The Tomato Feruloyl Transferase FHT Promoter Is an Accurate Identifier of Early Development and Stress-Induced Suberization

Anurag Kashyap [1,†], Álvaro Jiménez-Jiménez [1], Mercè Figueras [2], Olga Serra [2], Marc Valls [1,3] and Nuria S. Coll [1,4,*]

[1] Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, 08193 Bellaterra, Spain
[2] Laboratori del Suro, Biology Department, University of Girona, Campus Montilivi, 17003 Girona, Spain
[3] Department of Genetics, Universitat de Barcelona, 08028 Barcelona, Spain
[4] Consejo Superior de Investigaciones Científicas (CSIC), 08001 Barcelona, Spain
[*] Correspondence: nuria.sanchez-coll@cragenomica.es; Tel.: +34-935636600
[†] Current address: Department of Plant Pathology, Assam Agricultural University, Jorhat 785013, AS, India.

Abstract: As a wall polymer, suberin has a multifaceted role in plant development and stress responses. It is deposited between the plasma membrane and the primary cell wall in specialized tissues such as root exodermis, endodermis, phellem, and seed coats. It is formed de novo in response to stresses such as wounding, salt injury, drought, and pathogen attack and is a complex polyester mainly consisting of fatty acids, glycerol, and minor amounts of ferulic acid that are associated to a lignin-like polymer predominantly composed of ferulates. Metabolomic and transcriptomic studies have revealed that cell wall lignification precedes suberin deposition. The ferulic acid esterified to ω-hydroxy fatty acids, synthetized by the feruloyl transferase FHT (or ASFT), presumably plays a role in coupling both polymers, although the precise mechanism is not understood. Here, we use the promoter of tomato suberin feruloyl transferase (FHT/ASFT) fused to GUS (β-glucuronidase) to demonstrate that ferulate deposition agrees with the site of promoter FHT activation by using a combination of histochemical staining and UV microscopy. Hence, FHT promoter activation and alkali UV microscopy can be used to identify the precise localization of early suberizing cells rich in ferulic acid and can additionally be used as an efficient marker of early suberization events during plant development and stress responses. This line can be used in the future as a tool to identify emerging suberization sites via ferulate deposition in tomato plants, which may contribute to germplasm screening in varietal improvement programs.

Keywords: suberin; ferulates; stress; development; tomato

1. Introduction

The wall polymer suberin has a multidimensional role in plant development and stress responses [1,2]. Structurally, it is a glycerol-based, fatty-acid-derived polyester comprised primarily of ω-hydroxy acids, α, ω-dicarboxylic acids, fatty alcohols, and small amounts of hydroxycinnamic acids (mainly ferulic acid) [3]. It is deposited between the plasma membrane and the cell wall in specialized tissues, such as root exodermis, endodermis, seed coat, and phellem (cork), in root and aboveground tissue in woody plants [4]. Moreover, suberin can be formed de novo in response to stress such as wounding, salt injury, drought, and pathogen attack [5]. In addition to providing strength to the cell wall, suberin averts water loss and pathogen access by sealing off the layer of suberized cells. It acts as a potent barricade against pathogens, and in addition to providing strength to the cell wall, it may also act as an antimicrobial fence [6]. Recently, it has been shown that microbiota inhabiting the roots can also influence suberin deposition at the endodermis [7].

Hence, suberin has great potential for developing crops with multi-stress tolerance [8]. The polymer is also being explored for carbon sequestration applications using plants as carbon storage to mitigate global warming. Suberized cell walls also accumulate a lignin-like polymer rich in ferulates in the primary cell wall, which has also led to them being called suberin polyphenolic domains [2]. However, the configuration of suberin and lignin polymers and the progression of their deposition are poorly understood [2]. Metabolomics and transcriptomics data suggest that lignification precedes suberization, and ferulic acid has been identified to initiate suberin deposition, possibly offering connections to lignin or other cell wall polymers [2,9]. Suberin biosynthesis occurring during developmental processes has been studied in the last two decades using molecular genetic methods, particularly in the endodermis and seed coat model of *Arabidopsis thaliana* and in the tuber phellem of potato [10]. Precursors of the lignin-like polymer originate from the phenylpropanoid pathway, as does the ferulic acid of suberin, while the aliphatic monomers of suberin are synthesized through the fatty acid biosynthetic pathway [6]. Several genes encoding enzymes involved in the modification of these fatty acid precursors yielding the suberin monomers have been described, such as fatty acyl-CoA reductase (FAR1, FAR4, FAR5); fatty acid cytochrome P450 oxidases (CYP86A1/HORST, CYP86B1, CYP94B1, CYP94B3); glycerol-3-phosphate acyltransferase 5 (GPAT5/GPAT7); β-ketoacyl-CoA synthases (KCS1, KCS2/Daisy, KCS6, and KCS20); and genes involved in the conveyance of suberin monomers to the site of suberization (ABCG1, ABCG2, ABCG6, ABCG11, and ABCG20) [11–15]. In addition, a few upstream controllers of the suberin biosynthetic pathway have been recognized in Arabidopsis, such as the MYB transcription factors MYB41, MYB107, and SUBERMAN (MYB39) [2,16–18]. In Arabidopsis, suberin feruloyl transferase (FHT, also known as ASFT/HHT/RWP) has been functionally characterized and has been reported to be involved in the incorporation of ferulic acid into the suberin polyester. It specifically controls the accumulation of the ferulate esters of suberin in root endodermis, seeds, and phellem, but it has no effect on the content of p-coumarate or sinapate [19–21]. Knocking out FHT/ASFT causes abolition of the suberin-ester-linked ferulate, leading to altered permeability and sensitivity of seeds and roots to salt stress [19,20], and its activation has been reported as one of the earliest steps in suberin deposition [21]. Recently, a set of four MYB transcription factors (MYB41, MYB53, MYB92, and MYB93), each of which is individually regulated by developmental and exogenous signals, was shown to be sufficient to promote endodermal suberin deposition [22]. Moreover, the transcription factor WRKY9 has been demonstrated to regulate cytochrome P450 genes CYP94B3 and CYP86B1, leading to increased root suberin and salt tolerance in Arabidopsis [23]. The role of NAC transcription factor ANAC046 in suberin biosynthesis in *Arabidopsis thaliana* roots has also been experimentally validated [24].

