# INHIBITION OF ARABIDOPSIS O-ACETYLSERINE(THIOL)LYASE A1 BY TYROSINE-NITRATION*

**Consolación Álvarez[1§], Jorge Lozano-Juste[2§], Luís C. Romero[1], Irene García[1], Cecilia Gotor[1], José León[2]**

From Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC-Universidad de Sevilla, Avenida Américo Vespucio 49, 41092 Sevilla, Spain[1]; Instituto de Biología Molecular y Celular de Plantas, CSIC-Universidad Politécnica de Valencia, CPI Ed. 8E, Camino de Vera s/n, 46022 Valencia, Spain[2]

Running head: Nitration of OASA1

Address correspondence to: José León, Instituto de Biología Molecular y Celular de Plantas, CSIC-Universidad Politécnica de Valencia, CPI Ed. 8E, Camino de Vera s/n, 46022 Valencia, Spain, Tel: +34 963877882, Fax: +34 963877859, E-mail: jleon@ibmcp.upv.es

**The last step of sulfur assimilation is catalyzed by O-acetylserine(thiol)lyase (OASTL) enzymes. OASTLs are encoded by a multigene family in the model plant *Arabidopsis thaliana.* Cytosolic OASA1 enzyme is the main source of OASTL activity and thus crucial for cysteine homeostasis. We found that nitrating conditions after exposure to peroxynitrite strongly inhibited OASTL activity. Among OASTLs, OASA1 was markedly sensitive to nitration as demonstrated by the comparative analysis of OASTL activity in nitrated crude protein extracts from wild type and different *oastl* mutants. Furthermore, nitration assays on purified recombinant OASA1 protein led to 90 % reduction of the activity due to inhibition of the enzyme, as no degradation of the protein occurred under these conditions. The reduced activity was due to nitration of the protein because selective scavenging of peroxynitrite with epicatechin impaired OASA1 nitration and the concomitant inhibition of OASTL activity. Inhibition of OASA1 activity upon nitration correlated with the identification of a modified OASA1 protein containing 3-nitroTyr$^{302}$ residue. The essential role of the Tyr$^{302}$ residue for the catalytic activity was further demonstrated by the loss of OASTL activity of a Tyr$^{302}$Ala mutated version of OASA1. Inhibition caused by Tyr$^{302}$ nitration on OASA1 activity seems to be due to a drastically reduced O-acetylserine substrate binding to the nitrated protein, and also to reduced stabilization of the pyridoxal-5´-phosphate cofactor through hydrogen bonds. This is the first report identifying a Tyr nitration site of a plant protein with functional effect and the first post-translational modification identified in OASA1 enzyme.**

Sulfur is an essential nutrient for all living organisms as it is a component of the amino acids cysteine and methionine required for protein synthesis. Moreover, a major determinant in plant cellular redox control such as glutathione (GSH) also contained sulfur. Most plant sulfur-containing compounds, including GSH, are derived from Cys, which is the final product of the primary sulfate assimilation pathway. The Cys biosynthetic pathway involves two sequential reactions catalyzed by Ser acetyltransferase (SAT), which synthesizes the intermediary product, O-acetyl-Ser (OAS), from acetyl-CoA and Ser, and O-acetyl-Ser(thiol)lyase (OASTL), which incorporates sulfide, coming from the assimilatory reduction of sulfate, to OAS producing Cys. This reaction requires pyridoxal phosphate (PLP) as cofactor. There are nine genes coding for different isoforms of OASTL in the Arabidopsis genome (1). The most abundant OASTL transcripts correspond to the cytosolic OASA1, the plastidial OASB, and the mitochondrial OASC isoforms. Analysis of null alleles of different SAT and OASTL genes together with subcellular metabolite distributions in *A. thaliana* have recently shown that cysteine is predominantly formed in the cytosol, while OAS is produced in the mitochondria (2-6). The major cytosolic OASTL isoform and main responsible for cysteine biosynthesis, OASA1, is essential for heavy metal tolerance as its overexpression is sufficient to confer tolerance to elevated cadmium concentrations (7-9). By contrast, the mutant *oasa1.1* shows sensitivity to heavy metals but it is due to a constitutively reduced capacity to eliminate reactive oxygen species (ROS) under non-stressed conditions (9).

The uptake and assimilation of sulfate is strongly regulated by diverse regulatory mechanisms (for a recent review see 10). Some

components of the pathway are specifically regulated at the transcriptional level in plants, mainly the sulfate uptake and the reduction of 5'-Adenylylsulfate (APS). It has been characterized that OASA1 is regulated at the transcriptional level in different abiotic stresses such as salinity and the presence of heavy metal (7, 11). Besides, SAT and OASTL form the hetero-oligomeric cysteine synthase complex in such a way that SAT requires binding to OASTL for full activity, while bound OASTL becomes inactivated (12). It has been proposed this complex acting as a sensor of the sulfur status of the plant. Moreover, the activity of the cysteine synthase complex is also regulated at the level of the rate-limiting step catalyzed by SAT through cysteine-mediated inhibition of this enzyme, although depending on subcellular localization and plant species (13). Since cysteine biosynthesis requires the reduced sulfur in form of sulfide, which is exclusively produced through sulfate assimilatory pathway in plastids (14), the mitochondria provide the bulk of OAS (2), and the main site for Cys production is cytosol in Arabidopsis, an exchange of sulfide and OAS between subcellular compartments must be important in controlling the function of the enzyme components of the cysteine synthase complex.

Post-translational modification represents an increasingly interesting level of regulation of protein function in all living organisms. Among more than hundred different post-translational modifications characterized, those mediated by the action of nitric oxide (NO)-derived modifiers have attracted lately the attention of plant biology researchers. The most important post-translational modifications related to NO action are S-nitrosylation of Cys (15) and nitration of Tyr residues (16). Nitration of Tyr residues under physiological conditions is mostly the result of protein interaction with the strong nitrating agent peroxynitrite, which is formed by the reaction of NO with superoxide anion (17). There is a second alternative mechanism of tyrosine nitration based on the generation of $NO_2$• radicals by various hemoperoxydases in the presence of hydrogen peroxide and nitrite (18). Whatsoever, the nitration of tyrosine residues is a selective process with respect to the proteins that are nitrated and the affected tyrosine residues in a given protein (19, 20).

Despite the well documented regulation of OASTL and SAT function at the protein-protein interaction level, to our knowledge nothing has been reported about post-translational regulation of those proteins. Here we address whether post-translational modification of OASA1 may modulate its activity. Peroxynitrite-mediated nitration of crude extracts and purified recombinant protein as well as plant treatments with peroxynitrite inhibited OASTL activity through nitration of tyrosine residues. Mass spectrometry analysis of nitrated recombinant OASA1 protein allowed the identification of nitrated $Tyr^{302}$, a catalytically essential amino acid residue that is close to the previously reported key $Asn^{77}$ in O-acetylserine binding site of OASA1 (21).

## EXPERIMENTAL PROCEDURES

*Plant growth conditions and treatments*- *Arabidopsis thaliana* seeds of Col-0 wild type or different *oastl* mutants (9) were sown in moistened soil and grown under photoperiodic conditions (cycles of 16 h day and 8 h night for long days, at 22 ºC and 20 ºC). Plants were illuminated with 150 $\mu E\ m^{-2}\ s^{-1}$ cool-white fluorescent lamps and grown in 60 % relative humidity. Alternatively, surface sterilized seeds were germinated and grown in agar-supplemented Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands).

