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RESEARCH PAPER

Molecular cloning and characterization of a novel tomato xylosyltransferase specific for gentisic acid

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Abstract

The importance of salicylic acid (SA) in the signal transduction pathway of plant disease resistance has been well documented in many incompatible plant–pathogen interactions, but less is known about signalling in compatible interactions. In this type of interaction, tomato plants have been found to accumulate high levels of 2,5-dihydroxybenzoic acid (gentisic acid, GA), a metabolic derivative of SA. Exogenous GA treatments induce in tomato plants a set of PR proteins that differ from those induced by salicylic acid. While SA accumulates in tomato plants mainly as 2-*O*-β-D-glucoside, GA has only been found as 5-*O*-β-D-xyloside. To characterize this step of the GA signalling pathway further, the present work focuses on the study of the GA-conjugating activity in tomato plants. A gentisate glycosyltransferase (*GAGT*) cDNA has been isolated and overexpressed in *Pichia pastoris*, and GA-conjugating activity was confirmed by detecting the xylosylated GA. The purified plant protein is highly specific for GA, showing no activity toward many other phenolic compounds, including SA. In addition, it shows an outstanding selectivity for UDP-xylose as the sugar donor, which differentiates this enzyme from most glycosyltransferases. Both the GA-conjugating activity and the corresponding mRNA show a strong, rapid, and transient induction upon treatment of tomato plants with GA or SA. Furthermore, its expression is rapidly induced by compatible infections. However, neither the gene nor the activity seems to respond to incompatible infections or wounding. The unique properties of this new glycosyltransferase suggest a specific role in regulating the free GA levels in compatible plant–pathogen interactions.

Key words: Compatible interactions, gentisic acid, glycosylation, plant pathogens, salicylic acid, *Solanum lycopersicum*, xylosyltransferase.

Introduction

Because of their sessile condition, plants have evolved a very efficient defence system against all sorts of potential environmental aggressions, either of a biotic or an abiotic nature. Some of these defence mechanisms are constitutive, and are present in the plant before pathogen entrance, whereas others are pathogen-inducible (van Loon *et al.*, 2006). In the latter case, once the plant recognizes pathogen arrival, a very complex signalling network is established which involves signal molecules such as salicylic acid (SA), jasmonic acid (JA), or ethylene (ET) (Lorenzo and Solano, 2005; Broekaert *et al.*, 2006; Loake and Grant, 2007). Recent studies indicate that other hormones such as abscisic acid, auxins, gibberellic acid, cytokinins, brassinosteroids, and peptide hormones are also implicated in different aspects of plant defence signalling pathways (Bari and Jones, 2009).

Depending on the nature of the plant–pathogen interaction, the resulting infection can be localized or systemic. When the specific gene-for-gene recognition occurs between the plant and the pathogen, an incompatible interaction takes place (Flor, 1971). In this case, the plant activates the

so-called hypersensitive response (HR), which mainly consists in rapid cell death around the infection point, causing pathogen confinement, and the infection is referred to as necrotizing. This defence response is very often associated with the activation of systemic acquired resistance (SAR), which is non-specific and long-lasting along the whole plant. This SAR-mediated protection is based on a selective and coordinated activation of a number of genes (SAR genes) that are directly implicated in the establishment and maintenance of this resistance (Ryals *et al.*, 1996; Grant and Lamb, 2006). Among these SAR genes, Pathogenesis-Related Proteins (PRs) are low-molecular-weight proteins that not only display antimicrobial properties, but also accumulate locally and systemically in the plant upon infection (Granell *et al.*, 1987; Rodrigo *et al.*, 1993; Sels *et al.*, 2008). On the other hand, when no gene-for-gene recognition occurs, the resulting interaction is considered as compatible. In this case, although the plant may activate an antipathogenic response, the pathogen escapes from local defences and a systemic infection is established (Staskawicz *et al.*, 1995).

To date, HR and SAR have been broadly studied, and SA has been proposed as the signal molecule that mediates these defence responses in incompatible interactions (Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Loake and Grant, 2007). However, very little is known about the signalling of the defence response in compatible interactions. SA accumulates in compatible interactions (O'Donnell *et al.*, 2001; Huang *et al.*, 2003), but a general role has not been established in tomato. This contrasts with findings in *Arabidopsis*, where SA-deficient plants are generally more susceptible to pathogens (Nawrath and Métraux, 1999). Gentisic acid (GA) has been described to accumulate at higher levels than SA in tomato, Gynura, and cucumber plants subjected to different compatible infections (Bellés *et al.*, 1999, 2006). Moreover, exogenous GA elicits the accumulation of the antifungal PR proteins P23, P32, and P34 in tomato (García Breijo *et al.*, 1990; Rodrigo *et al.*, 1993; Bellés *et al.*, 1999). These proteins are not induced by exogenous SA, which is able to elicit other PR proteins in the same plant. Thus, GA has been proposed to play a role as an intermediary in compatible, non-necrotizing interactions (Bellés *et al.*, 1999, 2006). Interestingly, GA is an effective antifungal plant compound (Lattanzio *et al.*, 1994), and GA behaves as a strong antioxidant molecule in mammalian cells, exerting a protective effect against certain bacteria (Belicova *et al.*, 2001).

Similar to other hydroxybenzoates, GA accumulates in the plant as a glycoconjugate. However, unlike other related phenolics such as SA or benzoic acid, which are conjugated to glucose after their accumulation upon infection (Silverman *et al.*, 1995; Lee and Raskin, 1998, 1999; Chong *et al.*, 2001), GA accumulates exclusively as 5-*O*-β-D-xylopyranoside (Fayos *et al.*, 2006). This xylose conjugate of GA has recently been found to be the most important induced metabolite in tomato plants upon viroid infection (López-Gresa *et al.*, 2010).

In plants, glycosylation is one of the most common modifications of secondary metabolites, which is implicated in stabilization, the increase of solubility, and in the storage and regulation of levels of certain hormones and signal molecules as well as in the detoxification of xenobiotics (Yalpani *et al.*, 1992; Szerszen *et al.*, 1994; Gachon *et al.*, 2005). Glycosylation is carried out by glycosyltransferases (GTs) which transfer nucleotide-diphosphate-activated sugars (known as the 'glycosyl donor') to low-molecular-weight substrates. Increasing evidence suggests that glycosylation is an important mechanism to regulate plant cellular homeostasis with the identification of a large variety of GTs capable of recognizing many different compounds (Bowles *et al.*, 2006). According to the CAZy database (http://www.cazy.org/), glycosyltransferases can be classified into 91 families, depending on substrate specificity and sequence similarity (Osmani *et al.*, 2009). Currently, many GTs have been sequenced, although only a few of them have been characterized biologically.

In this work, the focus is on the purification and characterization of the tomato xylosyltransferase responsible for the conjugation of GA. A cDNA clone was isolated (AJ889012) and expressed in *Pichia pastoris*. The enzyme displays outstanding selectivity toward the sugar donor, using mainly UDP-xylose. Furthermore, gentisic acid seems to be the only phenolic compound specifically to accept the sugar. The protein and its mRNA show a rapid and transient induction upon systemic infections, and GA and SA treatments. Nevertheless, tomato GAGT apparently does not respond to incompatible interactions or wounding. The unique properties of this novel glycosyltransferase suggest a very specific role for this protein in the regulation of GA levels in compatible plant–pathogen interactions.

Materials and methods

Plant materials, chemicals, and pathogen treatments

Tomato (*Solanum lycopersicum* L. cv. Rutgers or Rio Grande) plants were grown under standard greenhouse conditions (20–25 °C and 16/8 h light/dark photoperiods).

Treatments and wounding were performed with 3–4-week-old plants. For the SA and GA treatments, fully expanded leaves were excised and immersed by the petiole in 2 mM SA or GA solutions. Ethylene treatments of full plants were carried out in air-tight plexiglass chambers under a continuous flow of gas at 50 ppm. Methyl jasmonate was applied by spraying plants with a 2 mM solution in water containing 0.02% (v/v) TWEEN-20. Wounding was performed by crushing one composite leaf per plant using forceps. The immediate upper leaves were also used to analyse the systemic response. Plant material was harvested at different times, then used immediately or stored frozen at –80 °C.

