Apple fruit periderms (russeting) induced by wounding or by moisture have the same histologies, chemistries and gene expressions

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Abstract

Russeting is a cosmetic defect of some fruit skins. Russeting (botanically: induction of periderm formation) can result from various environmental factors including wounding and surface moisture. The objective was to compare periderms resulting from wounding with those from exposure to moisture in developing apple fruit. Wounding or moisture exposure both resulted in cuticular microcracking. Cross-sections revealed suberized hypodermal cell walls by 4 d, and the start of periderm formation by 8 d after wounding or moisture treatment. The expression of selected target genes was similar in wound and moisture induced periderms. Transcription factors involved in the regulation of suberin (MYB93) and lignin (MYB42) synthesis, genes involved in the synthesis (CYP86B1) and the transport (ABC20) of suberin monomers and two uncharacterized transcription factors (NAC038 and NAC058) were all upregulated in induced periderm samples. Genes involved in cutin (GPAT6, SHN3) and wax synthesis (KCS10, WSD1, CER6) and transport of cutin monomers and wax components (ABCG11) were all downregulated. Levels of typical suberin monomers (ω-hydroxy-C20, -C22 and -C24 acids) and total suberin were high in the periderms, but low in the cuticle. Periderms were induced only when wounding occurred during early fruit development (32 and 66 days after full bloom (DAFB)) but not later (93 DAFB). Wound and moisture induced periderms are very similar morphologically, histologically, compositionally and molecularly.

Introduction

Russeting occurs on the skins of many fruit crop species, including of apples. In the smooth-skinned apple cultivars, russetting is perceived as a cosmetic impairment and so results in a quality downgrade in the packhouse, and so is the cause of significant economic loss for producers. In addition to cosmetic impairment, a russeted fruit skin is also more permeable to water vapor [1]. In this way, russeted fruit suffer increased rates of postharvest water loss in
transit and storage and so a greater loss of packed weight and, hence, a yield loss at point of sale (apples are commonly priced on a per fresh weight basis). A further problem associated with increased postharvest water loss is an increased incidence of shrivel, so is a further cause of quality downgrade at point of sale.

In botanical terms, russeting represents the replacement of a relatively simple primary surface, an epidermis and hypodermis, by a more complex secondary surface, a periderm. This comprises a phellem, a phellogen and a phelloderm [2]. It is the suberized cell walls of the phellem that are responsible for the rough-textured, dull-brown appearance of a russeted fruit.

The etiology of russeting in fruit is complex and not entirely clear. Russeting can be triggered by mechanical damage caused by external biotic factors, such as feeding insects [3] or external abiotic factors such as abrasion—e.g., leaf rub [4] or the use of some agrochemicals [5]. More commonly, the causes of russeting are developmental, the first visible symptoms of the disorder being the appearance of cuticular microcracks [6–8]. Such microcracks result from various sources including from strain of the fruit surface caused by growth [9, 10] or exposure to surface moisture [11–15]. The latter includes exposure either to vapor-phase water (high humidity) or to liquid-phase water (fog, dew, rain) [16].

The formation of microcracks impairs the barrier properties of the cuticle. By a yet unknown mechanism, microcracks can then trigger the formation of a periderm in the hypodermis, just below the epidermis [17–20]. When fully formed, the periderm partially restores the barrier properties of the impaired primary surface [21]. From an evolutionary perspective, formation of a periderm is an effective repair mechanism [21].

A periderm is also formed in response to mechanical wounding of the fruit surface. Like microcracking, mechanical wounding impairs the barrier function of the cuticle. It is thus not unlikely, that the subsequent processes leading to periderm formation may therefore be the same. If this were the case, one would expect a periderm formed after wounding and after moisture induction of microcracking to have similar histologies, chemistries and gene expressions.

The objective of this study was to test the above hypothesis. We employed abrasion, using fine sandpaper, to induce periderm formation after wounding. This was compared to moisture induced periderms. Moisture often plays a role in the natural development of russeting. It can be induced experimentally by exposing the surfaces of a developing apple fruit to water [11, 12, 22].

Material and methods

Plant materials

‘Pinova’ apple (Malus × domestica Borkh.) grafted on M9 rootstocks were cultivated in an experimental orchard of the horticultural research station of Leibniz University Hannover at Ruthe (lat. 52˚14'N, long. 9˚49'E) according to current regulations for integrated fruit production. All fruit were selected to uniformity of size and color and freedom from defects, tagged and assigned to one of two treatments. A total of 125 trees in two adjacent rows were used for randomized sampling.

Treatments and experiments

Fruit were subjected to one of two treatments. To induce a wound periderm, the fruit skin was gently rubbed in the equatorial plane with sandpaper (grit size 1000; Bauhaus, Mannheim, Germany). The opposite surface of the same fruit served as the control.

To induce a moisture periderm we followed the procedure established earlier [12]. Briefly, a tube cut from the tip of a disposable Eppendorf reaction tube (8 mm inner diameter, cut to ~
17 mm in length) was mounted on the fruit surface using a non-phytotoxic, fast-curing silicone rubber (Dow sil™ SE 9186 Clear Sealant, Dow Toray, Tokyo, Japan). After curing, deionized water was injected into the tube through the hole in the tip. Thereafter, the hole was sealed with silicone rubber to prevent evaporative water loss. The tube was removed and resealed to the fruit surface every 2 d to avoid loosening as a result of surface expansion growth. Again, the opposite side of the fruit remained without treatment to serve as the control. Moisture exposure was terminated by carefully removing the tube and blotting the surface dry using a soft paper tissue. The attachment/detachment procedures themselves caused no visible damage to the fruit surface and, importantly, no russetting [12].