*Expression, extraction and purification of recombinant His-tagged OASA1*- The complete cDNA of OASA1 was cloned in pDEST17 vector (Invitrogen) to express a hexahistidine-tagged version of OASA1 by transformation of BL21-AI *Escherichia coli* competent cells (Invitrogen). Site-directed mutagenesis to generate $Tyr^{203}$Ala and $Tyr^{302}$Ala versions of OASA1 was performed as previously reported (22) with slight modifications. For protein induction, cell cultures with OD= 0.4 were treated with 0.1 % L-Arabinose overnight at 22º. Recombinant protein production was checked by SDS-PAGE and Western-blot analysis. Recombinant protein purification was carried out with the Ni-NTA Purification System (Invitrogen) following manufacturers recommendations. His-tagged proteins were detected by Western blot with a polyclonal anti-6His antibody (Santa Cruz Biotechnology).

*Protein extraction, immunoprecipitation and nitrating treatments*- For activity assays, around 100 mg of Col-0 and *oastl* mutant leaves were frozen and ground in liquid nitrogen and then extracted by adding extraction buffer [50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 10

μM PLP, 0.5 mM PMSF, 1 % (v/v) protease inhibitor cocktail from Sigma, USA] with or without 1 mM DTT, as described in each case, and briefly vortexing. Protein extracts were obtained by 13000 x g centrifugation at 4 ºC. For immunoprecipitation purposes, proteins were extracted in [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % (v/v) protease inhibitor cocktail from Sigma, USA] buffer. Protein extracts (1 mg) were pre-cleared with 50 μL of Protein A-agarose (EZView Sigma, USA) for 8 hours at 4 ºC. The unbound fractions were incubated overnight with 0.1 μg of monoclonal anti-3-NY antibody (Cayman, USA) at 4 ºC. To recover 3-NY-containing proteins, 60 μL of Protein A-agarose were added and incubated for 8 hours at 4 ºC. After extensive washing with extraction buffer, proteins were eluted at 95 ºC with elution buffer (1% SDS, 100 mM DTT, 50 mM Tris-HCl pH=7.6) three times. Protein was quantified by Bradford´s method (23).

Nitrating treatments on crude extracts or purified recombinant protein was performed by treatment with peroxynitrite generated from sodium nitrite plus hydrogen peroxide, or 3-morpholinosydnonimine hydrochloride (SIN-1; Invitrogen) at the indicated concentrations, as previously reported (24, 25). *In planta* peroxynitrite treatments were performed by infiltrating leaves with SIN-1. Selective scavenging of peroxynitrite with epicatechin (26) was used to assess the specificity of protein nitration and not oxidation and the subsequent inhibition of OASTL activity.

*Measurement of OASTL activity*- OASTL activity was measured using the method described previously (27) in protein extracts obtained as described above. Cysteine was determined by measuring optical density at 560 nm after the formation of a complex with nynhidrine (28).

*Western blots*- Protein extracts were separated by 10 % SDS-PAGE, blotted onto nitrocellulose membrane, stained with Ponceau-S and probed with antibodies at the followed dilutions: monoclonal anti-3-NY (Cayman Chemicals) 1:1000, anti-His (Santa Cruz) 1:500, and custom-made polyclonal anti-recombinant OAS-A1 antibody (Biomedal S.L.) 1:10000. Secondary antibody was anti-mouse or anti-rabbit, for monoclonal or polyclonal primary antibodies respectively, coupled to horseradish peroxidase (GE, UK) at 1:10000 dilution, and ECL or ECL advance kit (GE, UK) was used for immunoreactive protein detection.

*MS analysis*- Samples were digested with sequencing grade trypsin, chymotrypsin or the glutamic acid specific V8 protease (Promega, USA) to improve peptide sequence coverage. Peptide separation by LC-MS/MS was performed using an Ultimate nano-LC system (LC Packings) and a QSTAR XL Q-TOF hybrid mass spectrometer (MDS Sciex-Applied Biosystems). Samples (5 μL) were delivered to the system using a FAMOS autosampler (LC Packings) at 40 μL min-1, and the peptides were trapped onto a PepMap C18 pre-column (5 mm, 300 m i.d; LC Packings). Peptides were then eluted onto the PepMap C18 analytical column (15 cm, 75 m i.d; LC Packings) at 200 nl min-1 and separated using a 55 min gradient of 15–50% ACN (120 min for the mixtures). The QSTAR XL was operated in information-dependent acquisition mode, in which a 1-s TOF MS scan from 400–2000 m/z, was performed, followed by 3-s product ion scans from 65–2000 m/z on the three most intense doubly or triply charged ions. Database search on Swiss-Prot and NCBInr databases was performed using MASCOT search engine (Matrix- Science). Searches were done with the different proteases specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.8 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met, deamidation of Asn and Gln, and nitration of Tyr as variable modifications.

*Structural analysis of OASA1*- Three-dimension structure of OASA1 (At4g14880) was obtained from protein data bank (PDB code access 1Z7W). The structure was visualized with Yasara (www.yasara.org) or PyMol (www.pymol.org) software. Distance between residues in Amstrongs (Å) and the presence of hydrogen bonds was carried out with both programs with default settings.

## RESULTS

*Inhibition of OASTL activity under nitrating conditions*- Nitration of many different cellular acceptors including tyrosine residues of proteins is the result of either peroxynitrite action or $NO_2$• radicals attack on the corresponding targets. Peroxynitrite can be produced *in situ* by different donors including 3-morpholinosydnonimine (SIN-1). Similarly, $NO_2$• radicals can be produced *in vitro* by treatment with nitrite and hydrogen peroxide.

We have tested whether nitrating treatments altered the OASTL activity of crude protein extracts from Arabidopsis. Figure 1A shows that nitration with 1 mM nitrite / peroxide treatment led to a reduction of 81 % in OASTL activity levels in wild type extracts without DTT, and to around 37 % in the presence of DTT, commonly used in the extraction buffer for OASTL activity (7-9). OASTL activity associated to OASA1 represented around 65 % to 70 % of total activity present in wild type Arabidopsis crude extracts, as *oasa1.1* extracts had 30 % to 35 % of the activity present in wild type plants (Fig. 1A) (9). Similar treatments on *oasa1.1* extracts led to a reduction of OASTL activity of 38 % and 53 % with and without DTT, respectively (Fig. 1A). The reduction of OASTL activity upon nitration must be due to inactivation of the enzyme as no changes in the OASA1 protein levels could be detected in any of the described treatments (Fig. 1A). Moreover, crude extracts from wild type and mutants of the most abundant OASTLs were also nitrated with 0.5 mM SIN-1 in the absence of DTT. Nitrated wild type, *oasb* and *oasc* mutant extracts retained 22 %, 19 % and 40 %, respectively, of the OASTL activity present in non-nitrated controls (Fig. 1B). However, when nitrated the two allele mutants *oasa1.1* and *oasa1.2,* extracts retained 69 % of the activity of its corresponding control (Fig. 1B). As shown with nitrite / peroxide treated samples, SIN-1 treatment did not produce OASA1 protein degradation as tested by Western blot (data not shown). These data suggest that OASA1 is more sensitive to nitration-mediated inhibition than other OASTLs. Based on wild type and *oasa1* allele activities upon nitration, SIN-1 and nitrite / peroxide treatments inhibited 37 % and 47 % of the OASTL activity catalyzed by non OASA1 proteins (TABLE ONE). In turn, OASA1 activity was 88 % and 95 % inhibited by both nitrating treatments (TABLE ONE). Differential effect of nitration in inhibiting activity of OASA1 and other OASTLs moved us to analyze the effects of nitrating treatment on purified recombinant OASA1.