The tissues commissioning suberization undergo a series of genetic and metabolic reprogramming comprising a web of metabolic conduits to yield the precursors of the polymer and subsequently their polymerization into the matrix [25]. In tomato, suberin plays vital roles both as a constitutive barrier as well as an inducible fence against diverse environmental stresses [26]. However, the visualization of suberin in tomato tissues mostly relies on stains such as Sudan IV or fluorol yellow, which target the aliphatic constituents of the polymer [27,28] and cannot capture early suberization events when suberizing walls accumulate ferulates, presumably playing a role in coupling the aromatic and aliphatic domains.

FHT is a key acyltransferase of the suberin biosynthesis pathway which catalyzes the conjugation of feruloyl-CoA and oxidized fatty acids, both of which are suberin monomers [19–21,29]. Hence, it acts as a linker of phenolic to aliphatic compounds to build the suberin polyester (Figure 1). The *FHT* gene encodes an acyltransferase of the BAHD superfamily named after the first four biochemically characterized enzymes of the group, which are plant-specific enzymes that catalyze the transfer of coenzyme A-activated donors onto various acceptor molecules [30].
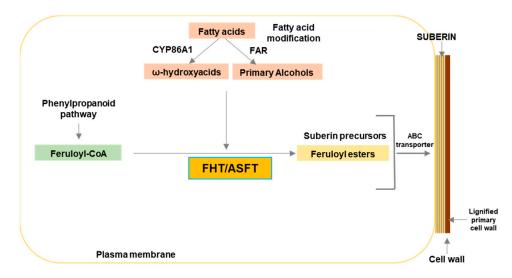
Figure 1. Schematic representation of the enzymatic function of suberin feruloyl transferase (FHT). FHT catalyzes the conjugation of feruloyl-CoA to aliphatic chains such as ω-hydroxy acids and primary alcohols to form feruloyl esters [19–21], potentially acting as a linker of suberin to lignin-like or other cell wall polymers.

Here, we present a method that allows precise localization of early suberization sites using the promoter of tomato suberin feruloyl transferase (FHT) fused to GUS (β-glucuronidase). The aim of this study is to determine whether the sites of FHT promoter activation correspond to those of ferulate deposition and thus can function as a robust marker for early suberization events. Transgenic tomato plants expressing *Pro_{SlFHT}::GUS* showed ferulate deposition at the position of promoter induction, visualized using alkali UV microscopy and in accordance with the predicted SlFHT functions. This tool can be used as an efficient marker of early suberization events during plant development and stress responses. Such markers will aid in the fundamental understanding of the suberization process and germplasm screening for varietal improvement programs in tomato against diverse stress responses.

2. Results

2.1. FHT Proteins of Different Plant Species Have a Conserved HxxxD Motif Involved in Catalysis and a DFGWG Motif Located at the C-Terminal End

In agreement with the characteristics of BAHD acyltransferases, all FHT proteins of different plant species have a conserved HxxxD motif involved in catalysis and a DFGWG motif located at the C-terminal end, the latter of which is presumed to have a structural function [21]. Protein homologs of tomato *FHT* gene (Solyc03g097500) were used for amino acid sequence alignment in ClustalW and visualized by Mview using the BAR (http://bar.utoronto.ca/, accessed on 2 May 2023) webpage (Figure 2). Phylogenetic analysis showed that the tomato *FHT* gene is very close in ancestry to the potato *FHT* (*StFHT*) gene (PGSC0003DMG400031731), which has been characterized in deeper detail [28].

2.2. Putative Cis Elements Found in Tomato FHT Promoter

The putative *SlFHT* promoter region (1710 bp upstream of the translation initiation) was examined using the PLACE [31] and the PlantCare [32] databases [29]. In agreement with the reported function of the *FHT* gene as a key acyltransferase involved in suberin biosynthesis, sequence analysis showed the presence of cis-regulatory motifs specific to abiotic stresses such as wounding, salt injury, water stress, etc. (e.g., WBOXNTERF3) and biotic stresses such as pathogenesis and salicylic acid receptiveness (e.g., GT1GMSCAM4, WBOXATNPR1). As expected, a number of ABA responsive motifs were present (e.g., WB-BOXPCWRKY1, MYB and MYC binding sites, SORLIP1, and WRKY), since suberin biosynthesis is known to be regulated by this hormone [33–35]. In addition, motifs corresponding

to organ-/cell-/and tissue-specific activation of phenylpropanoid genes (e.g., EBOXBN-NAPA) and root and seed inducible motifs were present (e.g., ROOTMOTIFTAPOX1, RYREPEATBNNAPA) (Table 1).
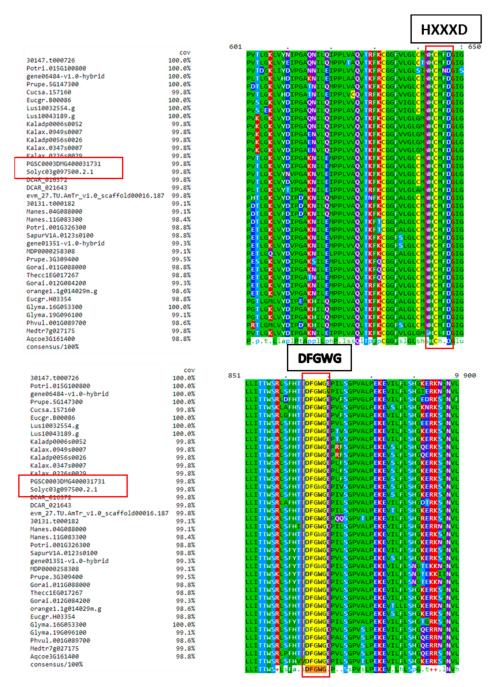

Figure 2. Sequence alignment of feruloyl transferase (FHT) proteins in different plant species. Protein homologs of tomato *FHT* gene (Solyc03g097500) were obtained from www.phytozome.jgi.doe.gov, accessed on 2 May 2023, and matches with more than 85% similarity were used for amino acid sequence alignment in ClustalW and visualized by Mview using BAR (http://bar.utoronto.ca/, accessed on 2 May 2023) webpage. In agreement with the characteristics of BAHD acyltransferases, all FHT proteins of different plant species have conserved HxxxD motif involved in catalysis and DFGWG motif located at the C-terminal end, the latter of which is presumed to have a structural function [21].