The following experiments were conducted:

A **time course study of periderm formation** following wounding or moisture treatments was conducted. Two batches of fruit were selected and tagged on the tree, 28 days after full bloom (DAFB). The first batch was wounded at 40 DAFB. The second batch was used for moisture induction, beginning at 28 DAFB. After 12 d of induction (at 40 DAFB), moisture treatment was terminated. For microcracking assessment, fruit were sampled at 0, 1, 2, 3, 4, 8 and 16 d after wounding or after termination of moisture treatment. For histology and analysis of gene expression, the sampling dates were 0, 2, 4, 8 and 16 after wounding or termination of moisture treatment.

The **compositions of periderms** induced by wounding, by moisture treatment, and that of a naturally russeted surface were investigated. In the subsequent season fruit reached a stage of development that was comparable to the time course study slightly earlier (at about 32 DAFB). Wounding was carried out at 32 DAFB and the fruit left on the tree until maturity (156 DAFB). The corresponding moisture treatment began at 31 DAFB and continued for 12 d. All fruit were harvested at maturity, photographed (Canon EOS 550D, lens: EF-S 18–55 mm, Canon Germany, Krefeld, Germany) and then either stored (sections of the fruit) in Karnovsky fixative or used for isolation of CMs and PMs, as described above.

The **developmental time course of periderm formation** following wounding was investigated by wounding fruit at 32 DAFB ('early'), 66 DAFB ('intermediate') or 93 DAFB ('late'). Samples for histology were taken 8 d after wounding and at maturity (156 DAFB).

**Methods**

**Microscopy.** Fruit surfaces were inspected for microcracks following exposure to wounding and to moisture [12]. For this, a fruit was dipped in 0.1% (w/v) aqueous acridine orange (Carl Roth, Karlsruhe, Germany) for 10 min, then rinsed with deionized water and blotted dry using a soft paper tissue. The treated and the control areas were then inspected using fluorescence microscopy (MZ10F; GFP-plus filter, 440–480 nm excitation wave length, ≥510 nm emission wave length; Leica Microsystems, Wetzlar, Germany). Three to four digital images were taken (DP71; Olympus Europa, Hamburg, Germany) on six to ten fruit, at each sampling date.

Periderm development was assessed by microscopy using thin anticlinal sections prepared from tissue blocks embedded in paraffin [11]. Briefly, excised tissue blocks (about 6×3×3 mm, two blocks per fruit per tree) comprising the fruit skin and some of the outer flesh were excised from the treated and control areas and fixed in Karnovsky fixative [23]. Blocks were then rinsed in deionized water, incubated in 70% (v/v) aqueous ethanol overnight (16 h) and then dehydrated in an ascending series of ethanol (70, 80, 90 and 96% v/v, for 30 min each). The ethanol was then displaced by isopropanol (100%, 40 min ×2) followed by a xylene substitute (AppliClear; AppliChem, Münster, Germany; 40 min ×2). For paraffin infiltration, blocks were transferred to a 1:1 (v/v) mixture of paraffin/xylene substitute (Carl Roth; 40 min ×1) at
60 °C followed by fresh paraffin wax (40 min × 2). All the incubation steps were carried out at reduced pressure (10.8 kPa). Finally, the blocks were cast in paraffin wax in a metal mold. Embedded blocks were then cooled and stored at 4 °C pending analysis.

Thin sections (10 μm) were cut using a rotatory microtome (Hyrax M 55; Carl Zeiss, Oberkochen, Germany). Sections were transferred to glass microscope slides, dried at 38 °C for 16 h and then rehydrated in xylene substitute (10 min, ×2) followed by a descending series of ethanol (96, 80, 70 and 60%; v/v; 10 min each) and finally in deionized water (5 min, ×2). Sections were stained in the dark using Fluorol Yellow (0.005%, w/v; Santa Cruz Biotechnology, Texas, USA) dissolved in glycerol (90%, v/v; Carl Roth) and melted (~ 90 °C) polyethylene glycol 4000 (PEG 4000; w/v; Carl Roth) in a ratio of 1:1 for 1 h [24]. Following washing in deionized water, the sections were viewed under transmitted white light or incident fluorescent light (filter U-MWB; 450–480 nm excitation; ≥520 nm emission wavelength; Olympus) using a fluorescence microscope (BX-60 equipped with a DP 73 digital camera; Olympus). We examined a minimum of 50 sections per block. Two blocks from the same fruit represented a single replication and there were a minimum of three replications.