Inoculation of Rutgers tomato plants with Citrus Exocortis Viroid (CEVd) or with Tomato Mosaic Virus (ToMV) was carried out according to the indications of Granell *et al.* (1987) and Bellés *et al.* (1999), respectively. Rio Grande (*PtoR*) tomato plants were inoculated with *Pseudomonas syringae* pv. *tomato* (*AvrPto*$^{+}$) at 10^{8} cfu ml^{-1} to produce a necrotizing infection. The bacterial culture was infiltrated into leaves, as previously described (Anderson *et al.*, 2006). Rio Grande tomato plants and bacteria were kindly supplied by GB Martin (The Boyce Thompson Institute for Plant Research, Ithaca, NY).

Extraction and quantification of SA and GA from tomato leaves

The preparation and analysis of free and conjugated SA and GA were performed according to Bellés *et al.* (1999, 2006). An HPLC

analysis of phenolics was done following the protocol detailed in Yalpani *et al.* (1992). A 20 µl aliquot from the final methanolic sample was injected into a reverse-phase Symmetry 5 µm C18 (4.6×150 mm; Waters) column equilibrated in 1% acetic acid. Eluents were 1% acetic acid (eluent A) and 100% methanol (eluent B). A lineal gradient starting with 100% eluent A and 0% eluent B and ending with 0% of eluent A and 100% eluent B was applied over 20 min at a flow rate of 1 ml min^{-1}. SA and gentisic acid were detected with a Waters 470 fluorescence detector (λ excitation=313 nm; λ emission=405 nm), and were quantified with the Waters Millennium^{32} software using authentic standards.

Xylosyltransferase activity assay and detection

The standard assay for GA xylosyltransferases was performed as follows: the reaction mixture contained an appropriate volume of the protein extract and a final concentration of 0.5 mM GA (Sigma) and 1 mM UDP-xylose (acquired from CarboSource Services, Complex Carbohydrate Research Center, University of Georgia, USA). In the radioactive assays, the reaction mixture contained 21 µM GA (Sigma) and 21 µM UDP-[^{14}C]-xylose (American Radiolabeled Chemicals Inc.). To test substrate specificity, 21 µM GA were replaced with the same concentration of the different acceptor substrates: salicylic acid, benzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 2,3,4-trihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, caffeic acid, ferulic acid, coumaric acid, scopoletin, esculetin, or umbelliferone. All these phenolics were purchased from Sigma. To study the specificity of the glycosyl donor substrate, UDP-[^{14}C]-xylose was replaced with UDP-[^{14}C]-glucose (American Radiolabeled Chemicals Inc.). The different preparations were incubated at 37 °C for 15–60 min. Then, samples were centrifuged for 15 min and analysed either by high performance liquid chromatography (HPLC, Waters), according to the indications of Yalpani *et al.* (1993) and Bellés *et al.* (1999) or thin-layer chromatography (TLC) on silica gel (Alugram SIL G/UV plates, Macherey-Nagel). To detect the conjugated products by HPLC, 40 µl of reaction volume were injected into a C18 reverse-phase column (5 µm, 4.6×150 mm; Waters, Milford, MA) with a linear gradient of methanol (0–100%) at a flow rate of 1.5 ml min^{-1} for 20 min. Conjugated phenolics were detected with either a spectrofluorescence detector as described above (λ excitation=313 nm; λ emission=405 nm) or a radioactivity detector (LB 509 EGG Berthold, Bad Wildbad-Germany). For the TLC analysis, 1–5 µl of each sample was applied to silica gel plates and separated using a solvent consisting of 1-butanol/acetic acid/water (4:1:1 by vol.). Sugars were detected by spraying the dried TLC plates with 15% (v/v) sulphuric acid containing 5 mM ceric sulphate, and were developed at 120 °C for 15 min. Radioactive spots were visualized by autoradiography.

Cloning of tomato GAGT

A sequence alignment of the different GTs of the Solanaceae family was carried out to build a set of degenerate oligonucleotide primers: sense (5′-GTTT(AC)(CT)GAT(AC)(CT)(AG)TT(CT)CTTCC-3′) and antisense (5′-TGGC(AC)(AT)TG(CT)(AC)A(CT)CATTGG-TAC-3′). Five µg of total RNA from GA-treated tomato leaves were reverse-transcribed in a final volume of 50 µl using oligo$(dT)_{18}$ and M-MLV reverse transcriptase (Promega). Then, 5 µl of RT product was amplified using *Pfu* DNA polymerase (TaKaRa) and the degenerate primers described. The amplified DNA (about 700 bp) was recovered from the agarose gel using the QIAquick Gel Extraction kit (Qiagen) and cloned in pGEM-T Easy (Promega) according to the manufacturer's recommendations. Several random clones were picked up and sequenced. To obtain the full-length cDNA clone, the selected DNA sequence was labelled with [^{32}P]-dCTP using the Ready-To-Go kit (GE Healthcare), and was used as a probe to screen a λ-ZAP (Stratagene) cDNA library constructed in our laboratory from the mRNA of gentisic acid-treated tomato leaves (our unpublished results).

RNA isolation, blotting, and hybridization

Total RNA was prepared by using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. For the Northern blot analysis, 30 µg of RNA were separated in formaldehyde-agarose gels and transferred onto Nytran membranes (Schleicher & Schuell). Hybridization with [^{32}P]-labelled probes and washing conditions were performed as described in Church and Gilbert (1984).

Overexpression and purification of the recombinant GAGT protein in Pichia pastoris

The coding region of the tomato GAGT cDNA was amplified by PCR using the primer pairs: sense (5′-CCGGTACCAGTATGGC-CATGACTACTCACAAAGCTC-3′) and antisense (5′-CCG-GGCCCGGAAATAGTAACCAACTTGG-3′); and the Expand High Fidelity PCR system (Roche) under the following conditions: one cycle of 95 °C for 3 min, 40 cycles of 1 min at 55 °C, 3 min at 72 °C, and 1 min at 95 °C, with a final extension step at 72 °C for 7 min. The PCR fragment was gel-purified and digested with *Kpn*I and *Apa*I. The pPICZ plasmid (Invitrogen) was cut under the same conditions and treated with shrimp alkaline phosphatase (Roche). The cDNA sequence was then ligated into pPICZ using T4 DNA ligase (Promega). After transformation into *E. coli* DH5α cells and plating on LB/half-salt agar containing zeocin (25 µg ml^{-1}, Invitrogen), positive clones were selected and sequenced to confirm the reading frame. Two to 10 µg of the expression construct plasmid DNA were linearized according to the supplier's instructions, and were used to electroporate the *P. pastoris* competent cells, obtained as described by Gietz and Woods (2002). The expression of the recombinant protein was induced by methanol according to the manufacturer's directions. Yeast cells were centrifuged and the pellet was resuspended in extraction buffer (20 mM sodium phosphate, pH 7.5, 500 mM NaCl, and 25 mM imidazole). Cells were mechanically broken using glass beads (0.5 mm diameter), and the suspension was centrifuged for 20 min at 10 000 *g* to obtain the crude protein extract for both the uninduced and methanol-induced yeast cells.

Xylosyltransferase activity purification

Preparation of the crude leaf extract: One hundred grams of frozen tomato leaves were ground to a fine dust in liquid nitrogen, and resuspended in 200 ml extraction buffer consisting of 25 mM MES (pH 6.5), 1 mM PMSF, 0.2% β-mercaptoethanol, and 0.05% PVP. The plant material was then homogenized using a Polytron. The tissue debris was removed by centrifugation at 15 000 *g* for 20 min and the supernatant was filtered through Miracloth. The filtrate was used for further purification.

Ammonium sulphate fractionation: The protein fraction precipitating between 35% and 65% saturation of ammonium sulphate was recovered by centrifugation. The pellet was resuspended in 25 mM MES (pH 6.5) containing 0.2% β-mercaptoethanol (MES-M buffer), and was desalted through PD-10 columns (Amersham Pharmacia Biotech) equilibrated with MES-M buffer.

Anion exchange chromatography: After desalting, protein samples were chromatographed through a Q-Sepharose Fast Flow column (Amersham Pharmacia Biotech), which was equilibrated with 25 mM MES-M buffer containing 0.05 M NaCl. Proteins were eluted with a 0.05–0.6 M lineal NaCl gradient in MES-M buffer at a flow rate of 1 ml min^{-1}. Fractions were collected and assayed for enzyme activity and protein concentration. The fractions containing enzyme activity were pooled, desalted using a PD-10 column, concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore) and then rechromatographed through Q-Sepharose under the same conditions.

