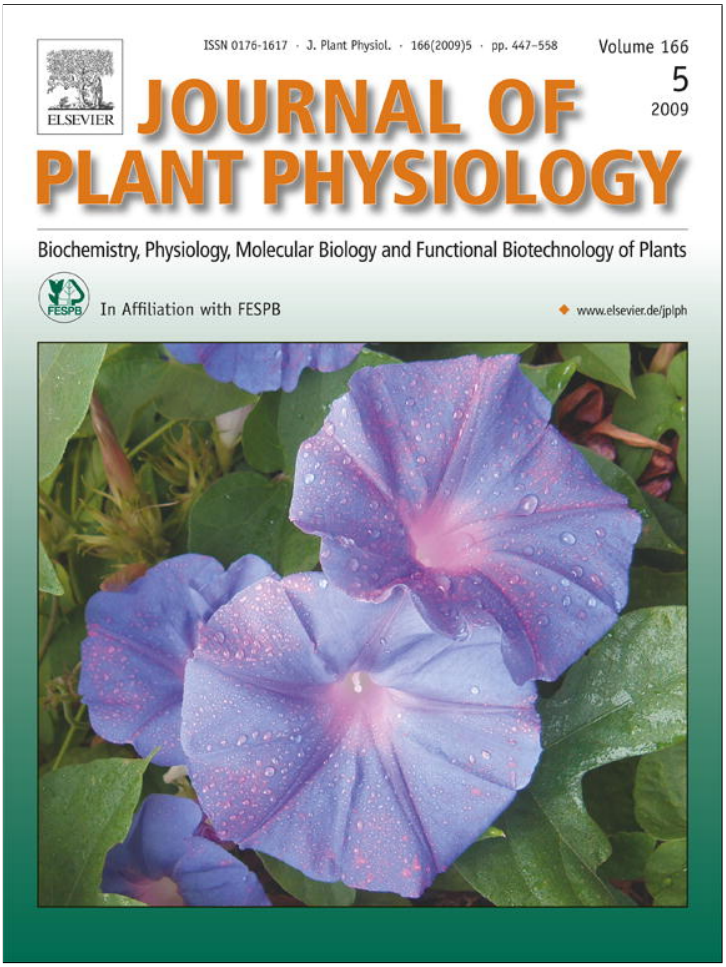

Journal of Plant Physiology **166** (2009) 543—547

Available online at www.sciencedirect.com

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JOURNAL OF PLANT PHYSIOLOGY

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SHORT COMMUNICATION

# Phloem sap of tomato plants contains a DIR1 putative ortholog

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Received 19 May 2008; received in revised form 8 July 2008; accepted 8 July 2008

**KEYWORDS**
Lipid transfer protein;
Phloem;
*Solanum lycopersicon*;
Systemic acquired resistance

## Summary

*Arabidopsis thaliana* defective in induced resistance 1 (At-DIR1) has been characterized as a protein responsible for the generation or transmission of the still unknown signal involved in systemic acquired resistance. This acidic apoplastic protein is a member of the family of lipid transfer proteins and was detected in vascular fluids. To our knowledge, no DIR1-like protein has been described in other plant species. Hence, we have performed data mining to identify a putative ortholog of DIR1 in tomato. This strategy allowed the detection of a few gene products displaying sequence similarity to At-DIR1 whose structural features were further analysed *in silico*. The best match (unigene SGN-327306) encoded a protein with an acidic p*I*, a peculiar characteristic of DIR1 among lipid transfer proteins, and was hence selected as a putative tomato ortholog of At-DIR1. This sequence, named Le-DIR1, served for the design of a specific antigenic peptide and the generation of polyclonal antibodies. The antiserum anti-Le-DIR1 recognized a peptide of the expected size (7 kDa) in phloem sap of tomato plants, hence confirming the existence of the predicted protein in vascular fluids. This result supports the notion of the existence of common systemic acquired resistance (SAR) signaling molecules in different species.


## Introduction

Systemic acquired resistance (SAR) is a whole-plant resistance response that occurs following an earlier localized exposure to a pathogen. SAR requires the translocation of a signal through the phloem and is associated with the induction of a wide range of genes (Durrant and Dong, 2004).

*Abbreviations:* BSA, bovine serum albumin; DIR1, defective in induced resistance 1; LTP, lipid transfer protein; SA, salicylic acid; SAR, systemic acquired resistance.

