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RESEARCH PAPER

The potato suberin feruloyl transferase FHT which accumulates in the phellogen is induced by wounding and regulated by abscisic and salicylic acids

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Abstract

The present study provides new insights on the role of the potato (*Solanum tuberosum*) suberin feruloyl transferase FHT in native and wound tissues, leading to conclusions about hitherto unknown properties of the phellogen. In agreement with the enzymatic role of FHT, it is shown that its transcriptional activation and protein accumulation are specific to tissues that undergo suberization such as the root boundary layers of the exodermis and the endodermis, along with the tuber periderm. Remarkably, *FHT* expression and protein accumulation within the periderm is restricted to the phellogen derivative cells with phellem identity. FHT levels in the periderm are at their peak near harvest during periderm maturation, with the phellogen becoming meristematically inactive and declining thereafter. However, periderm FHT levels remain high for several months after harvest, suggesting that the inactive phellogen retains the capacity to synthesize ferulate esters. Tissue wounding induces *FHT* expression and the protein accumulates from the first stages of the healing process onwards. *FHT* is up-regulated by abscisic acid and down-regulated by salicylic acid, emphasizing the complex regulation of suberin synthesis and wound healing. These findings open up new prospects important for the clarification of the suberization process and yield important information with regard to the skin quality of potatoes.

Key words: ABA, BAHD suberin feruloyl transferases, cell wall suberization, *FHT* promoter, phellem, phellogen, potato periderm, suberin, wound-healing periderm.

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Introduction

The potato enzyme FHT (fatty ω-hydroxyacid/fatty alcohol hydroxycinnamoyl transferase) and the respective *Arabidopsis* orthologue ASFT/RWP1/AtHHT (At5g41040) have previously been characterized both *in vitro* and *in planta* (Gou *et al.*, 2009; Molina *et al.*, 2009; Serra *et al.*, 2010b). Categorized as acyltransferases of the BAHD family capable of undertaking the *in vitro* catalytic transfer of ferulic acid from feruloyl-CoA to ω-hydroxyfatty acids and fatty alcohols, both enzyme orthologues are responsible for supplying monomers to suberin (reviewed by Liu, 2010; Serra *et al.*, 2010a). Suberin consists of a complex cell wall polymer which is used by land plants to regulate the apoplastic transport of water (see, among others, Bernards, 2002; Ranathunge *et al.*, 2011; Beisson *et al.*, 2012), being composed of an aliphatic domain cross-linked with a lignin-like aromatic domain that is fixed to the primary cell wall. The aliphatic domain is made up of a glycerol-based fatty acid-derived polyester which forms a matrix in which soluble lipids or waxes are embedded.

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Ferulate esters are structural yet minor components of the aliphatic suberin, and are also constituents of the waxes embedded within the suberin (Schreiber *et al.*, 2005; Graça, 2010). Suberin (bound) and wax (soluble) alkyl ferulates play crucial roles with regard to the apoplastic barrier. Potatoes deficient in FHT are characterized by a large reduction of ferulate in both suberin and waxes, displaying a periderm that is a 14-fold more permeable to water compared with wild-type potatoes (Serra *et al.*, 2010b). The above statement is in agreement with an increased permeability of the suberized tissues of *Arabidopsis rwp1* mutants (Gou *et al.*, 2009). On the other hand, potato tubers deficient in FHT present a rough skin similar to that of russet potato varieties, and are unable to complete the periderm maturation process correctly and consequently remain prone to suffer from skinning injury over a long period after harvest (Serra *et al.*, 2010b).

The periderm consists of the dermal structure that replaces the plant epidermis of secondary (mature) organs and tubers (Peterson and Barker, 1979). It comprises three tissues: the phellem, the phellogen or mother layer, and the phelloderm. The phellem or cork layer is composed of 6–12 layers of dead cells with suberized walls that prevent water loss and act as an effective barrier to plant pathogens. The phelloderm connects the periderm to storage tissues (tuber flesh) and consists of one or a few layers of cells with cellulosic walls which can hardly be distinguished from the cortical parenchyma. The phellogen functions as a meristem given that consecutive new layers of phellem are produced as the outer layers are sloughed off during tuber growth. While the phellogen continues to be physiologically active, its cell walls remain thin and prone to fracture, leading to potato skinning. Nonetheless, when tuber growth ceases by vine killing or harvest, the periderm enters a maturation period during which the phellogen becomes meristematically inactive, with cell walls thickening and becoming resistant to excoriation (Lulai and Freeman, 2001), while at the same time the adjacent phellem cells complete their full suberin and wax load (Schreiber *et al.*, 2005). Once mature, no new phellem cell layers are added nor are further changes observed in the periderm (Sabba and Lulai, 2005; Lendzian, 2006). However, very little is known about changes in phellogen cells during periderm maturation except for the modifications in cell wall composition studied by Sabba and Lulai (2005) and Neubauer *et al.* (2013).

Potatoes react to skinning or other types of injury by forming a wound periderm beneath the wound surface (Morris *et al.*, 1989). Native and wound periderms are similar in structure and composition, and follow analogous maturation processes (Lulai and Freeman, 2001), although the wound periderm is more permeable to water and is proportionally enriched by wax alkyl ferulates (Schreiber *et al.*, 2005). The wound healing ability that includes suberin deposition at the wound site is essential to extend the storage life of potatoes. Abscisic acid (ABA) is a potent phytoregulator that reduces evapotranspiration and hastens the wound-associated deposition of suberin (Soliday *et al.*, 1978; Lulai *et al.*, 2008), in contrast to ethylene which is not required for wound suberization (Lulai and Suttle, 2004, 2009). Furthermore, jasmonic acid (JA) is rapidly induced by wounding, but neither JA treatment nor inhibition of JA accumulation have any effect on suberin deposition (Lulai *et al.*, 2011). Clarifying the effects of plant hormones in wound-associated suberization may contribute further to better understanding of the healing processes and might help to improve the quality and storage life of potatoes.

Notwithstanding the crucial role played by FHT with regard to the water barrier function coupled to the external appearance of the tuber periderm, an in-depth study of the role of FHT as regards suberized tissues is still awaited. The present work was designed to provide experimental evidence for *FHT* promoter activity and protein accumulation in the native periderm together with other constitutively suberized tissues, as well as to widen FHT studies into the wound-induced suberization process. For these reasons a polyclonal antibody was produced and potato plants stably transformed with a *FHT* promoter::GUS–GFP (β-glucuronidase–green fluorescent protein) construct were obtained. FHT temporal and spatial profiles in normal and mechanically injured tissues are reported. The results show that *FHT* is specifically expressed in cells undergoing suberization and that it is induced by wounding and regulated by ABA and salicylic acid (SA). Information is presented on FHT accumulation in the periderm, providing a new significant insight with reference to phellogen cells once tuber growth ceases, which might be useful to improve potato storage.

Materials and methods

Plant material

Potato plants (*Solanum tuberosum*) subspecies *tuberosum* (cv. Désirée) and *andigena* were propagated as described by Serra *et al.* (2010b). For the *andigena* plants, tuber induction was performed in soil when plants reached the 14-leaf stage by setting short-day conditions (8 h light/16 h dark) and *in vitro* as described by Dobránszki (2001). The commercial potato cv. Kennebec used for the wound healing and hormone experiments was purchased from a local supermarket.