HiTrap Blue affinity chromatography: The fractions recovered from the previous step were further purified with a HiTrap Blue affinity column (Amersham Pharmacia Biotech) equilibrated with 25 mM MES-M buffer. Proteins were eluted with a 0.2–0.6 M NaCl gradient in MES-M buffer at a flow rate of 2 ml min^{-1}. Active fractions were desalted in a PD-10 column and concentrated using Amicon Ultra-15 filter units.

Protein analysis

Samples from each protein purification step were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 as described by Conejero and Semancik (1977). The Bradford method (1976) was employed for protein quantification using bovine serum albumin as a standard.

Protein modelling

The amino acid sequence of tomato GAGT was submitted to the SWISS-MODEL server (http://swissmodel.expasy.org) using the crystal structure data of *Medicago truncatula* UGT85H2 glycosyltransferase (Li *et al.*, 2007) as a template (PDB ID 2PQ6). The DeepView (http://spdbv.vital-it.ch/) software was used to visualize, align, and prepare the structures for submission. Final rendering was done with the UCSF Chimera software (http://www.cgl.ucsf.edu/chimera).

Results

Conjugation of gentisic acid in tomato

Upon a systemic infection, tomato plants undergo a strong accumulation of GA, whose levels increase considerably more than the corresponding SA levels (Bellés *et al.*, 1999). The production of GA or SA in the plant has been reported to be followed by a rapid conjugation of these phenolics (Lee and Raskin, 1998; Bellés *et al.*, 1999; Schuhegger *et al.*, 2006). Like most hydroxybenzoates, SA accumulates mainly as 2-*O*-β-D-glucoside (Edwards, 1994; Lee and Raskin, 1999), whereas GA is conjugated as 5-*O*-β-D-xyloside (Fayos *et al.*, 2006). To study the GA-conjugating activity in tomato, tomato leaves were treated with 2 mM GA, and the levels of the free and conjugated GA were analysed at 4 h and 24 h of treatment, since glycosyltransferases have been shown to be induced very quickly (Lee and Raskin, 1999; Park *et al.*, 2003). As shown in Fig. 1A, more than half the total GA was conjugated 4 h after treatment. By 24 h, most of the GA was conjugated, reaching up to 97%. An analogous experiment was carried out by treating plants with 2 mM SA (Fig. 1B). After 4 h, no conjugated SA was detected, and only half the total SA was conjugated at 24 h. All this indicates in relative and absolute terms that GA conjugates to a much greater extent than SA at 4 h and 24 h after the corresponding treatments.

In order to detect the corresponding GA xylosyltransferase activity, crude extracts of tomato leaves were incubated with UDP-xylose and GA as substrates. After 15 min, the accumulation of phenolics was analysed by HPLC. The chromatograms showed two peaks (Fig. 1C): one corresponds to free gentisic acid (13.8 min), while the more polar one corresponds to GA 5-*O*-β-D-xyloside (13.1 min). The standard for GA 5-*O*-β-D-xyloside was obtained in our laboratory (Fayos *et al.*, 2006). Using this activity assay, it was possible to study the induction pattern of the GA xylosyltransferase activity present in tomato leaves upon different treatments or infections.

Cloning the cDNA of GAGT

To obtain a cDNA corresponding to the xylosyltransferase activity detected, a comparative sequence analysis between different glycosyltransferases was performed. Since our activity was induced by either GA or SA (see below), several GTs that have been described to be induced by salicylic acid or by other phenolic compounds were used to perform a DNA sequence alignment. Specifically, the GTs used for the sequence comparison were *SAGT*, *IS5*, and *Togt1* from *Nicotiana tabacum*, and *Twi1*, from *Solanum lycopersicum*, both species belong to the Solanaceae family (Horvath and Chua, 1996; Fraissinet-Tachet *et al.*, 1998; O'Donnell *et al.*, 1998; Lee and Raskin, 1999). Based on this analysis, a set of degenerate primers was designed and used in a RT-PCR of the RNA of tomato plants which were either healthy or infected by the Citrus Exocortis Viroid. In both cases, a band of the predicted 700 bp was obtained and was more intense in the infected plants (not shown), which is in accordance with an increase in the accumulation of GA 5-*O*-β-D-xyloside in plants infected with this viroid as compared with control plants (Fayos *et al.*, 2006). This PCR band was cloned in a pGEM-T vector and, since the primers used were highly degenerated, a number of clones were sequenced. The sequences obtained fell into two different categories: the previously described *Twi1*, a salicylic acid- and wound-induced glycosyltransferase (O'Donnell *et al.*, 1998) and a new clone (GenBank accession number AJ889012). We focused on this clone as the putative GA glycosyltransferase (GAGT). To obtain the complete cDNA of GAGT, the PCR product was used as a probe to screen a cDNA library constructed from the mRNAs of GA-treated tomato leaves which had been previously obtained in our laboratory. The complete cDNA obtained contains an ORF of 1370 bp that codes for a 51.5 kDa protein (see Supplementary Fig. S1A at *JXB* online). This size is similar to that of most of the GTs implicated in secondary metabolism (Vogt and Jones, 2000). The protein has a deduced isoelectric point of approximately 5.7 and a net charge of –7.8 at pH 7. It contains the consensus sequence PSPG (Plant Secondary Product Glycosyltransferase motif) described by Hughes and Hughes (1994). This sequence has been proposed to be the binding site for the sugar donor UDP (Shao *et al.*, 2005). According to the CAZy database (http://www.cazy.org/), GAGT belongs to family 1 of the glycosyltransferases (GT1). This is the largest family and includes GTs involved in many different processes, such as conjugation and the regulation of signalling molecules (indole acetic acid, zeatin or SA). The GAGT sequence displays an 85% identity with the tobacco SAGT (Lee and Raskin, 1999), which also belongs to the GT1 family. However, its similarity to the rest of the GTs included in this family hardly exceeds 30%, and its identity with tomato

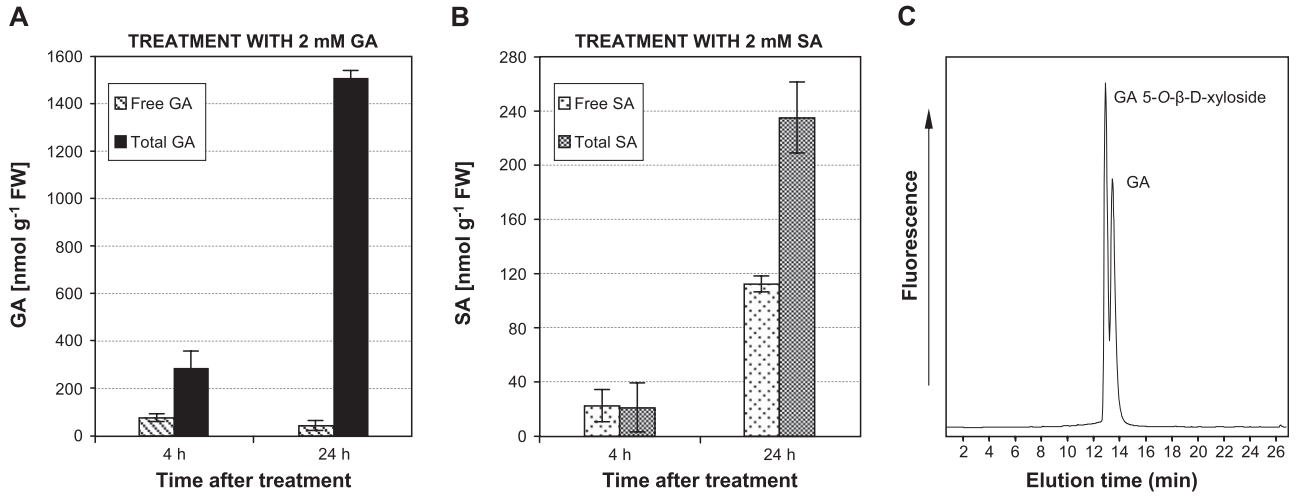

Fig. 1. Conjugation of GA and SA in tomato. Tomato leaves were treated with 2 mM gentisic acid or salicylic acid for 4 h and 24 h, and the contents of the free and total GA and SA are shown in (A) and (B), respectively. Results are the means of three independent assays ±SD (standard deviation). (C) HPLC detection of the GA conjugate. Gentisic acid and UDP-xylose were incubated with crude tomato leaf extracts as described in the Material and methods. After 10 min, phenolics were extracted and analysed by HPLC. The peak corresponding to the more polar GA 5-*O*-β-D-xyloside appears at 13.1 min, while free GA peaks later at 13.8 min.

