The impaired barrier function of the cuticle triggers russeting

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Abstract

Russeting is a physiological disorder that occurs in several economically important crops. Among these, apple is a prominent case. Previous studies on the etiology of russeting in apple indicate that (1) moisture and wounding are common inducers of russeting; (2) russeting is associated with cuticular microcracking; and (3) the early stage of fruit development is more susceptible to russeting than the later stages. However, the initial events of russeting remain unknown. To elucidate the etiology of russeting, the objectives of the present study were to investigate: (1) the effect of prolonged surface moisture on apple fruit; (2) the sequence of events during exposure to surface moisture and after its removal; (3) the similarity between the events caused by wounding and moisture and their resulting periderm; (4) the initial processes of russeting using a transcriptomic approach; (5) the potential role of atmospheric oxygen (O_2) in russeting.

Exposure of young apple fruit to surface moisture for 12 days (d) induced microcracking, increased water loss, and led to russeting. Using the same exposure technique, moisture-induced periderm was found to be biphasic: a periderm initiated only after moisture removal (Phase II) and required at least 6 d of exposure (Phase I) to change cuticle properties to form an impaired barrier and induce russeting. The moisture-induced and wound periderm shared similarities in their initiation at the histological level, the initial expression pattern of several lignin, suberin, cutin and wax-related genes and the chemical constituents at fruit maturity. Additional phase-specific expression profiles of the moisture-induced periderm are revealed by transcriptome. Further efforts were made to establish detached fruit systems in young apple and tomato fruit to study the role of O_2 in russeting under anoxia. Unfortunately, the apple system failed due to various problems, but a promising tomato system, which behaved similarly to the attached fruit was later used for this purpose. The exclusion of O_2 successfully prevented the formation of a wound periderm. This was demonstrated by the absence of a periderm after 8 d of wounding and the suppression of selected suberin and lignin-related genes.

In conclusion, the results of the present study provide evidence that an impaired barrier caused by microcracking triggers the process of russeting. This triggering is O_2-dependent.

Keywords: atmospheric oxygen, cuticle, cutin, detached fruit, fruit surface, periderm, lignin, *Malus* × *domestica*, microcracking, russeting, suberin, surface moisture, *Solanum lycopersicum*, wax, wounding

Zusammenfassung

Die Berostung ist eine physiologische Störung, die bei mehreren wirtschaftlich relevanten Kulturpflanzen auftritt. Unter diesen ist der Apfel ein bekanntes Beispiel. Frühere Studien zur Ätiologie der Berostung zeigen, dass (1) Feuchtigkeit und Verwundung häufig zu Berostung führen, (2) Berostung mit Mikrorissen in der Kutikula assoziiert ist und (3) das frühe Fruchtentwicklungsstadien anfälliger für Berostung sind als spätere Stadien. Die auslösenden Prozesse sind jedoch unbekannt. Um die Ätiologie der Berostung aufzuklären, waren die Ziele der Studie die Untersuchung folgender Prozesse: (1) die Auswirkungen anhaltender Oberflächenfeuchtigkeit auf Apfelfrüchte; (2) die Abfolge der Ereignisse während der Einwirkung von Oberflächenfeuchtigkeit und nach deren Entfernung; (3) die Ähnlichkeit zwischen den durch Verwundung und Feuchtigkeit erzeugten Periderm; (4) die anfänglichen Prozesse der Berostung unter Verwendung eines transkriptomischen Ansatzes; (5) die mögliche Rolle von Luftsauerstoff (O_2) bei der Berostung.

Die Exposition junger Apfelfrüchte gegenüber Oberflächenfeuchtigkeit für 12 Tage führte zu Mikrorissen, erhöhtem Wasserverlust und zur Berostung. Unter Verwendung derselben Expositionstechnik wurde festgestellt, dass die feuchtigkeitsinduzierte Berostung in zwei Phasen abläuft: Peridermbildung begann nach dem Entfernen der Feuchtigkeit (Phase II) und erforderte mindestens 6 Tage Exposition (Phase I), um die Barriereeigenschaften der Kutikula zu verändern und Berostung zu induzieren. Periderm induziert durch Oberflächenfeuchtigkeit sowie Verwundung ähnelten sich in ihrer Initiierung auf histologischer Ebene, dem Expressionsmuster von Lignin-, Suberin-, Cutin- und Wachs-assoziierten Genen und den chemischen Bestandteilen zur Fruchtreife. Transkriptomanalysen zeigten phasenspezifische Expressionsprofile während der Entwicklung der Berostung durch Oberflächenfeuchte auf. Abgetrennte Apfel- und Tomatenfrüchte wurden genutzt um die Rolle von O_2 bei der Berostung unter Anoxie zu untersuchen. Berostung in abgetrennten Tomaten und an der Pflanze verhielten sich ähnlich, jedoch nicht bei Äpfeln. Weitere Analysen in Tomaten zeigten, dass der Ausschluss von O_2 die Bildung eines Wundperiderms verhinderte. Dies wurde durch das Fehlen eines Periderms 8 Tage nach Verwundung und durch die Unterdrückung ausgewählter Suberin- und Lignin-assoziierter Gene nachgewiesen.

Zusammenfassend belegen die Ergebnisse der vorliegenden Studie, dass eine gestörte Barriere, die durch Mikrorisse verursacht wird, den Prozess der Berostung auslöst. Diese Auslösung ist O_2 abhängig.

Schlüsselwörter: abgetrennte Frucht, atmosphärischer Sauerstoff, Berostung, Cutin, Fruchtoberfläche, Kutikula, Lignin, *Malus* × *domestica*, Mikrorisse, Oberflächenfeuchtigkeit, Periderm, *Solanum lycopersicum*, Suberin, Verwundung, Wachs

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List of abbreviations

Name	Stands for
ABA	Abscisic acid
ABCG	*ATP Binding Cassette Transporter G family member*
ACLSV	Apple chlorotic leaf spot virus
AP2B3	*AP2/B3-like transcription factor family protein*
CAD	*Cinnamyl-alcohol dehydrogenase*
CER	*ECERIFERUM*
CO_2	Carbon dioxide
CYP86A1	*Cytochrome P450, family 86, subfamily A, polypeptide 1*
CYP86A22	*Cytochrome P450, family 86, subfamily A, polypeptide 22*
CYP86B1	*Cytochrome P450, family 86, subfamily B, polypeptide 1*
d	Day
DAFB	Days after full bloom
DAF	Days after fertilization
DEGs	Differentially expressed genes
GPAT	*Glycerol-3-phosphate acyl transferase*
HSP	*Heat shock protein*
L	Liter
LG	Linkage group
LEA	*Late embryogenesis abundant hydroxyproline-rich glycoprotein*
LIM	*GATA zinc type finger transcription factor family protein*
Md	*Malus* × *domestica*
MYB	*myb (myeloblastosis) domain protein*
mg	Milligram
NAC	*NAM, ATAF1/2, and CUC2*
O_2	Atmospheric oxygen
OSC	*Oxidosqualene cyclase*
PAL	*Phenylalanine ammonia lyase*
pH	Potential of hydrogen
Phase I	The period during the surface moisture exposure
Phase II	The period after the moisture removal
POX/PRX	*Peroxidase*
ppm	Part per million
QTL	Quantitative trait locus

List of abbreviations (continuous)

Name	Stands for
ROS	Reactive oxygen species
SGNH	*SGNH hydrolase-type esterase superfamily protein*
SHN	*SHINE*
TF	Transcription factor
VAS	*Vascular Tissue Size*
wk	Week
WOX	*WUSCHEL-related homebox*
WRKY	A category of transcription factors that have the amino acids tryptophan. (W), arginine (R), lysine (K), and tyrosine (Y)
WSD	*wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase*
yr	Year

1. General introduction

1.1 Russeting in apple

Russeting is a physiological disorder that forms a brownish and coarse surface and occurs in various plant species, including apples (Tukey, 1959), pears (Scharwies et al., 2014), prunes (Michailides, 1991), grapes (Goffinet and Pearson, 1991) and mangoes (Athoo et al., 2020). Among them, the apple crop is a prominent case and most of the literature on russeting refers to it (Winkler et al., 2022).

The susceptibility to russeting varies among apple cultivars (Khanal et al., 2013b). In the marketplace, russeting is accepted and considered "normal" for cultivars that frequently produce russeting., such as 'Reinette' (Winkler et al., 2022). Many commercial cultivars are even named with the suffix 'russet' to indicate their russeted skin, e.g. 'Sergeant Russet' (Gutierrez et al., 2018), 'Egremont Russet', 'Fall Russet' and 'Brownlees Russet' (CJR Fruit Trees, 2024). On the contrary, in non-russted apple cultivars, russeting is viewed unfavorably by customers, traders and growers due to its consequences. First, a waxy and smooth apple fruit surface is expected to appear on the market for non-russted apple cultivars. The brownish and coarse appearance of russeting is undesirable and less preferred by customers. Second, storage of the fruit extends the supply of fruits and fills the gap in market demand for months and is necessary before available fruit is imported from another hemisphere. Water loss through the fruit surface is an important determinant of fruit storage quality (Hasan et al., 2024). Unfortunately, russeting leads to water loss and consequent shrinkage of the fruit (Baker, 1931; Pieniazek, 1944; Tukey, 1959; Khanal et al., 2019). Any water loss during storage and transportation is an economic loss to traders, as the fresh weight of the fruit determines the market price. Third, the absence of russeting on the fruits of non-russted cultivars has been a quality indicator for years (Urmson, 1950; USDA Agricultural Marketing Service, 2019). If russeting occurs during fruit development, it would increase the time spent and effort made on cultural practices, fruit selection and sorting. Based on this, it is important to understand russeting in a prominent crop such as apple, and this knowledge can be applied to other crops where the same disorder occurs.

1.2 Etiology of russeting in apple

The nature of russeting in apple is the replacement of a periderm (secondary fruit surface) to the original waxy cuticle, epidermis and hypodermis (primary fruit surface) (Khanal et al., 2013a). Its etiology is often thought to be associated with microcracking (microscopic cracks; Peschel and Knoche, 2005) which causes an impaired barrier (Faust and Shear, 1972a; Winkler et al., 2022).

Strains in the cuticle and the underlying cell layers during fruit growth and development can lead to microcracking. Naturally, apple fruits grow and increase in volume (Skene, 1966) and cuticle thickness gradually from full bloom to maturity (Glenn et al., 1985; Knoche et al., 2018; Meyer, 1944). Meanwhile, the fruit surface is under considerable strains and must maintain surface integrity (Knoche and Lang, 2017; Meyer, 1944; Tetley, 1930). Tangential strain plays an important role in this, as evidenced by the fact that the pattern of microcracks propagates only in the ridge (anticlinal cell wall) and not in the lamellae (periclinal cell wall) (Knoche et al., 2018). The coincidence of (1) growth-induced reorientation of epidermal cell division (Bell, 1937a; Meyer, 1944) and (2) non-synchronization of fruit growth/expansion with lower cuticle deposition (Lai et al., 2016) results in such a higher tangential strain. In addition, the reason for the higher susceptibility of certain apple cultivars to russeting (Khanal et al., 2013b) could be explained by the tendency to microcracking due to (1) the uneven thickness of the cuticle and epidermis (Meyer, 1944; Tetley, 1930) and (2) the lower density and greater size variability of epidermal and hypodermal cells (Khanal et al., 2020). However, not all microcracks result in russeting. In mature apples, microcracks can occur without the formation of a periderm (Meyer, 1944) and can be "repaired" by filling with waxes (Curry, 2009). Since more developed fruit is much less susceptible to both microcracking and russeting than early development (Knoche et al., 2011; Meyer, 1944; Skene, 1981), microcracking that induces russeting appears to occur during early fruit development.

