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Molecular cloning and expression analysis of 12-oxophytodienoate reductase cDNA by wounding in *Solanum tuberosum*

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Abstract Jasmonic acid (JA) and 12-oxophytodienoic acid (OPDA) are signal molecules involved in the stress and defense responses in plants. A full-length cDNA clon of *OPR3* encoding 12-oxophytodienoate reductase 3, key enzyme involved in the biosynthesis of JA from linolenic acid was obtained from a *Solanum tuberosum* cDNA library. Sequence analysis showed that *OPR3* encoded a polypeptide of 400 amino acids with a predicted molecular mass of 43.9 kDa and pI of 7.72. The deduced amino acid sequence of OPR3 showed high similarities to other 12-oxophytodienoate reductases. A peroxisomal signal sequence indicates OPR3 probable location in peroxisome. Levels of *OPR3* mRNA accumulated in potato leaves reaching maximum levels within 1 hr of mechanical wounding. Elevated levels of JA were correlated to expression of the *OPR3* gene.

Keywords: 12-oxophytodienoate reductase, jasmonic acid, OPDA, *Solanum tuberosum*, stress, wounding

INTRODUCTION

The response of plants to abiotic and biotic stresses involves changes in gene expression patterns. These changes largely depend on signal molecules such as abscicic acid (ABA), salicylic acid (SA) as well as jasmonic acid (JA) and its biosynthetic precursors (Bari and Jones, 2009; Leon-Reyes et al. 2010). The role that JA plays as signal molecule has been shown by evidence through molecular and biochemical studies. Plants increase JA levels when they are subjected to wounding or attack from insects and pathogens (Schilmiller and Howe, 2005). JA and OPDA induce the expression of pathogenesis related genes like *PIN2*, *THI2.1* and *PDF1.2* (Farmer et al. 2003; Taki et al. 2005). Molecular mechanisms of JA signalling have been dissected by discovering protein factors like JAR1 and COI1 from *Arabidopsis* mutants that are deficient in JA synthesis or unable to respond to JA (Tani et al. 2008). The biosynthesis of JA through the octadecanoic pathway (Vick and Zimmerman, 1984) is initiated from *α*-linolenic acid (LA), which is thought to be released from chloroplast membrane lipids by specific lipases. This is followed by the action of lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) to form 12-oxophytodienoic acid (OPDA). Subsequently OPDA is transported from chloroplast to the peroxisome where the 12-oxophytodienoate reductase (OPR) reduces it to 12 oxophytoenoic acid (OPC-8:0). Three cycles of *β*-oxidation are then required for the shortening of the OPC-8:0 side chain leading to JA (Li et al. 2005). Evidence support the existence of three isoforms: OPR1, OPR2 and OPR3 (Schaller et al. 2000). Different studies have shown that OPR3 catalyzes the reduction of the 9S,13S-OPDA, the precursor physiologically relevant to synthesize JA (Schaller et al. 2000). Since OPDA and JA appear to be different signalling molecules (Stinzi et al. 2001) OPR3 seems to play a key role in controlling the pool of OPDA and JA to respond to different stress. *Solanum tuberosum* is a major carbohydrate source in human and animal diets around

the world. Monoculture and the lack of genetic diversity have helped the emergence and threat to potato production caused by pathogens. Current disease control relies on the use of pesticides that are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production (Savenkov et al. 2003). This infection process can be suppressed by secretion of effectors like protease inhibitors (Odeny et al. 2010). Mechanical damage or infection of potatoes with *Phytophthora infestans* causes an accumulation of protease inhibitors in exudates of potato tubers, and the treatment with JA intensified the accumulation of these inhibitors in response to the wound stress (Valueva et al. 2003). Inducing systemic resistance responses in crop plants is a promising way of disease management. In this work the aim was to clone the *OPR3* cDNA from *Solanum tuberosum* and evaluate the expression response of the gene and its correlation with JA levels and the systemic response of the plant involving the proteinase inhibitor *Pin2*, when the plant was subject to mechanical wounding. In this way our work contributes to the knowledge of the presence and importance of the *OPR3* gene in the JA pathway and the possibility of inducing systemic resistance responses in potato plants.