Table 1. Putative cis elements found in tomato FHT promoter.

Group	Motif Name	Sequence	Motif N^o	Function
Stress				
	WBOXNTCHN48	CTGACY	1	"W box." Elicitor-responsive element
	WBOXNTERF3	TGACY	5	"W box." Response to wounding
	OSE1ROOTNODULE	AAAGAT	5	Organ-specific elements (OSE) in infected cells of root nodules
	OSE2ROOTNODULE	CTCTT	2	Organ-specific elements (OSE) in infected cells of root nodules
	PREATPRODH	ACTCAT	1	Pro- or hypo-osmolarity-responsive element
	RAV1AAT	CAACA	5	RAV1 binding site, cold responsiveness
	MYB1AT	WAACCA	3	Element involved in dehydration responsiveness
	MYBCORE	CNGTTR	2	Element involved in response to water stress
	GT1GMSCAM4	GAAAAA	9	Pathogenesis and salt-induced element
	CURECORECR	GTAC	10	Copper-response element
	CCAATBOX1	CCAAT	5	HSE (heat shock element)
	ANAERO1CONSENSUS	AAACAAA	2	Response to anaerobiosis
	ACGTATERD1	ACGT	4	Involved in etiolation-induced dehydration
Hormones				
ABA	WBBOXPCWRKY1	TTTGACY	1	WRKY proteins binding site, responsive to ABA
	MYB2CONSENSUSAT	YAACKG	1	MYB recognition site involved in dehydration and ABA response
	MYCATERD1	CATGTG	1	MYC binding site involved in response to dehydration and ABA
	MYCATRD22	CACATG	1	MYC binding site involved in response to dehydration and ABA
	MYCCONSENSUSAT	CANNTG	6	MYC binding site involved in response to dehydration and ABA
	SORLIP1AT	GCCAC	4	Light-inducible, root-specific, and ABA-responsive element
Salicylic acid	WBOXATNPR1	TTGAC	3	"W-box"; binding site for SA-induced WRKY transcription factor
Ethylene	ERELEE4 CGTCA-motif	AWTTCAAA	1	Ethylene-responsive element
Auxin	CATATGGMSAUR	CATATG	2	Element involved in auxin responsiveness
Gibberellin	GARE2OSREP1	TAACGTA	1	Gibberellin-responsive element (GARE)
	GAREAT	TAACAAR	2	GARE (GA-responsive element)
	MYBGAHV	TAACAAA	2	Central element of gibberellin (GA) response complex (GARC)
	PYRIMIDINEBOXOSRAMY1A	CCTTTT	2	Gibberellin-response cis element of GARE and pyrimidine
	TATCCACHVAL21	TATCCAC	1	Conserved cis-acting response complex (GARC)
	PYRIMIDINEBOXHVEPB1	TTTTTTCC	2	"Pyrimidine box". Required for GA induction
Organ-/Tissue-/Cell type-specific				
	ROOTMOTIFTAPOX1	ATATT	12	Root specific element from rolD gene
	SP8BFIBSP8BIB	TACTATT	3	Tuberous root specific element

Table 1. *Cont.*

Group	Motif Name	Sequence	Motif N^o	Function
	RHERPATEXPA7	KCACGW	1	Right part of RHEs (root-hair-specific cis-elements)
	EBOXBNNAPA	CANNTG	6	RRE element. Tissue-specific activation of phenylpropanoids biosynthesis genes
	RYREPEATBNNAPA	CATGCA	1	Required for seed-specific expression
	POLLEN1LELAT52	AGAAA	12	Element required for pollen-specific expression
	CACTFTPPCA1	YACT	28	Element related to mesophyll expression
	BOXIINTPATPB	ATAGAA	3	Conserved element in promoters of plastids genes.
Others	TAAAGSTKST1	TAAAG	12	Dof1 protein controlling guard cell-specific gene expression
	MYBPZM	CCWACC	3	Core of consensus myb homolog binding site
	MYBST1	GGATA	2	Core motif of MybSt1 (a potato MYB homolog) binding site
	RYREPEATLEGUMINBOX	CATGCAY	1	RY repeat found in seed-storage protein genes

2.3. Induction of Pro~SlFHT~:GUS in Tomato Tissues Undergoing Developmental Suberization

We hypothesized that the tomato *FHT* promoter (*Pro~SlFHT~*) could be used as a good marker to study early suberization at the sites of suberin nucleation in different tissues. Hence, we generated *Pro~SlFHT~::GUS* lines in tomato in order to elucidate the participation of tomato FHT promoter in tissues known to deposit suberin. For this, we used the tomato variety Hawaii 7996 (H7996), which has been shown to induce ligno-suberin vascular coating to restrict infection of the vascular pathogen *Ralstonia solanacearum* [28].

A fragment consisting of 1713 bp upstream of the initial ATG codon of *SlFHT* was amplified from genomic DNA and fused to the reporter β-glucuronidase (GUS) to generate the *Pro~SlFHT~::GUS* construct, which was transformed into H7996 tomato. In agreement with the critical role reported for FHT/ASFT/RWP in suberization [19–21], we clearly observed the induction of *Pro~SlFHT~::GUS* in tissues known to accumulate suberin. Tomato root exodermal cells are known to deposit suberin [36]. We observed strong induction of *Pro~SlFHT~::GUS* in the root outer layers of young seedlings (Figure 3A). Further, prior to emergence, we observed strong induction of *Pro~SlFHT~::GUS* in lateral root primordia, indicating a hardening process by suberin deposition in the cells of the developing lateral root cap (Figure 3B). To analyze whether the induction of *Pro~SlFHT~::GUS* in tomato tissues corresponded to an increase in ferulates, in accordance to the function of FHT, we used a technique whereby ferulates can be detected by emission of blue fluorescence with UV excitation at neutral pH that characteristically changes to a stronger green emission under conditions of high pH such as in the presence of alkali [37–39]. We observed that the UV autofluorescence detected in root epidermal cells and lateral root primordia changed from blue to a strong green color upon treatment with alkali (1N KOH pH above 10) (Figure 3C,D), indicating the accumulation of ferulates in these tissues (Figure 3C,D). As expected, this pattern was highly coincidental to the observed induction of *Pro~SlFHT~::GUS*, highlighting the robustness of this method to report dual ferulate/FHT promoter activation.
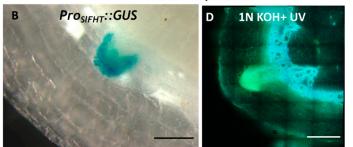

Figure 3. Induction of *Pro~SlFHT~::GUS* in tissues undergoing developmental suberization. Strong induction of *Pro~SlFHT~::GUS* was observed in the (**A**) root outer layers of young seedlings and (**B**) in the emerging lateral root primordia. Correspondingly, in similar samples, the ferulate signal was observed in (**C**) root outer layers of young seedlings and (**D**) lateral root primordia, as observed by alkali UV microscopic technique. Scale bar A = 500 μm, B = 200 μm, C = 40 μm, D = 500 μm.