Water has been identified as the most common environmental factor causing microcracking and russeting in the field. First, a relationship between water and russeting is clear. Tukey (1959) showed that bagging young apples in closed plastic bags induced russeting at maturity. In terms of russeting severity, high humidity (>60%) contributed the most to russeting in an 8-year (yr) observation (Creasy, 1980). Induction of russeting by water is more effective in early fruit development, most likely before 44 days after full bloom (DAFB). This has been demonstrated by immersing (Knoche et al., 2011), sprinkling (Winkler et al., 2014) or bagging (Creasy and Swartz, 1981) the fruit. Second, there is a link between water treatment and microcracking. Verner (1935) conducted an experiment in which mature apple fruits attached to the branch were immersed in water for 4 days (d), which resulted in cracking. Similarly,

Knoche and Grimm (2008) showed that exposing mature apple epidermal segments to water for 2 d induced microcracking. In addition, microcracks can also be induced by moist environment at an early stage of development (40 DAFB; Faust and Shear, 1972b).

Wounding also causes microcracking and russeting. Simons and Aubertin (1959) used three methods of wounding: cutting and scraping with a knife and sanding with sandpaper to induce fruit at developmental stages ranging from 2 d to 8 weeks (wks) after fruit set. The authors pointed out that the early development (before 3 wks after fruit set) was more susceptible to wounding and results russeting. De Vries (1968) mentioned that (1) there was a difference in the resulting russeting when wounded by cutting in early June, July and August. The healing ability decreased throughout the time and the wounding in August had no suberized cell formed; and (2) the wounded (skin) spot was mostly like the naturally russeted spot macroscopically and microscopically. A similar observation showed that the transition of the response to wounding by razor blade probably occured in mid-July (about 60 DAFB), and any developmental stages later than this were not capable of forming russeting (Skene, 1981).

Other factors like frost, acid rain, and agrochemical spraying have also been reported as causes for russeting in apple in the field. Late spring frosts have been observed to cause russeting (Baker, 1931; Dalhaus et al., 2020; Simons, 1957; Simons and Chu, 1978) in which the epidermis is destroyed (MacDaniels and Heinicke, 1930). Weekly application of simulated acid rain (at pH from 3.0 to 5.6) for 18 weeks from before fruit set (early March) to end of June damaged the epidermis and forms cracks, flaky wax and russeting (Rinallo 1992a,b; Rinallo and Mori, 1996) and the lowest pH even reduces the fruit quality (Rinallo, 1992a; Rinallo et al., 1993). Application of the Bordeaux mixture, a copper-containing fungicide, around full bloom (Bell, 1941) or at early developmental stages (Ross et al., 1970) caused russeting. This may be attributed to the induced cracks by histological observation (Bell, 1941).

Another category of factor that cause russeting is insects and microorganisms. In the case of the apple rust mite (*Aculus schlechtendali*), feeding the young fruit with this insect destroyed the cuticle, epidermis and hypodermis and caused russeting (Easterbrook and Fuller, 1986). Similarly, inoculation of the flower bud with yeast isolates led to russeting (*Rhodotorula glutinis*, *Sporidiobolus pararoseus* and *Aureobasidium pullulans*; Heindenreich et al., 1997; Gildemacher et al., 2006). Although the mechanism is not fully understood, it is most likely that the microorganism degrades the cuticle by digesting cutin (e.g. cutinase activity of *A. pullulans* Gildemacher et al., 2004; Goffinet et al., 2002; Kunz et al., 2023). Russet-like symptoms such as russet ring, star crack and russet wart on apple fruit can be caused by a group of viruses

(Cropley, 1968; Welch and May, 1967; Wood, 1972). A recent study showed that apple chlorotic leaf spot virus (ACLSV) caused such russet rings (Li et al., 2020).

In summary, all the above factors likely share a common step - an impaired barrier via microcracking that leads to russeting.

1.3 Chemical constituents of an apple fruit periderm

An intact normal apple fruit skin consists of the cuticle, the epidermis, and the hypodermis (Bell, 1937b). The cuticle contributes to the waxy appearance of the fruit. The major chemical constituents of the apple fruit cuticle can be divided into cutin and wax (Richmond and Martin, 1959). The polymer cutin consists mainly of fatty acids with carbon chain lengths of 16 and 18 (C16 and C18; Eglinton and Hunneman, 1968) and is enriched in 9,10,18-trihydroxy-octadecanoic acid, 10,20-dihydroxy-icosanoic acid, 10,16-dihydroxy-hexadecenoic acid, 9,10-epoxy-12-octadecenoic acid, and 9,10-epoxy-18-hydroxy-12-octadecenoic acid as monomers (Arrieta-Baez et al., 2020). On the other hand, wax is rich in triterpenoids such as ursolic acid and oleanolic acid (Belding et al., 1998; Leide et al., 2018).

Anatomically, the periderm in russeted skin consists of three distinct layers: phellem, phellogen, and phelloderm (Evert, 2006). The phellogen, also known as the cork cambium, is generally a single-layered meristematic cell that divides phellem cells outward and phelloderm cells inward (Serra et al., 2022). The phellem contributes to the typical brownish and rough appearance of the periderm. The role of the parenchymatic phelloderm cells is largely unknown (Serra et al., 2022). The polymer suberin impregnated into the phellem cell wall (i.e., suberization) is the key to distinguishing the chemical composition of russeted skin from that of waxy skin. Although there are some similarities between suberin and cutin, phenolic compounds derived from the lignin synthesis pathway, such as ferulic acid, and aliphatic fatty acids, such as α,ω-bifunctional fatty acids, are characteristic of suberin monomers (Graça, 2015; Phillipe et al., 2020; Xin and Herburger, 2021a).

To date, there have been few studies of the chemical constituents of the periderm of apple fruit. The pioneering work of de Vries (1969; 1970) on 'Golden Delicious' apples showed similarities in the cutin acids between normal smooth skin (cuticle) and russeted skins resulting naturally from wounding and copper oxychloride. The difference between them was quantitative rather than qualitative, and wounding resulted in a higher percentage of mono-, di-, and tri-hydroxymonobasic acids being deposited in the skin than in the smooth skin. Recently, Legay et al. (2017) provided a more comprehensive understanding of the chemical constituents of the mature 'Cox Orange Pippin' apple skin. In this work, the russeted skin accumulated more: (1) ferulic acid as hydroxycinnamic acid; (2) C20 to C24 carboxylic acids; (3) C22 to C26 primary alcohols; (4) C16 to C24 α,ω-dicarboxylic acids. On the other hand, the waxy skin contained more coumaric acid as hydroxycinnamic acid and C16, C18 and C18:2 ω-hydroxy acids and 10,16-dihydroxy-C16 and 9,10,18-trihydroxy-C18:1 acids. The wax of the two types of fruit skin also differs. The russeted skin was characteristic of alkyl-

hydroxycinnamates, lupeol and betulinic acids but had very few aldehydes. Ursolic and oleanolic acids and C26 and C28 aldehydes were rich in the waxy skin.

1.4 Genetic factors associated with russeting in apple

From the initiation (of a periderm) to a mature, developed and functional status, the whole process of russeting relies on (1) A cuticle prone to microcracking (2) The initiation of a phellogen and the formation of phellem and phelloderm cells and (3) the suberization of the phellem cells. Based on this, the russeting-related genes may be categorized into the following groups: (1) cuticle formation and integrity-related genes; (2) genes involved in the transcriptional regulation of the initial processes of russeting and (3) genes involved in the synthesis, transport and polymerization of suberin monomers. To identify those, various methodologies, including genome-wide identification, transcriptomic, proteomic, metabolomic and functional analyses, can be adopted experimentally.

Analysis of apple cultivars that differ in russeting susceptibility is a fundamental and direct way to identify the genetic factors that control russeting. In the last decade, several excellent studies have shed some light on this. The work of Legay et al. (2015) showed the differentially regulated genes in a comparison between russeted and waxy fruit skins of different genotypes at maturity: the cuticle-related genes are downregulated in the russeted skin, and some transcription factors (TFs) and genes involved in suberin and lignin metabolism were upregulated in the russeted skin. In the same year, segregating population studies by Falginella et al. (2015) and Lashbrooke et al. (2015) identified important quantitative trait loci (QTL) *ABCG11* and *SHN3* (*SHINE*), which are relevant for the regulation of cutin monomer and waxes transport and cuticle formation, respectively. Linkage groups (LGs) 2, 12, and 15 found in these two studies reappeared in the results of Powell et al. (2023), who identified a total of seven QTL on linkage groups associated with russeting in apple.

Research into the critical developmental stages when the fruit is more susceptible to russeting using techniques such as transcriptomic analysis would identify important events in russeting. Recently, Falginella et al. (2021) pointed out that the loss of cuticle integrity in early fruit development (between 31 and 40 DAFB) is the key to russeting, and that a russeted skin is characteristic of certain triterpenes that differ from a waxy skin. Furthermore, three genetically close apple clones with different skin types were compared for a change in triterpene metabolism in the fruit skin and a pattern of activation of an oxidosqualene cyclase (*OSC5*) in the russeted skin by the TFs *MYB52* and *MYB66* was proposed. André et al. (2022) combined metabolomic, proteomic and transcriptomic data and compared developmental stages to filter out genes that might be involved in russeting. The genes identified were one associated with cuticle integrity (*lipid transfer protein 3*, *LTP3*) and a group of genes belonging to the *BAHD (HXXXD-motif) acyltransferase* family involved in triterpene metabolism. Further, a bagging experiment conducted by Yuan et al. (2019) to study its effect on the young apples (20 DAFB)

in terms of russeting. The authors' results showed that bagging successfully reduced microcracks and russeting, and three genes involved in lignin synthesis [one *cinnamyl alcohol dehydrogenase* (CAD) and two peroxidases (*POX/PRX*)] were identified by both transcriptomic and proteomic analyses and could be regulated by TFs such as *LIM1* (belongs to the *GATA zinc type finger transcription factor family protein*).

Evidence from functional analysis or mutants allows interpreting of the putative role of a gene in specific biological or cellular processes. The apple gene *MYB93* [*myb (myeloblastosis) domain protein*] was previously identified by the study of Legay et al. (2015) and its involvement in the regulation of suberin deposition was further confirmed by heterologous overexpression in tobacco (*Nicotiana benthamiana*) leaves (Legay et al., 2016). Lashbrooke et al. (2016) used the chemical and gene expression analyses in apple and tomato (*Solanum lycopersicum*) skins as a basis and later identified two TFs, *MYB9* and *MYB107*, through the conserved co-expression patterns among seven plant species in a co-expression analysis. Their roles in regulating suberin deposition were confirmed by the phenotypes and suberin composition of the seed coats of corresponding Arabidopsis mutants. Recent studies in *N. benthamiana* (Xu et al. 2022, 2023) showed the importance of the two apple TFs *MYB52* and *MYB68*. The former is a positive regulator of lignin synthesis and the latter is involved in the regulation of suberin deposition.

1.5 Gap of Knowledge

In summary, previous studies on russeting in apple indicate that (1) russeting is associated with an impaired barrier via microcracking; (2) several factors, including surface moisture and wounding, can induce russeting; and (3) early fruit development is more susceptible to russeting. However, the nature of russeting is poorly understood. To date, there is no evidence that addresses a direct and causal relationship between the onset of barrier impairment, the initiation of a periderm and the expression of induced genes (e.g. transcriptional regulation, cutin, waxes and suberin synthesis-related) at shorter intervals (i.e. a few days) in young apples.