Twi1 is also very low (see the phylogenetic tree in Supplementary Fig. S1B at *JXB* online). In addition to its high similarity to tobacco SAGT, tomato GAGT appears close to another SA glycosyltransferase from rice (accession number BAD34358) and two glycosyltransferases induced by jasmonic acid from maize (Szerszen *et al.*, 1994) and tobacco (accession number AB000623).

Expression and enzyme activity of the recombinant protein

In order to determine whether the isolated putative glycosyltransferase sequence encodes an active GA xylosyltransferase, the coding region of the corresponding cDNA was expressed in *Pichia pastoris* using the pPICZ overexpression vector. The transformed yeast was grown in a methanol-induced media culture to express the recombinant protein. Detection of the enzyme activity was performed by using TLC and autoradiography as described for the other GTs expressed in yeast (Bencúrová *et al.*, 2003) and for many others expressed in *E. coli*, such as the tobacco SAGT (Lee and Raskin, 1999; Kohara *et al.*, 2007). Following the addition of UDP-[^{14}C]-xylose and GA, a [^{14}C]-xylosylated metabolite of GA was formed only in the reaction mixture corresponding to the methanol-induced yeast lysates containing the recombinant protein (Fig. 2A). The spot corresponding to the [^{14}C]-xylosylated GA shows the same mobility as the standard 5-*O*-β-D-xylopyranoside of GA obtained in our laboratory (Fayos *et al.*, 2006) and the xylosylated GA produced using crude plant extracts (Fig. 2B, lanes 2 and 3, respectively). No GA glycoside was produced when UDP-[^{14}C]-glucose was used as a sugar donor. In addition, no activity was detected when SA was tested as a sugar acceptor using UDP-[^{14}C]-xylose or UDP-[^{14}C]-glucose (data not shown). Therefore, these results indicate that the isolated clone encodes a GA-specific xylosyltransferase.

Expression of GAGT mRNA and induction of activity

The tomato GAGT cDNA was used as a probe to study the effect of different signal molecules, as well as compatible and incompatible pathogen interactions, on the induction of the mRNA, and gentisate-5-*O*-β-D-xylosyltransferase enzyme activity was measured in parallel for all treatments.

Effect of GA and SA: Xylosyltransferase activity was enhanced by both GA and SA treatments as compared to the control water-treated plants (Fig. 3A). This enhanced activity remained 24 h later. Interestingly, the effect of GA on enzyme activity was much stronger than that produced by SA treatment. This early induction pattern matches the behaviour described for many GTs induced by SA (Yalpani *et al.*, 1992; Fraissinet-Tachet *et al.*, 1998; O'Donell *et al.*, 1998; Lee and Raskin, 1999; Park *et al.*, 2003; Griesser *et al.*, 2008). In general, GTs are rapidly inducible enzymes when compared with other proteins that respond to SA, such as PR defence proteins, which typically begin to accumulate later (Granell *et al.*, 1987; Sels *et al.*, 2008). The Northern blot analysis indicates a rapid and transient induction of *GAGT* mRNA (Fig. 3B, C). Very low levels of *GAGT* mRNA were constitutively present prior to treatment. Message accumulation began at approximately 1 h after treatment with SA or GA, and reached a peak at between 4 h and 6 h, then returned to the basal levels at 24 h. These results are in agreement with the enzyme activity profiles. However, a high xylosyltransferase activity was still detected 24 h after starting treatments when the mRNA levels had returned to the basal levels.

Compatible interactions: Citrus Exocortis Viroid (CEVd) and Tomato Mosaic Virus (ToMV), which produce a systemic non-necrotizing infection in tomato, have been shown strongly to induce the accumulation of free and conjugated

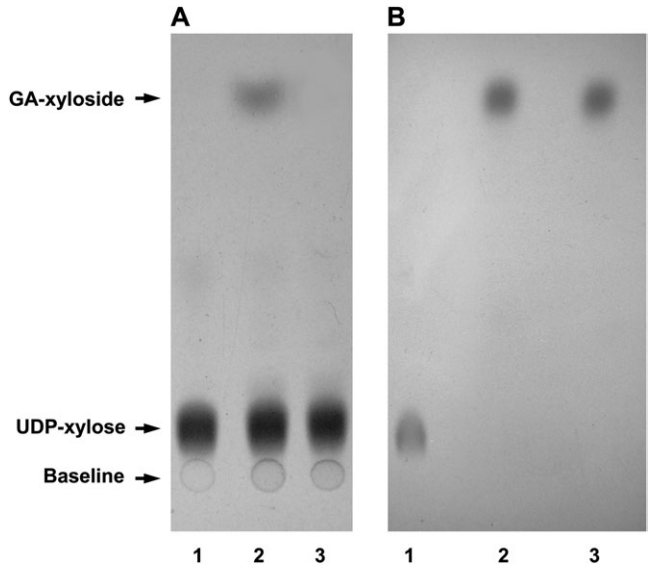

Fig. 2. Xylosyltransferase activity of recombinant GAGT. Left panel: *GAGT* cDNA was expressed in *Pichia pastoris* cells under the control of a methanol-inducible promoter (see the Materials and methods). Extracts from the uninduced (lane 1) and methanol-induced cells (lane 2) were incubated with UDP[^{14}C]-xylose and GA. Lane 3 corresponds to UDP[^{14}C]-xylose. Right panel: samples of UDP-xylose (lane 1), standard GA-5-*O*-β-D-xyloside, previously obtained in our laboratory (lane 2; Fayos *et al.*, 2006) and the GA xyloside produced using the crude tomato leaf extracts (lane 3) were separated by TLC under the same conditions, and were chemically revealed as described in the text.

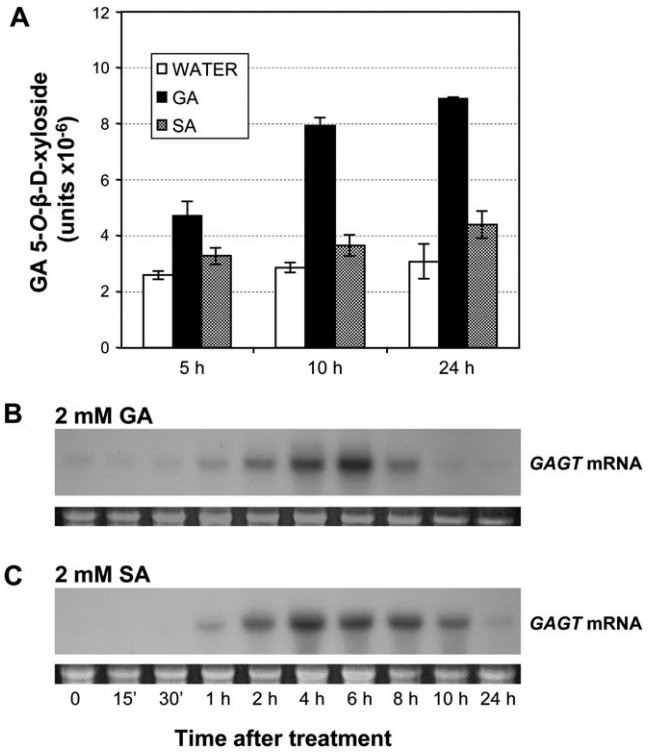

Fig. 3. GAGT induction by SA or GA treatment. Tomato leaves were incubated with water, 2 mM salicylic acid (SA) or 2 mM gentisic acid (GA), and plant material was collected at the indicated times. (A) The GAGT activity of the crude protein extracts was measured at 5, 10 or 24 h after treatment. The crude leaf extracts were incubated for 15 min with GA and UDP-xylose, and the amount of 5-*O*-β-D-xyloside formed was determined by fluorescence HPLC as described in the Materials and methods. Results are the means of three independent assays ±SD (standard deviation). (B, C) Northern blot analysis of *GAGT* mRNA accumulation in tomato leaves in response to GA (B) and SA (C) treatments. Samples were harvested at 0, 15 min, 30 min, 1, 2, 4, 6, 8, 10, and 24 h after treatment.