Samples of purified recombinant OASA1 obtained from *E. coli* expressing an N-terminal hexahistidine-tagged version were tested for OASTL activity after treatment with increasing concentrations of SIN-1. At 250 μM SIN-1 significant inhibition was already detected and 87 % inhibition occurred at 500 μM (Fig. 2A). This percentage of inhibition is fully consistent with the estimation of inhibition of the OASTL activity corresponding to OASA1 detected in nitrated crude extracts (TABLE ONE). Reduction of OASTL activity under these nitrating conditions was not due to degradation of protein as demonstrated by unaltered levels of proteins present in samples treated with increasing SIN-1 concentrations (Fig. 2B). Correlating with the strong inhibition of OASTL activity of OASA1 the corresponding Y-nitrated form of OASA1 was detected by Western blot using an anti-3-nitroY antibody (Fig. 2A). We checked that inhibition of OASA1 activity was specifically associated to nitration and not to oxidation by using the peroxynitrite scavenger epicatechin (26). Increasing concentrations of epicatechin blocked SIN-1 mediated OASA1 nitration and the concomitant inhibition of OASTL activity (Fig. 2B). Moreover, the protective effect of epicatechin specifically on OASA1 was supported by the negligible effect caused by epicatechin on crude extracts from the *oasa1.1* mutant (Fig. 2C).

*Identification of Y-nitration site in nitrated OASA1*- Nitrated and non-nitrated samples of purified OASA1 used for inhibition assays described above were further analyzed by mass spectrometry. Samples were digested with chymotrypsin or trypsin and analyzed by LC-MS/MS. Nitrated and non-nitrated samples were confirmed to be OASA1 with MASCOT scores (sequence coverages) of 725 (79 %) and 1207 (88 %) for trypsin-digested samples and 248 (24 %) and 163 (40 %) for chymotrypsin-digested samples, respectively. By LC-MS/MS, the nitrated peptide Y($NO_2$)LSTVLFDATR (Z = 2; m/z = 665.828096; MASCOT Ion Score: 76; Expect: 1.5e-006; Monoisotopic mass of neutral peptide: 1329.6565) was identified with an increase mass of 44.99 Da compatible with acquisition of a nitro group in Y302 (Figure 3). OASA1 contains 7 Tyr residues distributed all along the amino acid sequence but most of them are located far away from the active site in the three-dimensional structure, except $Tyr^{302}$ which is directed to the active site (Fig. 4A). $Tyr^{302}$ is located at 4.7 Å from the $Asn^{77}$ (Fig. 4B), and forming a hydrogen bond with this amino acid (Fig. 4C), which has been previously reported to be essential for the activity (21). $Asn^{77}$ interacts with the O-acetylserine and SAT binding site and through hydrogen bond stabilizes pyridoxal phosphate (PLP) cofactor anchored by $Lys^{46}$ (21). Changes in spatial conformation of PLP and substrate microenvironment sites may also alter the efficiency of substrate binding to the

active site. Upon binding of O-acetylserine to the active site of the enzyme, the substrate reacts with PLP yielding the α-aminoacrylate intermediate with an absorbance maximum at 470 nm (21, 29). We measured the increase in absorbance at 470 nm of purified OASA1 in the presence of increasing concentrations of O-acetylserine and compared to the values obtained using the same amount of nitrated protein. The non-nitrated OASA1 increases its absorbance at 470 nm with increasing OAS concentration as expected. By contrast, no increase in absorbance was detected in nitrated OASA1 (Fig. 4D), suggesting that either binding of O-acetylserine is severely hindered by nitration of $Tyr^{302}$ or binding occurs but far enough from the PLP site to avoid productive interaction between substrate and cofactor.

We have further confirmed the essential role of $Tyr^{302}$ for OASTL activity of OASA1 by constructing a site-directed $Tyr^{302}$Ala mutant version that retained less than 3 % of the activity of the unmodified recombinant OASA1 protein (Fig. 5). This effect was specific for $Tyr^{302}$ as another mutated version $Tyr^{203}$Ala, with a mutation in a Tyr residue that was not identified above as a functional target of nitration, retained 88 % of the activity and responded to SIN-1 nitrating treatment with nitration of the mutated protein and consequent inhibition of the activity (Fig. 5) similarly to that observed for unmodified OASA1 (Fig. 2A).

*In vivo detection of nitrated OASA1*- To test whether the nitration of Y detected in OASA1 *in vitro* could be physiologically relevant, we performed an immunoprecipitation assay of wild type protein extracts with anti-3-nitroY antibody. The immunoprecipitated proteins were analyzed by Western blot with an antibody against OASA1. Figure 6A shows that a small fraction of the OASA1 protein present in the crude extract was detected in the immunoprecipitated fraction, suggesting that OASA1 may be Y-nitrated under physiological conditions in the absence of an exogenous nitrating treatment. Despite efforts to identify *in vivo* Tyr-nitrated peptides we did not success, likely because of the low amounts of nitrated protein that are expected under physiological conditions. We then proceed to perform *in planta* nitration experiments by infiltrating leaves with SIN-1 and further protein extraction and OASTL activity analysis. Figure 6B shows that by 90 min after treatment with 2 mM SIN-1, OASTL activity was 34 % inhibited. Inhibition of activity correlated with detection of a significant fraction of nitrated OASA1 in SIN-1 treated leaf samples (Fig. 6B).

## DISCUSSION

Cysteine biosynthesis is a crucial process because this amino acid is a constituent of proteins. Moreover, Cys is a precursor for a huge number of essential bio-molecules, such as many plant defense compounds formed in response to different environmental adverse conditions. Among them, glutathione is the most determinant molecule in controlling the redox state of the cells. The main site of cysteine biosynthesis is the cytosol in *A. thaliana*, and therefore the major contributor to this synthesis is OASA1, but in its unbound form, when it is fully active. Association and dissociation of the cysteine synthase complex appears to be a level of regulation of cysteine biosynthesis in response to sulfur nutrition perturbations (12). The gene coding for OASA1 is also transcriptionally regulated by stress conditions (7, 11). Sulfate assimilation has been proposed to be extensively regulated by abiotic stress at the post-transcriptional level acting on plant adenosine 5'-phosphosulfate reductase (APR) through a complex network of multiple signals (30). However, besides transcriptional control of *OASTL* genes and interaction with SAT, no other level of regulation has been reported for OASTL function. A rapid way to modulate the function of OASTL may be through post-translational modification of the protein. However, to date no such modification has been identified in plants. Many stress activated responses have in common the participation of redox processes. Under oxidative, nitrosative or nitrative conditions plant proteins can undergo several modifications including oxidation of Met, Cys or Trp residues, nitration of Tyr and nitrosylation of Cys (31). The main physiological nitrating molecules are the radicals $NO_2$• and $ONOO^-$, which are in turn produced from nitric oxide by further oxidation (32). Although $NO_2$• and $ONOO^-$ are extremely reactive, their interactions with proteins do not lead to stochastic nitration of Y residues of the target protein. Factors determining the selectivity of Y nitration in proteins include the exposure of the aromatic ring to the surface of the protein, the location of the Y on a loop structure, its association with a neighbouring negative charge, the proximity of the proteins to the site of generation of nitrating