The knowledge gap is as follows:

(1) Water/moisture has been proven as a factor for russeting in the field condition (Creasy, 1980; Faust and Shear, 1972a; Tukey, 1959). However, its effect on microcracking, properties of the cuticle and underlying cell layers and how these consequences link to the initiation of a periderm (onset of russeting) are still unknown.

(2) In addition to moisture, wounding is another important factor that induces russeting in apple fruit (Simons and Aubertin, 1959; de Vries, 1968; Skene, 1981). A wound-induced periderm is almost indistinguishable from a native periderm macroscopically or microscopically by electron microscopy at maturity (de Vries, 1968). Also, there is only a quantitative but not a qualitative difference between the cutin constituents of wound-induced periderm and native periderm (de Vries, 1969, 1970). This information suggests that periderm from different origins may have similar underlying cellular and molecular mechanisms, resulting in similar chemical constituents at maturity. However, a detailed and comprehensive study is needed to understand this.

(3) Microcracking generates an impaired barrier in the cuticle and has been considered as a conserved step for russeting in apple (Baker, 1930; Chen et al., 2022; Faust and Shear, 1972a; Meyer, 1944; Tetley, 1930). As the impaired barrier exposes the underlying cell layers to the atmosphere, the three resulting consequences resulted from it may be involved in triggering of russeting: a more negative water potential, an elevated oxygen (O_2) and decreased carbon dioxide (CO_2). Till now there is no knowledge regarding the role of these three factors in russeting of apple.

1.6 Objectives

The main objective of the present study was to elucidate the etiology and the mechanism(s) of russeting in apple: how microcracking caused by different induction methods is related to the initiation of a periderm. The present study included the appropriate developmental stage and cultivar and verified induction methods in order to draw the correct conclusion about the nature of russeting: The experiments focused mainly on early fruit development, as this is the period most susceptible to russeting. 'Pinova' is chosen as the main and initiating cultivar because of its intermediate susceptibility to russeting and its known induction of russeting by moisture exposure in a preliminary test. According to previous studies, surface moisture and wounding are the two most common factors inducing russeting that can be artificially manipulated. Both induction methods and their respective untreated controls on the same fruit ("split fruit system") were included in the study for comparison to test the methods for their actual effect on russeting.

The following specific objectives were designed for individual chapter:

(1) To investigate the effect of prolonged surface moisture on apple fruit, including microcracking and russeting (**Chapter. 2.1**).
(2) To investigate the sequence of events that occur during the exposure to surface moisture and after the removal of surface moisture on the apple fruit skin (**Ch. 2.2-2.3**).
(3) To investigate the similarity between the events caused by wounding and moisture and their resulting periderm (**Ch. 2.4**).
(4) To investigate the initial processes of russeting using a transcriptomic approach (**Ch. 2.5**).
(5) To investigate the potential role of O_2 in russeting on the apple and tomato fruit surface (**Ch. 2.6 and 2.7)**.

2. Publications and Manuscripts

2.1 Surface moisture increases microcracking and water vapour permeance of apple fruit skin

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Type of authorship	Third author
Type of article	Research article
Contribution to the article	Y.H.C. was involved in conducting time-course experiments to investigate the effect of moisture exposure on fruit skin transpiration and microscopic observations of fruit skin.
Author contributions	M.K. and B.P.K. designed the research, Y.I., B.P.K., Y.H.C. and J.S. performed the experiments. B.P.K., Y.I. and M.K. analysed the data. B.P.K. and M.K. wrote the manuscript.
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RESEARCH PAPER

Surface moisture increases microcracking and water vapour permeance of apple fruit skin

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Keywords
cuticle; Malus × domestica; microcrack; moisture; transpiration.

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ABSTRACT

- Surface moisture induces microcracking in the cuticle of fruit skins. Our objective was to study the effects of surface moisture on cuticular microcracking, the permeance to water vapour and russeting in developing 'Pinova' apple fruit.
- Surface moisture was applied by fixing to the fruit a plastic tube containing deionized water. Microcracking was quantified by fluorescence microscopy and image analysis following infiltration with acridine orange. Water vapour permeance was determined gravimetrically using skin segments (ES) mounted in diffusion cells.
- Cumulative water loss through the ES increased linearly with time. Throughout development, surface moisture significantly increased skin permeance. The effect was largest during early development and decreased towards maturity. Recovery time courses revealed that following moisture treatment of young fruit for 12 days, skin permeance continued to increase until about 14 days after terminating the moisture treatment. Thereafter, skin permeance decreased over the next 28 days, then approaching the control level. This behaviour indicates gradual healing of the impaired cuticular barrier. Nevertheless, permeance still remained significantly higher compared with the untreated control. Similar patterns of permeance change were observed following moisture treatments at later stages of development. The early moisture treatment beginning at 23 DAFB resulted in russeting of the exposed surfaces. There was no russet in control fruit without a tube or in control fruit with a tube mounted for 12 days without water.
- The data demonstrate that surface moisture increases microcracking and water vapour permeance. This may lead to the formation of a periderm and, hence, a russeted fruit surface.

INTRODUCTION

The cuticle is a biopolymer that envelopes all primary surfaces of terrestrial plants. It covers the fruits of most species and all leaf surfaces. The cuticle performs important functions as a barrier to pathogen invasion (Yeats & Rose 2013; Guan *et al.* 2015) and in regulating the passage of water and other substances across the surface. Depending on organ, circumstances and chemistry of the penetrant, the transcuticular movements can be either inwards or outwards (Kerstiens 1996; Schreiber & Schönherr 2009; Dominguez *et al.* 2011; Yeats & Rose 2013). Obviously, the maintenance of an appropriate level of regulatory function throughout fruit development requires the cuticle to remain intact. Compared with a leaf, maintenance of cuticular integrity in a fruit is particularly challenging. This is because fruits differ from leaves in that fruit expansion commonly occurs over a lengthy period – commonly around 5 months (Knoche & Lang 2017). The ongoing growth subjects the fruit cuticle and its subtending dermal layers (which together make up the skin) to continuous tangential strain (Skene 1982). The epidermal and hypodermal cell layers can accommodate this strain by ongoing anticlinal cell divisions and by gradual changes in cell anticlinal aspect ratio, from portrait to landscape (Tukey & Young 1942). The polymeric cuticle, however, is not 'alive' in the same sense and so must sustain the ongoing strain, which sometimes leads to thinning as the surface area increases (Lai *et al.* 2016). If critical thresholds in the rate of strain are exceeded, cuticular failure occurs; microcracks develop that compromise the cuticle's barrier function. Moreover, exposure of the strained cuticle to surface moisture, or even just to high humidity, can exacerbate microcracking in a number of fruit crop species, including apple (Knoche & Grimm 2008; Knoche *et al.* 2011) and sweet cherry (Knoche & Peschel 2006). Incidentally, extended periods of surface wetness or high humidity are also conducive to this russeting (Tukey 1959; Creasy 1980; Winkler *et al.* 2014).

Microcracking of the cuticle is the first step in the development of a number of fruit skin disorders, including shrivelling (Knoche *et al.* 2019), macrocracking (Schumann *et al.* 2019), russeting (Faust & Shear, 1972a,b; Winkler *et al.* 2014) and skin spotting (Grimm *et al.* 2012; Winkler *et al.* 2014). Taken together, these skin disorders are of considerable commercial importance. Although in most cases they do not affect the nutritional quality of the fruit or the taste, etc., they do affect fruit appearance and so compromise fruit value at the point of sale.

Chapter 2.1 Surface moisture increases microcracking and water vapour permeance of apple fruit skin

Many fruit crop species are capable of repair processes that restore the functionality of the damaged cuticle barrier (Knoche & Lang 2017). For example, in russeting, a periderm is formed in the subtending hypodermal layer when the cuticular surface is breached by multiple microcracks (Meyer 1944; Faust & Shear, 1972a,b). The phellogen divides and produces stacks of cork cells that replace the barrier function of the primary surface. From a biological perspective, the formation of a periderm is beneficial as it restores (in part at least) the lost barrier functions of the primary surface in respect to the passage of water (Khanal *et al.* 2019). Unfortunately, the rough, brownish appearance of a russeted fruit usually leads to its downgrading and even rejection in high-end markets.

A second repair process is the deposition of wax in the microcracks. The filling of cracks with wax has been documented using scanning electron microscopy (SEM) for apple fruit surfaces on a number of occasions (Roy *et al.* 1999; Curry 2009; Curry & Arey 2010). In contrast to russeting, such wax deposition does not involve a morphological change in skin structure. Hence, this process is more rapid than the formation of a periderm. In addition, wax deposition in the strained cutin polymer alleviates stress by strain fixation (Khanal *et al.* 2013a). It is known that microcracks increase the water vapour permeance of the apple fruit surface (Maguire *et al.* 1999), but whether this secondary wax deposition and filling of microcracks completely restores the barrier properties of the fruit skin is not known. Also, it is not known, whether a filling of wax alters the subsequent susceptibility of the fruit surface to russeting.

The objectives of this study were: (a) to establish the effect of surface moisture on the formation of microcracks and the permeance of the skin to water vapour in developing apple fruit, and (b) to identify the effects of repair processes thereon. Because of the significance of russeting in commercial apple fruit production, (c) the relationship between microcracking and russeting was also quantified.

MATERIAL AND METHODS

Plant material

'Pinova' apple (*Malus* × *domestica* Borkh.) grafted on M9 rootstocks were grown in the experimental orchards of the Horticultural Research Station of Leibniz University in Ruthe, Germany (52° 14′ N, 09° 49′ E). Trees were cultivated according to the current regulations for integrated fruit production.

Fruit growth measurement

Fruits were sampled at 1- to 3-week intervals between full bloom and maturity (two fruits per tree, one from each side, for a total of 15 trees). Fruit mass was determined using a digital balance and fruit diameter was calculated from fruit mass, assuming a spherical shape and a density of 1. A sigmoidal regression model was fitted through the plot of fruit surface area *versus* time. Surface area growth rate ($cm^2 \cdot day^{-1}$) was calculated as the first derivative of this regression model. The relative growth rate at any time ($cm^2 \cdot cm^{-2} \cdot day^{-1}$) was obtained by dividing the growth rate at that time by the surface area at that time.

Moisture treatment

Fruits, free of visual defects, were selected and tagged at representative stages of development. For the moisture treatment, a polyethylene tube (8-mm inner diameter) was cut from the tip of a disposable Eppendorf reaction tube and glued to the fruit surface in the equatorial plane using fast-curing silicone rubber (Silicone RTV; Dow Toray, Tokyo, Japan).

After curing, tubes were filled with 1 ml deionized water using a disposable syringe. The hole in the tip of the tube was then sealed with silicone rubber. The tubes were inspected every 2 days and resealed when necessary. A untreated area in the equatorial region – usually opposite the tube – was left unprotected (without tube) on the same fruit and served as control.

To assure that water and not the tube was causal in inducing microcracking and subsequent russeting, an independent control experiment was conducted with three treatments: untreated control (no tube, no water), control with tube attached without water (with tube, no water), moisture treatment (with tube, with water). To prevent the accumulation of high humidity or rainwater inside the tube, the tube was cut in half and the cylindrical, non-tapered portion was glued to the equatorial surface of the fruit at 28 days after full blooming (DAFB). The tube was left open. After 12 days, the tubes were removed. Digital photographs of the surface of developing fruits were taken at 105 DAFB to document the presence or absence of a periderm.