GA (Bellés *et al.*, 1999; Fayos *et al.*, 2006). This has also been observed in other plant–pathogen compatible interactions (Bellés *et al.*, 2006). The GA-xylosyltransferase activity was measured in tomato plants infected with CEVd or ToMV. Tissue samples were collected 4 weeks after inoculation for CEVd-infected plants and 7 d after inoculation for ToMV-infected plants. As shown in Fig. 4A, a dramatic increase in activity was observed in both the virus- and viroid-infected plants, and higher 5-*O*-β-D-xylosyltransferase levels were found in the CEVd-infected plants as compared to the ToMV-infected tissues. These results are in agreement with those obtained by Bellés *et al.* (1999), where levels of total GA upon viroid infection were higher than the GA levels present in the virus-infected plants. Thus, there seems to be a correlation between GA accumulation and the enhanced GA xylosyltransferase activity in these plants. The Northern blot analysis shows that the *GAGT* mRNA apparently followed the severity of symptoms caused by viroid infection (Fig. 4B). Viroid disease symptoms appeared in the 'Rutgers' tomato plants 2 weeks after inoculation with CEVd. Accordingly, no accumulation of *GAGT* mRNA was detected 7–10 d after CEVd inoculation, when the mRNA levels progressively accumulated along the viroid disease. ToMV inoculation was performed in 5-week-old tomato plants. Samples of local and distal leaves were collected at 3 d and 7 d post-inoculation, coinciding with the absence or presence of disease symptoms, respectively. The presence of the viral capsid was confirmed by SDS/PAGE, and disease progress was concomitant with the induction of the *GAGT* mRNA (Fig. 4C). These results indicate that, in both the CEVd and ToMV infections, the induction of the *GAGT* runs parallel to the progress of the disease, resulting in enhanced GA 5-*O*-β-D-xylosyltransferase activity.

Incompatible interactions: In order to establish a necrotizing infection, tomato plants were inoculated with *Pseudomonas syringae*. Samples were taken at several times post-inoculation and tested for GA xylosyltransferase activity. As Fig. 5A shows, *Pseudomonas* infection induced no increase in GAGT activity. Consequently, only basal levels of *GAGT* mRNA were detected by Northern blot analysis (Fig. 5B). Since the appearance of local, necrotic lesions is accompanied by an increase in SA (Malamy *et al.*, 1990; Métraux *et al.*, 1990; Uknes *et al.*, 1993), the content of free SA and GA was determined throughout the bacterial

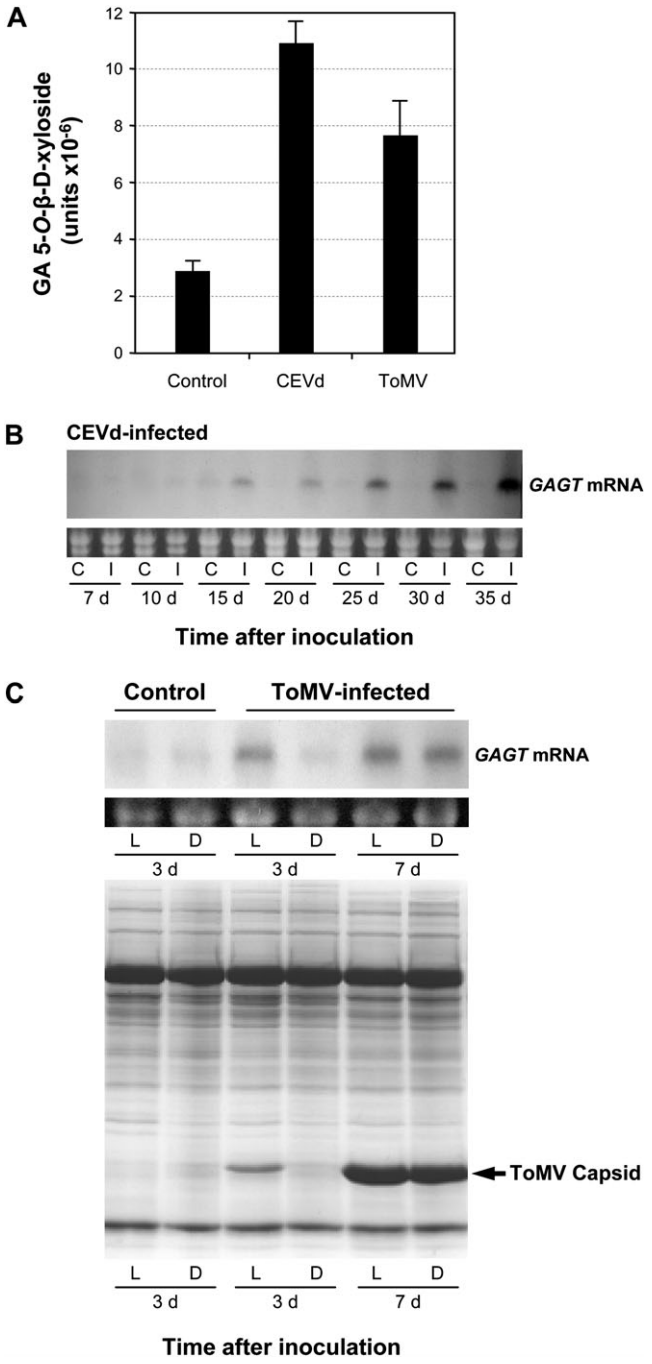

Fig. 4. GAGT induction by compatible infections. Tomato plants were inoculated with Citrus Exocortis Viroid (CEVd) or Tomato Mosaic Virus (ToMV), and infected leaves were collected at different days post-inoculation. (A) The GAGT activity of the tomato protein extracts was measured 35 d after CEVd inoculation or on day 7 after ToMV inoculation. The crude leaf extracts were incubated for 15 min with GA and UDP-xylose, and the amount of 5-*O*-β-D-xyloside formed was determined by fluorescence HPLC as described in the Materials and methods. Results are the means of three independent assays ±SD (standard deviation). (B) The time-course analysis by Northern blot of the *GAGT* mRNA accumulation in tomato leaves, in response to CEVd infection. C: control plants; I: CEVd-infected plants. Samples were collected on days 7, 10, 15, 20, 25, 30, and 35 post-inoculation. (C) Upper panel: time-course analysis by Northern blot of the *GAGT* mRNA accumulation. The local (L) or immediate upper (Distal, D) leaves were collected on days 3 or 7 post-inoculation. The first lanes correspond to the control, non inoculated plants. Lower panel: SDS-PAGE analysis of the total leaf proteins from the ToMV-infected tomato plants for the same samples shown in the upper panel. The protein band corresponding to the ToMV capsid is indicated by n arrow.

infection. Figure 5C shows that, although the infection led to an expected increase in the SA levels, the GA levels remained practically unchanged. However, SA treatment did induce *GAGT* (Fig. 3C). The results obtained with *P. syringae* question the effect of SA on *GAGT*, and suggest that induction by SA may occur through GA, which is a metabolic derivative of SA. This is in contrast with many glycosyltransferases which are induced in an SA-dependent way after infection with *Pseudomonas syringae* pv. tomato (Langlois-Meurinne *et al.*, 2005).

Wound and jasmonic acid treatment: Contrary to the results reported for other GTs such as tomato *Twi1* (O'Donnell *et al.*, 1998), whose mRNA is accumulated by wounding, no increase in GA xylosyltransferase activity was detected upon wounding or treatment with jasmonic acid (Fig. 6A); furthermore, the Northern blot analyses (Fig. 6B, C, D) confirm this observation.

Substrate specificity

The *in vitro* substrate specificity of recombinant GTs often differs from the native activity. The ability of the recombinant protein to conjugate diverse substrates makes it difficult to study the physiological role of GT in the plant (Jones and Vogt, 2001; Achnine *et al.*, 2005; Bowles *et al.*, 2006). Consequently, the GA 5-*O*-β-D-xylosyltransferase activity from Rutgers tomato plants was purified to study its substrate specificity properly. The enzyme in the crude extracts was stable for months when stored at –80 °C. However, the enzyme was sensitive to oxidation; therefore, a reducing agent (β-mercaptoethanol) was always included in the extraction buffer. Activity was purified as described in the Materials and methods, and resulted in a 68-fold purification factor (Table 1). The purified enzyme preparation was assayed for substrate specificity towards a variety of phenolic sugar acceptors, using UDP-[^{14}C]-xylose as a sugar donor (Fig. 7A, B). When UDP-[^{14}C]-glucose was used as a sugar donor, the purified enzyme showed no conjugating activity. This is in agreement with most GTs described to date, which are fairly specific to the sugar donor substrate (Warnecke and Heinz, 1994; Lee and Raskin, 1999; Vogt and Jones; 2000). By contrast, GTs are usually less selective toward sugar acceptors (Warnecke and Heinz, 1994; Fraissinet-Tachet *et al.*, 1998; Lee and Raskin, 1999; Jackson *et al.*, 2001; Griesser *et al.*, 2008). It is worth noting that, in our case, tomato GAGT shows a surprisingly narrow specificity toward GA as the sugar acceptor.