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doi:10.1016/j.jplph.2008.07.002

Its activation involves the accumulation of endogenous salicylic acid (SA), but experimental evidence has shown that SA is not the SAR mobile signal. Despite extensive research in the field, the nature of that signal still remains elusive (Grant and Lamb, 2006; Park et al., 2007). Nevertheless, a novel player in SAR signaling has been discovered in the last years. Maldonado et al. (2002) have demonstrated that an *Arabidopsis thaliana* mutant impaired in SAR response was defective in *dir1*, a gene encoding a putative lipid transfer protein (LTP). Dir1-1 mutants (defective in induced resistance 1) have been shown to be unable to produce and/or transmit an essential SAR signal from the inoculated leaf to distal places. DIR1 has been characterized as an apoplastic LTP detected in petiole exudates, but experimental evidence suggest that it is not itself the signal, because overexpression of DIR1 is not sufficient to induce SAR (Maldonado et al., 2002). On the other hand, LTPs are able to bind and translocate lipids (Carvalho and Gomes, 2007), and lipids seem to play a central role in SAR (Nandi et al., 2004; Grant and Lamb, 2006). This evidence has prompted to suggest that DIR1 may interact with a lipid molecule to promote long-distance signaling through the phloem (Maldonado et al., 2002). Despite the relevance of this finding, no progress on the mode of action of DIR1 has been reported since then, although the protein was crystallized and attempts were made to solve its structure (Lascombe et al., 2006). Moreover, according to our knowledge, even the presence of DIR1 orthologs in other species has not been reported yet. In this context, the aim of this work was to determine the presence of a DIR1-like protein in tomato using bioinformatic tools and further demonstrate the existence of the corresponding protein in phloem sap.

## Materials and methods

### Data mining

*Arabidopsis thaliana* (L.) Heynh. DIR1 sequence (accession AF342726) was analysed for the presence of a signal peptide using SignalP 3.0 (Bendtsen et al., 2004). At-DIR1 homologues were then identified in tomato with TBLASTN using its mature protein sequence as query against the TIGR tomato gene Index (http://www.tigrblast.tigr.org/tgi), Sol Genomics Network tomato unigene (http://www.sgn.cornell.edu) and Plant Genome Database (http://www.plantgdb.org). Multiple sequence alignment was performed with Clustal W (Higgins et al., 1994). ProtParam (Gasteiger et al., 2005) was used for primary sequence analyses, and other bioinformatic resources were from the Expasy Proteomics server.

### Plant material and protein extracts

Tomato plants *Solanum lycopersicon* (formerly *Lycopersicon esculentum*) cv. Platense were grown for 5–8 weeks under standard greenhouse conditions. Phloem sap was collected by dripping during 12 h as described by Madey et al. (2002). Briefly, stems were cut below the second leaf in the presence of 20 mM EDTA and allowed to exude at room temperature. Protein samples were concentrated by acetone precipitation and suspended in sample buffer (Laemmli, 1970) for electrophoretic analyses. Total protein extracts from leaves and seeds were extracted as detailed in Gonorazky et al. (2005). The medium of tomato cell suspensions for protein gel blot analysis was collected as described elsewhere (Gonorazky et al., 2008).

### Antibody production

An antigenic peptide of 14 amino acids was selected using antigen design tools (http://www.genscript.com; http://www.4adi.com), and synthesized coupled to bovine serum albumin (BSA) at Genscript Corporation, USA. The antigenic peptide (200 μg) was subcutaneously inoculated with Freund Adjuvant emulsion (500 μL) and two booster immunizations (200 μg) were repeated every 20 d. Serum collection (10 d after each inoculation) was performed by bleeding the marginal vein of the rabbit ear. Antiserum title was tested by dot blot on synthetic peptide samples. Preimmune serum was obtained before the first inoculation and used for control experiments. The antiserum used in protein gel blot analyses was incubated with and excess of BSA to prevent cross reaction due to putative BSA epitope recognition.

### Immunoblot analysis

Protein gel blot assays were performed as described previously (Regente and de la Canal, 2000), except that SDS-PAGE was developed on 15% Tricine gels for better resolution of low molecular weight proteins (Schägger and von Jagow, 1987). The blots were incubated with 1:2000 dilutions of antiserum (second boost) and alkaline phosphatase was used for signal visualization.

## Results and discussion

### A putative tomato ortholog of At-DIR1

The identification of tomato sequences displaying identity to At-DIR1 was performed with the mature sequence of At-DIR1 as query, using the TBlastn tool on three databases: TIGR, Plant Genome and Sol Genomics Network. Similar results were obtained in the three databases and hence only those obtained from Sol Genomics Network are presented in Table 1. Four matches were above the

cut off score of 60 imposed for the selection of the putative ortholog of At-DIR1.