The time course of moisture-induced microcracking was studied beginning at 29 DAFB. The duration of moisture exposure was 0, 2, 4, 8 or 12 days. Thereafter, the tubes were removed from the surface. The tubes detached very easily, there was no physical stress or damage to the fruit surface associated with tube removal. The effect of development stage on moisture-induced microcracking was studied beginning at 23, 44, 73 or 100 DAFB over 12-day periods of moisture exposure. Moisture-treated fruits were either harvested immediately after treatment or left on the tree to monitor the progress of any repair processes of the microcracked surfaces or to assess the extent of russeting at maturity. The fruits were processed immediately on the day of harvest or held overnight at 2 °C and 95% RH.

Water vapour permeance

The loss of water vapour through excised skin segments (ES) was quantified using stainless steel diffusion cells similar to those described by Geyer & Schönherr (1988). The ES (1.0- to 1.5-mm thick) were excised from the moisture-treated area or a untreated control area in the equatorial plane of the fruit. The cut surface of the ES was carefully blotted using soft tissue paper. The ES were then mounted on the diffusion cells using high-vacuum grease (Korasilon-Paste; Kurt Obermeier, Bad Berleburg, Germany). Diffusion cells were filled with deionized water through a port in the base and then sealed using clear transparent tape (Tesa film; Beiersdorf, Norderstedt, Germany). Following equilibration overnight, diffusion cells were incubated in a polyethylene box containing freshly dried silica gel at 24 °C. The diffusion cells in the polyethylene box were placed upside down on a metal grid such that the ES faced the silica gel. The amount of water loss from the diffusion cells was

quantified gravimetrically by weighing cells at regular intervals up to 4.5 h or 8.0 h. The rate of water loss (F in $g \cdot h^{-1}$) was obtained as the slope of a linear regression line fitted through a plot of cumulative transpiration *versus* time. The permeance (P; $m \cdot s^{-1}$) of the ES was calculated using the following equation:

$$\text{Permeance}\,(P) = \frac{F}{(\Delta C \times A)}.$$

In this equation, F represented the flow rate ($g \cdot h^{-1}/3600$) of water vapour, A the area of the transpiring surface of the ES (m^2) and ΔC the difference in water vapour concentration between the inside and the outside of the diffusion cells ($g \cdot m^{-3}$). Because the water vapour concentration above dry silica gel is close to zero (Geyer & Schönherr 1988), the water vapour concentration at saturation at 24 °C (21.8 $g \cdot m^{-3}$; Nobel 1999) represents the driving force for transpiration.

Microcracks

Microcracking of the cuticle was followed using the fluorescent tracer acridine orange. Fruits were dipped in a 0.1% (w/w) aqueous solution of acridine orange (Carl Roth, Karlsruhe, Germany) for 10 min. Subsequently, fruits were removed from the solution, rinsed with deionized water and blotted using soft tissue paper. Fruits were viewed under a fluorescence binocular microscope (MZ10F; Leica Microsystems, Wetzlar, Germany). Calibrated images of the moisture-exposed and of the untreated control regions were prepared under incident fluorescence light (Camera DP71; GFP-plus filter, 480–440 nm excitation, ≥510 nm emission wavelength). Three to four images per fruit and per treatment (control *versus* moisture treatment) were taken on a total of seven to ten fruits. The area infiltrated by the acridine orange solution was quantified using image analysis (Cell^P; Olympus Europa, Hamburg, Germany). Under the above-mentioned conditions, tissue infiltrated with acridine orange exhibits yellow and green fluorescence. Following setting of appropriate colour thresholds, all images were processed using the same thresholds. The areas exhibiting yellow and green fluorescence were quantified.

Using the experimental setup described above, the time course for different moisture exposure durations at 29 DAFB, the developmental time course of a 12-day moisture exposure period imposed at 23, 44, 73 or 100 DAFB and the recovery time courses following a 12-day moisture exposure that began at 23, 44, 73 or 100 DAFB were studied.

Russeting

Developing fruits exposed to moisture were tagged and harvested at 159 DAFB, when the fruit was fully mature. To identify the region treated with surface moisture through until harvest, the area of skin included within the tube was marked when the tube was removed by applying four dots on the fruit surface at approximately equal intervals around the perimeter using a black permanent marker. Calibrated images of the portion of the fruit surface that was exposed to moisture were taken (Canon EOS 550D, lens: EF-S 18-55 mm, Canon Germany, Krefeld, Germany). Images of the untreated surface on the same fruit served as control. The proportion of russeted area was quantified with image analysis (software package Cell^P; Olympus).

Statistical analysis

Data are presented as means ± SE. Where error bars are not visible, they were smaller than the data symbols. Pairwise t-tests and regression analyses were carried out using the statistical software package SAS (version 9.1.3; SAS Institute, Cary, NC, USA). Significance of the coefficient of determination at 0.05, 0.01 and 0.001 is indicated by *, ** and ***, respectively.

RESULTS

Fruit mass and surface area increased in a sigmoidal pattern with time (Fig. 1). The growth rate in surface area reached a maximum of 1.6 $cm^2 \cdot day^{-1}$ at about 77 DAFB (Fig. 1 upper left inset). The relative area growth rate (the rate of expansion per unit surface area) was maximal at the start of fruit development and decreased thereafter (Fig. 1 lower right inset).

The cumulative water loss through the ES exposed to moisture for up to 12 days increased linearly with time, indicating a constant rate of water loss (Fig. 2). The rate of water loss from an ES after 12 days of exposure to surface moisture was five-times higher than from a untreated control (Fig. 2).

When exposed to moisture at 29 DAFB, skin permeance increased rapidly, whereas the skin permeance of a untreated control surface on the same fruit decreased only slightly. After 2 days of moisture exposure (31 DAFB), the permeance increase was significant compared to the untreated control. After 8 days of moisture exposure (37 DAFB), the permeance reached a maximum and remained constant thereafter up to 12 days (41 DAFB), when the moisture treatment was terminated (Fig. 3a).

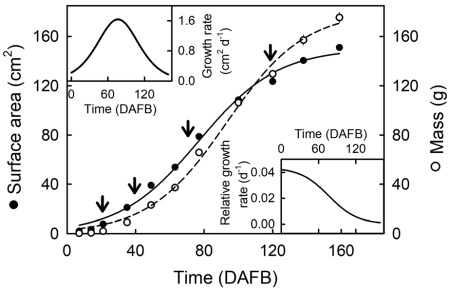

Fig. 1. Time course of changes in surface area and mass in developing 'Pinova' apple (main graph). The equations for the sigmoidal regression models were:Surface area (cm^2) = 180.26/(1 + exp(–(time(DAFB) – 93.19)/22.77; R^2 = 0.99,Mass (g) = 150.12/(1 + exp(–(time(DAFB) – 76.80)/22.96; R^2 = 0.99. Insets: Surface area growth rate (inset upper left corner) and relative surface area growth rate (inset lower right corner) in developing fruit. Arrows indicate the development stages when moisture treatments were imposed. Data represent mean ± SE, n = 30, *x*-axis scale in days after full bloom (DAFB).

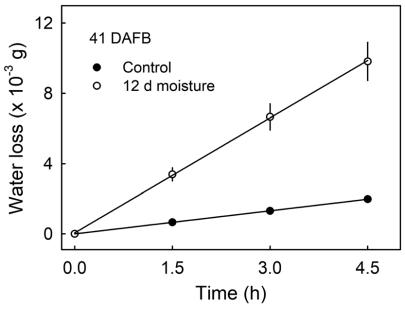

Fig. 2. Time course of water loss through excised skin segments (ES) of apple fruit exposed to moisture for 12 days, beginning at 29 days after full bloom (DAFB) until 41 DAFB. ES from the untreated surface of the same fruit served as control. Data represent mean ± SE of 15 fruits.

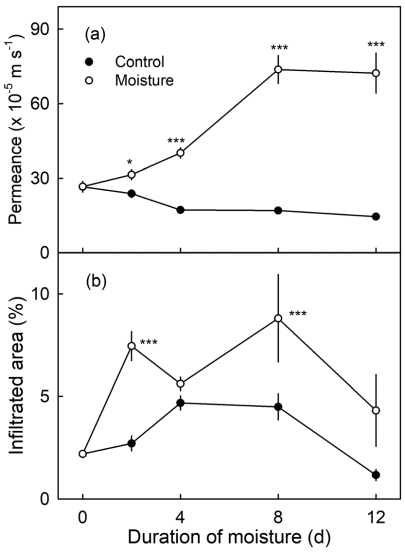

Fig. 3. Permeance (a) and acridine orange infiltrated area (b) as affected by the duration of exposure of the fruit surface to moisture. The surface was exposed to moisture beginning at 29 days after full bloom (DAFB) until 41 DAFB. Untreated surface of the same fruits served as control. Values represent mean ± SE, n = 12–15 (a) or 7–10 (b). * and *** indicate significant difference between control and moisture treatment at $P < 0.05$ and 0.001, respectively.

Moisture treatment increased the area infiltrated by acridine orange, indicating increased microcracking of the fruit surface. After 2 days of moisture treatment (31 DAFB), numerous, small, spot-like microcracks appeared (Fig. 4a–d). After 8 days, networks of long, wide microcracks had formed which were all infiltrated by the acridine orange (Fig. 4e,f). After 12 days, the area of infiltration of microcracks with acridine orange was reduced; many microcracks were visible, but they were not infiltrated by acridine orange (Fig. 4g–j). Quantifying the areas infiltrated by acridine orange indicates that the extent of infiltration varied markedly with time. At all times, the infiltrated areas were larger for moisture-treated fruit than for untreated control fruit (Fig. 3b).

When fruits were treated with moisture for 12 days at later stages of development (44 to 56 DAFB, 73 to 85 DAFB and 100 to 112 DAFB), the increases in permeance due to moisture treatment were markedly smaller, but they were still significant relative to the controls, even between 100 and 112 DAFB (Fig. 5a). Also, the area infiltrated by acridine orange was largest when young fruits (from 23 to 35 DAFB) were treated with moisture. At later stages of development (44 to 56 DAFB, 73 to 85 DAFB or 100 to 112 DAFB), the effect of moisture was smaller and not significant (Fig. 5b).

Interestingly, following the moisture treatment of young fruit from 23 to 35 DAFB, skin permeance continued to increase and peaked at about 49 DAFB; this was 14 days after termination of the moisture treatment. Thereafter, permeance decreased rapidly within 28 days, but remained significantly higher than the untreated controls (Fig. 6a). The change in area infiltrated by acridine orange essentially mirrored the change in permeance (Fig. 6b).

Performing the same experiment, but at later stages of fruit development, resulted in similar qualitative changes, *i.e.* decreases in permeance, but at markedly reduced levels following termination of the moisture treatment (Fig. 6a inset, b inset). Recovery of permeance was complete when microcracks were induced by moisture treatments between 73 to 85 DAFB and 100 to 112 DAFB, but not between 44 and 56 DAFB. As during early microcrack induction, the permeance remained higher in the moisture-treated fruits than in the untreated controls.

Monitoring infiltration of the ES with acridine orange revealed the same general trends – a transient increase in the infiltrated area up to about 49 DAFB (Fig. 7a,b). At this time, a dense network of open cracks had formed (Fig. 7c,d); the infiltrated area then decreased (Fig. 6b). The microcracks remained visible but they were not infiltrated by acridine orange (Fig. 7e–h). The fruits which were treated with moisture at 23 to 35 DAFB developed a significant amount of russet (Table 1, Fig. 8). There was no russet in the two control treatments regardless of the presence of the tube on the fruit surface, indicating that water exposure and not the tube was causal in russet formation (Fig. 8). Fruits which were treated at later stages of development (44 to 56 DAFB, 73 to 85 DAFB and 100 to 112 DAFB) did not produce russet at maturity (Table 1).