**RNA extraction.** Using a razor blade, thin patches of skin were excised from wounded, or moisture-treated, or un-treated (control) surfaces [22]. Skin patches from six fruit taken from six trees (one apple per tree) were collected within 15 min of picking and combined to obtain one replicate. The patches were immediately frozen in liquid nitrogen and held at -80 °C. For RNA extraction, the patches were ground in liquid nitrogen using a pestle and mortar. The RNA was extracted using the Invitrap Spin Plant RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer’s protocol. Genomic DNA was removed using the DNA-free™ Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The purity and quantity of the RNA was determined by measuring the absorbances at 230, 260 and 280 nm (Nanodrop 2000c; Thermo Fisher Scientific, Waltham, Massachusetts, USA). The RNA integrity was determined on a 1.5% agarose gel. Following dilution, the RNA samples (30 ng/μl) were converted into cDNA (LunaScript® RT SuperMix Kit; New England Biolabs, Ipswich, Massachusetts, USA). A standard PCR with a pair of actin primers (EB127077) [25] and the DCSPol DNA polymerase kit (DNA Cloning Service, Hamburg, Germany) was carried out. The amplification was checked on a 1.5% agarose gel. Samples were stored at -80 °C pending further use.

**Quantitative real-time PCR.** Twelve key genes associated with periderm formation, and suberin, cutin and wax metabolism were analyzed by qPCR (for details see in S1 Table, Selected transcription factors and genes analyzed in the present study). These genes were selected because they all play key roles in moisture-induced periderm formation [22]. Specific primer pairs were designed on Primer3 (http://primer3.ut.ee/) (for details see in S2 Table, Primers sequences of the genes analyzed in the present study). A total of 900 ng of RNA in a 60 μl reaction vial were reverse transcribed into cDNA (LunaScript® RT SuperMix Kit; New England Biolabs, Ipswich, Massachusetts, USA). Later, an 8 μl reaction volume containing 1 μl cDNA, primers (at 200 nM final concentration) and the Luna® Universal qPCR Master Mix (New England Biolabs) were used to carry out quantitative real-time PCRs (QuantStudio™ 6 Flex Real-Time PCR System; Applied Biosystems, Waltham, Massachusetts, USA). Conditions were: one cycle at 95 °C for 60 s, 40 cycles at 95 °C for 15 s and 40 cycles at 60 °C for 60 s. A melting curve analysis (95 °C for 15 s, 60 °C for 60 s, 60 to 95 °C in 0.5 °C increments) was carried out after the final amplification.

All expression values were obtained from the QuantStudio™ Real-Time PCR Software v1.3 (Applied Biosystems) and normalized using the two reference genes Protein disulfide isomerase (PDI) (MDP0000233444) and MdeF-1 alpha (AJ223969.1) [26, 27].
Isolation of cuticular membranes and periderm membranes. Cuticular membranes (CMs) and periderm membranes (PMs) were isolated enzymatically [28] from skin patches of wounded or moisture treated fruit. Skins of naturally russeted or non-russeted fruit served as controls. Excised skin segments (ES) were punched using a biopsy punch (12 mm diameter; Acuderm, Terrace, FL, USA). The ES were incubated in an isolation medium containing pectinase (9%, v/v; Panzym Super E flüssig; Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark), cellulase (0.5% v/v; Cellubrix L.; Novozymes A/S) and NaN₃ (30 mM) in 50 mM citric acid buffer adjusted to pH 4.0. The isolation medium was replaced periodically until CMs and PMs separated from the subtending tissues. The CMs and PMs were cleaned using a soft camel-hair brush, rinsed in deionized water, dried at 40 °C and kept above dry silica gel.

Quantification and identification of wax constituent by gas chromatography. Wax constituents of CM or PM were quantified and identified following the protocol of Baales et al. [29]. The CM and PM discs were cut into small fragments using a razor blade. Wax was extracted by incubating 0.5 to 1 mg of CMs and PMs in CHCl₃ (5 ml per replicate) at room temperature on a horizontal rolling bench (RM; Ingenieurbüro CAT, M. Zipperer, Staufen, Germany) overnight. Tetracosane (100 μl of 10 mg tetracosane in 50 ml CHCl₃) was added to the wax extract as an internal standard. The volume of the extract was reduced under a gentle stream of N₂ at 60 °C. The extracted dewaxed CM and PM were removed from the extract and dried on Teflon discs for analysis of cutin and suberin monomers.

To avoid interference of wax constituents containing polar hydroxyl- and carboxyl groups with the GC column, waxes were derivatized by silylation. This process yields trimethylsilyl ethers and–esters of the respective constituents. Samples were derivatized at 70 °C for 45 min following addition of 20 μl BSTFA (N, O-bis(trimethylsilyl)-trifluoracetamid; Machery-Nagel, Düren, Germany) and 20 μl pyridine (Sigma Aldrich, Deisenhofen, Germany). Wax constituents were quantified using a gas chromatograph equipped with a flame ionization detector (GC-FID; CG-Hewlett Packard 5890 series H, Hewlett-Packard, Palo Alto, CA, USA; 307 column-type: 30 m DB-1 inner Diam. 0.32 mm, film thickness 0.2 μm; J&W Scientific, Folsom, CA, USA). For quantification, the peak areas were normalized using the tetracosane internal standard and the areas of the PMs or CMs.

For identification, a GC coupled to a mass spectrometer was used (GC-MS; Quadrupole mass selective detector HP 5971; Hewlett-Packard, Palo Alto, CA, USA). Individual constituents were identified by comparing the fragmentation patterns with published data and with our own data library. The number of replicates was two to three.