Unigenes SGN-U327306 and SGN-U3220476 presented the highest score with acceptable *E* values, indicating their similarity to At-DIR1. The scores displayed by these sequences seem to reflect the limited degree of conservation characteristic of the members of the LTP family (Kader, 1996) but, as At-DIR1, these hits belong to the LTP2 subfamily of approximately 7 kDa (Carvalho and Gomes, 2007). A more detailed analysis was then performed on the first four matches from Table 1. Hence, the features of the primary sequences of these putative candidates were analysed *in silico* and compared to those of At-DIR1. Table 2 reveals that the protein encoded by unigene SGN-U327306 is the most similar to At-DIR1, basically including the same number of amino acids, similar predicted MW and a theoretical acidic p*I*. The latest is a relevant feature as all the other candidates are predicted to be basic proteins (as most LTPs) and the p*I* is expected to be related to the function of a protein. In addition, SGN-U327306 presents eight cysteine residues arranged in the typical pattern of LTPs (Kader, 1996) (Figure 1), a hydrophobic profile similar to that shown by At-DIR1 and a putative signal peptide (not shown). On the other hand, the second match (SGN-U320476) is the most similar clone to At5g48490, another 7 kDa DIR1-like protein present in *Arabidopsis*. Finally, a reversal verification was performed submitting SGN-U327306 sequence as query on the *Arabidopsis thaliana* database. This analysis showed DIR1 (At5g48485) as the best match. Taken together, these results strongly suggest that SGN-U327306 (TIGR TC128612) is the putative ortholog of At-DIR1 in tomato and is further referred as Le-DIR1. It is interesting to note that the unigene SGN-U327306 was built from four EST clones sequenced in three different tomato

**Table 1.** Tomato unigenes similar to At-DIR1 (AF342726). Sequences were detected using TBLASTN on Sol Genomics Network database using the procedure described in Materials and methods

| Unigene | Score | *E*-value |
|---|---|---|
| SGN-U327306 | 88 | 1e−18 |
| SGN-U320476 | 86 | 5e−18 |
| SGN-U322554 | 75 | 8e−15 |
| SGN-U316923 | 64 | 2e−11 |
| SGN-U322838 | 46 | 4e−06 |
| SGN-U319206 | 39 | 0.0005 |
| SGN-U338880 | 39 | 0.0006 |
| SGN-U338875 | 39 | 0.0006 |
| SGN-U338874 | 39 | 0.0006 |
| SGN-U338536 | 39 | 0.0006 |

**Table 2.** Comparison of At-DIR1 with tomato similar sequences. Best tomato matches from Table 1 (score >60) were analysed for primary sequence parameters using ProtParam software

| | At-DIR1 | SGN-U327306 | SGN-U320476 | SGN-U322554 | SGN-U316923 |
|---|---|---|---|---|---|
| MW (Da) | 7973 | 7966 | 8089 | 8165 | 8431 |
| aa | 76 | 76 | 76 | 77 | 78 |
| p*I* | 4.5 | 4.03 | 8.51 | 7.67 | 8.48 |

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SGN-U327306      ----MNICNMDDDGLTSCKPSVTQPNPVEPSASCCEALSGADLQCLCSYRNSFVLPSLGI
SGN-U320476      ----QGICNVSGEGLMSCRPSITPPYPTAPTAQCCNALSRADMACLCSYKNSQLLPSLGI
SGN-U322554      ----DSLCGLTIYDLMTCKSAVSGPKPLPPSDKCCAALTKADFPCLCTFKNSPMLSDFKI
SGN-U316923      AETPEVICKVTINDLMLCLPAVMGKRPPKPTPDCCAVLRKADLQCMCNQKSE--LGKFGI
At-Dir1-         ----IDLCGMSQDELNECKPAVSKENPTSPSQPCCTALQHADFACLCGYKNSPWLGSFGV
                       :* :    *  * .::    *  *:  ** .*  **: *:*  :.. * .: :