Across all development stages, permeances of fruit skins and the areas infiltrated by the fluorescent tracer acridine orange were positively related (Fig. 9). The regression equation for the relationships was:

$$\text{Permeance}\,(\times 10^{-5}\text{m}\cdot\text{s}^{-1}) = 8.3\,(\pm 0.7) \times \text{Area}\,(\%) - 4.6\,(\pm 2.4);\ R^2 = 0.78^{***},\ \text{n} = 40.$$

DISCUSSION

The most important findings of our study were:

1 A rapid increase in apple fruit skin microcracking and a corresponding increase in water vapour permeance in response to surface moisture.

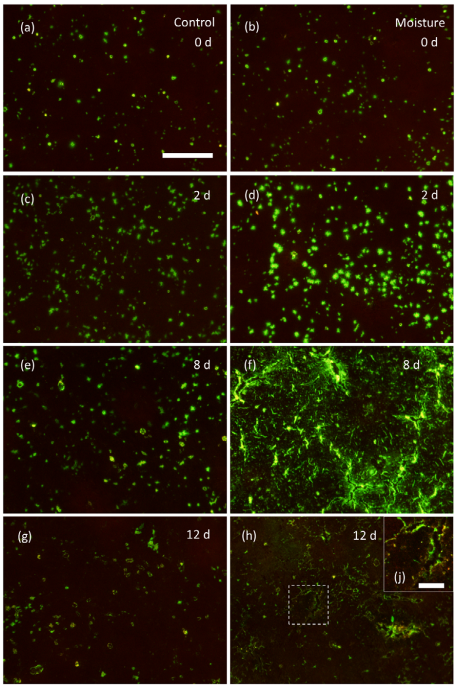

Fig. 4. Microscope images of fruit surfaces prepared after 10 min infiltration with a 0.1% aqueous solution of acridine orange. The surface was exposed to moisture beginning at 29 days after full bloom (DAFB) for 0 (b), 2 (d), 8 (f) or 12 (h) days. An untreated surface of the same fruit served as control (a, c, e, g). The image in (j) represents the magnified view of the area in (h) enclosed by the dotted rectangle. The scale bar (400 μm) in (a) is representative of images (b) to (f) of the composite. Scale bar in (j) = 100 μm.

2 A marked decrease (with some delay) in both microcracking and permeance following the termination of a moisture treatment; both values gradually approaching the control values.
3 A consistent effect of development stage on skin responses to exposure to moisture in terms of microcracking, of water vapour permeance and of russeting.

Microcracking and permeance to water vapour increase rapidly during and beyond the period of exposure to surface moisture

The effect of surface moisture observed in our *in vivo* study confirms earlier reports obtained *in vitro* using excised skin segments (Knoche & Grimm 2008; Knoche *et al.* 2011). As in earlier studies, the extent of moisture-induced microcracking depended markedly on the stage of fruit development (Knoche *et al.* 2011). Whole fruits and ES were most sensitive during early development (Wertheim 1982). During this stage, the growth strains are high as determined by the high relative area growth rates (Skene 1980; Lai *et al.* 2016).

Further indirect evidence for a relationship between russet and growth strain comes from studies in European pear (*Pyrus communis*), where a higher incidence of russet on the cheek as compared to the neck has been attributed to higher growth rates (Scharwies *et al.* 2014). Earlier studies established that the cuticle suffers from lower fracture strains compared to the underlying cellular layers of the dermis (Khanal & Knoche 2014), and that the fracture pattern of the cuticle is determined by the underlying cellular layers (Knoche *et al.* 2018). This is because the epidermal and hypodermal cell layers, and not the cuticle, represent the structural backbone of the apple fruit skin (Khanal & Knoche 2014). These arguments further suggest that microcracking, and the effect of surface moisture thereon, are also affected by the underlying cellular layers. It may be speculated that a swelling of anticlinal cell walls facilitates cell-to-cell separation along the abutting anticlinal walls as cell shape changes during growth from 'portrait' to 'landscape' (Meyer 1944; Maguire *et al.* 1999; Knoche *et al.* 2018). In sweet cherry, the swelling of cell walls reduces cell-to-cell adhesion, causing epidermal cells to partially separate at low rates of strain (Brüggenwirth & Knoche 2017). Whether this also applies for moisture-induced microcracking of apple fruit skin remains to be shown. The effect of moisture may be further exacerbated by decreases in the cuticle's fracture force and fracture strain due to hydration; this has often been reported for isolated cuticles (Knoche & Peschel, 2006; Khanal *et al.*, 2013b). In addition, surface wetness and high RH both decrease the biosynthesis and deposition of wax (Shepherd & Griffiths, 2006) and possibly also of cutin; this may lead to a thinner and mechanically weaker cuticle. However, direct evidence for effects of surface wetness and/or humidity on cutin and/or wax deposition in apple is lacking.

The changes in permeance observed in skins exposed to surface moisture throughout our study were a linear function of the extent of microcracking, as recorded by the areas infiltrated by acridine orange. This confirms an earlier report for Braeburn apples (Maguire *et al.* 1999).

It is interesting to note that the increase in microcracking and in water vapour permeance induced by surface moisture extended, and even increased further, well beyond the time when the surface moisture treatment was terminated. This observation is probably due to the ongoing growth strains causing gaping of the microcracks, before the cuticular repair processes were sufficiently active.

It could be argued that the moisture-induced russet is an artefact caused by the silicone and/or the Eppendorf tube. However, the following considerations make this possibility highly unlikely. First, when developing this technique, we also applied surface moisture using wet paper towels or wet tissue paper, or medical patches soaked and filled with water. All these rested loosely onto the fruit surface. These techniques were all equally effective in inducing russeting. However, these approaches were abandoned here because they were unreliable under field conditions. Second, natural moisture-induced microcracking and russeting can be seen in the stem cavity of most apple cultivars. During rain, the stem cavity fills with water. The area of skin beneath the 'puddle' so formed, remains wet for an extended period after the rain has stopped. Third, moisture-induced russeting has often been observed under field conditions (Tukey 1959; Creasy 1980); this is consistent with the findings reported herein. Fourth, we also

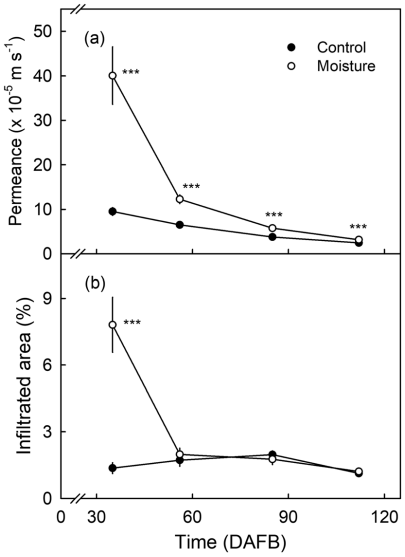

Fig. 5. Effect of surface moisture on permeance (a) and microcracking as shown by the area infiltrated by acridine orange (b) during fruit development. A selected area of the surface of a developing fruit was exposed to moisture for 12 days at four different stages of fruit development (from 23 to 35 days after full bloom (DAFB), 44 to 56 DAFB, 73 to 85 DAFB or 100 to 112 DAFB). The water vapour permeances and the surface areas infiltrated by acridine orange were quantified immediately after termination of the moisture treatment. Values represent mean ± SE of 18–20 (a) and 7–10 fruits (b). *** indicates significant difference between control and moisture treatment at $P < 0.001$.

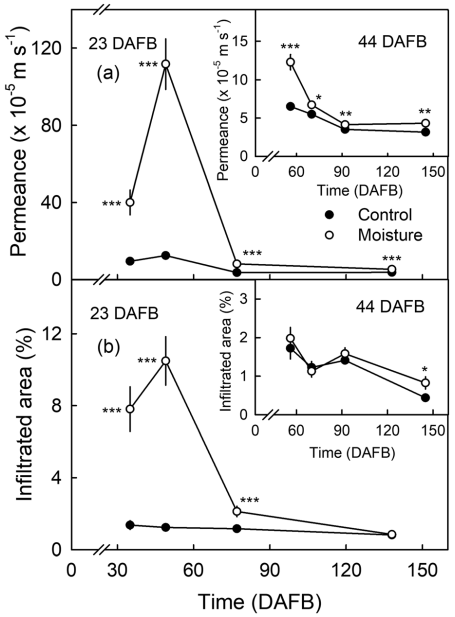

Fig. 6. Change in the permeances (a) main and inset) and acridine orange infiltrated areas (b) main and inset) of moisture-treated surfaces of developing fruits with time after termination of the moisture treatment. A selected portion of the fruit surface was exposed to moisture for 12 days, from 23 days after full bloom (DAFB) to 35 DAFB (main graphs) and from 44 DAFB to 56 DAFB (insets). Fruits were sampled at various stages of fruit development and the permeances and acridine orange-infiltrated areas of the fruit surface were quantified. Values represent mean ± SE of 18–20 (a, a inset) and 7–10 fruits (b, b inset). *, **, *** indicate significant difference between control and moisture treatment at $P < 0.05$, 0.01 and 0.001, respectively.

observed moisture-induced microcracking of the cuticle in earlier studies using excised epidermal segments of the apple fruit skin (Knoche & Grimm 2008). Fifth, if the silicone and/or the Eppendorf tube restricted growth, the fruit would be visibly deformed – it was not. Also, it would not be necessary to repeatedly reseal the tube to maintain surface wetness. The silicone we used attaches only very loosely to the fruit surface; it is thus very easily removed, without physical stress or damage to the fruit skin. Sixth, an empty tube (cylindrical, cut to only half length and left open) glued on the fruit did not produce any russet. Last, neither the silicone used nor the polyethylene Eppendorf tube release any chemicals that are phytotoxic. These arguments exclude possible artefacts due either to the silicone or to the Eppendorf tube.

For routine experimentation, we preferred to not mount empty tubes as control treatments. An empty tube may result in elevated humidity inside the tube and this would likely have induced microcracking and russeting (Knoche & Grimm 2008). Furthermore, condensation would likely have formed on the enclosed skin area due to the widely fluctuating temperatures in the field. Thus, unprotected exposure to the atmosphere (no tube) was selected as the most appropriate control.

Microcracking and permeance to water vapour decrease after removal of surface moisture

Our results demonstrate that following microcracking, fruit surface integrity recovers as demonstrated by parallel decreases in acridine orange infiltration and in water vapour permeance. Within 4 weeks of exposure to surface moisture, the barrier function was largely restored. Nevertheless, water vapour permeance remained slightly and significantly higher than in control fruit. Some microcracks remained visible but were not infiltrated by acridine orange. The decrease in the area of skin infiltrated by acridine orange was proportional to the decrease in skin permeance. The basis of this recovery effect may be twofold, as described below.