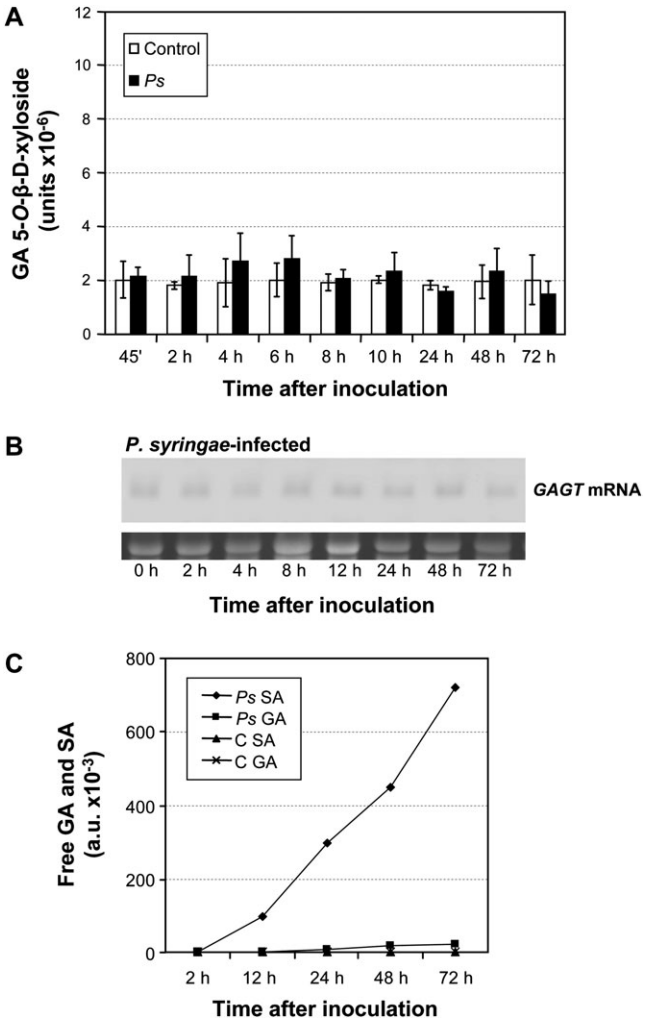

Fig. 5. GAGT induction by incompatible infections. (A) The GAGT activity of tomato protein extracts was measured at different times (45 min, 2, 4, 6, 8, 10, 12, 24, 48, and 72 h) post-inoculation with *Pseudomonas syringae*. Enzyme reactions were incubated for 15 min and 5-*O*-β-D-xyloside was analysed by fluorescence HPLC. Results are the means of three independent assays ±SD (standard deviation). (B) The time-course analysis by Northern blot of the *GAGT* mRNA accumulation in tomato leaves, in response to *Pseudomonas syringae* infection. Samples were harvested at 0, 2, 4, 6, 8, 10, 12, 24, 48, and 72 h post-inoculation. (C) The free gentisic acid and salicylic acid levels in the control or the *Pseudomonas syringae*-infected tomato leaves at 2, 12, 24, 48, and 72 h post-inoculation.

A similar specificity has been reported for another glycosyltransferase purified from *Catharanthus roseus*, which efficiently conjugates GA among other phenolics, but this enzyme uses UDP-glucose as the sugar donor instead of UDP-xylose (Yamane *et al.*, 2002).

Protein modelling

To date, the crystal structure of four plant glycosyltransferases is available: two from *Medicago truncatula* (MtUGT71G1 and MtUGT85H2), one from *Vitis vinifera*

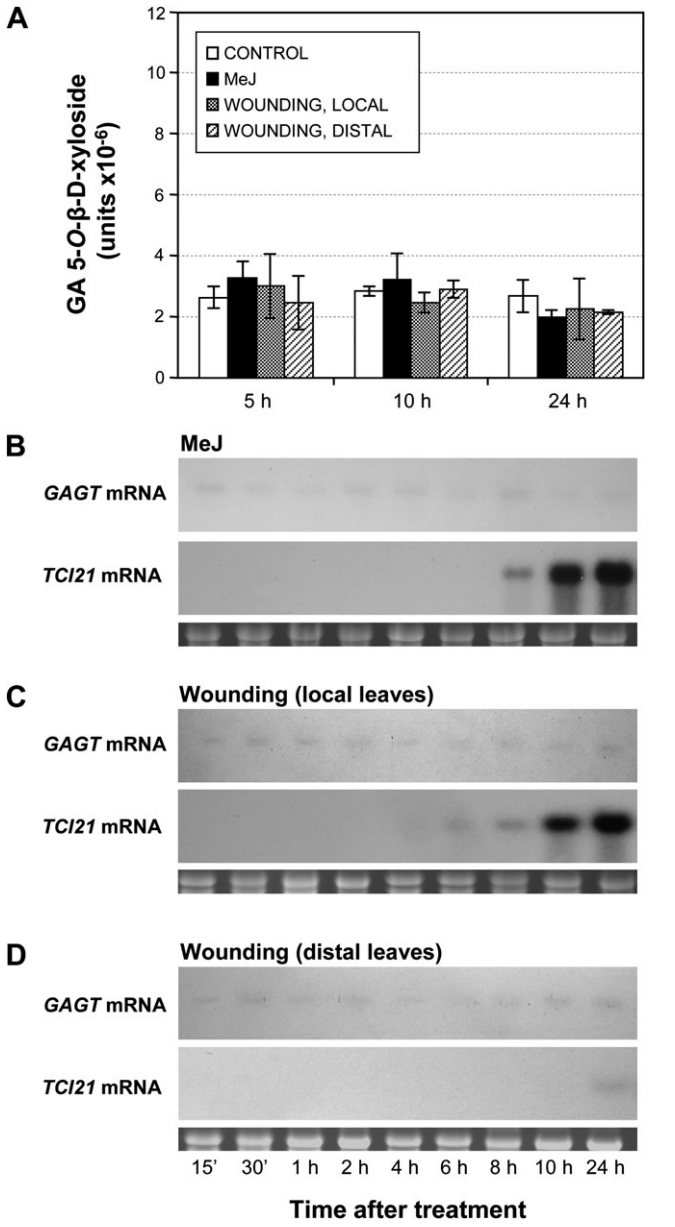

Fig. 6. *GAGT* induction by wounding or MeJ treatment. Tomato plants were wounded or treated with 2 mM methyl-jasmonate (MeJ, and leaves were collected at different times. (A) The GAGT activity present in the tomato protein extracts was measured at 5, 10, or 24 h after wounding or MeJ treatment. Activity was measured in the control leaves, MeJ-treated leaves, wounded leaves (local), and immediate upper leaves (distal). Enzyme reactions were incubated for 15 min and 5-*O*-β-D-xyloside was determined by fluorescence HPLC. Results are the means of three independent assays ±SD (standard deviation). (B) Northern blot analysis of the *GAGT* mRNA accumulation in tomato leaves in response to MeJ treatment. The mRNAs from tomato leaves were harvested at 15 min, 30 min, 1, 2, 4, 6, 8, 10, and 24 h after spraying plants with a 2 mM MeJ solution. The *TCI21* probe was used as a positive control (Lisón *et al.*, 2006). (C, D). Northern blot analysis of the *GAGT* mRNA accumulation in tomato leaves in response to wounding. The total mRNAs from tomato wounded leaves (C) or immediate upper leaves (D) were extracted from the plant material harvested at the indicated times. *TCI21* was used as a positive control.