2.4. Induction of Pro$_{SlFHT}$:GUS in Tomato Tissues Undergoing Wound Healing

Since suberin deposition is known to occur as part of the wound-healing response [29,40], we analyzed the induction of *Pro$_{SlFHT}$::GUS* upon injury. At 48 h post pin-prick injury on leaves, a strong induction of *Pro$_{SlFHT}$::GUS* was observed surrounding the injured region (Figure 4A,B). Further, when water imbalance or other factors lead to fruit cracks in tomato, the plants have a mechanism to seal this crack to prevent rotting due to the growth of saprophytes. We observed specific induction of *Pro$_{SlFHT}$::GUS* in the sealing region of the cracks, indicating suberization in this particular wound-healing response (Figure 4C,D). Further, we visualized ferulate deposition using alkali UV microscopy concomitantly with FHT promoter activation (GUS signal) during pin-prick injury and fruit cracks in tomato (Figure 4E,F).
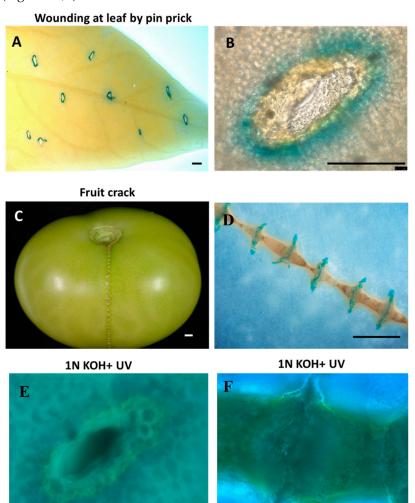

Figure 4. *Pro$_{SlFHT}$::GUS* is induced in leaves and fruits of tomato during wound healing. (**A**) *Pro$_{SlFHT}$::GUS* expression 48 h after pin-prick injury on the leaves. (**C**) Magnified image from (**A**). (**B**) Induction of *Pro$_{SlFHT}$::GUS* was observed in fruit cracks undergoing wound healing. (**D**) Magnified view of the crack showing GUS signal. Correspondingly, ferulate signal was observed in adjacent areas of (**E**) pin-prick injury on the leaves and (**F**) fruit cracks undergoing wound healing, as observed by alkali UV microscopic technique. Scale bar A = 400 μm, B = 200 μm, C = 20 μm, D = 20 μm, E = 200 μm, F = 20 μm.

2.5. Induction of Pro$_{SlFHT}$::GUS in Vascular Suberization Response in Tomato against Pathogens

Suberin vascular coating in response to the vascular pathogen *Ralstonia solanacearum* infection has been observed in the resistant tomato cultivar H7996 [28]. *Pro$_{SlFHT}$::GUS* transgenic H7996 tomato plants were inoculated through their roots by soaking the soil

with *R. solanacearum* with a concentration of ~1 × 10⁷ CFU mL⁻¹ and grown at 28 °C for 20 days. In water-treated plants, induction of *Pro_SLFHT_::GUS* was not observed in the xylem vasculature (Figure 5A). In infected resistant tomato plants, induction of *Pro_SlFHT_::GUS* was observed in the xylem vascular tissue as well as in the outer layers of the root (Figure 5B). Interestingly, we observed that the UV autofluorescence detected in vascular coatings in response to *R. solanacearum* infection in resistant H7996 (Figure 5D) changed from blue to a strong green color upon treatment with alkali (1N KOH pH above 10)—but not in response to mock treatment (Figure 5C)—indicating the presence of ferulates in these coatings (Figure 5F,G), as previously reported [28]. In contrast, no such vascular coating was observed in water-treated plants (non-infected) (Figure 5E,G). These data indicate that resistance to *R. solanacearum* in H7996 is mediated by induction of the *FHT* expression in the vasculature, which is accompanied by ferulate deposition in the same area. The lipophyllic fluorescent dye fluorol yellow, which stains the aliphatic portion of suberin, was also used in *Pro_SLFHT_::GUS* transgenic tomato plants infected with *R. solanacearum* and mock-treated plants. As shown in Figure S1, infection with *R. solanacearum* resulted in a clear increase in root fluorescence compared to mock-treated roots. However, this dye provided less zonal information regarding potential sites of *R. solancearum*-induced suberin deposition in tomato roots.
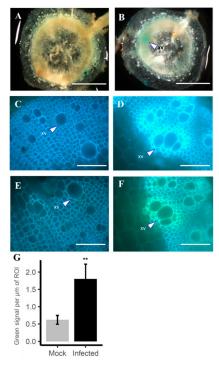

Figure 5. Pathogen-triggered induction of *Pro_SlFHT_::GUS* in xylem vasculature of tomato. *Pro_SlFHT_::GUS* transgenic tomato plants were inoculated through their roots by soaking the soil with 40 mL of suspension per plant of *R. solanacearum* with a concentration of ~1 × 10⁷ CFU mL⁻¹ and grown at 28 °C or with water as a control. GUS staining of (**A**) water-treated plants and (**B**) infected plants, where induction of *Pro_SlFHT_::GUS* could be observed in xylem vascular tissue (xv). UV autofluorescence before alkali treatment as observed in the vasculature of water-treated (**C**) or inoculated (**D**). Alkali UV microscopic technique was used to detect ferulate deposition in the vasculature of water-treated (**E**) or inoculated (**F**) samples at 20 days post-inoculation. Scale bar A, B = 2 mm; C, D, E, F = 150 μm. (**G**) Quantification of green fluorescence signal per μm in Region of Interest (ROI) from ferulate deposits upon alkaline treatment with KOH. Green color intensity calculation was performed with ImageJ software by selecting the vascular areas around the main vessels with localized fluorescence or green signal. Images from a representative experiment out of three with *n* = 5 plants per condition. Asterisks indicate statistically significant differences (**, *p*-value < 0.005, α = 0.05, Fisher's least significant difference test).