First, a likely candidate process is the deposition of wax in the microcracks. Indirect evidence comes from SEM images that show microcracks filled with wax crystals (Roy *et al.* 1999; Curry 2009; Curry & Arey 2010; Konarska 2013). Unfortunately, an attempt to gain direct quantification of microcrack infilling by wax crystals using interferometry was not successful

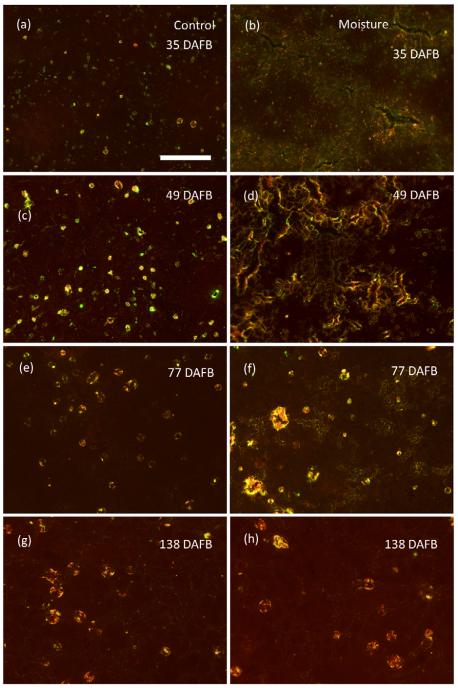

Fig. 7. Time course of changes in microcracking as recorded by acridine orange infiltration of the surface of developing apple fruit. Fruits were exposed to moisture for 12 days from 23 days after full bloom (DAFB) to 35 DAFB. Images were prepared from moisture-treated (b, d, f, h) and untreated (a, c, e, g) surfaces of the same fruit. The scale bar (400 μm) in (a) is representative of all images of the composite figure.

due to the high variability of microcracking over the apple fruit surface (B.P. Khanal, unpublished data). The wax that fills the microcracks in the cuticle surface is not necessarily derived from *de novo* synthesis in the epidermis and subsequent diffusion to the surface. Instead, wax deposition in microcracks is thought more likely derived from a redistribution of wax already within the cuticle. This view is based on the observation that wax is a highly dynamic structure that re-assembles itself if its structure is disturbed – either mechanically or by heat (Neinhuis *et al.* 2001; Koch *et al.* 2004). Also, the decrease in water vapour permeance of cuticles during storage has

Table 1. Effect of fruit development on surface moisture-induced russeting in 'Pinova' apple. Surface moisture was applied for 12 days at four stages of fruit development. The areas of russeting on the treated and untreated surfaces were quantified at harvest maturity. n = 21, DAFB = days after full bloom.

Stage of development (DAFB)	Frequency of fruit with russet (%)	Russeted area (% of treated area)	
		Moisture	Control
23 to 35	100	37.1 ± 7.3	0
44 to 56	0	0	0
73 to 85	0	0	0
100 to 112	0	0	0

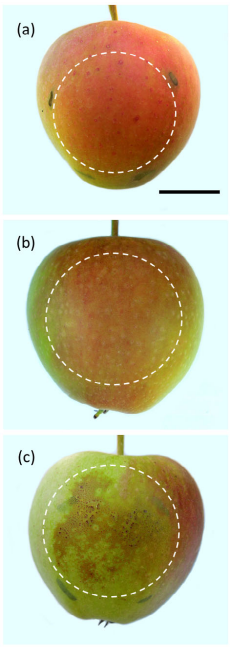

Fig. 8. Russet formation in 'Pinova' apple 105 days after full bloom (DAFB). (a) Untreated control fruit without tube and without water; (b) untreated control fruit with tube, but without water; (c) moisture-treated fruit with tube and with water. The tubes were mounted 28 DAFB, left on the fruit for 12 days and then removed. The dashed circle marks the original footprint of the tube. The moisture treatment, but neither of the two controls revealed marked russeting. The scale bar (2 cm) in (a) is representative of all images of the composite picture. For details see the Material and methods.

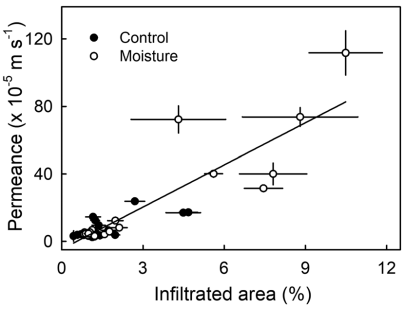

Fig. 9. Relationship between permeances and acridine orange infiltrations of the surface of apple fruits at various stages of development. Open circles are for moisture-treated skins, closed circles for untreated skins. Values represent mean ± SE of 12–20 (permeance) and 7–10 (infiltrated area) fruits.

previously been attributed to a recrystallization of pre-existing wax (Geyer & Schönherr 1990). This behaviour is also consistent with its function during growth as a filler in the cutin polymer (Knoche *et al.* 2018).

Second, the formation of a subtending periderm in response to cuticular microcracking may also contribute to a decrease in microcracking and in water vapour permeance. However, the water vapour permeance of the periderm remains significantly higher than that of the cuticle on the primary surface (Khanal *et al.* 2019).

Effect of fruit development on microcracking, water vapour permeance and russeting

The effect of surface moisture on cuticular microcracking, skin permeance and russeting is consistent with the view that microcracking is the first visible symptom of cuticular damage, with increased permeance being the immediate consequence and this the probable trigger for russeting. Because surface moisture-induced microcracking is substantially limited to the early stages of fruit development, so susceptibility to russeting is also highest during the early stages of fruit development (Wertheim 1982). In the later stages of fruit development, apple fruit skin does not respond to the presence of surface moisture to nearly the same extent – in respect either to microcracking or to russeting.

The decrease in the response to surface moisture with increasing fruit maturity may be a characteristic of the cultivar 'Pinova' fruit investigated here. We note that in 'Elstar' apples, late-season exposure to surface moisture results in a skin spot disorder and this is also a consequence of surface-moisture-induced microcracking (Grimm *et al.* 2012; Winkler *et al.* 2014).

The relationship between exposure to surface moisture and microcracking of apple fruit skin is important from a practical point of view. Because of their high capital and maintenance costs, the provision of rain shelters for apples is uneconomic. Instead, the method of choice to decrease the duration of surface moisture and, hence, the incidence of microcracking is to train the apple orchard to an open canopy structure. This could be augmented by a typical gibberellin (GA_3 or GA_{4+7}) spray application programme that works to minimize cuticular microcracking (Knoche *et al.* 2011) and russeting (Wertheim 1982).

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AUTHOR CONTRIBUTIONS

M.K. and B.P.K. designed the research, Y.I., B.P.K., Y.H.C. and J.S. performed the experiments. B.P.K., Y.I. and M.K. analysed the data. B.P.K. and M.K. wrote the manuscript.

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2.2 Russeting in apple is initiated after exposure to moisture ends—I. Histological evidence

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Russeting in Apple Is Initiated After Exposure to Moisture Ends—I. Histological Evidence

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Abstract: Russeting (periderm formation) is a critical fruit-surface disorder in apple (*Malus* × *domestica* Borkh.). The first symptom of insipient russeting is cuticular microcracking. Humid and rainy weather increases russeting. The aim was to determine the ontogeny of moisture-induced russeting in 'Pinova' apple. We recorded the effects of duration of exposure to water and the stage of fruit development at exposure on microcracking, periderm formation and cuticle deposition. Early on (21 or 31 days after full bloom; DAFB) short periods (2 to 12 d) of moisture exposure induced cuticular microcracking—but not later on (66 or 93 DAFB). A periderm was not formed during moisture exposure but 4 d after exposure ended. A periderm was formed in the hypodermis beneath a microcrack. Russeting frequency and severity were low for up to 4 d of moisture exposure but increased after 6 d. Cuticle thickness was not affected by moisture for up to 8 d but decreased for longer exposures. Cuticular ridge thickness decreased around a microcrack. In general, moisture did not affect cuticular strain release. We conclude that a hypodermal periderm forms after termination of moisture exposure and after microcrack formation. Reduced cuticle deposition may cause moisture-induced microcracking and, thus, russeting.

Keywords: russeting; periderm; *Malus* × *domestica*; surface moisture; cuticle; strain

1. Introduction

Russeting is a commercially important surface disorder of many fruit crop species, worldwide. Among other species affected are: apple [1], pear [2], grape [3] and prune [4]. The rough, brownish appearance of russeting renders a fruit unattractive to the consumer. Russeting also increases rates of postharvest moisture loss that lead to shriveling (fruit lose their fresh glossiness, so look old) and to higher rates of mass loss during storage, transport and retail (fruit are priced to the consumer on a per-kg basis) [5].

In anatomical terms, russeting represents a periderm comprising the phellem, a phellogen and a phelloderm [6,7]. The phellem cells (also referred to as cork cells) have suberized cell walls that are responsible for the dull and brownish color of a russeted fruit. These cork cells typically occur in stacks, resulting from division of the phellogen cells [8].

Information on how such a periderm is initiated in apple fruit skin is limited. Empirical evidence indicates that a range of factors may be involved. These include mechanical wounding [9], certain agrochemicals [10–12], epiphytic microorganism [13], insects (rust mites) [14] and diseases [15].

Of particular interest here is the effect of moisture on russeting in apple. Numerous studies indicate that exposure to surface wetness [16–18] or to high humidities [19] can be the cause of russeting in apple. Surface moisture, applied either as liquid-phase water or as vapor-phase water, induces microcracking in a number of fruit crop species, including apple [16]. Microcracks in the apple fruit skin are the first visible symptom of insipient russeting [20–22]. The mechanism of water-induced microcracking is not clear. It is possible that one of the factors is modification of the mechanical properties of the cuticle induced through changes in hydration [23].

We recently developed a system that reliably induces microcracking and russeting by local exposure of patches of the apple fruit surface to moisture [24]. Briefly, a length of tube is attached to the fruit surface using a non-phytotoxic silicone rubber. The tube is filled with water and periodically resealed to the fruit surface. The patch of skin included within the tube footprint first develops microcracks and, later, displays symptoms of russeting. These symptoms are microscopically identical to those observed on a fruit naturally exposed to surface moisture in the field. This system may be helpful in studying the mechanistic basis of russeting. It also avoids confusions associated with comparisons of different fruit genotypes or of different individual fruit or of different regions on the fruit surface. It allows critical comparisons to be made by imposing a moisture treatment to a defined patch of fruit skin, while an untreated (control) patch is defined in an equivalent region on the surface of the same fruit. It thereby allows standardization for a range of potential sources of response variability including stage of fruit development, differences in micro-environment, in orientation and in management (tree center vs. periphery etc.).

The specific objectives here were to identify the sequence of events that culminate in moisture-induced russeting. We were particularly interested to determine when and where a periderm is formed in relation to the location of moisture exposure. We focused on apple because apples are an important fruit crop species in both the northern and southern hemispheres and because russeting presents a problem to producers of this fruit crop.

2. Results

Following a 12 d exposure to moisture, a periderm had developed after an additional 8 d without moisture as indexed by stacks of fluorescing phellem cells visible in cross-sections of the skin (Figure 1). Furthermore, the typical russeting symptoms were visible at the fruit surface. There was no periderm and no russet visible in either of the moisture controls, regardless of the presence (or not) of the tube. Hence, we conclude that the periderm resulted from moisture exposure and not from the mounting of the tube. Because of this finding, there was no need to mount an empty tube as a control in subsequent experiments.

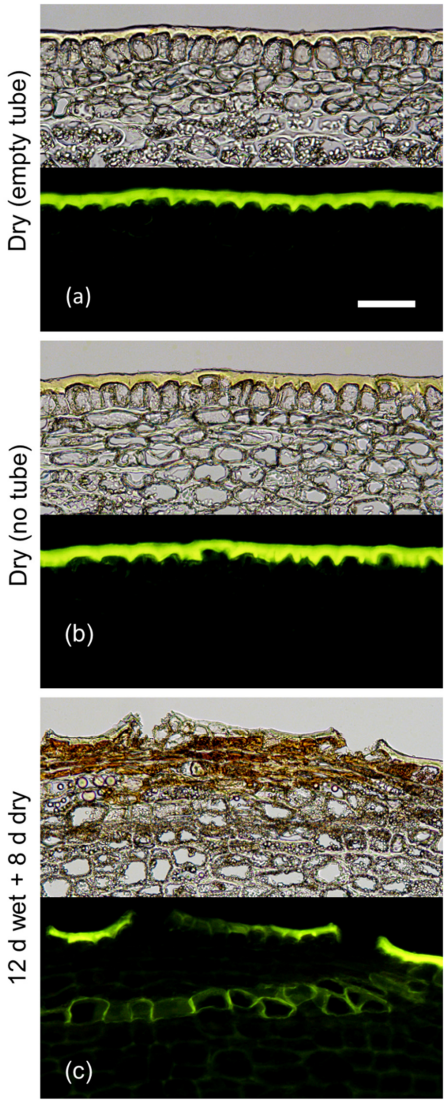

Figure 1. Effects of mounting tubes on the fruit surface without and with added moisture for 12 d, on the formation of periderm 8 d after removal of the tubes. (**a**) control that had a tube without water mounted for 12 d. (**b**) control without tube. (**c**) moisture treatment that had a tube containing water mounted for 12 d. The experiment comprised two phases: Phase I consisted of mounting the tube without or with water and Phase II marks the period after termination of moisture treatment. Micrographs taken under transmitted white light (upper) or incident fluorescent light (lower) (filter module U-MWB) following staining with Fluorol Yellow 088. The scale bar in (**a**) is 50 µm long and representative of all images in the composite (n = 3).