Phytohormone treatments

Potato discs (3 mm thick and 13 mm in diameter) were obtained by cutting cylinders of parenchyma tissue excised from tubers with a cork borer. Hormone stock solutions were prepared at 0.1 M ABA (Sigma, A-1049) in dimethylsulphoxide (Lulai *et al.*, 2008), 0.1 M JA (Sigma, J-2500), and 0.25 M SA (Sigma, S-7401) in ethanol. ABA, JA, and SA assays were performed on freshly cut discs at a final concentration of 0.1 mM diluted with milliQ water. Discs were placed in the hormone solutions (≤30 discs/100 ml of solution) and incubated at room temperature for 1 h on a rotatory shaker (50 cycles min^{-1}) to achieve uniform hormone permeation. After treatment, discs were removed from the solution and allowed to wound heal at room temperature in saturated humidity and dark conditions. As a control, the same protocol was applied to potato discs in treatments without phytohormones and with the respective dimethylsulphoxide or ethanol volumes. Control and treated discs were collected and frozen in liquid nitrogen for analysis.

Generation of ProFHT::GUS-GFP transgenic potatoes

The promoter of *FHT* was obtained by Genome Walker (Clontech) and using the *Solanum phureja* genome (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml; Potato Genome Sequencing

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Consortium, 2011). A fragment consisting of 2541 bp upstream of the initial ATG codon (KC695749) was amplified with the forward primer 5′-GCACGAAGTTTCCAAGCATT-3′ and the reverse primer 5′-TTCTCAAATTAAAAATCCTGTTT-3′. This sequence was cloned into the GATEWAY entry vector pENTR/D-TOPO (Invitrogen) and transferred into the GATEWAY destination vector pKGWFS7 (Karimi *et al.*, 2002) by LR reaction (Invitrogen). Potato leaves were infected with *Agrobacterium tumefaciens* strain GV2260 and stably transformed with the *ProFHT*::*GUS-GFP* recombinant plasmid according to Banerjee *et al.* (2006). Kanamycin-resistant plants were regenerated and grown *in vitro* until tuber development.

FHT polyclonal antiserum and western analysis

The FHT protein was purified as described by Serra *et al.* (2010b) and the polyclonal antibody was obtained from the Antibody Production Service of the CSIC (Barcelona). Following standard protocols, two rabbits were respectively immunized with 1 mg of purified FHT. To obtain reactivity of the antibody against both the native and non-native proteins, each injection contained both the native and the heat-denatured antigen (1:1). Dot-blot and western blot assays confirmed that an antiserum dilution of 1:10 000 was able to detect 1 ng of the native protein and 100 ng of the denatured protein. The antiserum was purified as follows: a membrane containing 100 µg of purified FHT was incubated with 100 mM glycine at pH 2.5 for 10 min to remove poorly bound proteins, blocked with 5% skimmed milk powder in TRIS-buffered saline–Tween (TBST) for 45 min, followed by overnight incubation with 10 ml of the antiserum, and subsequently washed thoroughly with TBST buffer. Purified antibodies were eluted with 100 mM glycine (pH 2.5) and then neutralized with TRIS-HCl (pH 8) until a pH of 7 was reached.

Soluble proteins were extracted from tissues with a buffer containing 56 mM $NaCO_3$, 56 mM dithiothreitol (DTT), 2% SDS, 12% sucrose, and 2 mM EDTA in a ratio of 1 ml per 0.5 g of fresh tissue. Protein concentrations were determined using the Bradford assay. Extracts were resolved in either 10% or 12% acrylamide SDS–polyacrylamide gels and blotted onto nitrocellulose membranes (Millipore) using 40 µg of total protein. The membranes were blocked and then probed overnight at 4 °C against a 1:10 000 dilution of crude rabbit anti-FHT serum and a 1:4000 dilution of mouse anti-actin (Agrisera) used as a loading control. Primary antibodies were detected by means of secondary antibodies against rabbit (Nordic Immunology) and mouse (Calbiochem), respectively, which were conjugated to a peroxidase. Peroxidase activity was detected by chemiluminiscence (Millipore) and images of the blots were used for quantification via densitometry (Flurochem SP, AlphaInnotech). Band quantification was performed by Quantity One Software (Bio-rad).

Detection of FHT promoter activity

Plant tissues were immersed in an ice-chilled 90% acetone (v/v) bath and incubated for 20 min on ice, after which they were rinsed with water. Tissues were infiltrated with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic sodium salt 3·H_2O (X-GlcA, Duchefa), 50 mM sodium phosphate buffer (pH 7), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 10 mM EDTA, and 0.05% (v/v) Triton X-100 for 20 min under vacuum, incubated at 37°C for a maximum of 48 h, and then cleared with 70% (v/v) ethanol. Stained tissues were washed 2–3 times with phosphate-buffered saline (PBS) and cryoprotected through a series of 0.1, 0.5, and 1 M sucrose in PBS solution in order to carry out sectioning in a Cryocut 1800 (Reichert-Jung) cryotome. Observations were made using a Nikon SMZ-1000 stereomicroscope and an Olympus Vannox microscope, and micrographs were obtained using a set of Infinity X, Deltapix, and Nikon digital cameras. Transgenic roots were observed using a Nikon CS1 90i Eclipse confocal laser-scanning microscope. For the visualization of GFP, fluorescent samples were excited at 488 nm with an argon ion laser and emission was monitored at 500–530 nm; images were obtained using the EZ-C1 software.

Immunohistochemical detection of FHT

Tissues fixed by vacuum infiltration for 90 min in 4% paraformaldehyde (w/v) in PBS were subsequently washed twice with PBS and twice with distilled water. Waxes were removed through an ethanol–xylol series (Sauer *et al.*, 2006) and cryosectioning was performed. Dried sections were incubated in PBS for 10 min, blocked with 2% bovine serum albumin (BSA) solution in PBS for 30 min, and then labelled by incubation with the purified FHT antibody diluted 1:50 in 2% BSA at 4 ºC overnight, followed by a secondary goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen) diluted 1:500 in 2% BSA. Whole-mount tissues were treated according to Sauer *et al.* (2006) and then incubated with the purified FHT antibody diluted 1:25 for 240 min at 37 ºC, followed by incubation with an Alexa Fluor 488-(Invitrogen) labelled secondary antibody diluted 1:500 for 180 min at 37 ºC. Fluorescence images were observed with an epifluorescence LEICA DMR-XA microscope and images were taken with a Jenoptik ProgRes C14 digital camera.

Subcellular fragmentation assay

Plant material was ground in liquid nitrogen, and protein extraction and subcellular fractionation were performed as described by Rautengarten *et al.* (2012). The extracted proteins in the supernatant and pellet fractions were analysed via western blot as described above. Blots were probed with rabbit anti-UGPase (Agrisera) at a 1:3000 dilution, rabbit anti-calreticulin (Abcam) at a 1:1000 dilution, and crude rabbit anti-FHT at a 1:10 000 dilution at 4 ºC overnight. After three consecutive washing steps, the membranes were incubated for 1 h at room temperature with a goat anti-rabbit antibody (Nordic Immunology) conjugated to peroxidase 1:40 000 dilution. Peroxidase activity was detected by chemiluminiscence as described above (Millipore).