agents, their abundance and the local environments of the Y residues (19, 33). Moreover, the physiological significance of a regulatory mechanism based on the differential effect of a post-translationally modified protein relies on the conservation of that residue in others proteins with the same activity. This work reports the negative effect of $Tyr^{302}$ nitration on the OASTL activity of OASA1. This protein, which represents the main isoform in Arabidopsis, contains 7 Tyr residues spanning the whole primary sequence of the protein. Three-dimensional structure of OASA1 allowed the spatial location of different Y residues respect to the surface molecule and proximity to the catalytic site (Fig. 4). TABLE TWO summarizes the parameters defining the position and the microenvironment of every Tyr residue in OASA1 protein. Among seven Tyr residues of OASA1 only two, $Tyr^{91}$ and $Tyr^{302}$, are located in loops, are conserved in the family of OASTL Arabidopsis proteins (3) and have both negatively and positively charged amino acids close enough (TABLE TWO) to fulfill theoretical requirements for being targets of nitration (19,33). In our approach using purified recombinant OASA1 protein nitrated in vitro, only $Tyr^{302}$ was identified as 3-nitroTyr (Fig. 3). The identification of $Tyr^{302}$ in nitrated OASA1 that lost most of its OASTL activity is consistent with the location of this residue very close to the active site of the enzyme (Fig. 4). In fact, $Tyr^{302}$ is only 4.7 Å from the $Asn^{77}$, which is a key amino acid for the efficient binding of the substrate O-acetylserine to OASA1 (21). Moreover, $Asn^{77}$ and other closely located amino acids including $Ser^{269}$, $Gly^{181}$, $Gly^{183}$, $Thr^{183}$ and $Thr^{185}$ are all involved in stabilizing PLP through hydrogen bonds with the phosphate group of the cofactor (21). It is tempting to propose that 3-nitro$Y^{302}$ might introduce a perturbation in the microenvironment of PLP cofactor leading to a more restricted accessibility of the substrates to the catalytic site or to unproductive substrate binding. Productive O-acetylserine binding to OASA1 can be easily tested because the conversion to cysteine is mediated by the formation of an α-aminoacrylate intermediate with PLP that show a maximum of absorbance at 470 nm. An increase in $A_{470}$ should be detected when appropriate O-acetylserine binding occurs and that is what we observed with purified OASA1 (Fig. 4C). However, a similar assay with the nitrated $Tyr^{302}$-containing protein led to no increase in $A_{470}$ (Fig. 4C), suggesting that modification of this amino acid either drastically hinder binding of the substrate to the enzyme, or the conformation of the active site has been altered enough to allow binding of the substrate but not close enough to PLP to form the α-aminoacrylate intermediate required to complete the transformation of O-acetylserine to cysteine. By contrast, $Tyr^{91}$ is located in a surface loop far from the catalytic site of the enzyme (Fig. 3A) and thus, even if nitrated under certain conditions, less effect on catalytic activity should be expected. Because phosphorylation of Tyr is a post-translational modification with a big impact on general plant signalling (34), and particularly in hormone-derived signalling (35-37), nitration of Tyr may have not only an effect itself but may interfere also with phosphorylation, thus being doubly important in regulating function of the modified protein. Interestingly, prediction of Tyr-phosphorylation sites in OASA1 gave a high score only to $Tyr^{158}$ and $Tyr^{302}$ (TABLE TWO). The potential modification of $Tyr^{302}$ by either phosphorylation or nitration may represent also a new still unexplored regulatory mechanism in controlling OASA1 function. Moreover, it has been demonstrated that O-acetylserine and the C terminus of SAT compete for the same binding site (38), and the modification of $Tyr^{302}$ (Fig. 4B) may have influence on the formation or disruption of the Cys synthase complex. It is clear whatsoever that $Tyr^{302}$ is an essential residue for OASTL activity of OASA1 as demonstrated by the drastically reduced activity detected in a $Tyr^{302}$Ala mutated version of OASA1 (Fig. 5).

The fact that even under an extensive *in vitro* nitration assay on OASA1 only one of the Y residues of the protein has been identified as nitrated tells about the selectivity of the Y nitration process. Alternatively, the ratio of nitrated molecules to the non-nitrated protein molecules present in the sample may be very low thus making the MS-based identification difficult. This may explain why we could not identify the 3-nitro $Tyr^{302}$ modification *in vivo*. Nevertheless, crude extracts that were not exogenously nitrated should contain nitrated forms of OASA1, as immunoprecipitation with anti-3-nitroTyr antibody and further detection by Western blot with a specific anti-OASA1 antibody allowed the recovery and detection of OASA1 protein in the immunoprecipitated fraction (Fig. 6A). Since immunoprecipitation did not certainly led to an OASA1 enrichment

(Fig. 6A) compared to levels detected in the crude extracts, it is likely that the amount of nitrated forms *in vivo* are far below the levels of non-nitrated form of the protein. We have anyway confirmed the in vivo effect of nitration on OASA1 by infiltration of leaves with SIN-1 and further protein and activity analysis.

Post-translational modification of OASA1 by Y-nitration may represent a rapid and efficient regulatory mechanism to control the biosynthesis of cysteine and glutathione in response to stress factors. Both abiotic and biotic stress factors lead often to the production of reactive oxygen and nitrogen species in the stressed plants. Simultaneous production of NO (39) and superoxide anion in cells of stressed plants correlates with the production of strong nitrating peroxynitrite, which can nitrate Tyr residues of proteins. Cytosolic OASA1 is the main OASTL enzyme so an efficient control of its activity is crucial for controlling cysteine homeostasis under stress conditions. Recently, new insights on the function of this enzyme as a determinant of the antioxidative capacity of the cytosol have been proposed (9). Heavy metals have been characterized as inducers of cysteine biosynthesis (7, 8), and also as activators of NO production in eukaryotic algae and plants (40, 41). NO-derived and peroxynitrite-mediated Tyr-nitration of OASA1 may represent a rapid mechanism to control the amplitude and duration of the responses triggered by heavy metals or any other stress factors in plants. Alternatively, this sort of rapid inactivation mechanism could be operative only in tissues undergoing the direct effect of the stress factor, thus allowing the execution of the stress-activated responses in the rest of the plant. This local inactivation mechanism of OASA1 could be useful to avoid an extra production of cysteine and/or glutathione, which may act as a strong scavenger of the reactive oxygen and nitrogen species in the stressed area, thus limiting the activation of downstream signaling processes required for an efficient stress-related response in the whole plant. Rapid and local inactivation of enzymes could represent not only an efficient mechanism to modulate responses to stress, but also as a way to restrict enzyme activities in certain plant organs or under specific developmental stages, what makes this post-translational modification a relevant level for the regulatory effects exerted by sulfur-containing compounds on development and responses to stress. The identification of the Tyr-nitration site in OASA1 protein and the subsequent inhibition of enzyme activity represent, to our knowledge, the first report identifying a post-translational modification of plant OASTLs and the first identification and further characterization of a nitration site in a plant protein with strong impact on its function.