Quantification and identification of suberin and cutin monomers by gas chromatography. Suberin and cutin monomers were quantified and identified following the protocol of Baales et al. [29]. The extracted CMs and PMs were transesterified by incubation in 1 ml BF₃/MeOH for 16 h at 70 °C. Thereafter, 20 μg of dotriacontane (100 μl of 10 mg dotriacontane in 50 ml CHCl₃) was added as an internal standard. Depolymerization was stopped and 2 ml of saturated NaHCO₃ was added.

The cutin and suberin monomers were extracted using CHCl₃ (×3, 2 ml each). The CHCl₃ phase was separated, washed with 1 ml HPLC grade water, dried with Na₂SO₄ and concentrated under a gentle stream of N₂ at 60 °C. Samples were derivatized as described above. The monomers and constituents were quantified by GC-FID and identified by GC-MS as described above. The data were normalized relative to the internal standard and to the fruit surface area. The fragmentation patterns were compared with published data and our in-house library. The number of replicates was two to three.
Data analysis

Total suberin, cutin and wax were calculated by summation of all individual constituents identified and quantified by gas chromatography. The PMs isolated from wounded, moisture treated or naturally russeted fruit often represent mixed polymers that comprise areas with patches covered by periderm adjacent to patches covered by cuticle and underlying epidermal and hypodermal cells. The area ratios may vary between replicates. Because suberin, cutin and wax share common monomers and constituents, it is impossible to attribute individual constituents obtained in the compositional analyses of these mixed polymers to either the cutin or the suberin fractions. However, in an earlier study we quantified the mass ratios for typical constituents of suberin from the trunk of ‘Pinova’ trees [22]. The constituents unique for suberin are the ω-hydroxy-C\textsubscript{20}, -C\textsubscript{22} and -C\textsubscript{24} acids. These ω-hydroxy-acids account for 17.6% of the total suberin. Using these constituents and the composition of a ‘pure’ native periderm, the composition of mixed PMs could be calculated and assigned to the PM. As pointed out by Straube et al. [22], the calculation is based on the assumption that the suberin composition of a ‘Pinova’ fruit PM is identical to that of the trunk periderm of the same cultivar. Due to the lack of PM-specific wax constituents, this calculation was not possible for the wax fraction.

Data are presented as means ± standard error (SE) of the means. Where error bars are not visible, they are smaller than data symbols. Data were subjected to analyses of variance, regression analysis or t-tests using the statistical software SAS\textsuperscript{®} Studio (SAS 9.4; SAS Institute, Cary, NC, USA). Significance of P-values at the 0.05 level is indicated by *.

Results

Wounding by abrading the skin of developing apple fruit resulted in numerous microcracks in the cuticle. The microcracks and the surrounding dermal tissue were infiltrated by aqueous acridine orange. As growth progressed after wounding, the microcracks widened (Fig 1). Microcracks also formed after a 12-d moisture treatment (Fig 1). Like the microcracks resulting from wounding, those caused by surface moisture treatment also traversed the cuticle as indexed by infiltration with aqueous acridine orange. In contrast to microcracks resulting from abrasion, those caused by moisture treatment were not straight and parallel to one another but followed the pattern of the anticlinal cell walls of groups of epidermal cells. Furthermore, moisture-induced microcracks branched at tricellular junctions.

Cross-sections of wounded apple fruit skins revealed browning and death of epidermal and some hypodermal cells shortly after abrasion (Fig 2). By 4 d after wounding, cell walls in the hypodermal cell layers began to suberize (marked with arrows) as indexed by staining with Fluorol Yellow. By 8 d after wounding, and even more so by 16 d, stacks of cells with suberized cell walls had formed that are characteristic of a periderm.

In cross-sections of moisture treated fruit skins of the same developmental stage, microcracks were present in the cuticle. These microcracks widened and the cuticle curled upwards as fruit growth continued, indicating the presence of considerable growth strain. At 4 d after termination of moisture treatment, the cell walls of the hypodermal cells below the microcrack began to suberize. By 8 d and 16 d after termination of moisture treatment, periderm formation had begun (Fig 2).

The two transcription factors involved in the regulation of the synthesis processes of suberin (MYB93) and lignin (MYB42), a gene involved directly in the synthesis of suberin monomers (CYP86B1) and a gene involved in the transport of suberin monomers (ABCG20) were all upregulated. The other two transcription factors (NAC038 and NAC058), that do not yet have assigned functions, were also upregulated. Relative normalized expressions of MYB42, CYP86B1 and NAC058 were highest at 4 d or at 8 d but then decreased slightly at 16 d
(Fig 3C, 3E and 3K) whereas the expressions of MYB93, ABCG20 and NAC038 increased continuously to 16 d (Fig 3A, 3G and 3I). The log fold changes in expression are provided in the S1 Dataset.

Very similar expression profiles, but at somewhat lower levels, were obtained in the moisture treated patches (Fig 3B, 3D, 3F, 3H, 3J and 3L). The only exception was the upregulation of MYB42 in moisture treated fruit at 4 d after termination of moisture treatment. This exceeded that in the wounded fruit (Fig 3D).

Genes involved in the synthesis of cutin (SHN3, GPAT6) and wax (KCS10, WSD1, CER6) and the transport of cutin monomers and wax components (ABCG11) were downregulated in both wounded and moisture treated skin patches (Fig 4). In general, the relative expressions
were qualitatively and quantitatively similar in the wounded and moisture treated skin patches.