Results

FHT localization in the native periderm and root tissues

In order to verify the FHT expression profile and test the FHT polyclonal antibody, protein extracts derived from potato tissues were analysed by western blot (Fig. 1). A band with an electrophoretic mobility corresponding to 55 kDa, in accordance with that predicted for the FHT protein, was only present in the periderm and root tissues which contain suberized tissues. This band was absent in stem, leaf, and tuber flesh (tuber parenchyma) which correspond to unsuberized tissues and also in the controls incubated with the pre-immune serum (data not shown). These results are in agreement with the *FHT* transcript profile carried out by northern blot analysis (Serra *et al.*, 2010b) and validate the use of the FHT antiserum in further studies.

The tuber periderm and the root tissues were analysed at a histological level to determine in which precise cells the *FHT* promoter is active and the protein accumulates. Plants of *S. tuberosum* ssp. *andigena*, chosen because tuberization can be induced by photoperiod, were stably transformed with a construct carrying the *FHT* promoter region (2541 bp upstream of the translation initiation codon) fused to the GUS and GFP coding regions. Potato tubers cut in half and stained for GUS activity showed the blue marker specifically at the region of the periderm that covers the tuber surface (Fig. 2A, arrowheads), while it was found to be absent from the apical bud region which had not yet developed a periderm

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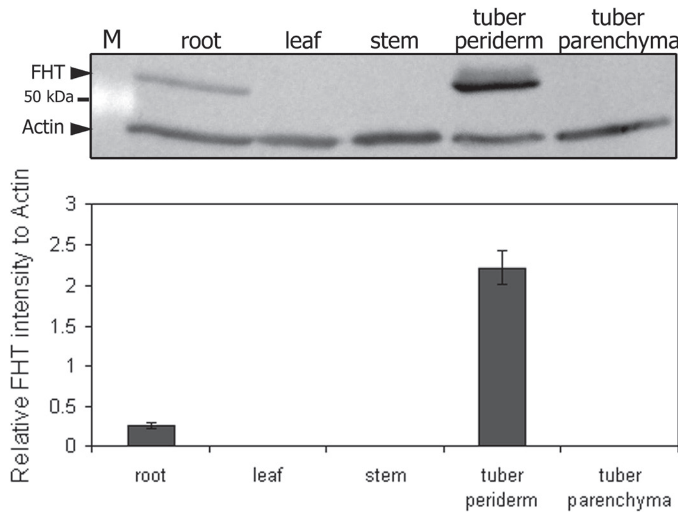

Fig. 1. FHT protein profile of potato tissues. Protein extracts derived from root, leaf, stem, tuber periderm, and tuber parenchyma separated by SDS–PAGE and analysed by western blot using antiserum against FHT. Actin was used as the internal control. The 50 kDa molecular mass marker is indicated to the left of the panel. Relative FHT accumulation with respect to actin is quantified for each lane. Relative intensity values are means ±SD of two independent biological replicates.

(Fig. 2A, arrow). The thin sections used for microscopy analysis allowed the distinction between the suberized phellem, made up of dead cells, and the adjacent non-suberized layers, the phellogen and phelloderm, by means of suberin autofluorescence (Fig. 2B). GUS activity was particularly localized beneath of the phellem innermost cell layer and concentrated in a single layer of live cells corresponding to the phellogen (Fig. 2B, C). The immunolocalization of FHT was performed using a secondary antibody conjugated to Alexa Fluor 488 as its green fluorescence contrasts with the faint dark-yellow autofluorescence emitted by suberin under blue excitation. In the immunostained periderm sections, the green fluorescence showed no overlap with the suberin autofluorescence and was restricted to a single cell layer of live cells corresponding to the phellogen (Fig. 2D–G). The antiserum and the FHT affinity-purified antibodies were both used in these experiments to rule out a possible cross-reactivity. No green fluorescence was observed in the negative controls performed with the pre-immune serum nor using only the primary or secondary antibodies; in the same way, green fluorescence was absent in tubers of FHT silenced lines (data not shown). Upon inspection of the periderm in some cork-warts that form spontaneously in stems of *in vitro* cultured potato plants, GUS activity restricted within the phellogen cell layer was confirmed (Supplementary Fig. S1 available at *JXB* online). Thus, the FHT transcriptional and translational activity of the native periderm is specific to the phellogen cells. On the other hand, root tissue was examined using primary roots of *in vitro* cultured plants carrying the *ProFHT::GUS-GFP* construct. In roots stained for GUS activity, the blue marker was restricted to the exodermis, located beneath the epidermis, as