SGN-U327306      DPELALALPTKCNLTSPSNC- 76
SGN-U320476      DPNLAIQLPQKCRLPNPPRC- 76
SGN-U322554      NSTLAMDLPSKCKLDSPNCSA 77
SGN-U316923      SPEAAMNLPKQCKIKVPDGC- 78
Dir1-            DPELASALPKQCGLANAPTC- 76
                 ..  *  ** :* :  .  .
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**Figure 1.** Sequence alignment of At-DIR with similar tomato protein sequences. The translated sequences of tomato unigenes presenting scores above 60 were aligned with At-DIR1 using Clustal W. The antigenic sequence selected in SGN-U327306 for the generation of antibodies is underlined. Below the alignment, a star denotes identical residues in all the sequences, a colon means that conserved substitutions have been detected and a dot corresponds to semi-conserved substitutions. Dashes indicate gaps inserted for better alignment.

libraries, indicating that the gene is actively expressed. In addition, TBLASTX searches on Genbank showed the existence of sequences similar to Le-DIR1 in various species such as *Medicago truncata*, *Vitis vinifera* and *Populus trichocarpa*, none of them still annotated as DIR1-like proteins.

### Detection of Le-DIR1 in tomato

The strategy chosen to verify the existence of DIR1 in tomato was to develop polyclonal antibodies against a fragment of the predicted sequence of Le-DIR1, to be used in protein blot assays. An antigen design tool was then used to select a 14 amino acids sequence, corresponding to positions 17 through 29 of Le-DIR1. This antigenic sequence (shown in Figure 1) displayed low conservation with the other tomato LTP sequences similar to Le-DIR1, hence reducing the probability of cross-recognition. In fact, only 7 out of 14 amino acids are conserved in SGN-U320476, 6 out of 14 in SGN-U322554 and 4 out of 14 in SGN-U316923 (Figure 1). So, the synthetic peptide SVTQPNPVEPSASC coupled to BSA was used as antigen to generate polyclonal antibodies in rabbit. The evolution of the antiserum title was analysed by dot blot, and the second boost was selected for further usage.

In order to verify the existence of DIR1 in tomato we have extracted phloem sap-enriched fractions following standard procedures, in addition to total extracts from leaves, seeds and medium from cell suspensions. Protein gel blot analyses allowed the detection of a reactive signal of 7 kDa in tomato phloem sap (Figure 2), in accordance with the predicted molecular weight of DIR1 and its expected localization, as At-DIR1 was detected in petiole exudates enriched in phloem sap of *Arabidopsis* (Maldonado et al., 2002). On the other hand, no positive signal of 7 kDa was detected in all the other extracts tested (not shown).

To our knowledge, this report constitutes the first evidence on the existence of a DIR1-like protein aside from *Arabidopsis*, suggesting that the function demonstrated for At-DIR may be a generalized mechanism in plants. Although the role of Le-DIR1 in SAR signaling awaits further experimental evidence, recent data support the idea of mechanistic conservation of SAR in *Arabidopsis* and tomato. Hence, petiole exudates enriched in phloem sap collected from *Arabidopsis* leaves inoculated with an avirulent pathogen promote resistance not only when applied to *Arabidopsis*, but also to tomato and wheat, indicating that common signaling molecules are present in different species

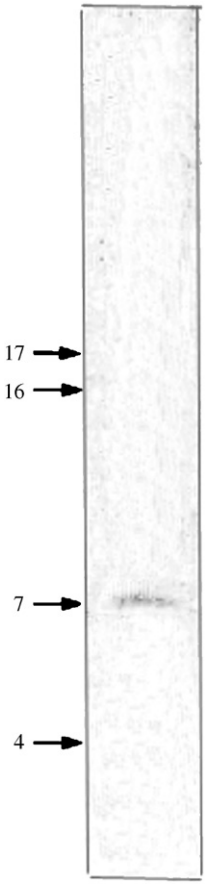


**Figure 2.** Immunodetection of DIR1 in tomato. A synthetic peptide deduced from the Le-DIR1 sequence was chemically synthesized and used to raise antibodies in rabbit. 1: Samples of phloem sap (28 μg of proteins) were fractionated on Tricine SDS-PAGE gels and submitted to Western blot using anti-Le-DIR1 antiserum in a dilution 1:2000. The arrows on the left indicate the low molecular weight markers (kDa). No signal was detected with preimmune serum.

(Chaturvedi et al., 2008). Besides, this work highlights the power of data mining and *in silico* analyses to assign putative identity to the vast number of sequences deposited in databases with deficient annotation.

## Acknowledgements

This work was supported by grants from the Argentine Research Council (CONICET), Agencia Nacional de Promoción Científica y Tecnológica and the University of Mar del Plata, from Argentina. LdlC is a member from the CONICET.

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