3. Discussion

Suberin deposition as a response to multiple stresses has recently been explored with renewed vigor to develop plants resilient to abiotic and biotic stresses [2]. In tomato, suberin plays vital roles against diverse environmental stresses [41]. However, the absence of markers or tools for localizing suberizing tissues remains as an impediment for the fundamental understanding of the process in tomato and other crop plants, particularly during the response to various stresses, as well as during germplasm screening for varietal improvement programs. In tomato, the visualization of suberin in tissues mostly relies on stains such as Sudan IV or fluorol yellow, which target the aliphatic components [42] and cannot capture the early accumulation of ferulate esters catalyzed by FHT, which is expressed earlier than the fatty acyl biosynthetic machinery [43,44] and before suberin lamellae structuring. Suberin undergoes enormous compositional changes as the lamella matures. In the initial stages, lignin-like polymer is laid down in advance to the suberin polyester [45]. In fact, ferulates constitute a crucial component of the lignin-like polymer, potentially acting as nucleating sites for suberin matrix polymerization [29,46]. Hence, our tool using the promoter of *SlFHT* fused to GUS allows precise localization of early suberization sites. FHT is a key acyltransferase, which catalyzes the conjugation of feruloyl-CoA to aliphatic chains to form feruloyl esters, which are suberin precursors [19–21,29].

The putative *SlFHT* promoter region showed presence of cis-regulatory motifs specific to abiotic stresses such as wounding, salt injury, water stress, etc. (e.g., WBOXNTERF3) and to biotic stresses such as pathogenesis and salicylic acid receptiveness (e.g., GT1GMSCAM4, WBOXATNPR1), and as expected, a number of ABA responsive motifs were present (e.g., WBBOXPCWRKY1, MYB and MYC binding sites, SORLIP1, and WRKY) since suberin biosynthesis is known to be regulated by ABA [33–35]. In addition, motifs corresponding to tissue-specific activation of phenylpropanoid genes (e.g., EBOXBNNAPA) and root and seed inducible motifs were present (e.g., ROOTMOTIFTAPOX1, RYREPEATBNNAPA). The tomato *FHT* gene is very close in ancestry to the potato *FHT* gene [28], and its promoter was also reported to contain similar cis-regulatory motifs [29]. In agreement with the critical role reported for FHT in suberization [17,18,24], we observed the induction of $Pro_{SlFHT}::GUS$ in the outer root layers, which could correspond to root exodermis, which is well known to deposit suberin [36]. Further, we observed strong induction of $Pro_{SlFHT}::GUS$ in the lateral root primordia, indicating a hardening process by suberin–ferulate deposition in the cells of the developing lateral root cap before emergence. Interestingly, the root caps of lateral roots have been reported to contain a cuticle-like polymer in Arabidopsis [47]. In contrast, we observed that lateral root primordia initiated suberization during their development in tomatoes. It is possible that both lipid-based polymers, suberin and cutin, having common ancestry [48], perform similar functions towards root cap hardening. However, ferulic acid, which is a chief component of suberin-associated phenolics, is a minor component in the chemically similar cutin polymer [1]. In accordance with the function of FHT, we observed ferulate deposition using alkali UV microscopy at the sites of promoter activation. Since suberin deposition is known to occur as part of the wound-healing response [29,40], we also observed a strong induction of $Pro_{SlFHT}::GUS$ surrounding the tissues at 48 h post pin-prick injury on leaves. Further, when water imbalance or other factors lead to fruit cracks in tomato, the plants have a mechanism to seal this crack to prevent rotting due to the growth of saprophytes. We could observe specific induction of $Pro_{SlFHT}::GUS$ in the sealing region of the cracks, indicating suberization in this particular wound-healing response. In all cases, a positive correlation between *FHT* promoter activity and ferulate deposition by alkali UV microscopy was observed. Additionally, induction of $Pro_{SlFHT}::GUS$ in the vasculature of resistant tomato cultivar H7996 against pathogen *R. solanacearum* was observed. Suberin vascular coating in response to vascular pathogen *R. solanacearum* infection has been previously reported in resistant tomato cultivar H7996 [28]. In infected resistant tomato plants, induction of $Pro_{SlFHT}::GUS$ was observed in the xylem vascular tissue, and ferulate vascular reinforcements were also observed, showing positive

correlation between induction of *Pro_{SlFHT}::GUS* and ferulate deposition after infection with *R. solanacearum*.

Together, these data indicate that this tool can be used as an efficient marker of early suberization events during plant development and stress responses. It can potentially be translated to other vascular pathogens to tomato as well as for studying other stress responses such as salt injury, drought stress, etc. Further, the tomato *FHT* promoter is a good candidate for tissue-specific expression of desired genes and metabolic engineering at targeted sites such as the exodermis, lateral root primordia, wound-healing zones, and other sites undergoing suberization to confer resilience to diverse stresses. In conclusion, we have developed a very effective tool to detect the sites for early suberization events linked to FHT activation and ferulate deposition.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

The tomato (*Solanum lycopersicum*) variety Hawaii 7996 (H7996) was used for all experiments. Seeds were germinated and plants were grown in pots containing soil (Substrate 2, Klasmann-Deilmann GmbH) mixed with perlite and vermiculite (30:1:1) in controlled growth chambers at 60% humidity and 12 h photoperiod with light intensity of 120–150 μmol·m^{-2}·s^{-1}. The temperature was set at 27 °C when using LED lighting and at 25 °C when using fluorescent lighting.

4.2. DNA Constructs

The *Pro_{FHT}::GUS* construct was generated using the Gateway system (Invitrogen, Waltham, MA, USA). A fragment consisting of 1713 bp upstream of the initial ATG codon (Solyc03g097500) was amplified from genomic DNA of H7996 tomato using the forward primer (ProFHTF1: ACAAGTTTGTACAAAAAAGCAGGCTAAACAACAAAATAAGATTG-CAC) and the reverse primer (ProFHTR1: ACCACTTTGTACAAGAAAGCTGGGTTTTCT-CAAAATTAATAAATCCTG) containing the attB flanking sequences. This sequence was cloned into the Gateway entry vector pDONR 207 using a BP reaction and then transferred into the Gateway destination vector pGWB3 using an LR reaction.