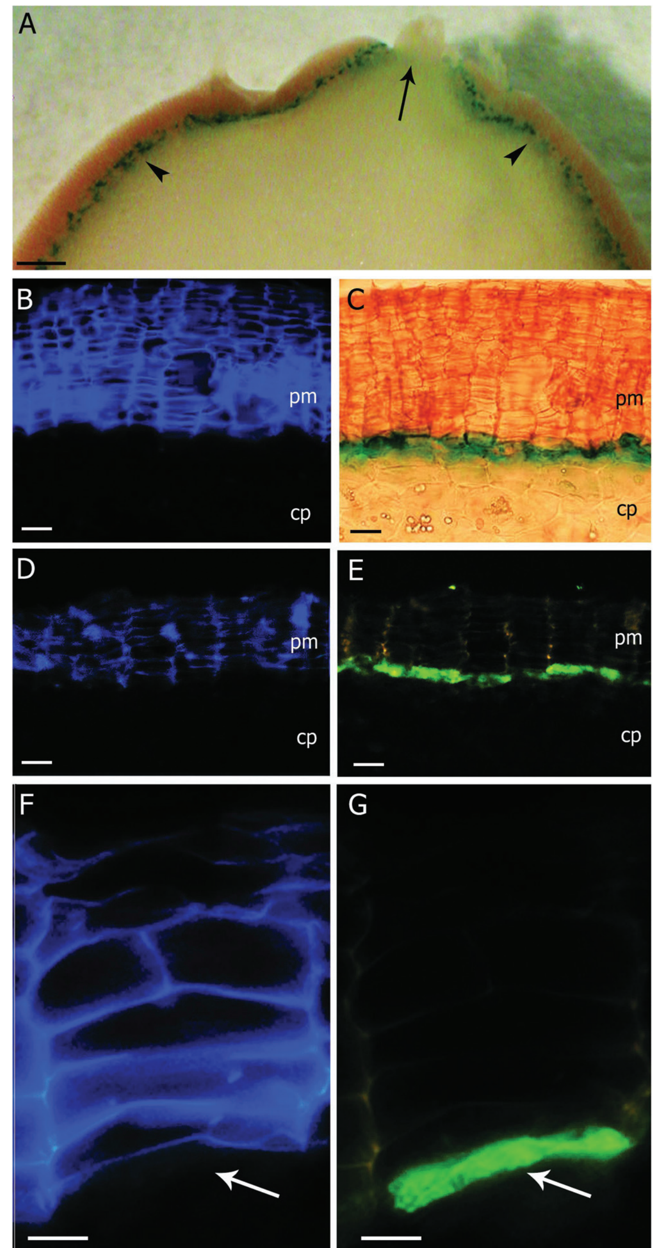

Fig. 2. FHT expression in native tuber periderm of potato. (A–C) GUS activity directed by the *FHT* promoter in transgenic tubers. (A) An *in vitro* cultured tuber cut in half and showing GUS staining specific to the periderm located beneath the phellem (arrowheads). No signal was detected in the apical bud region (arrow). (B) Cryosection of the GUS-stained periderm showing the suberin autofluorescence of the phellem and (C) the GUS blue marker located in a single cell layer beneath the phellem. (D–G) FHT immunolocalization using the Alexa Fluor 488-labelled FHT purified antibody. Sections observed under UV (D, F) showing the suberin autofluorescence and under blue excitation (E, G) showing the green fluorescence of labelled FHT antibody located in the phellogen cell layer (white arrow). Scale bars=500 μm (A), 50 μm (B–E), and 20 μm (F, G). cp, cortical parenchyma; pm, phellem.

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well as the endodermis, located between the cortex and the stele (Fig. 3). In root cross-sections, GUS staining overlapped with the autofluorescence signal (Fig. 3A, B). Whole-mount roots observed under bright field and confocal microscopy exhibited GUS activity, and GFP fluorescence localized in these suberized cell layers (Fig. 3C–E).

FHT expression throughout tuber development, maturation, and storage

Developing tubers of *ProFHT::GUS-GFP* plants were collected and stained for GUS activity at a number of main developmental stages according to Kloosterman *et al.* (2008): stolon tip, stolon swelling, tuber initiation, and early, middle, and late tuber growth stages. The blue marker begins to become visible through the skin when the developing tubers reach the stage of early tuber growth (Fig. 4A). The blue colour is first detected at the tuber basal end region and progressively extends upwards to cover the entire tuber surface (Fig. 4A, B). Lenticels showed up as deep blue dots indicative of an intense GUS activity (Fig. 4B) in agreement with a greater fluorescence intensity of FHT (Fig. 4C, D). These observations are in accordance with the periderm developmental gradient and confirm an intense activity in the lenticular phellogen of growing tubers. Furthermore, periderm samples obtained at different time points throughout the maturation and ageing process of tubers (up to 10 months of storage at 4 ºC) were analysed by western blot; as shown in Fig. 5, the level of FHT was greater in samples which were obtained near to harvest, coinciding with the periderm maturation period, while it decreased thereafter. However, the FHT level still remained high after 4 months of storage, and FHT was even detected after 10 months of storage. It is noteworthy that one tuber stained for GUS after a 7 month storage period at 4 ºC displayed a faint blue surface colour in contrast to an intense blue colour of the lenticels (Supplementary Fig. S2 at *JXB* online); however, two other tubers kept in the same conditions showed no visible GUS signals.

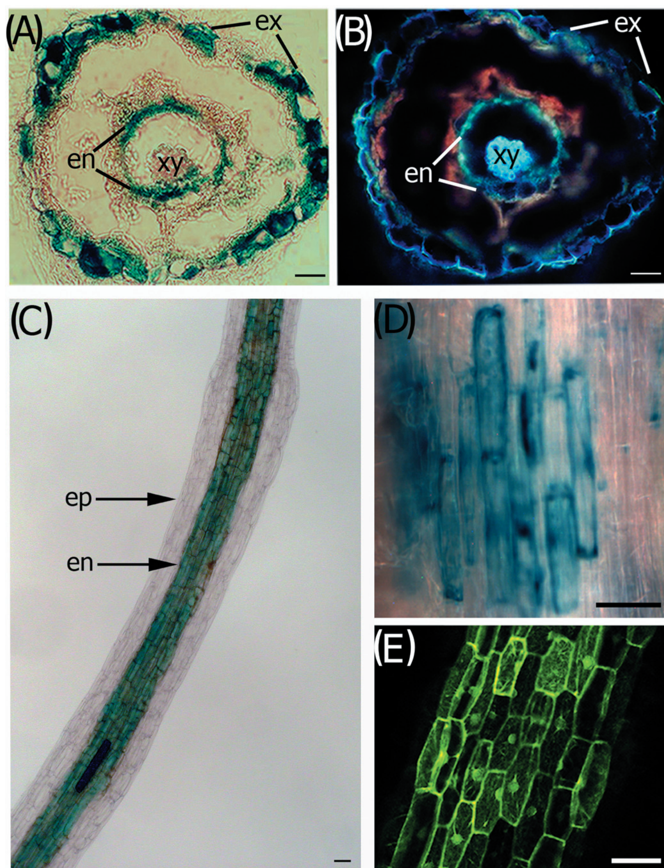

Fig. 3. *FHT* expression in root tissues of potato. GUS and GFP expression driven by the *FHT* promoter is restricted to the exodermis and endodermis. (A and B) Root cross-section under bright field (A) and UV excitation (B). In the endodermis and exodermis, the GUS signal overlaps with the suberin autofluorescence. (C–E) Whole mounts showing GUS activity localized (C) in the endodermal and (D) in the exodermal cells. (E) Confocal microscope image showing GFP accumulation in exodermal cells. Scale bars=25 μm (A, B), 50 μm (C, D, E). ex, exodermis; en, endodermis; ep, epidermis; xy, xylem vessels.

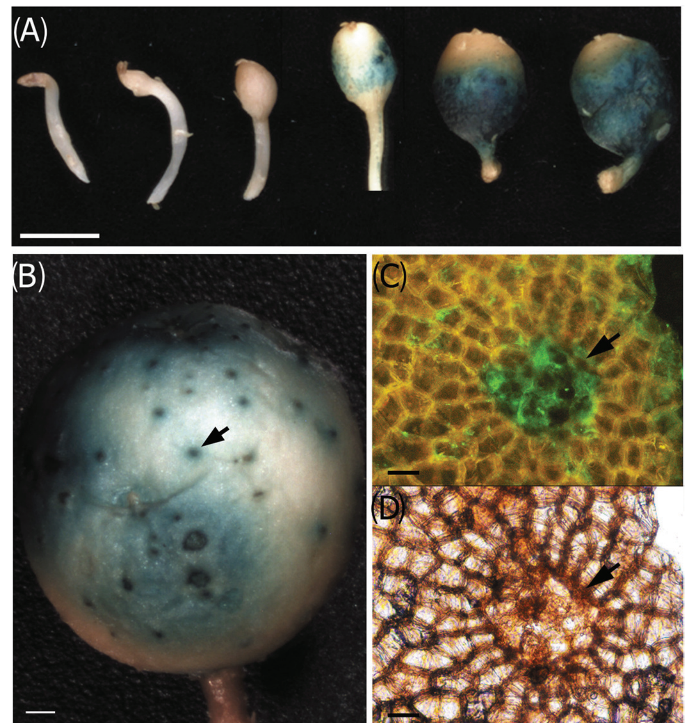

Fig. 4. *FHT* induction in developing tubers of potato. (A and B) GUS signal observed through the surface of tubers in *ProFHT::GUS-GFP* potato plants. (C and D) FHT immunolocalization in a lenticel. (A) Tubers grown in soil sampled at the stolon tip, stolon swelling, tuber initiation, and early, middle, and late tuber growth stages. The GUS staining starts to become visible at the basal end when tubers enter the growth stage and the signal progressively covers the whole tuber surface. (B) Tuber in a late growth stage showing lenticels as dark blue dots (arrow). (C and D) Detail of a lenticel stained for FHT under blue light excitation (C) and under bright light (D). Scale bars=5 mm (A), 1 mm (B), 50 μm (C, D).