MATERIALS AND METHODS

Screening of cDNA library

A cDNA library from potato leaves cloned in phage λ ZAP (Stratagene), was screened through phage hybridization. A solution of the cDNA library was laid over a lawn of *E. coli* XL1-Blue. Some of the cells were then transferred to a membrane of nylon HyBond N^+ (Porablot, Stratagene). This membrane was subject to hybridization with a cDNA probe of *OPR3* from *A. thaliana* (kindness to Mussig C), radioactively marked with ^{32}P using the kit RadPrime (GIBCO/BRL) to isolate putative clones of *OPR3* with complementary sequences.

Membranes were subject to prehybridization at 42ºC in hybridization buffer (25 mM sodium phosphate bufffer pH 7.2, 25 mM NaCl, 10 mM EDTA, 7% SDS, 30% formamide, 10% PEG 6000 and 0.25 mg/ml salmon sperm DNA) for 2 hrs without probe and then 12 hrs with the probe. Membranes were washed with wash solution (0.5% SDS, 2x SSC) for 10 min and exposed to autoradiography X-OMAT LS (Kodak) at-80ºC. Clones were later propagated in *E. coli* SOLR allowing the isolation of clones in phagemid Bluescript (pBS). Selected clones were subject to endonuclease restriction analysis and then subject to sequencing through the DNA sequencer ABI3700 at the Centro de Biotecnología e Ingeniería Genética from the Universidad Estatal de Campinas SP Brasil.

Sequence analysis

Predictions of open reading frames (ORFs) were made using ORF finder from NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and Translate Tool from ExPASy (http://us.expasy.org/tools/dna.html). Searches for homology of nucleotide sequences and deduced amino acid sequences to cDNA clones were identified by NCBI BLAST against GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence comparison was conducted using the ClustalW program from EMBnet (http://www.ch.embnet.org/software/ClustalW.html). Theoretical molecular weights of deduced polypeptides were made using the Protein property calculator (http://web.expasy.org/compute_pi/). From this bioinformatics analysis the selected cDNA clone was then used for the expression analysis of *OPR3* through Northern blot.

Plant materials

Potato plants (*Solanum tuberosum,* cultivar *Désirée*) were cultivated *in vitro* in MS media supplemented with 2% sacarose and solidified in 0.8% agar in a cultivation chamber with controlled temperature (22ºC), relative humidity (70-80%) and photoperiod (16 hrs light/8 hrs dark). Once the adequate size was reached (1 month) they were moved to earth in a greenhouse with the same controlled conditions. After 2 months of growth they were subject to mechanical damage stress. Samples recollected after the treatment were immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction.

Mechanical damage treatment

Potato plants were damaged by incisions on two leaves per plant and recollected at different times (0.5, 1, 3, 6 and 12 hrs). Control samples from plants that were not damaged were also recollected.

Expression analysis of *OPR3* through Northern blot

Total RNA was extracted from potato leaves from different treatments using guanidine hydrochloride-containing buffer followed by direct extraction with phenol/chloroform. The RNA was precipitated from the aqueous phase, washed with 3 M sodium acetate and 70% ethanol, and finally dissolved in water. 20 µg of total RNA were fractioned through electrophoresis gel in 1.1% agarose/formaldehyde and transferred to a membrane of nylon HyBond N^+ (Porablot). This membrane was subject to hybridization with different probes: cDNA from *OPR3* isolated previously, *AOS* from tomato and *Pin2* from potato radioactively marked with ^{32}P using RadPrime (GIBCO/BRL). Membranes were subject to prehybridization at 42ºC in hybridization buffer for 2 hrs without probe and then for 12 hrs with probe. Membranes were washed at 42ºC with wash solution for 10 min and subject to autoradiography X-OMAT LS (Kodak) at -80ºC.

Quantification of JA

Endogenous JA was extracted from potato plants with mechanical damage. For the quantification of JA levels leaves were homogenized together with an internal standard (dihidro jasmonic acid). An extraction was made with a mixture of acetone/citric acid and was evaporated for 12 hrs. The mixture was decanted and supernatant was recovered. This was followed by an ether extraction and evaporation. The ether phase was loaded on a solid phase column of amino propel, pre equilibrated with ether and washed with chloroform/IPA. An extraction with a mixture of ether/acetic acid was used to recover the fixed product in the column and completely evaporated. The product was passed to a chromatography vial, washed with dichloromethane and completely evaporated. Then the product was subject to derivatization, and the product was resuspended in absolute methanol and derivatization reagent (DMF, dimetilacetal) at 60ºC. Samples were subject to analysis with a GC-MS (HEWLETT PACKARD 5973 Mass Selective Detector), measuring the ions of endogenous JA m/z = 224 and 151 and from the internal standard (dihidro jasmonic acid) m/z = 226 and 153. The concentration of JA was determined with the relation between endogenous JA and the internal standard.

