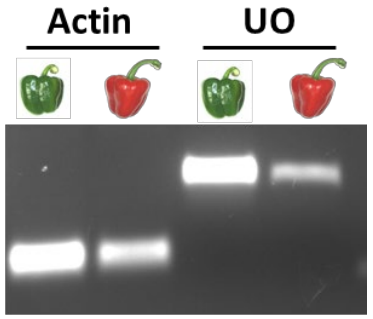


Gene Expression

RNA-Seq

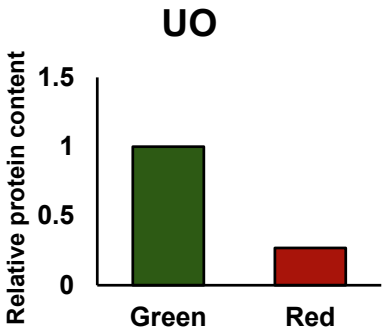


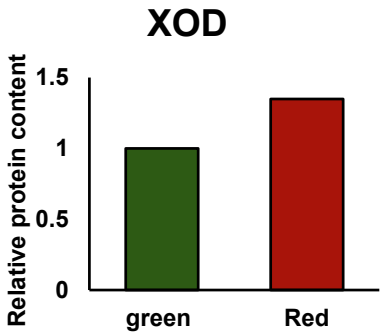
Semiquantitative RT-PCR



Relative Expression	1	1	0,89	0,40

Protein Content





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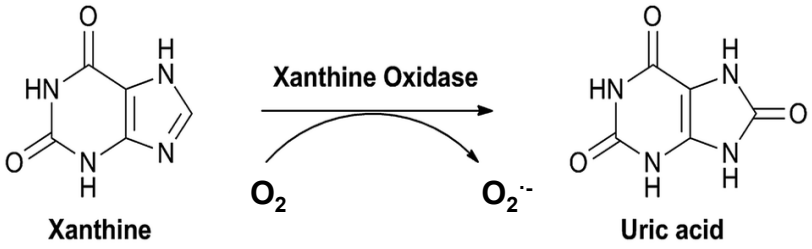
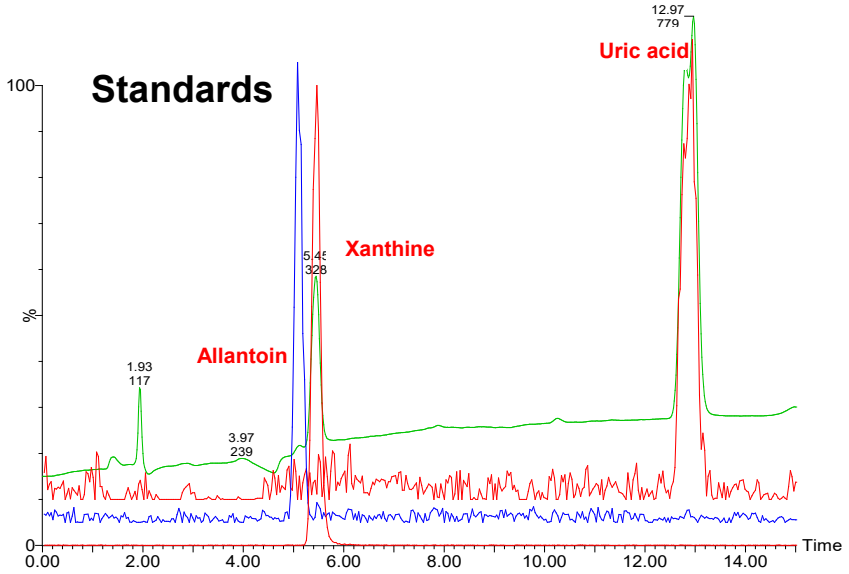
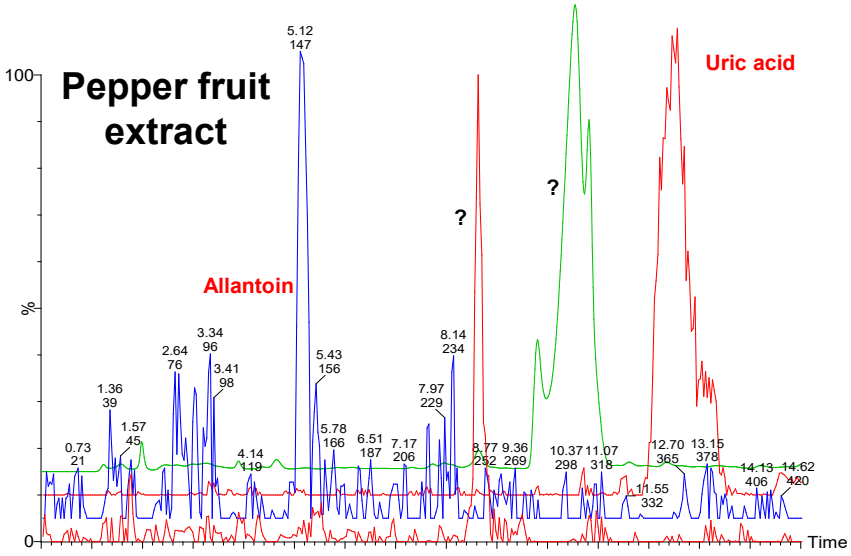
XOD in perosixomes from pepper fruits