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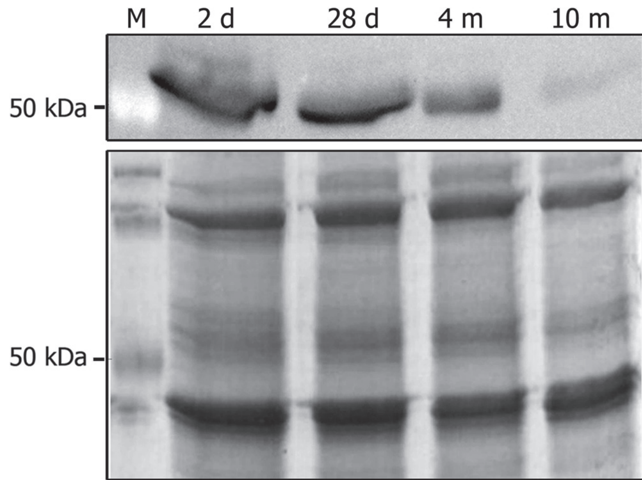

Fig. 5. FHT levels in the potato periderm during tuber maturation and ageing (storage). Western blot analysis (upper panel) shows that a higher level of FHT is observed close to the harvest period and thereafter decreases, although it is still detected after 10 months of storage at 4 ºC. SDS–polyacrylamide gel stained with Coomassie Brilliant Blue (lower panel) showing that equal total protein amounts were loaded in each lane. d, days; m, months.

Temporal and spatial FHT pattern in healing tissues

In order to elucidate the participation of FHT in the healing process, its expression in mechanically injured tissues was investigated. Fully expanded leaflets of plants bearing the *ProFHT*::*GUS–GFP* construct were injured with a 'dog brush' and left to heal. In wounded leaflets the FHT level peaks after 72 h and decrease subsequently by a half at 96 h following injury (Fig. 6A). When leaflets were examined for GUS activity 48 h after wounding, the blue marker appeared to be restricted to the scar tissues at the margin of wounds (Fig. 6B–D), corresponding to the suberin autofluorescence area (data not shown). Young (primary) stems were superficially injured with a scalpel and left to heal. In wounded stems 48 h after injury the GUS blue colour also appeared confined to the site of damage (Fig. 6E), being more intense at the wounded margins yet also detectable in the central areas in which only the epidermis was eroded. In tubers, the healing process was examined in single cuts or in excised parenchyma discs at 0, 24, 48, and 72 h, and 6 d after injury. A certain amount of FHT was detected 24 h after injury and levels increased as the healing process progressed (Fig. 7A). Compared with 24 h after injury, the amount of FHT relative to actin was increased by 9- to 10-fold after the sixth day. Tubers with single cuts were used to examine the *FHT* transcriptional activity 48 h after wounding. In these tubers, the entire severed surface showed a very intense GUS signal (Fig. 7B, arrows) which connects to the wounded edges, with the GUS signal being distinct in the intact periderm covering the undamaged surface (Fig. 7B, arrowheads). Microscopic examination revealed that the GUS staining localized within the live parenchyma cells closest to the injured surface (1–2 cells from the wounded surface) (Fig. 7C, D) as seen by the green fluorescent signal in FHT immunostained tissue

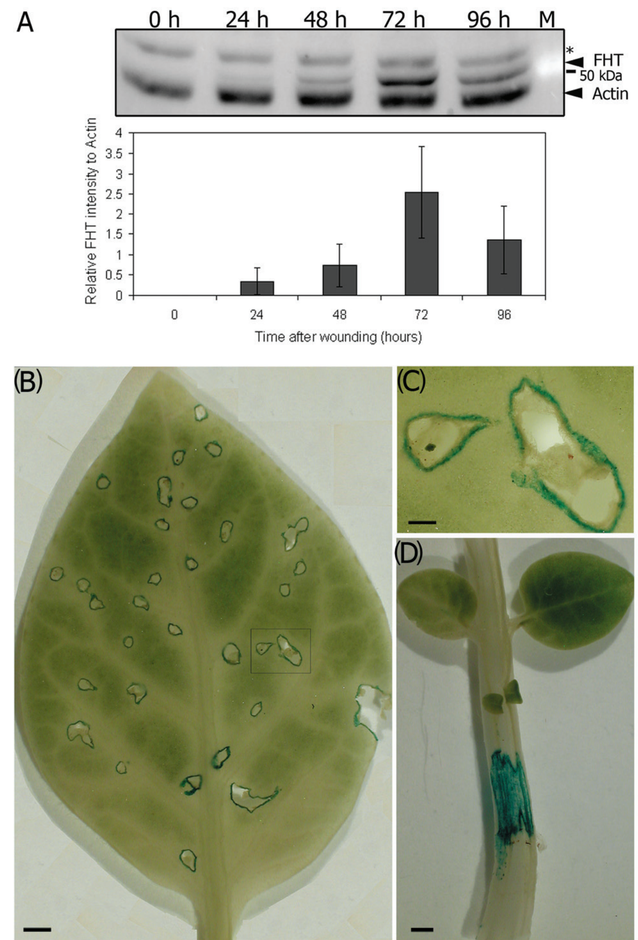

Fig. 6. FHT in wound-healing leaflets and stems of potato. (A) The upper panel shows the FHT protein profile in mechanically injured leaflets monitored by western blot using actin as a loading control. The 50 kDa molecular marker is shown to the right. The asterisk indicates an extra band not corresponding to the molecular weight of FHT or actin. The lower panel shows the FHT accumulation relative to actin as quantified for each lane (values are means ±SD of three independent biological replicates). (B) Injured leaflet stained for GUS activity 48 h after wounding. (C) Detail of wound lesions in B. (D) Injured stem stained for GUS activity 48 h after wounding. Scale bars=1 mm (B, D), 200 µm (C).

sections (Fig. 7E, F). Some of these parenchyma cells were not yet suberized although they showed signs of amyloplast depletion.