## REFERENCES


1. Wirtz, M., Droux, M., and Hell, R. (2004) *J. Exp. Bot*. **55**, 1785–1798
2. Haas, F.H., Heeg, C., Queiroz, R., Bauer, A., Wirtz, M., and Hell, R. (2008) Plant Physiol. **148**, 1055-1067
3. Heeg, C., Kruse, C., Jost, R., Gutensohn, M., Ruppert, T., Wirtz, M., and Hell, R. (2008). Plant Cell **20**, 168–185
4. Krueger, S., Niehl, A., Lopez Martin, M.C., Steinhauser, D., Donath, A., Hildebrandt, T., Romero, L.C., Hoefgen, R., Gotor, C., and Hesse, H. (2009) *Plant Cell Environ*. **32**, 349-367
5. Watanabe, M., Kusano, M., Oikawa, A., Fukushima, A., Noji, M., and Saito, K. (2008) *Plant Physiol.* **146**, 310-320
6. Watanabe, M., Mochida, K., Kato, T., Tabata, S., Yoshimoto, N., Noji, M., and Saito, K. (2008) *Plant Cell* **20**, 2484-2496
7. Dominguez-Solís, J.R., Gutierrez-Alcala, G., Vega, J.M., Romero, L.C., and Gotor, C. (2001) J. Biol. Chem. **276**, 9297–9302
8. Dominguez-Solís, J.R., Lopez-Martin, M.C., Ager, F.J., Ynsa, M.D., Romero, L.C., and Gotor, C. (2004) *Plant Biotechnol. J*. **2**, 469-476
9. López-Martín, M.C., Becana, M., Romero, L.C., and Gotor, C. (2008). *Plant Physiol*. **147**, 562–572
10. Yi, H., Galant, A., Ravilious, G.E., Preuss, M.L., and Jez, J.M. (2010) *Mol. Plant* **3**, 269-279
11. Barroso, C., Romero, L.C., Cejudo, F.J., Vega, J.M., and Gotor, C. (1999) *Plant Mol. Biol.* **40**, 729-736
12. Wirtz, M., and Hell, R. (2006) *J. Plant Physiol.* **163**, 273-286

13. Droux, M. (2004) *Photosynth. Res*. **79**, 331-348
14. Leustek, T., Martin, M.N., Bick, J.A., and Davies, J.P. (2000) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**, 141–165
15. Lindermayr, C., and Durner, J. (2009) *J. Proteomics* **73**, 1-9
16. Corpas, F.J., Chaki, M., Leterrier, M., and Barroso, J.B. (2009) *Plant Signal. Behav*. **4**, 920-923
17. Szabó, C., Ischiropoulos, H., and Radi, R. (2007) *Nat. Rev. Drug Discov*. **6**, 662-680
18. Thomas, D.D., Espey, M.G., Vitek, M.P., Miranda, K.M., and Wink, D.A. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12691–12696
19. Ischiropoulos, H. (2003) *Biochem. Biophys. Res. Commun*. **305**, 776-783
20. Abello, N., Kerstjens, H.A., Postma, D.S., and Bischoff, R. (2009) *J. Proteome Res.* **8**, 3222-3238
21. Bonner, E.R., Cahoon, R.E., Knapke, S.M., and Jez, J.M. (2005) *J. Biol. Chem.* **280**, 38803–38813
22. Atanassov, I.I, Atanassov, I.I, Etchells, J.P., and Turner, S.R. (2009) *Plant Methods* **5**:14
23. Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248–254
24. Radi, R. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 4003–4008
25. Zhang, Y., Lu, N., and Gao, Z. (2009) *Int. J. Biochem. Cell Biol*. **41**, 907-915
26. Schroeder, P., Klotz, L-O. Buchczyk,, D.P., Sadik, C.D. Schewe, T., and Sies, H. (2001) *Biochem. Biophys. Res. Commun.* **285**, 782–787
27. Barroso, C., Vega, J.M., and Gotor, C. (1995). *FEBS Lett*. **363**, 1–5
28. Gaitonde, M.K. (1967) *Biochem. J*. **104**, 627-633
29. Tai, C.H., and Cook, P.F. (2001) *Acc. Chem. Res*. **34**, 49-59
30. Koprivova, A., North, K.A., and Kopriva, S. (2008) *Plant Physiol*. **146**, 1408-1420
31. Rinalducci, S., Murgiano, L., and Zolla, L. (2008) *J. Exp. Bot*. **59**, 3781-3801
32. Durzan, D.J., and Pedroso, M.C. (2002) *Biotechnol. Genet. Eng. Rev*. **19**, 293-337
33. Souza, J.M., Daikhin, E., Yudkoff, M., Raman, C.S., and Ischiropoulos, H. (1999) *Arch. Biochem. Biophys.* **371**, 169-178
34. de la Fuente van Bentem, S., and Hirt, H. (2009) *Trends Plant Sci*. **14**, 71-76
35. Ghelis, T., Bolbach, G., Clodic, G., Habricot, Y., Miginiac, E., Sotta, B., and Jeannette, E. (2008) *Plant Physiol*. **148**, 1668-1680
36. Oh, M.H., Wang, X., Kota, U., Goshe, M.B., Clouse, S.D., and Huber, S.C. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 658-663
37. Kim, T.W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J.X., Sun, Y., Burlingame, A.L., and Wang, Z.Y. (2009) *Nat. Cell Biol.* **11**, 1254-1260
38. Francois, J.A., Kumaran, S., and Jez, J.M. (2006) *Plant Cell* **18**, 3647-3655
39. Lozano-Juste, J., and León, J. (2010) *Plant Physiol.* **152**, 891-903
40. Zhang, L.P., Mehta, S.K., Liu, Z.P., and Yang, Z.M. (2008) *Plant Cell Physiol*. **49**, 411-419
41. De Michele, R., Vurro, E., Rigo, C., Costa, A., Elviri, L., Di Valentin, M., Careri, M., Zottini, M., Sanità di Toppi, L., and Lo Schiavo, F. (2009) *Plant Physiol*. **150**, 217-228

## FOOTNOTES

* The work was supported by grants BIO2008-00839 and CONSOLIDER TRANSPLANTA CSD2007-00057 to J.L. and BIO2010-15201 to C.G. [§] These authors contributed equally to this work. C.A acknowledges JAE program for fellowship support. Helpful assistance with three dimension protein structure analysis by Rafael Ruiz-Partida from Centro de Investigación Principe Felipe (CIPF) is gratefully acknowledged. Proteomic analysis was performed in the Laboratorio de Proteómica from CIPF.

The abbreviations used are: APS, 5'-Adenylsulfate; APR, adenosine 5´phosphosulfate reductase; CE, crude extract; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; IP, immunoprecipitate; LC-MS/MS, Liquid Chromatography Tandem Mass Spectrometry; MS, Murashige and Skoog medium;

NO, nitric oxide; OAS, O-acetyl-Ser; OASTL, O-acetylserine(thiol)lyase; PLP, pyridoxal-5´-phosphate; PMSF, phenylmethanesulfonylfluorid; ROS, reactive oxygen species; SAT, Serine acetyltransferase; SDS, sodium dodecyl sulfate; SIN-1, 3-morpholinosydnonimine hydrochloride; Sup, supernatant; TCA, Trichloroacetic acid.