4.3. Stable Transformation of Tomato

Pro_{FHT}::GUS construct was transformed into H7996 using *Agrobacterium tumefaciens* strain C58C1. *A. tumefaciens* was used for co-culture with tomato cotyledons. Explant preparation, selection, and regeneration were performed following standard protocols [49]. Transformants were selected on kanamycin-containing MS medium and propagated into subsequent generations.

4.4. Detection of SlFHT Promoter Activity

Plant tissues or taproot–hypocotyl transition zone sections were immersed in an ice-chilled 90% (*v/v*) acetone bath and incubated for 20 min on ice, after which they were rinsed with water. Tissues were vacuum-infiltrated for 20 min with a solution containing 1 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc), 50 mM sodium phosphate buffer (pH 7), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, and 0.05% (*v/v*) Triton X-100. Samples were then incubated at 37 °C for a maximum of 48 h. Stained tissues were washed 2–3 times with phosphate-buffered saline (PBS) and then cleared with 70% (*v/v*) ethanol and stored in 70% (*v/v*) ethanol. Images were obtained using an Olympus SZX16 stereomicroscope equipped with a DP71 camera system.

4.5. Histological Methods

Thin tangential sections were made with a sterile razor blade of leaf discs, fruit section, or taproot–hypocotyl transition zone for histological assays. Autofluorescence from ferulates bound to the cell wall shows a pH-dependent blue-to-green color conversion [37–39]. Ferulates in the xylem vascular tissue were visualized by mounting cross-sections in 70%

ethanol (neutral pH) and illuminating them with UV with excitation bandpass filter in the range 340–380 nm to observe blue autofluorescence. These same sections were subsequently mounted in 1N KOH (pH above 10) to observe green autofluorescence from ferulates. Green color intensity calculation was performed with ImageJ software by selecting the vascular areas around the main vessels with localized fluorescence or green signal.

4.6. Wounding in Leaves

Injuries in leaves were performed by pin prick using a sterile 0.3 × 13 mm needle (30G × 1/2″, BD Microlance, Becton Dickinson, Franklin Lakes, NJ, USA).

4.7. Bacterial Inoculation in Plants

Four- to five-week-old tomato plants were inoculated through their roots with *Ralstonia solanacearum* GMI1000 using the soil drenching method [50]. For this, roots were wounded by making four holes in the soil at the corners of the pot with a 1 mL pipette tip and inoculated with a 1×10^7 CFU mL^{-1} (OD$_{600}$ = 0.01) suspension of bacteria [50]. Inoculated plants were kept in a growth chamber at 28 °C for 20 days.

References

1. Cohen, H.; Dong, Y.; Szymanski, J.; Lashbrooke, J.; Meir, S.; Almekias-Siegl, E.; Zeisler-Diehl, V.V.; Schreiber, L.; Aharoni, A. A Multilevel Study of Melon Fruit Reticulation Provides Insight into Skin Ligno-Suberization Hallmarks. *Plant Physiol.* **2019**, *179*, 1486–1501. [CrossRef] [PubMed]
2. Serra, O.; Geldner, N. The Making of Suberin. *New Phytol.* **2022**, *235*, 848–866. [CrossRef] [PubMed]
3. Beisson, F.; Li-Beisson, Y.; Pollard, M. Solving the Puzzles of Cutin and Suberin Polymer Biosynthesis. *Curr. Opin. Plant Biol.* **2012**, *15*, 329–337. [CrossRef] [PubMed]
4. Bernards, M.A. Demystifying Suberin. *Can. J. Bot.* **2002**, *80*, 227–240. [CrossRef]
5. Bernards, M.A.; Lewis, N.G. The Macromolecular Aromatic Domain in Suberized Tissue: A Changing Paradigm. *Phytochemistry* **1998**, *47*, 915–933. [CrossRef] [PubMed]

6. Vishwanath, S.J.; Delude, C.; Domergue, F.; Rowland, O. Suberin: Biosynthesis, Regulation, and Polymer Assembly of a Protective Extracellular Barrier. *Plant Cell Rep.* **2015**, *34*, 573–586. [CrossRef]

7. Salas-González, I.; Reyt, G.; Flis, P.; Custódio, V.; Gopaulchan, D.; Bakhoum, N.; Dew, T.P.; Suresh, K.; Franke, R.B.; Dangl, J.L.; et al. Coordination between Microbiota and Root Endodermis Supports Plant Mineral Nutrient Homeostasis. *Science* **2021**, *371*, eabd0695. [CrossRef] [PubMed]

8. Chen, A.; Liu, T.; Wang, Z.; Chen, X. Plant Root Suberin: A Layer of Defence against Biotic and Abiotic Stresses. *Front. Plant Sci.* **2022**, *13*, 1056008. [CrossRef]

9. Andersen, T.G.; Molina, D.; Kilian, J.; Franke, R.B.; Ragni, L.; Geldner, N. Tissue-Autonomous Phenylpropanoid Production Is Essential for Establishment of Root Barriers. *Curr. Biol.* **2021**, *31*, 965–977.e5. [CrossRef]

10. Ranathunge, K.; Schreiber, L.; Franke, R. Suberin Research in the Genomics Era—New Interest for an Old Polymer. *Plant Sci.* **2011**, *180*, 399–413. [CrossRef]

11. Höfer, R.; Briesen, I.; Beck, M.; Pinot, F.; Schreiber, L.; Franke, R. The Arabidopsis Cytochrome P450 CYP86A1 Encodes a Fatty Acid ω-Hydroxylase Involved in Suberin Monomer Biosynthesis. *J. Exp. Bot.* **2008**, *59*, 2347–2360. [CrossRef] [PubMed]

12. Vishwanath, S.J.; Kosma, D.K.; Pulsifer, I.P.; Scandola, S.; Pascal, S.; Joubès, J.; Dittrich-Domergue, F.; Lessire, R.; Rowland, O.; Domergue, F. Suberin-Associated Fatty Alcohols in Arabidopsis: Distributions in Roots and Contributions to Seed Coat Barrier Properties. *Plant Physiol.* **2013**, *163*, 1118–1132. [CrossRef] [PubMed]