Skin patches that were wounded or moisture treated for 12 d during early fruit development had formed a continuous periderm (marked with arrows) and developed a russeted surface by maturity. The periderms following wounding or moisture treatment were indistinguishable from the periderms of naturally russeted fruit of the same cultivar (Fig 5). At maturity, the skins of non-russeted patches had developed a thick cuticle (marked with arrows).

The monomer compositions of the periderms induced by wounding or by moisture treatment and that of the native periderm were very similar. The amounts of the typical suberin monomers ω-hydroxy-C_{20}, -C_{22} and -C_{24} acids were very similar in all three periderms (wound induced, moisture induced, and native), and were significantly lower in the cuticle. The contents of carboxylic-C_{22} acid were also very similar in the three types of periderms but were much lower in the cuticle (Fig 6). Minor differences between the three types of periderms were: (1) The 9,10-dihydroxydicarboxylic-C_{16} acid was similar in wound and moisture induced periderms but significantly lower in native periderm. (2) The 1-hydroxy-C_{18} acid was present in higher amounts in moisture induced periderm than in wound and native periderm. (3) The 2-hydroxy-C_{18} acid content was higher in wound periderm than in moisture induced or in native periderm. (4) The hydrocinnamic acid was higher in moisture induced periderm.
Fig 3. Time courses of change in the expressions of two transcription factors involved in the regulation of the synthesis of suberin (MYB93) and lignin (MYB42), a gene involved directly in the synthesis of suberin monomers (CYP86B1) and a gene involved in the transport of suberin monomers (ABCG20) and two uncharacterized transcription factors (NAC038 and NAC058) in the skin of 'Pinova' apple fruit following wounding or following exposure of the fruit surface to moisture. Patches of fruit skin were wounded 40 days after full bloom (DAFB) by abrading the cuticle using fine sandpaper ('Wounding'). For comparison, microcracks were induced by exposure of skin patches to surface moisture ('Moisture'). Here, the fruit surface was exposed to surface moisture from 28 to 40 DAFB. Non-treated fruit served as the respective controls ('Control'). Expression values are means ± SE of three biological replicates comprising six fruit each. The ‘*’ indicates significant differences between the wounded patch and its control or between the moisture exposed patch and its control, $P \leq 0.05$ (Student’s t-test).

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Fig 4. Time courses of change in the expression of genes involved in the synthesis of cutin monomers (SHN3, GPAT6) and wax constituents (KCS10, WSD1, CER6) and their transport (ABCG11) in the skin ‘Pinova’ apple fruit following wounding or following exposure of the fruit surface to moisture. Patches of fruit skin were wounded 40 days after full bloom (DAFB) by abrading the cuticle using fine sandpaper (‘Wounding’). For comparison, microcracks were induced by exposure of skin patches to surface moisture (‘Moisture’). Here, the fruit surface was exposed to surface moisture from 28 to 40 DAFB. Non-treated fruit served as control (‘Control’). Expression values are means ± SE of three biological replicates comprising six fruit each. The ‘*’ indicates significant differences between the wounded patch and its control or between the moisture exposed patch and its control, P ≤ 0.05 (Student’s t-test).

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than in the wound or native periderm. The abundances of this monomer were similar in the cuticle and moisture induced periderm (Fig 6).

Wax occurred in low amounts in the moisture induced and native periderm and was even lower in the wound periderm. The composition of wax was similar in the moisture induced and native periderm. Dominating wax components in moisture induced and native periderms and in the cuticle were C$_{28}$ aldehydes, oleanolic and ursolic acids (Fig 7).

Total suberin was higher and total wax was lower, in the three periderms compared with in the cuticle. Accordingly, cutin occurred in higher amounts in the cuticle than in any of the three periderms (Fig 8).

Marked differences were found in periderm formation between different stages of fruit development. Wounding during early fruit development (32 DAFB) resulted in a typical periderm characterized by stacked and suberized phellem cells after 8 d of wounding, and which were still visible at maturity (156 DAFB; Fig 9). When wounding occurred at 66 DAFB a layer of cells with suberized cell walls had formed in the cortex within 8 d. At maturity, a typical periderm had developed (Fig 9). Interestingly, following wounding at a late stage of development (93 DAFB) only cells with suberized cell walls had formed in the cortex at maturity, but not a complete periderm (Fig 9).

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**Fig 5.** Cross-sections through patches of ‘Pinova’ apple fruit skin at the mature stage (156 days after full bloom (DAFB)) that had been wounded or exposed to surface moisture during early fruit development. Patches of fruit skin were wounded at 32 DAFB by abrading the cuticle using abrasive paper (‘Wound periderm’). For comparison, microcracks were induced by exposure of skin patches to surface moisture (‘Moisture-induced periderm’) from 31 to 43 DAFB. Non-treated naturally russeted surfaces (‘Native periderm’) and non-russeted surfaces served as control (‘Cuticle’). The cross-sections were stained with Fluorol Yellow. Scale bars in A 10 mm (upper) and 50 μm (lower). Bars are representative for all bright field and all fluorescence images of the composite.

