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BRIEF COMMUNICATION

MtTdp1α-depleted *Medicago truncatula* plants show reduced cuticle permeability and altered expression of defense genes

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Abstract

The link between the *MtTdp1α* (tyrosyl-DNA phosphodiesterase) gene, involved in the repair of DNA topoisomerase I mediated DNA damage, and the plant defense response has been investigated in *MtTdp1α*-depleted *Medicago truncatula* transgenic lines obtained by intron-spliced hairpin RNA approach, compared to the control line (CTRL, empty vector). Reduction of cuticle permeability highlighted by chlorophyll efflux assays positively correlated with the level of *MtTdp1α* gene silencing. The increased cuticle thickness was confirmed by transmission electron microscopy, which revealed an apparent expansion of the epicuticular waxes deposited on the outer surface. RNA-Seq analysis, carried out in the *MtTdp1α*-depleted plants, revealed the different expression of resistance (R) genes, PAMP (pathogen-associated-molecular pattern) triggered immunity (PTI) genes and transcription factors (TFs) involved in the regulation of the plant defense response.

Additional key words: cuticular waxes, RNA-Seq, transgenic plants, tyrosyl-DNA phosphodiesterase.

The role played by tyrosyl-DNA phosphodiesterase 1 (Tdp1), responsible for the removal of DNA topoisomerase I (topo I)-mediated DNA lesions, in maintaining genome stability has been deeply investigated in animal cells (Murai *et al.* 2012). The *Tdp1* gene family from *Medicago truncatula* has been characterised by Macovei *et al.* (2010) while RNA-Seq carried out in *MtTdp1α*-depleted *M. truncatula* plants allowed the identification of differentially expressed genes involved in DNA damage sensing/repair (Donà *et al.* 2013). In the present work, the molecular characterization of the *MtTdp1α*-depleted *M. truncatula* lines has been expanded focusing on the plant defense response. Down-regulation of the *MtTdp1α* gene in *M. truncatula* Gaertn. cv. Jemalong (M9-10a genotype) was obtained using an intron-spliced hairpin RNA approach and plants were micropropagated *in vitro* as described by Donà *et al.* (2013). The Tdp1α-2° and Tdp1α-2a1 lines hereby analysed were derived from two independent transformation events.

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Abbreviations: APX - ascorbate peroxidase; bZIP - basic leucine ZIPper; C2H2 - cysteine2-histidine2; CC - coiled coil; CTRL - control line; HR - hypersensitive response; LTP - lipid transfer protein; NBS-LRR - nucleotide binding site-leucine-reach repeat; PAMP - pathogen-associated-molecular pattern; PTI - PAMP triggered immunity; PR - pathogenesis related; R - resistance; Tdp - tyrosyl-DNA phosphodiesterase; TEM - transmission electron microscopy, TFs - transcription factors; topo I - DNA topoisomerase I; ZFP - zinc finger protein.

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The Tdp1*α*-2a, Tdp1*α*-2a1, and control (CTRL, empty vector) lines were subjected to chlorophyll efflux assays as reported by Curvers *et al.* (2010). Equal amounts of leaf tissues were excised from 20-d-old plants grown *in vitro* and incubated in 80 % (v/v) ethanol at room temperature for 180 min. Aliquots (1 cm^3) were sampled at different time points from the beginning of the treatment, chlorophyll content was quantified as described by Wellburn (1994) and calculated from absorptions at 480, 645, and 663 nm. Statistically significant differences were determined using Student's *t*-test ($P < 0.05$).

Transverse sections of leaves collected from the *MtTdp1α*-depleted and CTRL plants grown *in vitro* for 20 d were examined by transmission electron microscopy (TEM). Leaf tissue was fixed with 2 % (v/v) glutaraldehyde for 3 h, rinsed in phosphate buffered saline (pH 7.2) overnight, and post-fixed in 1 % (m/v) aqueous solution of OsO_4. The specimens were dehydrated in acetone and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and observed with a *Zeiss EM900* electron microscope (EM) equipped with a 30 mm objective aperture and operating at 80 kV. For each condition (or treatment) four different samples were examined and for each sample 3 EM grids, carrying at least 5 thin sections each, were analyzed.