13. Franke, R.; Höfer, R.; Briesen, I.; Emsermann, M.; Efremova, N.; Yephremov, A.; Schreiber, L. The DAISY Gene from Arabidopsis Encodes a Fatty Acid Elongase Condensing Enzyme Involved in the Biosynthesis of Aliphatic Suberin in Roots and the Chalaza-Micropyle Region of Seeds. *Plant J.* **2009**, *57*, 80–95. [CrossRef]

14. Belsson, F.; Li, Y.; Bonaventura, G.; Pollard, M.; Ohlrogge, J.B. The Acyltransferase GPAT5 Is Required for the Synthesis of Suberin in Seed Coat and Root of Arabidopsis. *Plant Cell* **2007**, *19*, 351–368. [CrossRef] [PubMed]

15. Yadav, V.; Molina, I.; Ranathunge, K.; Castillo, I.Q.; Rothstein, S.J.; Reed, J.W. ABCG Transporters Are Required for Suberin and Pollen Wall Extracellular Barriers in Arabidopsis. *Plant Cell* **2014**, *26*, 3569–3588. [CrossRef]

16. Cohen, H.; Fedyuk, V.; Wang, C.; Wu, S.; Aharoni, A. SUBERMAN Regulates Developmental Suberization of the Arabidopsis Root Endodermis. *Plant J.* **2020**, *102*, 431–447. [CrossRef]

17. Kosma, D.K.; Murmu, J.; Razeq, F.M.; Santos, P.; Bourgault, R.; Molina, I.; Rowland, O. AtMYB41 Activates Ectopic Suberin Synthesis and Assembly in Multiple Plant Species and Cell Types. *Plant J.* **2014**, *80*, 216–229. [CrossRef]

18. Gou, M.; Hou, G.; Yang, H.; Zhang, X.; Cai, Y.; Kai, G.; Liu, C.J. The MYB107 Transcription Factor Positively Regulates Suberin Biosynthesis. *Plant Physiol.* **2017**, *173*, 1045–1058. [CrossRef]

19. Gou, J.-Y.; Yu, X.-H.; Liu, C.-J. A Hydroxycinnamoyltransferase Responsible for Synthesizing Suberin Aromatics in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18855–18860. [CrossRef]

20. Molina, I.; Li-Beisson, Y.; Beisson, F.; Ohlrogge, J.B.; Pollard, M. Identification of an Arabidopsis Feruloyl-Coenzyme A Transferase Required for Suberin Synthesis. *Plant Physiol.* **2009**, *151*, 1317–1328. [CrossRef]

21. Serra, O.; Hohn, C.; Franke, R.; Prat, S.; Molinas, M.; Figueras, M. A Feruloyl Transferase Involved in the Biosynthesis of Suberin and Suberin-Associated Wax Is Required for Maturation and Sealing Properties of Potato Periderm. *Plant J.* **2010**, *62*, 277–290. [CrossRef] [PubMed]

22. Shukla, V.; Han, J.P.; Cléard, F.; Lefebvre-Legendre, L.; Gully, K.; Flis, P.; Berhin, A.; Andersen, T.G.; Salt, D.E.; Nawrath, C.; et al. Suberin Plasticity to Developmental and Exogenous Cues Is Regulated by a Set of MYB Transcription Factors. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2101730118. [CrossRef] [PubMed]

23. Krishnamurthy, P.; Vishal, B.; Bhal, A.; Kumar, P.P. WRKY9 Transcription Factor Regulates Cytochrome P450 Genes CYP94B3 and CYP86B1, Leading to Increased Root Suberin and Salt Tolerance in Arabidopsis. *Physiol. Plant.* **2021**, *172*, 1673–1687. [CrossRef] [PubMed]

24. Mahmood, K.; Zeisler-Diehl, V.V.; Schreiber, L.; Bi, Y.M.; Rothstein, S.J.; Ranathunge, K. Overexpression of ANAC046 Promotes Suberin Biosynthesis in Roots of *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **2019**, *20*, 6117. [CrossRef] [PubMed]

25. Lashbrooke, J.; Cohen, H.; Levy-Samocha, D.; Tzfadia, O.; Panizel, I.; Zeisler, V.; Massalha, H.; Stern, A.; Trainotti, L.; Schreiber, L.; et al. MYB107 and MYB9 Homologs Regulate Suberin Deposition in Angiosperms. *Plant Cell* **2016**, *28*, 2097–2116. [CrossRef]

26. Ayaz, A.; Huang, H.; Zheng, M.; Zaman, W.; Li, D.; Saqib, S.; Zhao, H.; Lü, S. Molecular Cloning and Functional Analysis of GmLACS2-3 Reveals Its Involvement in Cutin and Suberin Biosynthesis along with Abiotic Stress Tolerance. *Int. J. Mol. Sci.* **2021**, *22*, 9175. [CrossRef]

27. Landgraf, R.; Smolka, U.; Altmann, S.; Eschen-Lippold, L.; Senning, M.; Sonnewald, S.; Weigel, B.; Frolova, N.; Strehmel, N.; Hause, G.; et al. The ABC Transporter ABCG1 Is Required for Suberin Formation in Potato Tuber Periderm. *Plant Cell* **2014**, *26*, 3403–3415. [CrossRef]

28. Kashyap, A.; Jiménez-Jiménez, Á.L.; Zhang, W.; Capellades, M.; Srinivasan, S.; Laromaine, A.; Serra, O.; Figueras, M.; Rencoret, J.; Gutiérrez, A.; et al. Induced Ligno-Suberin Vascular Coating and Tyramine-Derived Hydroxycinnamic Acid Amides Restrict Ralstonia Solanacearum Colonization in Resistant Tomato. *New Phytol.* **2022**, *234*, 1411–1429. [CrossRef]

29. Boher, P.; Serra, O.; Soler, M.; Molinas, M.; Figueras, M. The Potato Suberin Feruloyl Transferase FHT Which Accumulates in the Phellogen Is Induced by Wounding and Regulated by Abscisic and Salicylic Acids. *J. Exp. Bot.* **2013**, *64*, 3225–3236. [CrossRef]