Moisture exposure of the fruit surface at the young stage induced microcracks in the cuticle as indexed by increased infiltration of the fluorescent tracer acridine orange (Figure 2). Moisture exposure periods of 2 to 12 d resulted in significantly higher acridine orange infiltration as compared to the non-exposed control (Phase I, Figure 2). When the moisture exposure was terminated, the area infiltrated with acridine orange decreased to a level similar to that of the non-treated control (Phase II). The only exception was at 8 d after termination of the moisture treatment. By this time, rainfall had occurred in the orchard (Phase II, Figure 2).

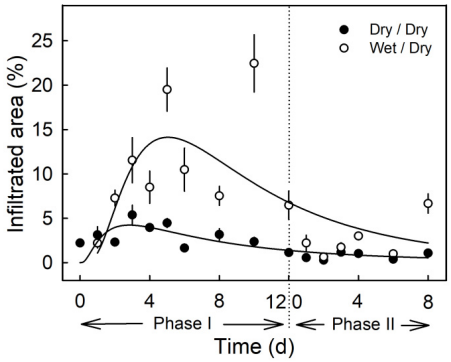

Figure 2. Time course of moisture-induced microcracking. Microcracking of the cuticle was indexed by quantifying the percentage of treated area infiltrated with acridine orange. The experiment comprised two phases: The first period of moisture exposure (Phase I) and the second period after termination of moisture exposure (Phase II). The end of Phase I and the beginning of Phase II is indicated by the dashed vertical line. The moisture treatment is referred to as 'wet/dry' and the control as 'dry/dry.' Data symbols present means ± SE (n = 6 to 20).

During exposure to moisture (Phase I), there was no indication of periderm formation from microscopy of cross-sections stained with Fluorol Yellow 088, regardless of exposure duration (6 or 12 d; Figure 3). Microcracks had formed that traversed the cuticle. Following termination of moisture exposure (Phase II), a periderm developed by 4 d below the epidermis in the hypodermal cell layers. Periderm formation was indexed by stacks of cells that stained with Fluorol Yellow 088. These cells represented the typical cork cells (phellem) that originate from an underlying phellogen. There was no apparent difference between the periderms that formed after a 6 d or a 12 d period of moisture exposure.

Varying the duration of moisture exposure (Phase I) revealed that a minimum moisture period of 6 d was needed to induce a periderm within 4 d after moisture termination (Phase II). As in the previous experiment, there were no detectable changes in the fruit skin during moisture exposure except for the formation of microcracks. These were observed after 4 d of moisture exposure (Figure 4).

The frequency of russeted fruit and the percentage of russeted area were low for moisture exposures up to 4 d (Phase I) at the young stage (from 31 DAFB onwards) but increased markedly for moisture exposures of 6 d or longer. There was little difference in frequency of russeted fruit beyond 6 d moisture exposure (Figure 5a). However, the russeted areas continued to increase from 6 to 16 d of moisture exposure (Figure 5b). There was no moisture-induced russeting at maturity (156 DAFB), when surfaces were exposed to moisture for 12 d at 66 DAFB or at 93 DAFB (n = 10–15; data not shown).

Fruit exposed to moisture for 12 d beginning at 31 DAFB had developed russet at maturity (156 DAFB) and a multistack phellem typical for russeted apples was visible (Figure 6). By maturity, the cuticle and the remains of the epidermis and hypodermis had sloughed off and the brown color of the periderm was fully exposed at the surface. Furthermore, the micromorphology of the

skin of moisture-treated fruit was identical to that of naturally russeted fruit of the same cultivar (data not shown).

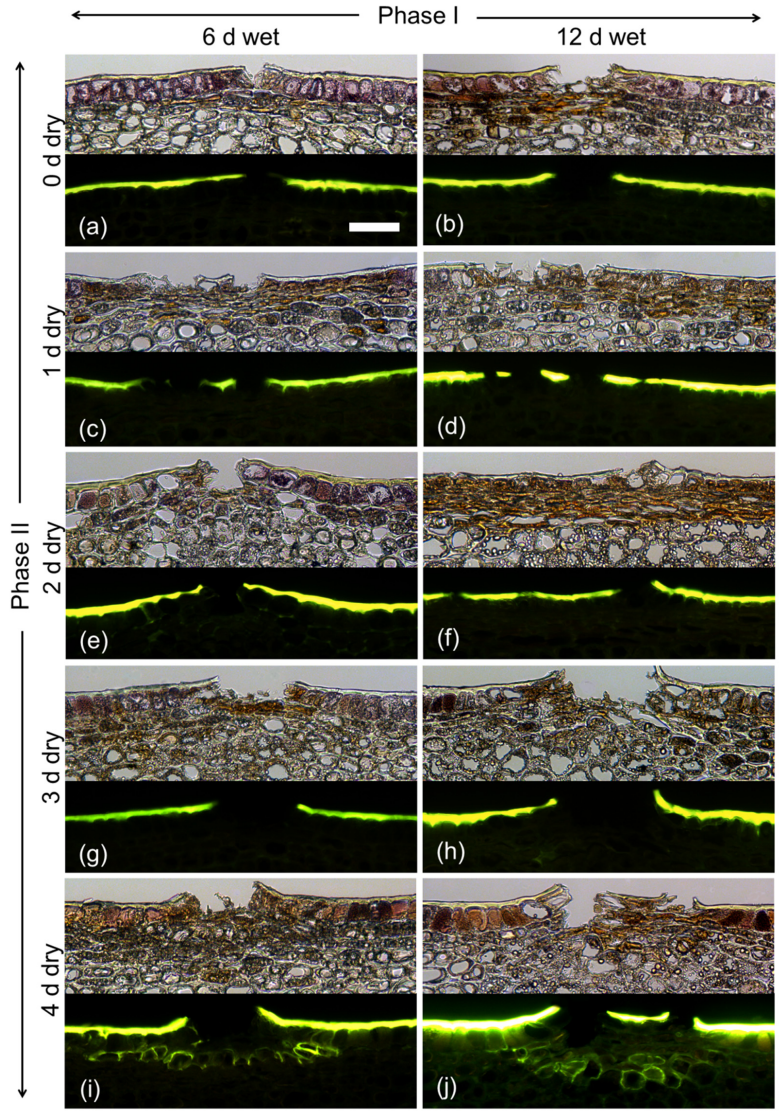

Figure 3. Effect of moisture exposure for 6 d (**a**,**c**,**e**,**g**,**i**) or for 12 d (**b**,**d**,**f**,**h**,**j**) on the time course of periderm development established at 0 d (**a**,**b**), 1 d (**c**,**d**), 2 d (**e**,**f**), 3 d (**g**,**h**) or 4 d (**i**,**j**) after termination of moisture exposure. The experiment comprised two phases: Phase I of moisture exposure and Phase II after termination of moisture exposure. Micrographs taken under transmitted white light (upper) or incident fluorescent light (lower) (filter module U-MWB) following staining with Fluorol Yellow 088. The scale bar in (**a**) is 50 µm long and representative of all images in the composite (n = 3).

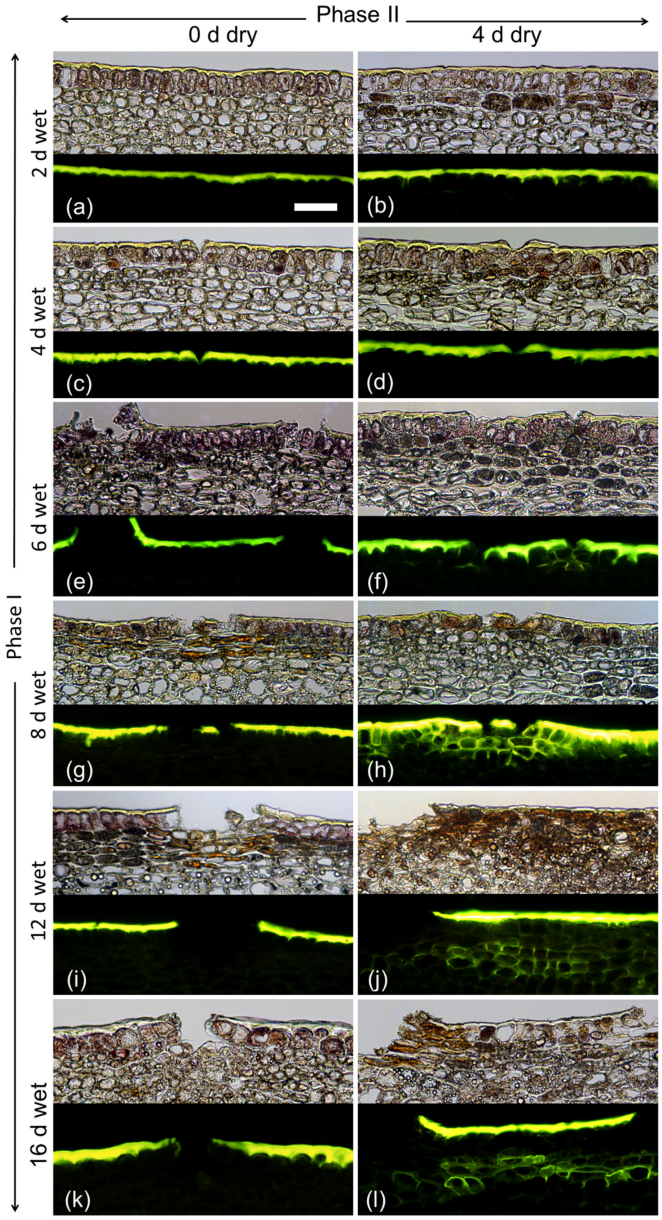

Figure 4. Effect of moisture exposure for 2 d (**a**,**b**), 4 d (**c**,**d**), 6 d (**e**,**f**), 8 d (**g**,**h**), 12 d (**i**,**j**) or 16 d (**k**,**l**) on periderm formation. The experiment comprised two phases: Phase I—time of moisture exposure and Phase II—time after termination of moisture exposure. Phase I was recorded immediately after termination of moisture exposure (0 d) (**a**,**c**,**e**,**g**,**i**,**k**). Phase II was recorded 4 d after termination of moisture exposure (**b**,**d**,**f**,**h**,**j**,**l**). Micrographs taken under transmitted white light (upper) or incident fluorescent light (lower) (filter module U-MWB) after being stained with Fluorol Yellow 088. The scale bar in (**a**) is 50 µm long and representative of all images in the composite (n = 3).