Phytohormones and FHT induction in healing tissues

In order to better understand the role of ABA in wound-induced suberization and to discern possible effects of JA and SA, FHT accumulation was examined in potato tuber discs treated with 0.1 mM hormone solutions for 1 h and afterwards left to heal. Upon examination 24 h and 48 h after wounding, the ratio between the intensity of the FHT and actin bands was greater in the ABA-treated discs than in the non-treated discs where the FHT band was barely visible (Fig. 8A). Thus, ABA treatment enhances the induction of FHT in healing

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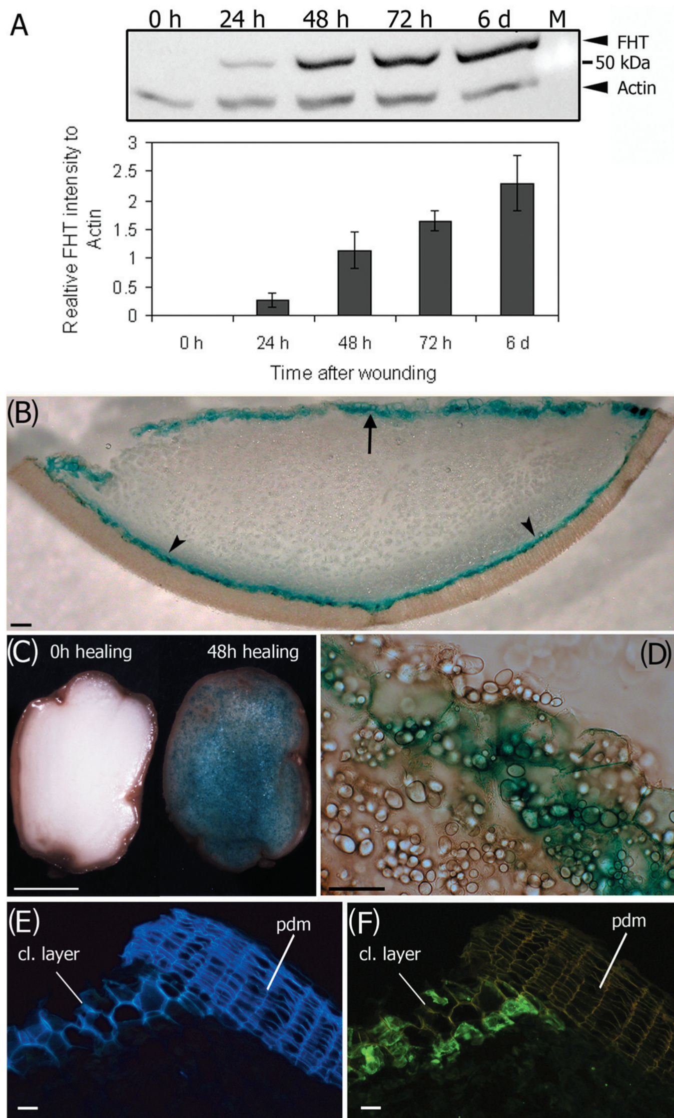

Fig. 7. FHT in wound-healing tubers of potato. (A) The upper panel shows the FHT protein profile in healing potato discs monitored by western blot using actin as a loading control. The lower panel shows FHT accumulation relative to actin as quantified for each lane (values are means ±SD of three independent biological replicates). FHT accumulation is observed 24 h after injury and increases progressively up to the sixth day. (B) Section of a transgenic tuber 48 h after injury showing GUS activity localized on the wound surface (arrow) and also in the native periderm (arrowheads). (C) A tuber cut in half stained for GUS activity at 0 h and 48 h after wounding. (D) Thin section of the wound showing *FHT* promoter activity localized in the live parenchyma cells closest to the wound surface. (E and F) Cryosection of the wound obtained 72 h after injury showing the contact zone between the wound and the native periderm. Observed under (E) UV excitation to show the suberin autofluorescence and (F) under blue light excitation to show the green fluorescence of the FHT. Scale bars=100 µm (B), 5 mm (C), 50 µm (D–F). cl. layer, wound closing layer; pdm; native periderm.

tissues of potato. Examination at the same time periods revealed that discs treated with JA showed no effects on FHT accumulation in comparison with the controls (Fig. 8B). In contrast, in the SA-treated discs, FHT protein expression was not detected at 24 h following wounding and the intensity of the band 48 h after wounding was lower compared with that of the control discs (Fig. 8C), thus pointing to a regulatory effect of SA in wound-induced suberization which is antagonistic to that of ABA.

Subcellular localization of FHT

Sequence analysis of FHT using TMHMM (Krogh *et al.* 2001), SignalP (Petersen *et al.*, 2011), and the WolfPSORT (Horton *et al.*, 2007) programs to predict the subcellular localization anticipated no transmembrane helices and no signal peptide; therefore, they forecast a cytosolic localization of the protein. The experimental evidence for the FHT subcellular localization was obtained by ultracentrifugation of the protein homogenates from native and wounded periderm as well as root tissue. The protein extracts were separated into supernatant and pellet fractions expected to contain soluble (cytosolic) and microsomal proteins, respectively. These fractions were analysed by western blot using antibodies against FHT, a cytosolic protein marker (the UDP-glucose pyrophosphorylase, UGPase) protein, and a microsomal protein marker calreticulin (Fig. 9). The calreticulin antibody reacted only with the pellet fractions, confirming that microsomal proteins are localized in the pellet. Conversely, the UGPase antibody reacted with the supernatant, although a faint reaction also appeared in the pellet of the tuber-wound periderm. The FHT protein behaved in a similar manner to UGPase, a result consistent with a cytosolic localization in accordance with the '*in silico*' predictions.

Discussion

FHT is accumulated in the phellogen

FHT encodes a potato feruloyl transferase involved in suberin and wax biosynthesis that is necessary for periderm integrity (Serra *et al.*, 2010b). FHT silenced tubers display a defective skin, lose large amounts of water, and remain prone to excoriation (skinning) for a long period after harvest (Serra *et al.*, 2010b). Here it is demonstrated that *FHT* is specifically expressed and that the protein accumulates in the phellogen cell layer (Fig. 2). No FHT protein—or only extremely faint traces—was observed in the innermost layers of the phellem. Thus, *FHT* becomes active in phellogen cells before suberin deposition starts or at least before it can be detected. It is remarkable that *ASFT*, the *FHT Arabidopsis* orthologue, is the only gene among seven other suberin reporter genes that is expressed much earlier than the start of suberin deposition in endodermal cells (Naseer *et al.*, 2012). Also worth mentioning is the fact that the aromatic suberin is laid down in the cell wall well in advance of the aliphatic suberin (Lulai and Corsini, 1998). The early accumulation of ferulate may be a critical aspect for the coupling of the aromatic and aliphatic suberin domains, considering that ferulate esters are able to form covalent bonds with cell wall polysaccharides and polyphenolics while leaving the aliphatic chain ready for