Mateos et al. (2003) J. Plant Physiol. 160: 1507-1516



Detection of ureides – HPLC/MS





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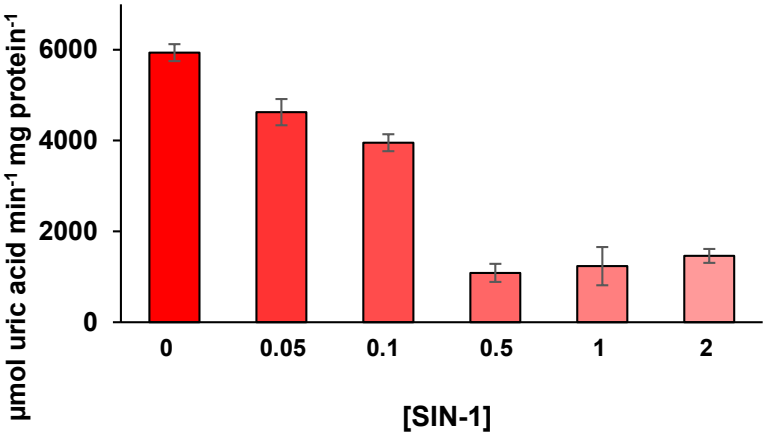
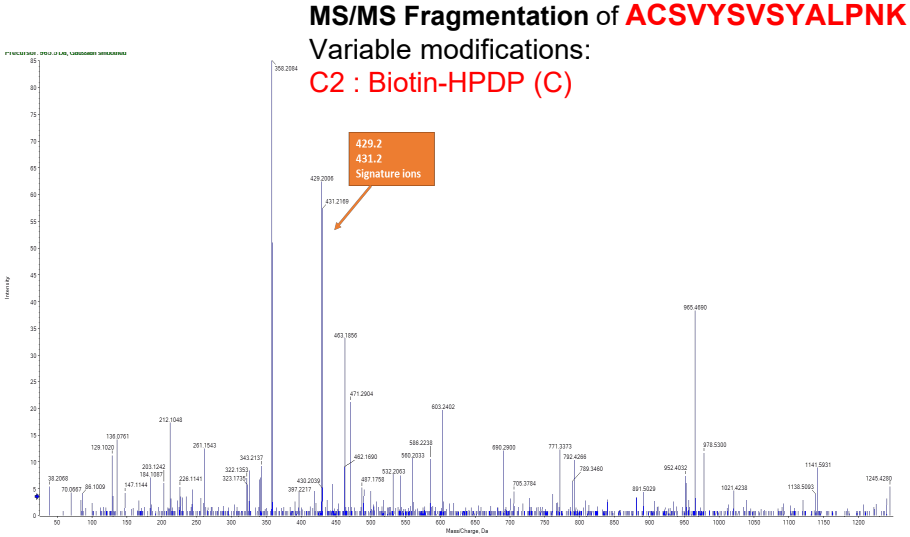
Results