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Fig 6. Composition of cutin and suberin of skins of mature apple fruit. Periderm formation in the fruit skin was induced during early development by abrading the cuticle using abrasive paper (‘Wound periderm’) (A) or by exposing the fruit skin to surface moisture for 12 d between 31 and 43 days after full bloom (DAFB; ‘Moisture-induced periderm’) (B). The treated patches of skin were excised at maturity 156 DAFB. Native periderm from naturally russeted fruit (C) and cuticles from non-treated non-russeted fruit served as controls (D). Data represent means ± SE of two to three replicates comprising periderms and cuticles of five fruit each. The data shown in (B) were taken from Straube et al. [22].

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Discussion

Our results demonstrate that periderms induced by wounding and by surface moisture are similar and do not differ from periderms found on naturally russeted fruit surface. This conclusion is based on the following arguments.

Fig 7. Wax constituents of the skins of mature apple fruit. Periderm formation in the fruit skin was induced during early development by abrading the cuticle using fine sandpaper ("Wound periderm") (A) or by exposing the fruit skin to surface moisture for 12 d between 31 and 43 days after full bloom (DAFB; "Moisture-induced periderm") (B). The treated patches of skin were excised at maturity 156 DAFB. Native periderm from naturally russeted fruit (C) and cuticles from non-treated non-russeted fruit served as controls (D). Data represent means ± SE of two to three replicates comprising periderms and cuticles of five fruit each. The data shown in (B) were taken from Straube et al. [22].

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Fig 8. Total masses of suberin (A), wax (B) and cutin (C) in patches of skin of mature apple fruit. Periderm formation in the fruit skin was induced during early fruit development by abrading the cuticle using fine sandpaper (‘Wound periderm’) or by exposing the fruit skin to surface moisture for 12 d between 31 and 43 days after full bloom (DAFB; ‘Moisture-induced periderm’; [22]). The treated patches of skin were excised at maturity 156 DAFB. Periderm from naturally russeted fruit (Native periderm) and cuticles from non-treated, non-russeted fruit (Cuticle) served as controls. Data represent means ± SE of two to three replicates comprising periderms and cuticles of five fruit each.

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First, there was no difference in morphology and histology between wound induced and moisture induced periderms and both were similar to those of a native periderm. The skin sections inspected revealed all typical characteristics of a periderm. These include stacks of phellem cells. These cells have suberized cell walls and therefore stain with Fluorol Yellow [30]. The ‘stacked’ arrangement indicates the cells in a stack originate from a single mother cell of the phellogen.

Second, gene expression was similar following wounding and following termination of moisture exposure. Genes related to the synthesis and transport of suberin monomers and transcription factors involved in periderm formation were all upregulated. Those involved in the synthesis and transport of cutin monomers and wax components were downregulated. In an earlier study, Straube et al. [22] observed an upregulated expression of CYP86B1, MYB42, ABCG20, NAC038, NAC058 and MYB93 in moisture exposed patches of apple skins after termination of the treatment. CYP86B1 is a key gene involved in the synthesis of very long chain ω-hydroxy and ω,ω-dicarboxylic acids, the monomers of suberin [22, 31]. The transcription factor MYB42 is involved in regulation of lignin synthesis [32]. ABCG20 is required for the
transport of suberin monomers [33]. NAC038 and NAC058 are transcription factors of the NAC family that are upregulated in russeted skins of apple [25, 34]. Expression of MYB93, another transcription factor, was also expressed in the russeted skin of apples [25, 34]. Its overexpression in N. benthamiana enhanced the expression of NAC038 and NAC058 [34]. Additionally a multispecies gene coexpression analysis highlighted a possible involvement of NAC038 and NAC058 in transcriptional regulation of suberin synthesis [35].

Third, there was little difference in composition between the wound induced, moisture induced or native periderms. While cutin and suberin share common monomers, the long chain ω-hydroxy acids (C_{20}, C_{22}, C_{24}) are unique for suberin [22, 36–38]. These dominated in all three periderms. Despite similarity in suberin composition, the wound periderm had a lower wax content compared to native and moisture induced periderms. The reason for this may be the following: the damage caused by abrading the cuticle was so harsh that most of the cuticle was removed and thus the developing periderm on the wounded surface contained no or very much less residual cuticle. In contrast, the native periderms contained significant amounts of dried cuticle residue on the surface [39, 40]. The moisture induced periderm is also expected to contain cuticle residues on the surface as the etiologies of periderm development and periderm morphology are similar to native periderm (Fig 5). The report of Schreiber et al. [41] for potato tubers, that the wound periderm contained 40 to 50% less wax than the native periderm, also supports of our findings.

Fourth, the ontogenies of formation of wound induced periderm and moisture induced periderm were similar. Periderms formed in developing fruit but did not develop in mature fruit. This observation is also consistent with earlier observations [4, 11, 12, 22, 42–44]. Also, Winkler et al. [15] reported that overhead sprinklers induced russet in ‘Elstar’ apples during early fruit development, but not shortly before maturity or at maturity. Apparently, the ability to form a periderm is lost by the later stages of fruit development. A possible explanation to account for this may be a decrease in the rate of growth strain. Towards maturity, the relative area growth rate of the fruit surface decreases continuously. Growth strain represents the main driver of microcracking [9].

The similarity of the periderms induced by wounding or by moisture and native periderms suggests the processes triggering periderm formation are likely similar. In all three periderms, the barrier properties of the cuticle are impaired due to microcracking, the only difference being the reason for the microcracking. While microcracking of the cuticle occurs at the surface, periderm formation begins by a de-differentiation of the subtending hypodermal cells. This requires some sort of signal which connects the two events. Potential signals resulting from impaired barrier properties include: (1) a decreased CO$_2$ concentration, (2) an increased O$_2$ concentration and (3) a more negative water potential of the flesh due to a more rapid dehydration at the fruit surface [8, 11, 22].