Nucleotide and amino acid sequences

OPR3 cDNA coding for 12-oxophytodienoate reductase from *Solanum tuberosum* is available at GenBank accession JN241968.

RESULTS AND DISCUSSION

Cloning and sequence analysis

In this study using screening of cDNA library we managed to clone the 12-oxophytodienoate reductase cDNA, GenBank accession JN241968. A full length clone revealed a putative 1203 bp open reading frame encoding a predicted polypeptide of 400 amino acid residues with an estimated molecular weight of 43.9 kDa and pI of 7.72. BLAST searches showed high similarities of the OPR3 from *S. tuberosum* with other OPR available in the GenBank database. The highest identity of OPR3 from *S. tuberosum* was with other OPR3, like *A. thaliana* with 75% and *L. esculentum* with 99%. OPR3 from potato also showed an identity of 51 and 55% with OPR1 and OPR2 from *A. thaliana* and 54 and 44% with OPR1 and OPR2 from tomato respectively. The amino acid sequence of OPR3 from *S. tuberosum* was aligned with OPR3 from *A. thaliana* and *S. lycopersicum* (Figure 1). The analysis of the amino acid sequence of OPR3 from *S. tuberosum* showed the peroxisomal signal peptide (SRL) at the C-terminus of the protein, a feature shared with OPR3 from *A. thaliana* and *S. lycopersicum* (Strassner et al. 2002) whereas OPR1 and OPR2 do not show the presence of the peroxisomal signal. This reinforces the hypothesis octadecanoic pathway involves the plastid and peroxisomal compartment. The comparison of the sequence with other OPR and OYE, all oxo-reductases dependent of the cofactor flavin mononucleotide (FMN), shows that the majority of the residues responsible for the binding of FMN and

NADPH are conserved; situation observed in other OPR described (Costa et al. 2000). The amino acid sequence comparison of OPR3 from *S. tuberosum* with that of OPR1 and OPR3 from *S. lycopersicum*, with known crystallographic structure (Breithaupt et al. 2001; Breithaupt et al. 2009) showed conserved residues at the catalytic site, but different residues at the selectivity site with OPR1, probably because OPR3 should recognize 9S,13S-OPDA instead of 9R,13R-OPDA recognized by OPR1 (data not shown).

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OPR3/L.esculentum      ----MASSAQDGNNPLFSPYKMGKFNLSHRVVLAPMTRCRALNNIPQAALGEYYEQRATA 56
OPR3/S.tuberosum       MANTTSSSAQDGNNPLFSPYKMGKFNLSHRIVLAPMTRCRALNNIPQAALGEYYEQRATA 60
OPR3/A.thaliana        ----MTAAQGNSNETLFSSYKMGRFDLSHRVVLAPMTRCRALNGVPNAALAEYYAQRTTP 56
                            :::  :.*:.***.****:*:****:************.:*:***.*** **:*.

OPR3/L.esculentum      GGFLITEGTMISPTSAGFPHVPGIFTKEQVREWKKIVDVVHAKGAVIFCQLWHVGRASHE 116
OPR3/S.tuberosum       GGFLITEGTMISPTSAGFPHVPGIFTKEQVEEWKKIVDVVHAKGAVIFCQLWHVGRASHE 120
OPR3/A.thaliana        GGFLISEGTMVSPGSAGFPHVPGIYSDEQVEAWKQVVEAVHAKGGFIFCQLWHVGRASHA 116
                       *****:****:** **********::.***. **::*:.*****..************

OPR3/L.esculentum      VYQPAGAAPISSTEKPIS-NRWRILMPDGTHGIYPKPRAIGTYEISQVVEDYRRSALNAI 175
OPR3/S.tuberosum       VYQPAGAAPISSTEMPIS-KRWRILMPDGTHGIYPKPRAIGTYEISQVVEDYRRSALNAI 179
OPR3/A.thaliana        VYQPNGGSPISSTNKPISENRWRVLLPDGSHVKYPKPRALEASEIPRVVEDYCLSALNAI 176
                       **** *.:*****: *** :***:*:***:*  ******: : **.:*****  ******