(VvGT1), and another one from *Arabidopsis* (AtUGT71B2) (reviewed in Osmani *et al.*, 2009). Our sequence data were submitted to the SWISS-MODEL server by considering these four GTs as a reference. No valid model was retrieved when AtUGT71B2 or VvGT1 were used as a template. The modelling using *M. truncatula* UGT71G1 provided only a partial folding at the C-terminal domain (not shown). However, a fairly good structure was obtained with *M. truncatula* UGT85H2 glycosyltransferase (see Supplementary Fig. S2 at *JXB* online). Although this procedure does not necessarily describe the real three-dimensional structure of the protein, the proposed model for tomato GAGT shares the structural features described for the Family-1 GTs by adopting the so-called GT B-fold formed by the C-terminal and N-terminal domains separated by an interdomain linker (Osmani *et al.*, 2009). The sugar donor is deeply buried in a narrow groove in the C-terminal domain and interacts with the highly conserved PSPG motif. Remarkably, tomato GAGT and *M. truncatula* UGT85H2 only share a 32% amino acid identity, but the model proposed for tomato GAGT fits the described structure of MtUGT85H2 well.

Discussion

Gentisic acid has been described as a very efficient antifungal compound in plants (Lattanzio *et al.*, 1994). Besides, GA has been proposed as a signal molecule in the activation of the plant defence response in systemic

Table 1. Purification of GAGT from tomato leaves

GAGT was extracted from 100 g of tomato leaves and activity was measured by integrating the HPLC fluorescence peak area corresponding to the GA-xyloside as detailed in the Materials and methods.

Step	Total protein (mg)	Total activity (milliunits)	Specific activity (milliunits mg^{-1})	Purification (Fold)	Yield (%)
Crude extract	527.4	7700.1	14.60	1.0	–
$(NH_4)_2SO_4$ (35–65%) precipitation	301.3	6544.2	21.72	1.5	84.9
Q-Sepharose, first step	3.7	1852.1	500.56	34.3	24.1
Q-Sepharose, second step	1.1	1082.4	983.96	67.4	14.1
HiTrap Blue	0.2	199.5	997.46	68.3	2.6

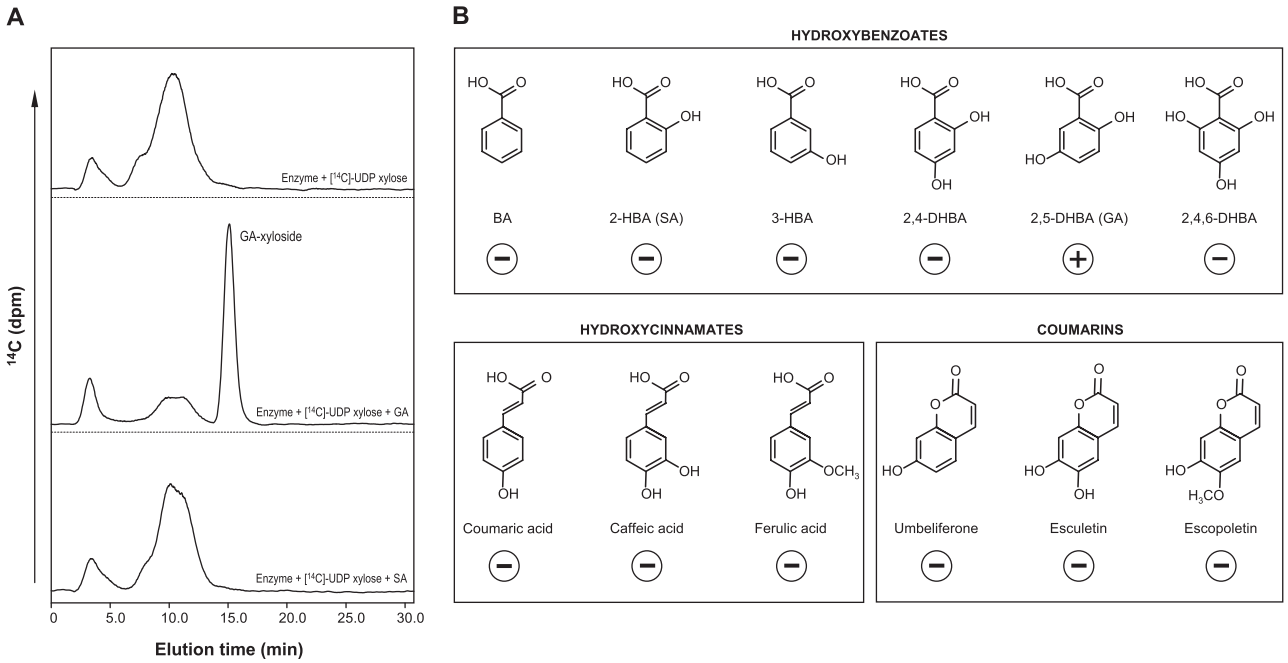

Fig. 7. Substrate specificity assay of sugar acceptors for GAGT. The purified plant enzyme was tested for substrate specificity using UDP-[^{14}C]-xylose and the following phenolics were used as sugar acceptors: benzoic acid (BA), 2-hydroxybenxoic acid (salicylic acid, SA), 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid (gentisic acid, GA), 2,6-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 2,3,4-trihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, caffeic acid, ferulic acid, coumaric acid, escopoletin, esculetin, and umbelliferone. Samples were HPLC-analysed and the eluted radioactivity was monitored. (A) Typical elution profiles obtained with UDP-[^{14}C]-xylose alone (top panel) or in the presence of GA (middle panel) or SA (bottom panel). A distinctive radioactive peak was detected using GA as a sugar acceptor, but not when the acceptor was SA. The same negative result was obtained with the rest of the phenolics assayed. The qualitative results for some representative phenolics are shown in (B).

infections (Bellés *et al.*, 1999, 2006). Although in lower levels than GA, in these compatible interactions the plant accumulates SA along with the appearance of symptoms, and both compounds conjugate quickly and efficiently (Bellés *et al.*, 1999, 2006; Fayos *et al.*, 2006; Schuhegger *et al.*, 2006). The same occurs with many other hydroxybenzoic acids derived from secondary metabolism. The sugar moiety with which these metabolites conjugate varies depending on plant species. Thus, in tobacco plants, SA has been found as either a glucosyl-ester or an *O*-β-D-glucoside (Lee and Raskin, 1998, 1999). In rice plants however, SA accumulates only as the 2-*O*-β-D-glucoside (Silverman *et al.*, 1995), which is the predominant and most stable conjugated form of SA in many plants (Edwards, 1994; Lee and Raskin, 1999). As regards GA, it has been seen to accumulate as 5-*O*-β-D-glucoside in both *Catharanthus roseus* (Yamane *et al.*, 2002) and *Fagopyrum esculentum*, where SA is detoxified by turning into GA which, in turn, is quickly glucosylated (Schulz *et al.*, 1993). Gentisic acid has also been found as 2-*O*-β-D-glucoside in *Cotoneaster orbicularis* (El-Mousallamy *et al.*, 2000). Similar to what has been described above, the acceptor substrate is bound to a glucose molecule in most cases. Very interestingly, GA accumulates as 5-*O*-β-D-xylopyranoside in systemic infections of tomato plants (Fayos *et al.*, 2006), and this compound is the principal differential metabolite in viroid-infected plants (López-Gresa *et al.*, 2010).

The conjugation of these phenolic compounds is carried out by glycosyltransferases (GTs). The main role of these enzymes is to regulate the free and active levels of different metabolites (Yalpani *et al.*, 1992; O'Donnell *et al.*, 1998; Lee and Raskin, 1998). To date, a large number SA-conjugating GTs have been characterized in plants. Some are able to use different hydroxybenzoic acids as a substrate, including GA (Yalpani *et al.*, 1992; Fraissinet-Tachet *et al.*, 1998; Lee and Raskin, 1999; Lim *et al.*, 2002). A GT that conjugates GA as a preferred phenolic substrate has been described in *Catharanthus roseus* (Yamane *et al.*, 2002). In addition, O'Donnell *et al.* (1998) have characterized a putative tomato GT gene (*twi1*), which is rapidly induced by SA and wounding. Nonetheless, no biochemical characterization of a tomato GT that conjugates SA and/or GA has been performed to date.