## TABLES

TABLE ONE

**Sensitivity of OASA1 and other OASTL enzymes to nitration-mediated inhibition.**

OASTL activity associated to OASA1 was estimated by the difference between the total activity measured in wild type seedlings and that measured in *oasa1.1* mutant. Nitration (N) was performed by treatments for one hour with nitrite/peroxide or SIN-1 and no DTT as indicated. Untreated samples were used as controls (C). Values (nmol $min^{-1}$) are the mean of three replicates ± standard deviation. Relative percentages of inhibition were calculated for OASA1 and the rest of OASTLs after nitration by both treatments.

| | wt C | *oasa1.1* C | OASA1 C | Other OASTLs C | wt N | *oasa1.1* N | OASA1 N | Other OASTLs N | Inhibition (%) OASA1 | Inhibition (%) Other OASTLs |
|---|---|---|---|---|---|---|---|---|---|---|
| Nitrite/ $H_2O_2$ | 3020±99 | 970±49 | 2050 | 970 | 559±85 | 458±22 | 101 | 458 | 95 | 47 |
| SIN-1 | 603±3 | 231±3 | 372 | 231 | 189±25 | 145±30 | 44 | 145 | 88 | 37 |

TABLE TWO

**Theoretical requirements of Tyr residues of OASA1 to be nitrated and *in silico* prediction of phosphorylation potential.**

Distances between residues were calculated from the three-dimension structure model shown in Figure 4 with the analysis performed with Yasara software as indicated in Experimental procedures. Prediction of phosphorylation potential was analyzed with the NetPhos 2.0 software from CBS (http://www.cbs.dtu.dk/services/NetPhos/). * Significant score for Tyr phosphorylation site prediction.

| Tyr | Conserved | Distance to Asp/Glu | Proximal basic amino acids in primary sequence | Location in loop | Tyr phosphorylation score |
|---|---|---|---|---|---|
| 20 | No | - | No | No | 0.156 |
| 91 | Yes | 3.9 Å to Glu58 | Lys92 | Yes | 0.043 |
| 143 | Yes | 8.1 Å to Asp56 | No | No | 0.050 |
| 158 | No | 4.3 Å to Glu195 | His157 | No | 0.912* |
| 192 | Yes | 7.6 Å to Glu195 | Lys191 | No | 0.138 |
| 203 | No | 5.7 Å to Glu242 | Lys201 | No | 0.032 |
| 302 | Yes | 11.2 Å to Glu300 | Arg301 | Yes | 0.718* |

## FIGURE LEGENDS

Fig. 1. Effect of nitration on OASTL activity and OASA1 protein levels of wild type and mutant extracts. Total OASTL activity and OASA1 protein levels were analyzed in wild type and *oasa1.1* extracts obtained either in the presence (+DTT) or absence of (-DTT), and either non-nitrated as control (C) or nitrated (N) by treatment with 1 mM nitrite / hydrogen peroxide mixture for one hour. Anti-OASA1 antibody crossreacted preferentially with OASA1 but showed also a weaker crossreaction with OASB isoform. Ponceau S staining of Rubisco is shown as loading control. OASTL activities are the mean values of three replicates ± standard deviation (A). OASTL activity in 0.5 mM SIN-1 treated extracts from wild type, *oasa1.1*, *oas1.2*, *oasb* and *oasc* mutants was measured after one hour. After SIN-1 removal, activity was measured along with non-treated samples as controls. Values are the mean ± SD of three replicates and are expressed relative to the corresponding non-nitrated control for every genotype (B).

Fig.2. OASTL activity and nitrated OASA1 protein levels in purified OASA1 protein treated with increasing concentrations of the nitrating reagent SIN-1. Samples of 12 μg of His-tagged purified recombinant OASA1 protein were treated with the indicated concentration of SIN-1 for one hour. After SIN-1 removal, OASTL activity and total and nitrated protein levels were quantified (A). Activity and protein levels in samples treated with 0.5 mM SIN-1 or untreated as a control (c) and epicatechin at the indicated concentrations (B). Western blot with anti-3nitroY antibody (α-3NY) and Ponceau staining as loading control are shown in panels A and B. Scavenging effect of epicatechin on the OASTL activity of SIN-1 (0.5 mM)-nitrated crude extracts from wild type (clear grey bars) and *oasa1.1* (dark grey bars) leaves (C). OASTL activities are the mean values of three replicates ± standard deviation.

Fig. 3. Identification of a Y-nitration site in OASA1. LC-MS/MS analysis of nitrated OASA1 and non-nitrated control allowed the identification of Y*LSTVLFDATR peptide with nitrated or non-nitrated Y302, respectively (A). The 22.49 Da shift of the double charged peptide indicates nitration of Y302. Ion score (MASCOT) of the nitrated and non nitrated peptides are shown. The MS spectra corresponding to Y($NO_2$)LSTVLFDATR (Z = 2; m/z = 665.82) is sown (B). $NO_2$-Y indicates the presence of the nitrated tyrosine immonium ion mass in the LC-MS/MS spectra. Identified y and b ion series are pointed.

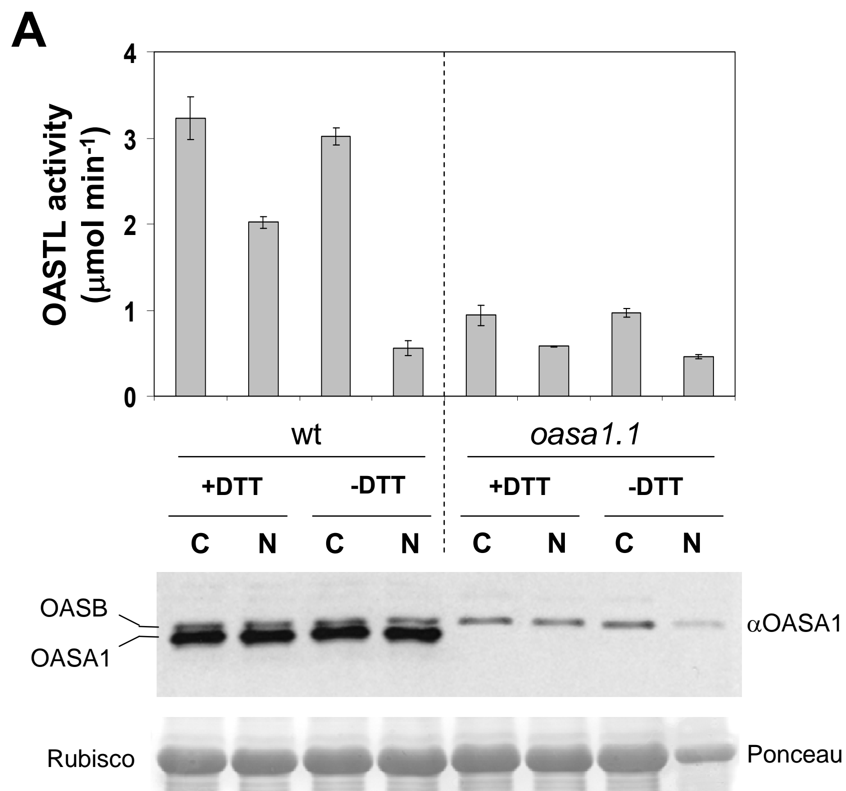
Fig. 4. Three-dimension model of OASA1 showing the position and potential interactions of Tyr residues. The conformation of OASA1 molecule showing the position of the seven Tyr residues (in red), the PLP binding site (in orange), O-acetylserine substrate binding site (in yellow) and SAT protein interaction site (in purple) are shown (A). Detail of the three-dimension structure showing $Y^{302}$ and the $N^{77}$ residues at a 4.7 Å distance, the amino acid residues interacting with the $K^{46}$ (in orange)-linked PLP (in yellow) through hydrogen bonds (in green), and the OAS and SAT binding site (in purple) (B). In silico analysis, using PyMol software, of the potential hydrogen bonds (yellow dashed line) between amino acid residues surrounding PLP (C). Spectrophotometric detection of the reaction of OASA1-bound O-acetylserine with PLP to form an α-aminoacrylate intermediate. Samples of 200 μg of non-nitrated (open circles) or nitrated (close circles) purified OASA1 were incubated with increasing concentrations of O-acetylserine as indicated, and the increase in the absorbance at 470 nm registered (D).