OPR3/L.esculentum      EAGFDGIEIHGAHGYLIDQFLKDGINDRTDEYGGSLANRCKFITQVVQAVVSAIGADRVG 235
OPR3/S.tuberosum       EAGFDGIEIHGAHGYLIDQFLKDGINDRTDEYGGSLANRCKFITQVVQAVVSAIGADRVG 239
OPR3/A.thaliana        RAGFDGIEIHGAHGYLIDQFLKDGINDRTDQYGGSIANRCRFLKQVVEGVVSAIGASKVG 236
                       .*****************************:****:****:*:.***:.*******.:**

OPR3/L.esculentum      VRVSPAIDHLDAMDSNPLSLGLAVVERLNKIQLHSGSKLAYLHVTQPRYVAYGQTEAGRL 295
OPR3/S.tuberosum       VRVSPAIDHLDAMDSNPLSLGLAVVERLNKIQLHSGSKLAYLHVTQPRYVAYGQTEAGRL 299
OPR3/A.thaliana        VRVSPAIDHLDATDSNPLSLGLAVVDMLNKLQDVNGLKLAYLHVTQPRYHAYGQTESGRQ 296
                       ************ ************: ***:*  .* ************ ******:**

OPR3/L.esculentum      GSEEEEARLMRTLRNAYQGTFICSGGYTRELGIEAVAQGDADLVSYGRLFISNPDLVMRI 355
OPR3/S.tuberosum       GSEEEEARLMRTLRNAYQGTFICSGGYTRELGIEAVAQGDADLVSYGRLFISNPDLVMRI 359
OPR3/A.thaliana        GSDEEEAKLMKSLRMAYKGTFMSSGGFNKELGMQAVQQGDADLVSYGRLFIANPDLVSRF 356
                       **:****:**::** **:***:.***:.:***::** **************:***** *:

OPR3/L.esculentum      KLNAPLNKYNRKTFYTQDPVVGYTDYPFLQGNGSNGPLSRL 396
OPR3/S.tuberosum       KLNAPLNKYNRKTFYTQDPVVGYTDYPFLQGNGSNGPLSRL 400
OPR3/A.thaliana        KIDGELNKYNRKTFYTQDPVVGYTDYPFLA------PSSRL 391
                       *::. ************************       * ***
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Fig. 1 Amino acid alignment of OPR3 from *S. tuberosum* (JN241968) with *A. thaliana* (AJ238149) and *S. lycopersicum* (AJ278332) using ClustalW. Identical amino acids are marked (*), strongly similar residues (:) and weakly similar residues (.).

Analysis of the expression of *OPR3* by mechanical wounding

Transcripts of *OPR3* in leaves wounded directly (local response) began to accumulate already at 30 min, reaching the maximum at 1 hr after treatment, beginning to decline after 3 hrs and disappearing after 12 hrs (Figure 2a). In leaves not wounded directly (systemic response) no transcript of *OPR3* accumulated (Figure 2b). This is in accordance with evidence that *OPR3* transcript accumulate after wounding in *A. thaliana* (Schaller et al. 2000) and tomato (Strassner et al. 2002). To verify the adequate mechanical wounding the expression of *AOS* and *Pin2* were analyzed. Transcript of *AOS* and *Pin2* accumulated both local (in mayor proportion) and systemically (in less proportion) in response to mechanical wounding confirming the adequate treatment. Evidence that support this is that *AOS* transcript accumulates both locally and systemically in response to mechanical damage (Lulai et al. 2011). *Pin2* transcripts also accumulate during mechanical damage (Peña-Cortés et al. 1995). To relate the expression of *OPR3* with the biosynthesis of JA, endogenous levels of JA were quantified. Not damaged leaves (control) presented levels of 420.90 ng gr^{-1}fw, while locally damaged leaves started to accumulate rapidly at 30 min, reaching a maximum of 1530.24 ng gr^{-1}fw after 1 hr, to later start to decline after 3 hrs, reaching control levels after 12 hrs (Figure 3). These results clearly shows that *OPR3* gene expression from *S. tuberosum* is inducible by mechanical damage and that its expression in related with an increase in endogenous JA levels.