NO-derived post-translational modifications (PTMs) of UO from *Candida utilis*

Nitration



Nitrosation

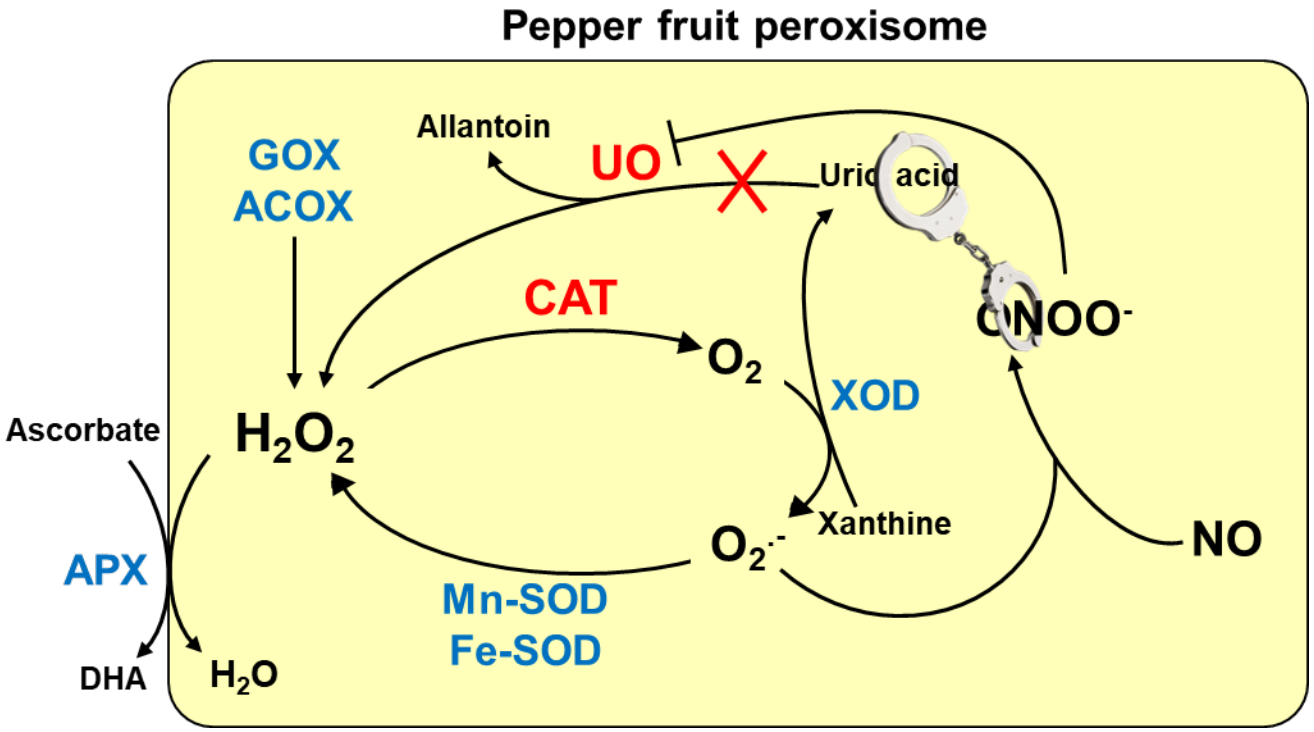


1- MSTTLSSSTY GKDNVKFLKV KKDPQNPKKQ EVMEATVTCL LEGGFDTSYT EADNSSIVPT
DTVKNTILVL AKTTEIWPIE RFAAKLATHF VEKYSHVSGV SVKIVQDRWV KYAVDGKPHD
HSFIHEGGEK RITDLYYKRS GDYKLSSAIK DLTVLKSTGS MFYGYNKCDF TTLQPTTDRI
LSTDVDATWV WDNKKIGSVY DIAKAADKGI FDNVYNQARE ITLTTFALEN SPSVQATMFN
MATQILEKAC SVYSVSYALP NKHYFLIDLK WKGLENDNEL FYPSPHPNGL IKCTVVRKEK
TKL - 303

Potentially nitrosylated

Results

Peroxisomal nitro-oxidative metabolism (Proposed model)



Modified from Palma et al. (2018) Subcell. Biochem.

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Conclusions

1) Although we were not able to detect urate oxidase activity in bell pepper fruits, our results suggest that this enzyme is actively involved in the ripening process.

1) Urate oxidase activity appears to be influenced by NO, through post-translational modifications such as nitration or *S*-nitrosation.

2) Further studies are needed to determine the influence of ureide metabolism during the ripening process of bell pepper fruit.