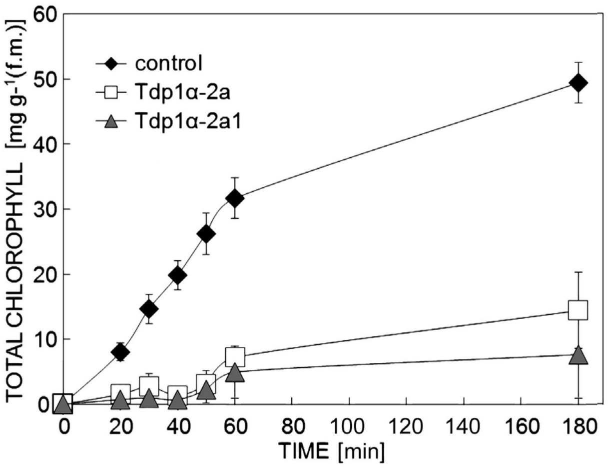

Fig. 1. Chlorophyll efflux assay. Total chlorophyll was extracted and measured from leaves of *MtTdp1α*-depleted and CTRL lines at different time points.

The amount of total chlorophyll extracted was significantly ($P < 0.05$) reduced in the Tdp1*α*-2a and Tdp1*α*-2a1 lines compared to the CTRL (Fig. 1*A*). The reduction of cuticle permeability highlighted by the chlorophyll efflux assays positively correlated with the level of *MtTdp1α* gene silencing. On the other hand, the pectinase assay performed on leaf tissues of the *MtTdp1α*-depleted lines did not reveal significant differences in cell wall properties (data not shown). In the CTRL, the cuticle was visible as an electron-dense layer on the outer part of the cell wall (Fig. 2*A*). In the Tdp1*α*-2a line, increased thickness of the cuticle, compared to CTRL, was observed (Fig. 2*B*). The most evident differences were visible in the external layer (Fig. 1*D*,*E*) since in the Tdp1*α*-2a line there was an expansion of the epicuticular waxes deposited on the outer surface. Similar observations were made with the Tdp1*α*-2a1 line (data not shown). The reduced cuticle permeability observed, as well as cuticle thickening in the *MtTdp1α*-depleted lines might be part of a general stress response constitutively activated in these plants. As shown for the topo I-deficient carrot cells (Balestrazzi *et al.* 2010), a significant reduction in the ascorbate peroxidase (*APX*) transcription was detected in the *MtTdp1α*-depleted lines (Donà *et al.* 2013). Low ascorbate amounts possibly resulting from impaired APX activity would also facilitate the onset of the oxidative burst, a key event in the plant response to pathogens.