30. Bontpart, T.; Cheynier, V.; Ageorges, A.; Terrier, N. BAHD or SCPL Acyltransferase? What a Dilemma for Acylation in the World of Plant Phenolic Compounds. *New Phytol.* **2015**, *208*, 695–707. [CrossRef]

31. Higo, K.; Ugawa, Y.; Iwamoto, M.; Korenaga, T. Plant Cis-Acting Regulatory DNA Elements (PLACE) Database: 1999. *Nucleic Acids Res.* **1999**, *27*, 297–300. [CrossRef] [PubMed]

32. Lescot, M.; Déhais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Van De Peer, Y.; Rouzé, P.; Rombauts, S. PlantCARE, a Database of Plant Cis-Acting Regulatory Elements and a Portal to Tools for in Silico Analysis of Promoter Sequences. *Nucleic Acids Res.* **2002**, *30*, 325–327. [CrossRef] [PubMed]

33. Woolfson, K.N.; Haggitt, M.L.; Zhang, Y.; Kachura, A.; Bjelica, A.; Rey Rincon, M.A.; Kaberi, K.M.; Bernards, M.A. Differential Induction of Polar and Non-Polar Metabolism during Wound-Induced Suberization in Potato (*Solanum tuberosum* L.) Tubers. *Plant J.* **2018**, *93*, 931–942. [CrossRef] [PubMed]

34. Negrel, J.; Lotfy, S.; Javelle, F. Modulation of the Activity of Two Hydroxycinnamoyl Transferases in Wound-Healing Potato Tuber Discs in Response to Pectinase or Abscisic Acid. *J. Plant Physiol.* **1995**, *146*, 318–322. [CrossRef]

35. Soliday, C.L.; Dean, B.B.; Kolattukudy, P.E. Suberization: Inhibition by Washing and Stimulation by Abscisic Acid in Potato Disks and Tissue Culture. *Plant Physiol.* **1978**, *61*, 170–174. [CrossRef]

36. Kajala, K.; Gouran, M.; Shaar-Moshe, L.; Mason, G.A.; Rodriguez-Medina, J.; Kawa, D.; Pauluzzi, G.; Reynoso, M.; Canto-Pastor, A.; Manzano, C.; et al. Innovation, Conservation, and Repurposing of Gene Function in Root Cell Type Development. *Cell* **2021**, *184*, 3333–3348.e19. [CrossRef]

37. Harris, P.J.; Trethewey, J.A.K. The Distribution of Ester-Linked Ferulic Acid in the Cell Walls of Angiosperms. *Phytochem. Rev.* **2010**, *9*, 19–33. [CrossRef]

38. Donaldson, L.; Williams, N. Imaging and Spectroscopy of Natural Fluorophores in Pine Needles. *Plants* **2018**, *7*, 10. [CrossRef]

39. Carnachan, S.M.; Harris, P.J. Ferulic Acid Is Bound to the Primary Cell Walls of All Gymnosperm Families. *Biochem. Syst. Ecol.* **2000**, *28*, 865–879. [CrossRef]

40. Graça, J. Suberin: The Biopolyester at the Frontier of Plants. *Front. Chem.* **2015**, *3*, 62. [CrossRef]

41. Kashyap, A.; Planas-marquès, M.; Valls, M. Blocking Intruders: Inducible Physico-Chemical Barriers against Plant Vascular Wilt Pathogens. *J. Exp. Bot.* **2021**, *72*, 184–198. [CrossRef] [PubMed]

42. Brundrett, M.C.; Kendrick, B.; Peterson, C.A. Efficient Lipid Staining in Plant Material with Sudan Red 7b or Fluoral Yellow 088 in Polyethylene Glycol-Glycerol. *Biotech. Histochem.* **1991**, *66*, 111–116. [CrossRef] [PubMed]

43. Naseer, S.; Lee, Y.; Lapierre, C.; Franke, R.; Nawrath, C.; Geldner, N. Casparian Strip Diffusion Barrier in Arabidopsis Is Made of a Lignin Polymer without Suberin. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 10101–10106. [CrossRef] [PubMed]

44. Wahrenburg, Z.; Benesch, E.; Lowe, C.; Jimenez, J.; Vulavala, V.K.R.; Shiyou, L.; Hammerschmidt, R.; Douches, D.; Yim, W.C.; Santos, P.; et al. Transcriptional Regulation of Wound Suberin Deposition in Potato Cultivars with Differential Wound Healing Capacity. *Plant J.* **2021**, *107*, 77–99. [CrossRef] [PubMed]

45. Lulai, E.C.; Corsini, D.L. Differential Deposition of Suberin Phenolic and Aliphatic Domains and Their Roles in Resistance to Infection during Potato Tuber (*Solanum tuberosum* L.) Wound-Healing. *Physiol. Mol. Plant Pathol.* **1998**, *53*, 209–222. [CrossRef]

46. Bernards, M.A.; Lewis, N.G. Alkyl Ferulates in Wound Healing Potato Tubers. *Phytochemistry* **1992**, *31*, 3409–3412. [CrossRef] [PubMed]

47. Berhin, A.; de Bellis, D.; Franke, R.B.; Buono, R.A.; Nowack, M.K.; Nawrath, C. The Root Cap Cuticle: A Cell Wall Structure for Seedling Establishment and Lateral Root Formation. *Cell* **2019**, *176*, 1367–1378.e8. [CrossRef]

48. Philippe, G.; Sørensen, I.; Jiao, C.; Sun, X.; Fei, Z.; Domozych, D.S.; Rose, J.K. Cutin and Suberin: Assembly and Origins of Specialized Lipidic Cell Wall Scaffolds. *Curr. Opin. Plant Biol.* **2020**, *55*, 11–20. [CrossRef]

49. Mazier, M.; Flamain, F.; Nicolaï, M.; Sarnette, V.; Caranta, C. Knock-down of Both EIF4E1 and EIF4E2 Genes Confers Broad-Spectrum Resistance against Potyviruses in Tomato. *PLoS ONE* **2011**, *6*, e29595. [CrossRef]

50. Planas-Marquès, M.; Kressin, J.P.; Kashyap, A.; Panthee, D.R.; Louws, F.J.; Coll, N.S.; Valls, M. Four Bottlenecks Restrict Colonization and Invasion by the Pathogen *Ralstonia Solanacearum* in Resistant Tomato. *J. Exp. Bot.* **2019**, *71*, 2157–2171. [CrossRef]