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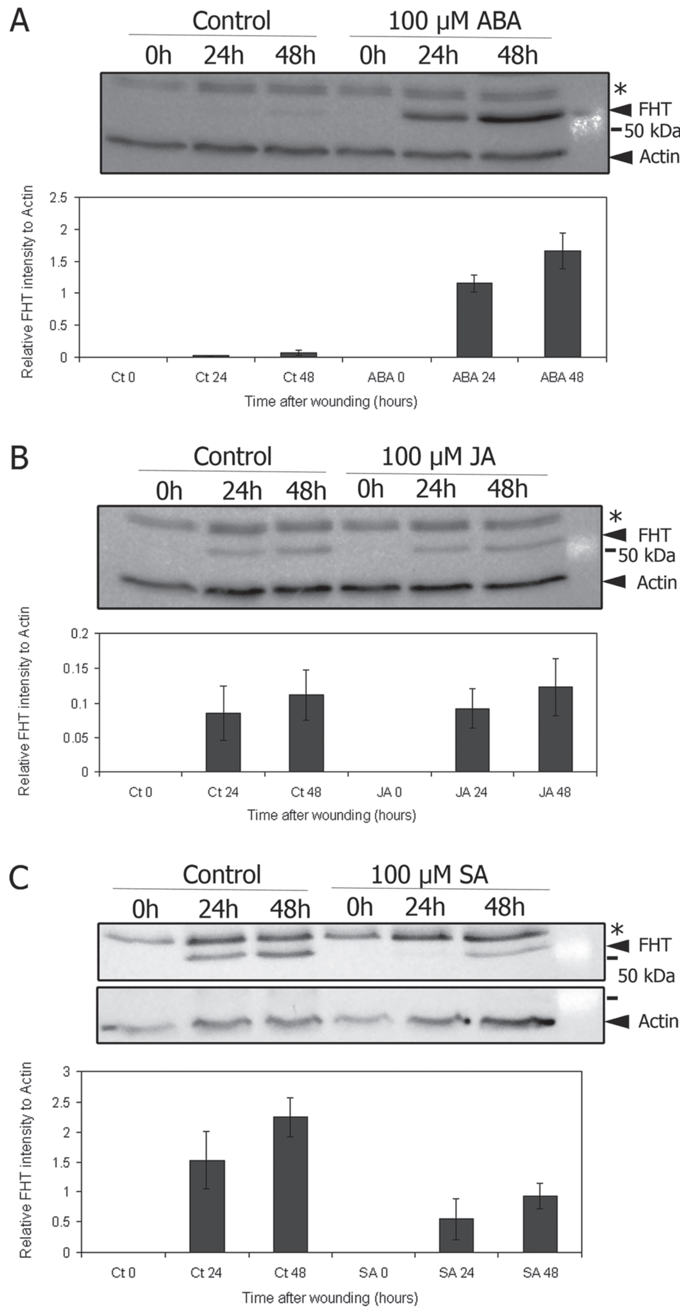

Fig. 8. ABA and SA but not JA modify FHT expression in healing potato discs. Protein extracts were analysed by western blot (upper panels) with FHT antiserum. Actin was used as a loading control. The lower panels show FHT accumulation relative to actin as quantified for each lane (values are means ±SD of three independent biological replicates). (A) FHT induction by ABA was monitored in wound-healing potato tuber discs. ABA treatment enhances FHT accumulation during the wound-healing process (*t*-test, $P < 0.01$). (B) No significant differences between JA treatment and the control treatment with regard to FHT protein accumulation were detected. (C) FHT protein accumulation is reduced in SA-treated discs compared with the control treatment (*t*-test, $P < 0.05$). The molecular marker is shown to the right. Asterisks mark extra bands that do not correspond to the expected molecular weights of the proteins analysed.

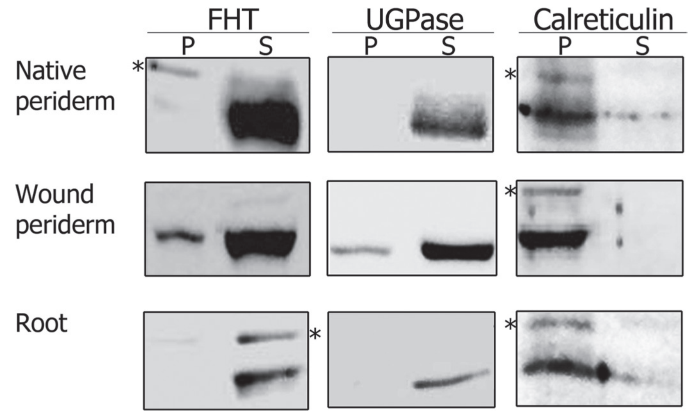

Fig. 9. FHT immunodetection in the subcellular fractions derived from suberized tissues. Protein fractions of native and wound periderm as well as root tissues were obtained by ultracentrifugation and analysed by western blot. In addition to the FHT antiserum, UGPase and calreticulin antibodies were also used as cytosolic and microsomal markers, respectively. S, soluble (cytosolic) fraction; P, pellet (microsomal fraction). The asterisks mark non-specific bands.

esterification (Liu, 2010). On the other hand, the maximum FHT accumulation in the periderm occurs during progression of the periderm maturation (Fig. 5), a complex physiological process that usually takes place at harvest and in which the phellogen becomes meristematically inactive (Lulai and Freeman, 2001), while at the same time the phellem completes its full suberin and wax load (Schreiber *et al.*, 2005). The mature periderm maintains the FHT levels although with a decreasing trend (Fig. 5). This sustained FHT presence suggests a continuous function of this protein in phellogen cells of the mature periderm which remain meristematically inactive. Such a function may be related to the maintenance of the integrity of the apoplastic barrier: a pool of FHT kept at a basal level may rapidly provide new ferulate esters if eventually the phellogen receives the appropriate stimuli to undergo phellem differentiation. Such a mechanism may be effective with regard to microfissures or small cracks that could promote water loss and the entry of microorganisms.

Lenticels are special areas of the periderm which are crucial to regulate gas exchange. They form early in developing tubers by periclinal divisions of cells beneath the stomata, giving rise to a particular phellogen which produces a type of suberized tissue that is permeable to water and gases (complementary tissue). The phellogen then extends from lenticels to build up a complete layer of native periderm (Adams, 1975; Tyner *et al.*, 1997). The preponderance of the FHT transcriptional activity and protein accumulation in lenticels (Figs 4, 5) agree with an intense activity of the lenticular phellogen in developing tubers. Moreover, the regulation of gas exchange by lenticels is based on the long-term structural changes which involve phellogen activity and suberin biosynthesis, namely the formation of a closing layer of highly suberized and dense cells to restrict gas exchange, or the enlargement of the lenticular area by proliferation to increase gas exchange

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(Lendzian, 2006). Environmental factors such as temperature and humidity have been related to the proliferation of the lenticular phellogen during tuber storage (Adams, 1975). Lenticel disorders in fresh market potatoes have been related to suberin deposition in lenticels (Makani, 2010).

FHT in the root boundary layers

FHT and its *Arabidopsis* orthologue ASFT (Molina *et al.*, 2009) are specifically expressed in root exodermal and endodermal cells where suberization occurs, although not in other cells (Fig. 3). Together the endodermis and exodermis are effective water and ion barriers while both possess Casparian strips and develop suberin lamellae (Enstone *et al.*, 2003). The strips develop earlier than lamellae and are important to prevent the apoplastic bypass of salts into the stele (Chen *et al.*, 2011). In addition, both the exodermis and endodermis are variable barriers that develop closer to or further from the root tip depending on abiotic stress (Enstone *et al.*, 2003) or pathogens (Thomas *et al.*, 2007). Moreover, the rate of suberization (Hose *et al.*, 2001) and the proportion between aliphatic and aromatic monomers in the root suberin (Zimmerman *et al.*, 2000) also depend on stress factors such as drought, anoxia, or salinity. In agreement with this, some genes involved in root suberin deposition are expressed under salt, osmotic treatment, or drought (Franke *et al.*, 2009; Lee *et al.*, 2009; Domergue *et al.*, 2010). In addition, suberin mutants, such as *GPAT5*, *esb1*, and the *FHT* ortholog *AtHHT*/*rwp* show modified sensitivities to salt stress (Beisson *et al.*, 2007; Baxter *et al.*, 2009; Gou *et al.*, 2009). Therefore, the contribution of *FHT* with regard to the regulation of root suberin deposition under stress cues such as anoxia, drought, or biotic stress could be surmised, taking into account the predicted *cis*-regulatory elements of the *FHT* promoter (Supplementary Table S1 at *JXB* online).