Among those potential signals, the roles of O$_2$ and CO$_2$ have been studied in kiwifruit and potato tuber. In kiwifruit, wound periderm formation was reduced significantly when O$_2$ was eliminated from the storage atmosphere [45]. Similarly, in potato tuber, there was nearly no periderm on the tuber stored at low (0.5 to 1%) O$_2$. In contrast, 2 to 4 layers of periderm cell had formed when tubers were stored at ambient (21%) O$_2$ concentrations [46]. Based on the observation in kiwifruit, the reduced suberization resulted from decreased activities of phenylalanine ammonia-lyase, peroxidase, catalase, and polyphenol oxidase [45]. Exposure to elevated CO$_2$ concentrations (10%) reduced periderm development in potato tuber [47]. To our knowledge, there are no reports of a potential role for a decreased water potential in the tissue surrounding a microcracked cuticle, in triggering periderm formation.
Conclusion

Periderms induced by wounding or moisture are similar from morphological, histological, compositional and molecular perspectives. Thus, the signal(s) linking the impaired barrier properties to the differentiation of a periderm in the hypodermis is likely to be the same after wounding and after moisture induced microcracking. These findings have important implications for experimental research. The data presented herein justify the use of wounding to study the relationship between the impaired barrier properties of the cuticle due to formation of microcracks and the beginning of periderm formation in the hypodermis, some cell layers below. The search for the linking signal may now begin.

Supporting information

S1 Table. Selected transcription factors and genes analyzed in the present study.
(DOCX)

S2 Table. Primer sequences of the genes analyzed in the present study.
(DOCX)

S1 Dataset. Excel file containing all data produced in figures throughout the manuscript.
(XLSX)

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References

1. Khanal BP, Ikigu GM, Knoche M. Russetting partially restores apple skin permeability to water vapour. Planta. 2019; 249:849–860. https://doi.org/10.1007/s00425-018-3044-1 PMID: 30448863

2. Evert RF. Periderm. In: Evert RF, editor. Esau’s Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body—Their Structure, Function, and Development. Hoboken: John Wiley & Sons Inc.; 2006, pp. 427–446.

3. Easterbrook MA, Fuller MM. Russetting of apples caused by apple rust mite Acarus schlechtendali (Acarina: Eriophyidae). Ann Appl Biol. 1986; 109:1–9. https://doi.org/10.1111/j.1744-7348.1986.tb03178.x

4. Simons RK, Aubertin M. Development of epidermal, hypodermal and cortical tissues in the Golden Delicious apple as influenced by induced mechanical injury. Proc Amer Soc Hort Sci. 1959; 74:1–9.

5. Goffinet MC, Pearson RC. Anatomy of russetting induced in Concord grape berries by the fungicide Chlorothalonil. Amer J Enol Vitic. 1991; 42:281–289.

6. Faust M, Shear CB. Russetting of apples, an interpretive review. HortScience. 1972; 7:233–235.

7. Faust M, Shear CB. Fine structure of the fruit surface of three apple cultivars. J Amer Soc Hort Sci. 1972; 97:351–355.

8. Winkler A, Athoo T, Knoche M. Russetting of fruits: Etiology and management. Horticulture. 2022; 8:231. https://doi.org/10.3390/horticulture8030231

9. Scharwies JD, Grimm E, Knoche M. Russetting and relative growth rate are positively related in 'Conference' and 'Condo' Pear. HorticScience. 2014; 49:746–749. https://doi.org/10.21273/HORTSCI.49.6.746

10. Skene DS. The development of russet, rough russet and cracks on the fruit of the Apple Cox's Orange Pippin during the course of the season. J Hort Sci. 1982; 57:165–174. https://doi.org/10.1080/00221589.1982.11515037

11. Chen YH, Straube J, Khanal BP, Knoche M, Debener T. Russetting in apple is initiated after exposure to moisture ends-I. Histological evidence. Plants. 2020; 9:1293. https://doi.org/10.3390/plants9101293 PMID: 33008020

12. Khanal BP, Imoro Y, Chen YH, Straube J, Knoche M. Surface moisture increases microcracking and water vapour permeance of apple fruit skin. Plant Biol. 2021; 23:74–82. https://doi.org/10.1111/plb.13178 PMID: 32881348

13. Knoche M, Grimm E. Surface moisture induces microcracks in the cuticle of 'Golden Delicious' apple. HorticScience. 2008; 43:1929–1931. https://doi.org/10.21273/HORTSCI.43.6.1929

14. Tukey LD. Observations on the russetting of apples growing in plastic bags. Proc Amer Soc Hort Sci. 1959; 74:30–39.

15. Winkler A, Grimm E, Knoche M, Lindstaedt J, Köpcke D. Late-season surface water induces skin spot in apple. HorticScience. 2014; 49:1324–1327. https://doi.org/10.21273/HORTSCI.49.10.1324

16. Creasy LL. The correlation of weather parameters with russet of Golden Delicious apples under orchard conditions. J Amer Soc Hort Sci. 1980; 105:735–738.