Fig. 5. OASTL activity of mutated recombinant versions of OASA1. Purified samples of wild type (WT) and mutated (Y302A and Y203A) versions of OASA1 were assayed for OASTL activity in the absence or presence of the indicated concentrations of SIN-1. OASTL activities are the mean values of three replicates ± standard deviation. Western blot with anti-3nitroY antibody (α-3NY) and Ponceau staining as loading control (Ponceau) are shown for nitrated Y203A samples.

Fig. 6. Analysis of *in vivo* Tyr-nitration of OASA1. Crude extracts from wild type *A. thaliana* seedlings were immunoprecipitated with anti-3-nitroY antibody, and the presence of OASA1 in crude

extract (CE) input and the resulting supernatant (Sup) and immunoprecipitate (IP) was analyzed by Western blot with anti-OASA1 antibody (A). Leaves were infiltrated with 2mM SIN-1 for 90 minutes and then crude protein extracts were prepared for OASTL activity measurement (bottom panel) and for immunoprecipitation with 3-nitroTyr (IP 3-NY) antibodies. Western blot with anti-OASA1 (α-OASA1) was performed with protein extracts (left up panel) or IP (right up panel). OASTL activity values are the mean values of three replicates ± standard deviation (B).

Figure 1



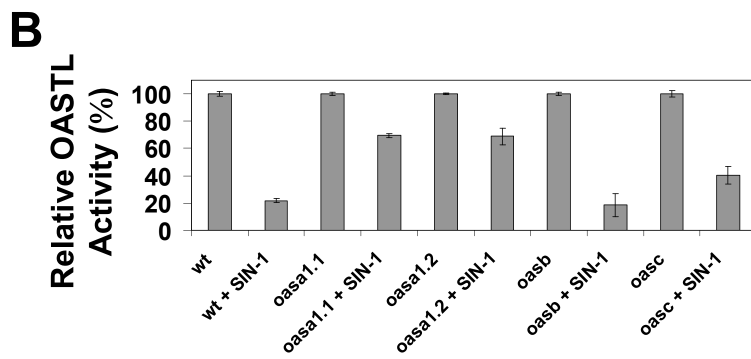



Figure 2

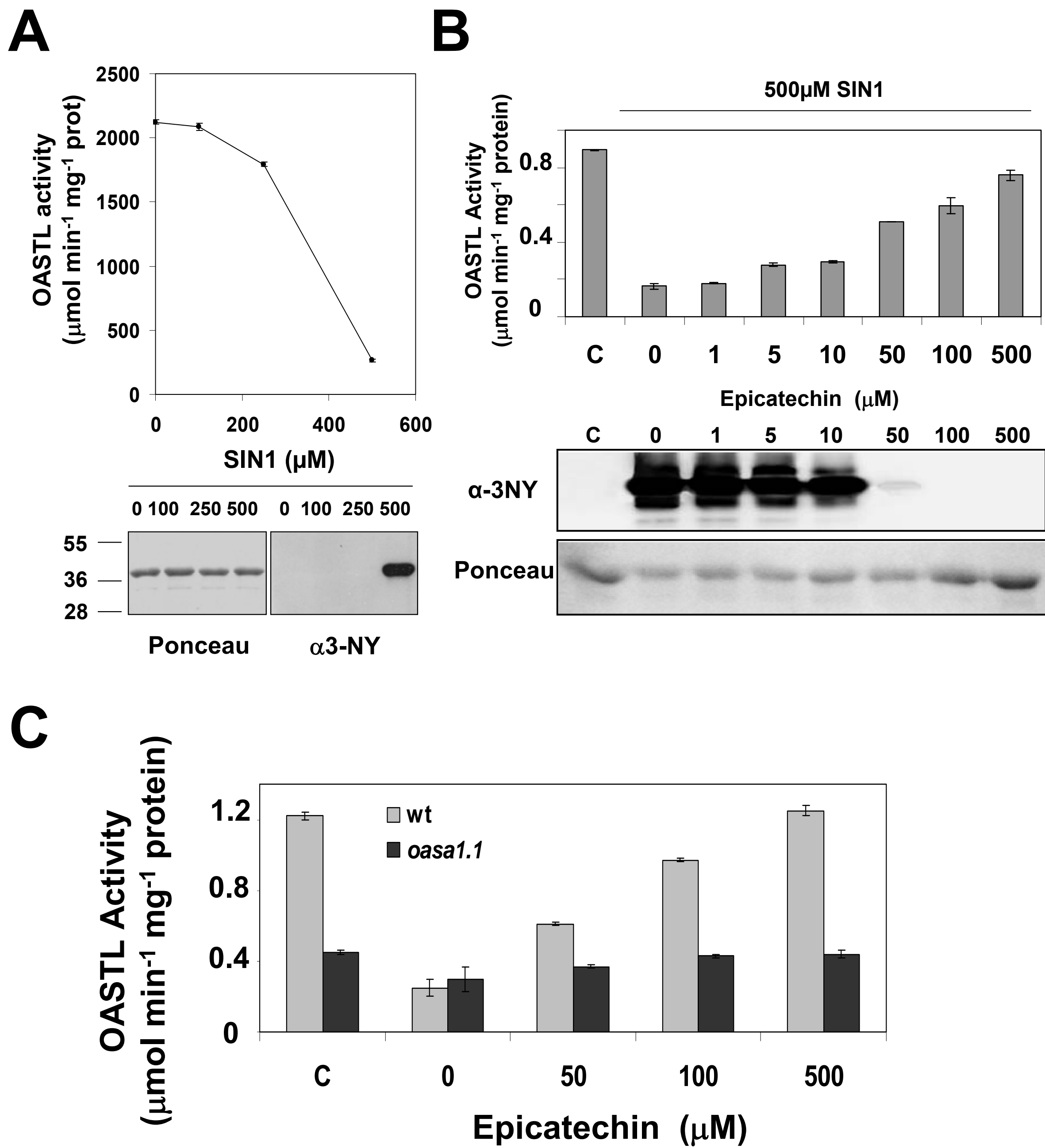


Figure 3

**A**

| Peptide | Score | Expect | [$MH^{2+}$] | [MH] | Shift | Treatment |
|---|---|---|---|---|---|---|
| YLSTVLFDATR | 82 | $7.1\ 10^{-6}$ | 643.33 | 1286.66 | | Control |
| $^{NO2}$YLSTVLFDATR | 76 | $1.5\ 10^{-6}$ | 665.82 | 1331.64 | +44.98 | Nitrated |