Bionformatic analysis was conducted using RNA-Seq data (Donà *et al.* 2013) available at http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi under accession number SRP013555. The accession numbers of differentially expressed transcripts were blasted into the *LegumeIP* database (http://plantgrn.noble.org/LegumeIP/) to retrieve their functional annotations. Accessions with the *GO* (gene onthology) terms, such as ‘defense response to fungus’, ‘wounding’, ‘response to chitin’, ‘cell wall modification’, ‘defense response to bacterium’, and ‘plant-type hypersensitive response’, were retrieved. Different *R (RESISTANCE)* gene families are represented in Table 1 Suppl., *e.g.*, *NBS-LRR* (nucleotide binding site-leucine-reach repeat) and *CC* (coiled coil)-*NBS-LRR* (Burkhard *et al.* 2001). Ten *R* genes were differentially expressed between the two *M. truncatula* lines. Five *R* genes were less expressed in the CTRL compared to the *MtTdp1α*-depleted line. Three *PTI* [PAMP (pathogen-associated-molecular pattern) triggered immunity] genes encoding expansins, which participate in the pathogenesis of necrotrophic fungi (Abuqamar *et al.* 2013), were less expressed in the *MtTdp1α*-depleted cells (Table 2 Suppl.). The differential expression of two genes encoding pectin methylesterases and one gene coding for a cellulose synthase-like enzyme was also observed (Table 2 Suppl.). The finding that the *Medtr5g029190.1* gene was less expressed in the *MtTdp1α*-depleted line suggests increasing defence response since impairment of cellulose synthases enhances disease resistance (Vega-Sanchez *et al.* 2012). Four *PR (PATHOGENESIS RELATED)* genes encoding chitinases were more expressed in the *MtTdp1α*-depleted line, which might have activated the defence response despite the absence of pathogens (Table 2 Suppl.). A key defence gene encoding the antifungal protein thaumatin (Chu and Ng 2003) was also more expressed in the *MtTdp1α*-depleted cells (13-fold) compared to the CTRL (Table 2 Suppl.). Lipid transfer proteins (LTPs) are involved in the delivery of wax components during cuticle assembly (DeBono *et al.* 2009). The *Medtr3g106740.1* gene coding for a lipid transfer protein was less expressed (11-fold) in the *MtTdp1α*-depleted tissues (Table 2 Suppl.). At the moment, it is difficult to figure out the role of LTPs in this context of defective DNA repair, since besides