17. MacDaniels LH, Heinicke AJ. To what extent is spray burn of apple fruit caused by freezing of the flowers? Phytopathology. 1930; 20:903–906.

18. Meyer A. A study of the skin structure of Golden Delicious apples. Proc Amer Soc Hort Sci. 1944; 45:105–110.

19. Pratt C. Periderm development and radiation stability of russet-fruited sports of apple. Hort Res. 1972; 12:5–12.

20. Simons RK. The origin of russetting in russet sports of the 'Golden Delicious' apple. Hort Res. 1965; 5:101–106.

21. Knoche M, Lang A. Ongoing growth challenges fruit skin integrity. Crit Rev Plant Sci. 2017; 36:190–215. https://doi.org/10.1080/07352689.2017.1369333

22. Straube J, Chen YH, Khanal BP, Shumbusho A, Zeisier-Diehl V, Suresh K, et al. Russetting in apple is initiated after exposure to moisture ends: Molecular and biochemical evidence. Plants. 2021; 10:65. https://doi.org/10.3390/plants10010065

23. Karnovský MJ. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol. 1965; 27:1A–149A.

24. Brundrett MC, Kendrick B, Peterson CA. Efficient lipid staining in plant material with Sudan Red 7B or Fluoral Yellow 088 in polyethylene glycol-glycerol. Biotech Histochem. 1991; 66:111–116. https://doi.org/10.3109/010520299109110562
25. Legay S, Guerriero G, Deleruelle A, Lateur M, Evers D, André CM, et al. Apple russetting as seen through the RNA-seq lens: Strong alterations in the exocarp cell wall. Plant Mol Biol. 2015; 88:21–40. https://doi.org/10.1007/s11103-015-0303-4 PMID: 25786603

26. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 2001; 29:e45. https://doi.org/10.1093/nar/29.9.e45 PMID: 11328886

27. Chen YH, Khanal BP, Linde M, Debener T, Akio M, Knoche M. Expression of putative aquaporin genes in sweet cherry is higher in flesh than skin and most are downregulated during development. Sci Hort. 2019; 244:304–314. https://doi.org/10.1016/j.scienta.2018.09.065

28. Orgell WH. The isolation of plant cuticle with pectic enzymes. Plant Physiol. 1955; 30:78–80. https://doi.org/10.1104/pp.30.1.78 PMID: 16654733

29. 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. https://doi.org/10.1073/pnas.1205726109 PMID: 22665765

30. Franke R, Schreiber L. Suberin—a biopolyester forming apoplastic plant interfaces. Curr. Opin. Plant Biol. 2007; 10:252–259. https://doi.org/10.1016/j.pbi.2007.04.004 PMID: 17434790

31. Geng P, Zhang S, Liu J, Zhao C, Wu J, Cao Y, et al. MYB20, MYB42, MYB43, and MYB85 regulate phenylalanine and lignin biosynthesis during secondary cell wall formation. Plant Physiol. 2020; 182:1272–1283. https://doi.org/10.1104/pp.19.101070

32. Yadav V, Molina I, Ranathunge K, Castillo IQ, Rothstein SJ, Reed JW. ABCG transporters are required for suberin and pollen wall extracellular barriers in Arabidopsis. Plant Cell. 2014; 26:3569–3588. https://doi.org/10.1105/tpc.114.129049

33. Legay S, Guerriero G, André C, Guignard C, Cocco E, Charton S, et al. MdMyb93 is a regulator of suberin deposition in russeted apple fruit skins. New Phytol. 2016; 212:977–991. https://doi.org/10.1111/nph.14170

34. Schreiber L, Franke R, Hartmann K. Wax and suberin development of native and wound periderm of potato (Solanum tuberosum L.) and its relation to peridermal transpiration. Planta. 2005; 220:520–530. https://doi.org/10.1007/s00425-004-1364-9

35. de Vries HAMA. Development of the structure of the russeted apple skin. Acta Bot. Neerl. 1968; 17:405–415.

36. Knoche M, Khanal BP, Stopar M. Russetting in apple and pear: A plastic periderm replaces a stiff cuticle. Aust Plants. 2013; 5:pls048. https://doi.org/10.1093/aobpla/pls048 PMID: 23350024

37. Simons RK, Chu MC. Periderm morphology of mature ‘Golden Delicious’ apple with special reference to russetting, Sci Hort. 1978; 8:333–340. https://doi.org/10.1016/0304-4238(78)90055-9

38. Schreiber L, Franke R, Hartmann K. Wax and suberin development of native and wounded periderm of potato (Solanum tuberosum L.) and its relation to peridermal transpiration. Planta. 2005; 220:520–530. https://doi.org/10.1007/s00425-004-1364-9

39. Wei X, Mao L, Han X, Lu W, Xie D, Ren X, et al. High oxygen facilitates wound induction of suberin polyphenolics in kiwifruit, J Sci Food Agric. 2018; 98:2223–2230. https://doi.org/10.1002/jsfa.8709 PMID: 28963774
46. Lipton WJ. Some effects of low-oxygen atmospheres on potato tubers. Amer Potato J. 1967; 44:292–299. https://doi.org/10.1007/BF02862531.

47. Wigginton MJ. Effects of temperature, oxygen tension and relative humidity on the wound-healing process in the potato tuber. Potato Res. 1974; 17:200–214. https://doi.org/10.1007/BF02360387