FHT is induced by injury

Tissues react to injury by forming a suberized and lignified closing layer which in most tissues is followed by active cell division that gives rise to a new phellogen and thereafter a wound periderm. In potato, leaves are characterized by the formation of a closing layer which is adjacent to the wounded margin and lacks cell division (Bloch, 1941), while tubers develop a wound periderm as has been widely documented (see, among others, Morris *et al.*, 1989; Sabba and Lulai, 2002). In leaves, FHT protein accumulation peaks after the third day following wounding when the formation of the closing layer is completed (Fig. 6A). In tubers, FHT accumulates early but keeps increasing at least up to the sixth day after injury (Fig. 7A) when the formation of the wound periderm is almost completed. These observations prove a rapid and massive induction of FHT during the healing process concomitant with suberin deposition. It has been shown that deposition of the aromatic suberin precedes that of the aliphatic suberin (Yang and Bernards, 2006). In mechanically injured potato leaves, the gene encoding phenylalanine ammonia lyase (*PAL*), an enzyme that operates at the very early steps of the phenylpropanoid biosynthesis, peaks 2 h after wounding and returns to its original level ~6 h afterwards (Joos and Halborck, 1992). In wounded potato tubers, suberization-associated anionic peroxidases appear after day 2 post-wounding and gradually increase until day 8 (Chaves *et al.*, 2009). In leaves of *Arabidopsis*, the *DAISY* transcript which encodes a fatty acid elongase peaks 1 h after wounding (Franke *et al.*, 2009), while transcripts encoding fatty acid reductases (*FAR*) peak 48 h after injury (Domergue *et al.*, 2010).

FHT is regulated by ABA and SA

Injury and pathogen attack activate JA, ethylene, ABA, and SA production, and these signals are transduced to a number of genes which are essential for plant protection (Bruxelles and Roberts, 2001). Moreover, interactions among these pathways allow for antagonistic and synergistic effects (Atkinson and Urwin, 2012). Suberin and lignin deposition are involved in most defence reactions (Thomas *et al.*, 2007). FHT is induced by wounding (Figs 6, 7) and responds to ABA and SA treatments (Fig. 8), presenting predicted *cis*-regulatory motifs for biotic and abiotic stress as well as ABA, JA, and SA responsiveness (Supplementary Table S1 at *JXB* online). A positive effect of ABA with regard to the induction of suberin genes and suberin deposition has been documented in potato (Soliday *et al.*, 1978; Roberts and Kolattukudy, 1989; Lulai *et al.*, 2008), *Arabidopsis* (Lee *et al.*, 2009), and tomato (Leide *et al.*, 2011). Moreover, Suttle *et al.* (2013) showed that endogenous ABA concentrations in potato tubers decrease after injury and reach a minimum after 24 h; nonetheless, the concentration then increases from the third to the seventh day in a pattern parallel to that of FHT (Fig. 7A). Furthermore, Lulai *et al.* (2008) reported that endogenous ABA concentrations increase after tuber harvest and then decrease during tuber storage, displaying an age-dependent pattern also similar to that of FHT (Fig. 5). According to Kumar *et al.* (2010), treatment with ABA partly restores the healing ability of older tubers by enhancing the accumulation of suberin aromatics. These authors also demonstrated that the age-induced loss of the healing ability is partly due to a reduced capacity to accumulate ABA and modulate the production of suberin aromatics through PAL. A similar modulation might also be contemplated through FHT. On the other hand, injury of potato tubers triggers a rapid increase (by 5-fold) of the basal JA content which peaks 4–6 h after wounding and thereafter returns to basal levels, a pattern compatible with a role in the early wound response (Koda and Kikuta, 1994). However, Lulai *et al.* (2011) showed no effect of JA treatment or inhibition of JA accumulation on suberin biosynthesis in the wound closing layer, in agreement with the lack of an enhancing or inhibiting effect of JA with regard to FHT induction (Fig. 8B). In contrast, Ozeretskovskaya *et al.* (2009) reported a positive effect of exogenous JA in reference to periderm proliferation, but this finding opposes the more general view that one of the functions of the wound-induced JA is related to the inhibition of growth by mitotic suppression (Zhang *et al.*, 2008). Concerning SA, its role in wound responses has

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so far not been elucidated (Vlot *et al.*, 2009). Previous experiments using potato discs have to date been unable to detect any effect of exogenous SA in connection with the healing process (Ozeretskovskaya *et al.*, 2009). However, SA impedes FHT induction after injury (Fig. 8C), acting in an antagonistic manner with respect to ABA. The antagonistic interaction among the ABA and SA signalling pathways has already been reported in several stress and defence responses (Jiang *et al.*, 2010; Sánchez-Vallet *et al.*, 2012).

FHT is located in the cytosol

Most factors that contribute to the transport and polymerization of suberin monomers are still unknown and the subcellular organization of the enzymes of the suberin biosynthesis pathway remains unclear (Pollard *et al.*, 2008; Beisson *et al.*, 2012). The endoplasmic reticulum (ER) has been reported as the location of some suberin, cutin, and wax enzymes, such as CER4/FAR3, CYP86A1/Horst KCS, KCR, and LACS (Rowland *et al.*, 2006; Höfer *et al.*, 2008; Joubès *et al.*, 2008; Beaudoin *et al.*, 2009; Weng *et al.*, 2009). Thus, the ER is supposed to be the place where reduction, hydroxylation, and elongation of the very long fatty acid chains occur. It is noteworthy that FAR proteins 1, 4, and 5 provide the fatty alcohols required for FACT, a feruloyl transferase closely related to FHT (Kosma *et al.*, 2012). However, subcellular fractionation indicates that FHT is absent from the ER but present in the cytosol. Moreover, two cutin BAHD acyltransferases also localize in the cytoplasm, and one of them, DCF (defective in cutin ferulate), has a homologous enzyme activity to that of FHT (Panikashvili *et al.*, 2009; Rautengarten *et al.*, 2012). The cytosolic localization of these enzymes is intriguing when the hydrophobic nature of their substrates is considered. In fact, Pascal *et al.* (2012) hypothesized an interaction with microsomal proteins as a requirement for CER2, which is annotated as BADH acyltransferase and is localized only in the cytosol.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. GUS activity driven by the *FHT* promoter in a cork-wart of the stem of an *in vitro* cultured plant.

Figure S2. GUS expression in freshly harvested tubers and in tubers stored for 7 months.

Table S1. Putative *cis*-regulatory elements of the *FHT* promoter.

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