The main objective of this work is to characterize the glycosyltransferase responsible for the conjugation of GA to xylose in tomato in order to gain a better understanding of the role of this phenolic compound in the signalling of the plant defence response. To study the GA conjugation in tomato, the glycosylation of GA and/or SA in tomato leaves treated with these two phenolics were compared first. Our results reveal how GA accumulates mainly as a glycoconjugate, whereas only a small fraction of the total GA is present as a free form (Fig. 1). By contrast, no conjugated SA is detected at 4 h of treatment, and only half the total SA takes a conjugated form after 24 h. Such a glycosylation might be performed by the same enzyme or by different enzyme activities. According to our data, if a single enzyme activity is responsible for conjugating both phenolics, GA will be the preferential substrate. Alternatively, we could speculate about the existence of a specific gentisate-5-*O*-β-D-xylosyltransferase. In any case, these results encouraged us to characterize this potent glycosyltransferase which is responsible for the conjugation of GA to xylose in tomato.

The GA conjugating activity was monitored by the HPLC detection of GA 5-*O*-β-D-xyloside. In many cases, glycosyltransferases have been seen to be rapidly induced by their own substrate (Fraissinet-Tachet *et al.*, 1998; Lee and Raskin, 1999; Lim *et al.*, 2002). An increase in tomato GA xylosyltransferase was detected at 5 h of GA or SA exogenous treatments. This increase was much higher in the GA-treated plants (Fig. 3A) while the mRNA levels are similar (Fig. 3B, C). This could indicate that there is another gene which is only activated by GA. However, the possibility that the enzyme activity or stability is positively regulated by GA cannot be disregarded. Enzyme activity also increased in those tomato plants systemically infected with CEVd or ToMV, and the conjugation of the GA was much higher in the CEVd-infected plants (Fig. 5A). These results are in accordance with those previously obtained in our laboratory (Bellés *et al.*, 1999). In that former work, the free and conjugated GA levels in the tomato leaves infected with CEVd were higher than the levels detected in the ToMV-infected plants. GA-conjugating activity is not apparently induced in incompatible infections, such as the Rio Grande tomato plants infected with *Pseudomonas syringae* pv. *tomato AvrPto* (Fig. 5A). In this kind of infection, the analysis of phenolic compounds did not reveal an increase in free or conjugated GA, although an outstanding increase in free and conjugated SA has been widely described in the HR response (Malamy *et al.*, 1990; Métraux *et al.*, 1990; Uknes *et al.*, 1993). These results indicate that the induction of the GA conjugating activity observed in SA-treated tomato plants (Fig. 3A) may not be due to the SA itself, but to the conversion of SA into GA, which could be the effective inducer of the xylosyltransferase. In fact, similar to the results previously described by Schulz *et al.* (1993), it was observed that the SA-treated tomato leaves accumulate conjugated GA (Fig. 3A).

The complete cDNA sequence of the tomato GA-glycosyltransferase was isolated from a λ-ZAP library built from GA-treated tomato plants, and codes for a 51.5 kDa protein whose amino acid sequence is very similar to the tobacco SA-glycosyltransferase described previously by Lee and Raskin (1998, 1999). GAGT cDNA has been expressed in *Pichia pastoris* and the recombinant protein was active when GA and UDPX were used. However, it showed no activity toward SA when either UDPX or UDPG were used as sugar donors.

The substrate specificity of recombinant proteins may differ from the specificity of the native protein, which makes it difficult to ascertain the physiological role of the glycosyltransferase *in planta* (Jones and Vogt, 2001; Achnine *et al.*, 2005; Bowles *et al.*, 2006). Consequently, it was decided to purify the corresponding activity from tomato plants in order to perform specificity studies. A large number of phenolic compounds, such as sugar

acceptors, were tested using either UDPG or UDPX as sugar donors, and it was observed that the purified tomato glycosyltransferase was only active when GA and UDPX were used as substrates. The results obtained with both the purified and the recombinant protein show that, unlike the rest of the GTs previously characterized (Warnecke and Heinz, 1994; Fraissinet-Tachet *et al.*, 1998; Lee and Raskin, 1999; Jackson *et al.*, 2001; Griesser *et al.*, 2008), tomato GAGT displays very high substrate specificity. In addition, these results indicate that the glycosylation of GA and SA would be carried out by different enzymes. Such an outstanding specificity would allow the protein selectively to regulate the free and conjugated GA levels. Why the plant is so selective in the conjugation of GA remains an interesting open question.

Tomato GAGT seems to be regulated by pathogen signalling. In parallel with GA xylosyltransferase activity, the *GAGT* mRNA is induced quickly by an exogenous treatment of either SA or GA, and declines a few hours later. It is also induced in tomato plants infected with ToMV or CEVd while symptoms appear. This mRNA induction explains not only the accumulation of 5-O-β-D-xyloside previously described in these infections (Bellés *et al.*, 1999; Fayos *et al.* 2006), but also the increased GAGT activity detected. However, this tomato GA xylosyltransferase is not apparently involved in the response to wounding, even though the phylogenetic study indicates that *GAGT* is in close vicinity to the other GT genes induced by jasmonic acid.

Taken together, our results indicate that the GA-conjugating xylosyltransferase that we have characterized is involved in the plant defence response, specifically in non-necrotizing compatible interactions where GA has been described as a major signal molecule. Unlike this tomato GAGT, most of the previously described GTs have been implicated in incompatible or necrotizing interactions (Fraissinet-Tachet *et al.*, 1998; O'Donnell *et al.*, 1998; Lee and Raskin, 1999; Park *et al.*, 2003), where SA has been involved in the induction of PR proteins and the stablishment of SAR (Delaney *et al.*, 1994; Sticher *et al.*, 1997). SA appears to be the immediate precursor of GA biosynthesis (Bellés *et al.*, 1999). The absence of a GA signal in incompatible interactions, despite the SA levels being high, could indicate that the activity which converts SA into GA (a salicylate-5-hydroxylase) would not be induced or would be inhibited. Should this be the case, the expression of both SA-5-hydroxylase and GAGT could be co-ordinated and implicated in systemic infections. Therefore, it is hypothesized that a rapid induction of the salicylate-5-hydroxylase during compatible interactions would occur, thus provoking the accumulation of GA. After having induced PR proteins or other defence genes, GA would be quickly inactivated by GAGT, thus preventing its possible toxicity.

To gain a better understanding of the role of GA in plant defence, the generation of transgenic tomato plants that either overexpress or silence GAGT would be a powerful tool. In this respect, some results have been reported in the literature. Transgenic tobacco plants that overexpress or down-regulate the biosynthesis of a tobacco glucosyltransferase (TOGT1), which acts on the hydroxycoumarin scopoletin, have been obtained. The down-regulation of *TOGT1* led to a reduced accumulation of scopoletin glucoside, enhanced oxidative stress, and weakened virus resistance (Chong *et al.*, 2002). Conversely, the overexpression of *TOGT1* led to precocious lesion formation during the hypersensitive response to tobacco mosaic virus (Gachon *et al.*, 2004), and also to increased resistance against Potato virus Y (Matros and Mock, 2004). In potato, the ectopic expression of an anthocyanin 5-*O*-glucosyltransferase (5-UGT) improved plant defence against *Erwinia carotovora* subsp. *carotovora* (Lorenc-Kukuła *et al.*, 2005). In *Arabidopsis thaliana*, the overexpression of a deoxynivalenol-glucosyltransferase (DOGT1) led to enhanced tolerance against deoxynivalenol, which is a mycotoxin from *Fusarium*, and the T-DNA tagged mutants (*ugt73b3* and *ugt73b5*) exhibited less resistance to *P. syringae* pv. tomato-AvrRpm1, indicating that the expression of the corresponding UGT genes is necessary during the hypersensitive response (Langlois-Meurinne *et al.*, 2005). Recently, the down-regulation of a *Capsicum annuum UGT* by VIGS suggests the implication of this gene in the resistance response against TMV infection by controlling SA accumulation (Lee *et al.*, 2009). All these results emphasize the importance of plant secondary metabolite glycosyl transferases in plant–pathogen interactions.

The expression pattern of tomato GAGT and its narrow substrate specificity suggest that this protein plays a specific role in defence signalling. As speculated for other glycosyl transferases (O'Donnell *et al.*, 1998; Roberts *et al.*, 1999), the substrate of such a rapidly induced, defence-related enzyme may be an important signal molecule. Since gentisic acid has been found to be involved in different systemic plant–pathogen interactions, the early and transient induction of this novel xylosyltransferase may indicate its important role in selectively regulating the free levels of this phenolic in plants.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. (A) Sequence alignment of tomato GAGT and different plant glycosyltransferases; (B) phyllogenetic tree for the sequence alignment of Fig. S1A.

Supplementary Fig. S2. Proposed folding for tomato GAGT.

Acknowledgements

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