**B**

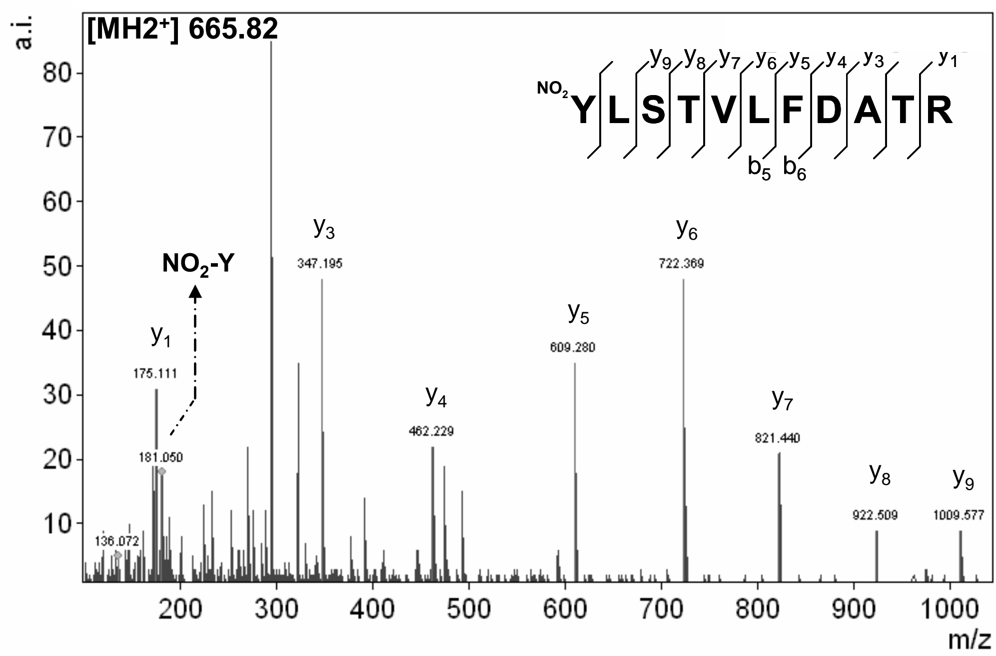


Figure 4

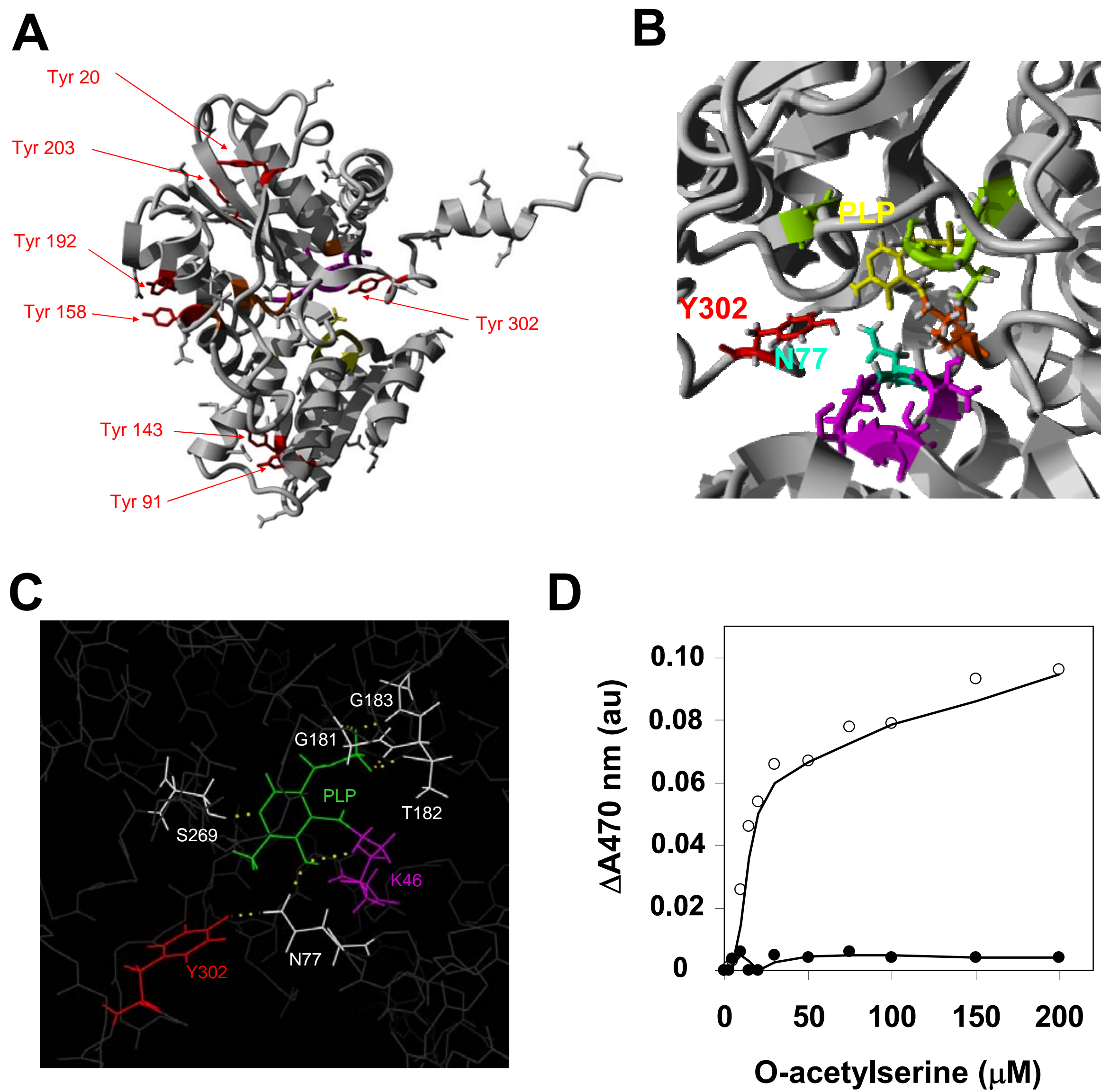


Figure 5

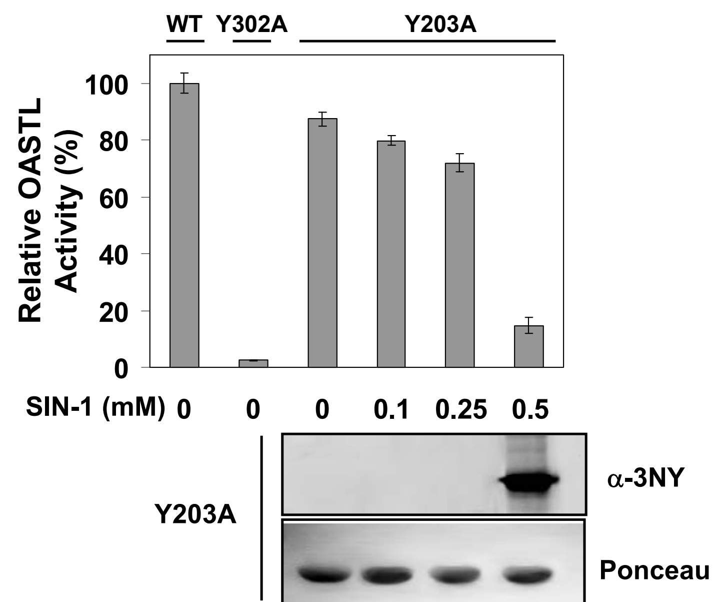


Figure 6