These patterns of *OPR3* gene expression and JA levels are consistent with a function of OPR3 in the octadecanoid metabolism in *S. tuberosum*. The cloning of the *OPR3* gene opens the way for future work involving transgenic potato plants carrying constructs for overexpression of the *OPR3* cDNA and for antisense inhibition of *OPR3* gene expression. These will help clarify the specific function of OPR3 and octadecanoids in stress responses.

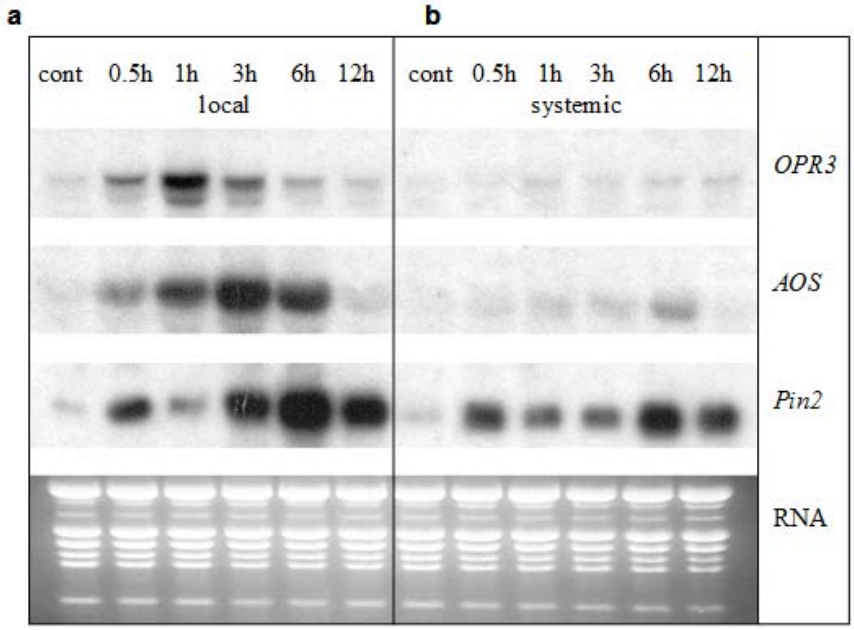

Fig. 2 Northern blot analysis of the gene expression of *OPR3* in potato plants subject to mechanical damage in a) local response and b) systemic response. 20 µg of total RNA was used to hybridization with probes *OPR3*, *AOS* and *Pin2*. Total RNA is visualized with ethidium bromide as loading control for RNA blot.

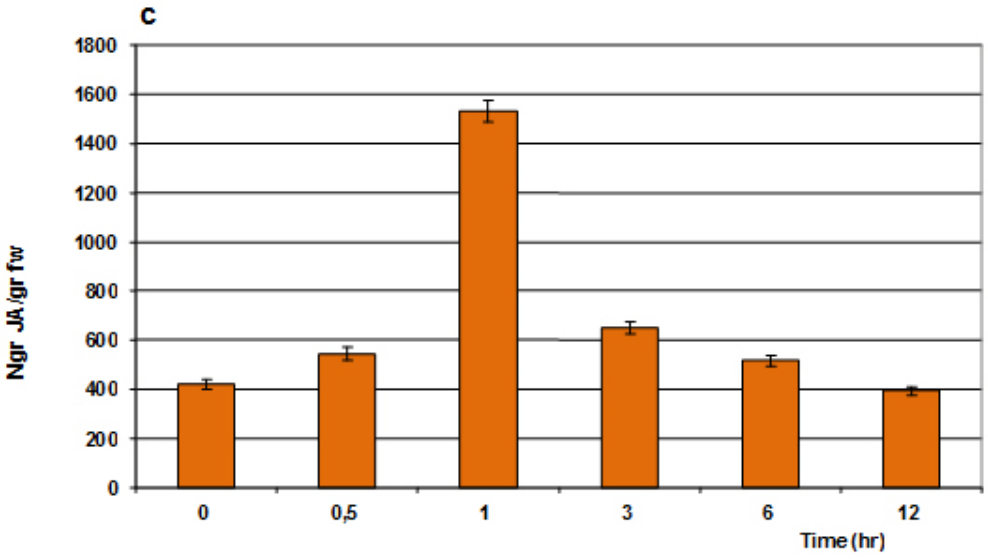

Fig. 3 JA levels in locally damaged potato plants subject to mechanical damage. The concentration of JA was analyzed by GC-MS. Data are in triplicate and correspond to the average ± SD.

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