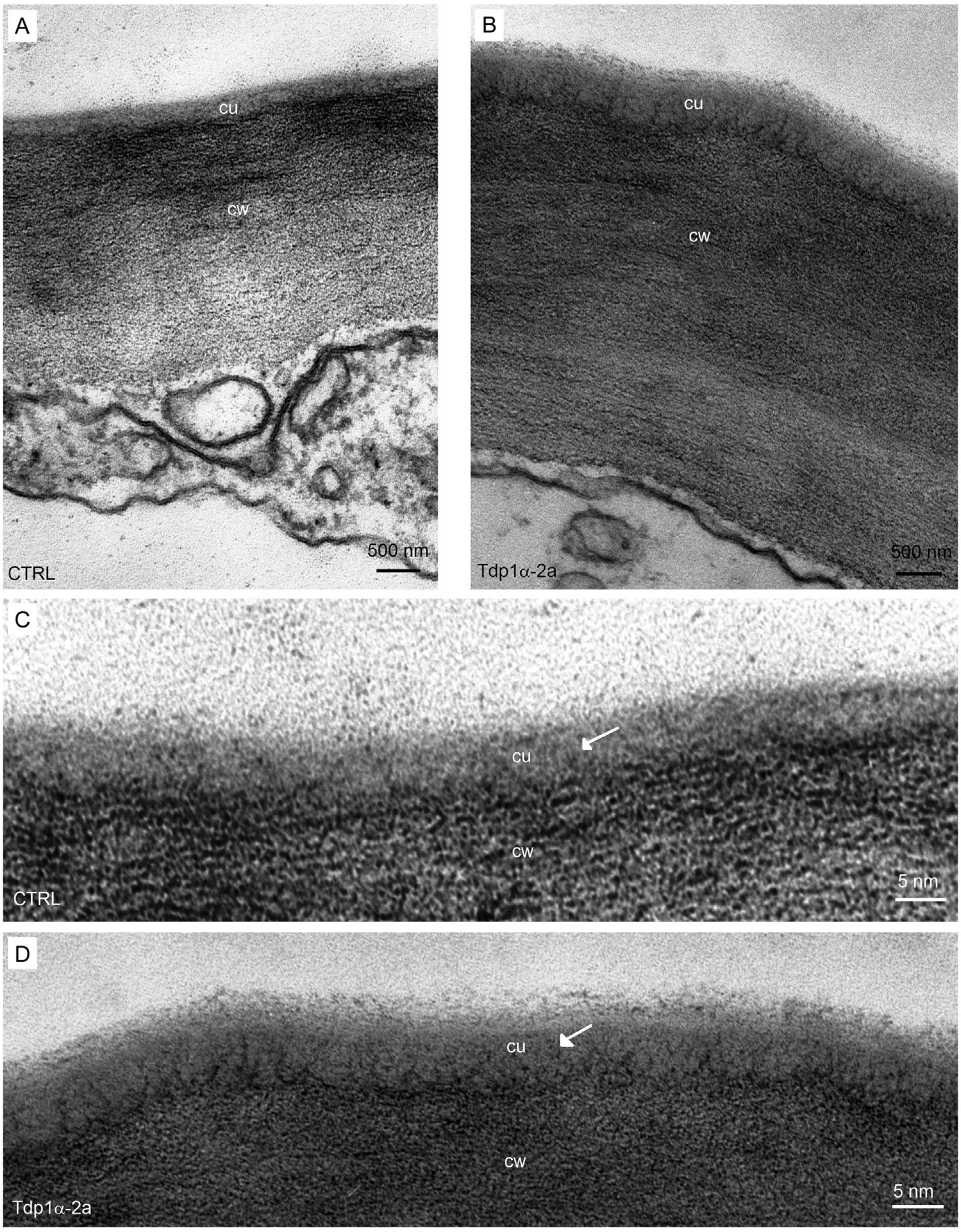

Fig. 2. Ultrastructural aspects of cuticle (cu) and cell wall (cw) revealed by TEM in *MtTdp1α*-depleted and CTRL lines. *A* - CTRL line, *B* - Tdp1α2a line, *C* - detail of the cell wall of CTRL line, *D* - detail of cell wall of Tdp1α2a line.

cuticle assembly, these proteins can also act within the signalling pathways leading to the hyper-sensitive (HR) response, thus triggering defence mechanisms (Buhot *et al*. 2001). While the *Medtr1g072420.1* gene coding for a putative endoglucanase inhibitor was less expressed in the *MtTdp1α*-depleted cells (Table 2 Suppl.), there was a significant enhancement in the expression of the *Medtr4g128580.1* and *Medtr4g076490.1* genes encoding a putative xyloglucan endotransglucosylase/hydrolase and endo-β-1,3-glucanase, respectively. As for transcription factors, lack of *MtTdp1α* gene expression resulted into differential expression of zinc finger proteins (ZFPs), which play a role in the response to biotic stress (Li *et al*. 2013). Zinc finger and bZIP C2H2-type genes were more expressed in the *MtTdp1α*-depleted plants compared to the CTRL (Table 3 Suppl.). A similar response was observed for several other transcription factors (TFs) implicated in the plant disease resistance response (Libault *et al*. 2007) (Table 3 Suppl.). Other *TF* genes were less expressed, *e.g*., the *NF-X1* homologue of the human transcription repressor *NF-X1*, the regulator of the trichothecene phytotoxin-induced defense response (Asano *et al*. 2008) (Table 3 Suppl.). The characterisation of the *MtTdp1α*-depleted plants hereby reported indicates the constitutive activation of multiple defence mechanism, including the reinforcements of the cell physical barriers. Since surface chemical and physical signals are known to affect adhesion, germination, and differentiation of preinfection structures in pathogenic fungi (Uppalapati *et al.* 2012), it would be interesting in the near future to conduct a study in which changes in the cuticule lipid and wax composition will be assessed in CTRL and *MtTdp1α*-depleted lines. The reported data highlight changes in the expression of several genes involved in cell wall modifications under stress conditions occurring in *MtTdp1α*-depleted *M. truncatula* plants. Some genes identified in this study have been identified in other legume plants as differentially expressed in response to pathogen attack (Almeida *et al.* 2015). Although our study was conducted in healthy and non-infected plants of *M. truncatula*, it provides us with a range of possible biomarkers to be assessed in future studies, in which the plant/pathogen interaction will be explored. Additionally, strong evidences of the potential of manipulating *Tdp1* genes as an approach to improve plant responses to biotic stresses are provided.

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