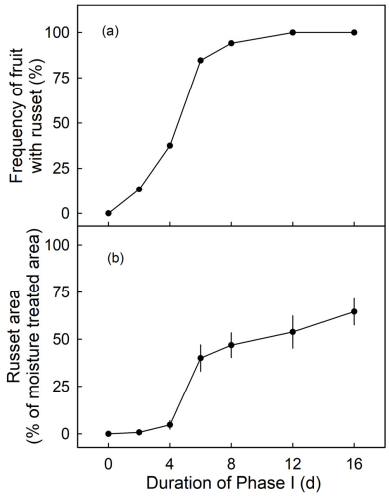

Figure 5. Effect of duration of moisture exposure (Phase I) on the frequency of russeted fruit (**a**) and the percentage of the moisture-exposed area that is russeted at maturity (156 days after full bloom; DAFB) (**b**). Fruits were exposed to moisture starting from 31 DAFB for 0, 2, 4, 6, 8, 12 or 16 d. Data represent means ± SE (n = 9–31).

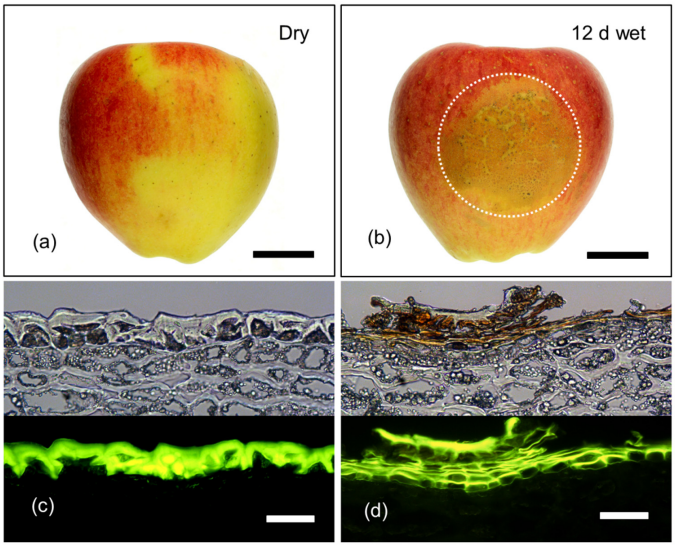

Figure 6. Macrographs (**a**,**b**) and micrographs (**c**,**d**) of mature (156 days after full bloom; DAFB) 'Pinova' apple fruit following exposure to surface moisture for 12 d at 31 DAFB (wet). Fruit without moisture-exposure, served as controls (dry). Micrographs represent cross-sections of the fruit skin in the moisture-exposed region and the dry region. Micrographs were taken under transmitted white light (upper) or incident fluorescent light (lower) (filter module U-MWB) after being stained with Fluorol Yellow 088. The area enclosed by the dotted circle represents the footprint of the moisture-treated patch of skin that subsequently developed russet. Scale bar in (**a**) and (**b**) is 2 cm long and that in (**c**) and (**d**) is 50 μm long.

The developmental time course revealed that 12 d moisture exposure induced periderm at 31 DAFB but not at 66 or 93 DAFB (Figure 7). Interestingly, microcracks were observed only following moisture exposure at 31 DAFB but not at 66 or 93 DAFB (Figure 7).

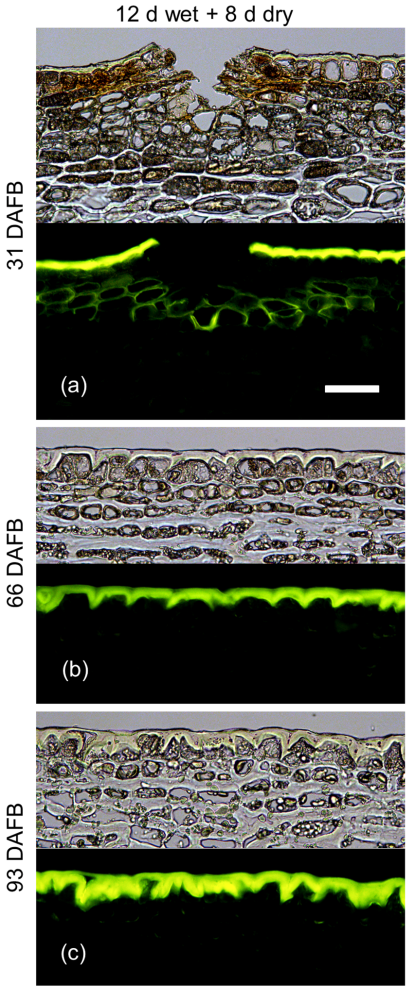

Figure 7. Effect of a 12 d moisture exposure (wet; Phase I) on periderm development in the skin of apple fruit. Cross-sections were prepared 8 d after termination of moisture exposure (dry; Phase II). The fruit surface was exposed to moisture starting at 31 days after full bloom (DAFB) (**a**) or 66 DAFB (**b**) or 93 DAFB (**c**). Cross-sections were prepared from the moisture-treated surface of the fruit. Images were taken under transmitted white light (upper) or incident fluorescent light (lower) (filter module U-MWB) after being stained with Fluorol Yellow 088. The scale bar in (**a**) is 50 µm long and representative of all images in the composite (n = 3).

Moisture had no effect on cuticle thickness during the first 8 d of exposure, nor on the ridges of the cuticular membrane (CM) above the anticlinal cell walls, nor on the lamellae above the periclinal cell walls (Phase I, Figure 8). From the day of moisture removal onwards, the thickness of the cuticle of the previously exposed patch increased at a lower rate comparable to that of the non-exposed control patch (Phase II, Figure 8).

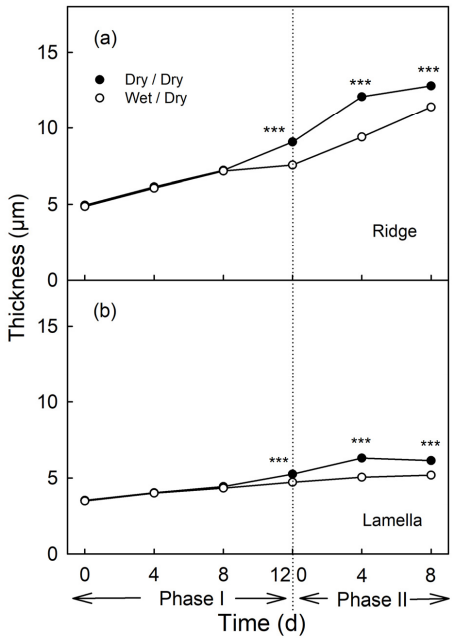

Figure 8. Effect of moisture exposure on the thickness of the cuticle above the anticlinal cell walls (ridge) (**a**) and above the periclinal cell walls (lamella) (**b**) of the apple fruit skin. In Phase I, the fruit was exposed to moisture for 12 d. Phase II began following termination of moisture exposure (indicated by the dotted vertical line) and the surface remained dry thereafter (wet/dry). Fruit surface without moisture exposure served as control (dry/dry). *** indicate significant difference between 'dry/dry' and 'wet/dry' treatment at $p < 0.001$. Data represent means ± SE ($n = 6$).

The thicknesses of the CM ridges were lowest in the immediate vicinity of a microcrack. As distance increased, the CM thickness increased and approached the mean thickness averaged across the micrograph. This was also the case 4 d and 8 d after termination of the moisture treatment (Phase II, Figure 9).

Neither moisture exposure (Phase I) and nor the termination of moisture exposure (Phase II) had an effect on strain release following preparation of the excised skin segments (ES) and isolation of the CM (Figure 10a). However, the strain release after wax extraction was higher during Phase I and after exposure to moisture (Phase II) than of the non-exposed control (Figure 10b). The difference in strain release between exposed and non-exposed CM increased up to about 6 d after the beginning of exposure and then remained approximately constant (Figure 10b). Calculating total strain from the two component strains revealed that the ε_{tot} increased during moisture exposure (Phase I). The rate of increase was somewhat higher for the ε_{tot} from the moisture treatment than for the control. The difference in ε_{tot} decreased slightly when moisture exposure was terminated (Phase II; Figure 10c).

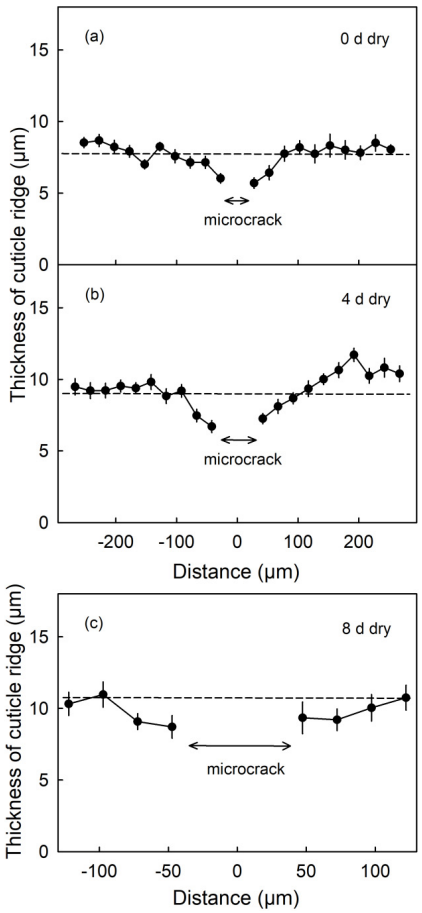

Figure 9. Thickness of the cuticle above the anticlinal cell walls (ridge) as affected by the distance from a moisture induced microcrack. Microcracks were induced by 12 d of moisture exposure. Thickness was measured on cross-sections of the fruit skin prepared from fruit sampled on the day of termination of moisture exposure (0 d) (**a**) and 4 d (**b**) and 8 d (**c**) after moisture termination (during Phase II). The distance '0' represents the center of the microcrack. Thickness was measured in both directions from the microcrack. The dashed line is the grand mean thickness of all cuticle ridges within the micrograph. The arrows indicate the mean width of the microcrack. Data represent means ± SE of 14 to 19 microcracks on a total of six fruits.

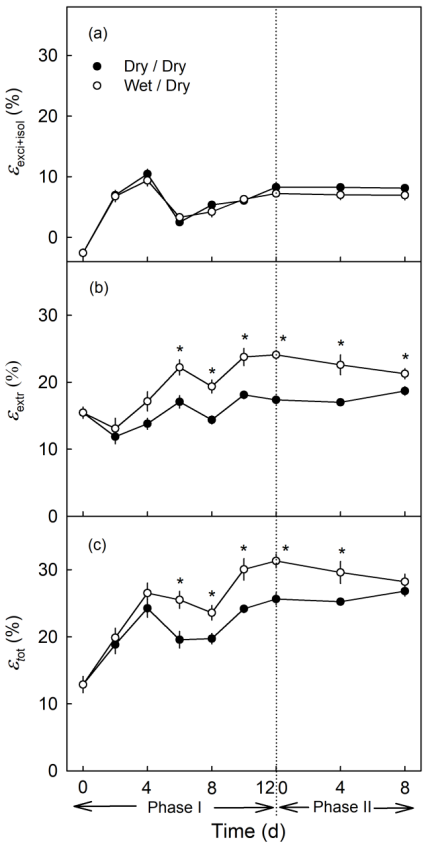

Figure 10. Effect of 12 d of moisture exposure (Phase I) on the elastic strain of the cuticular membrane (CM). Strain was quantified as the strain release during excision and isolation of the CM ($\varepsilon_{exci+isol}$; **a**) and following wax extraction of the CM (ε_{extr}; **b**) and the sum of $\varepsilon_{exci+isol}$ plus ε_{extr} (ε_{tot}; **c**). Phase I represents the period of moisture exposure (wet). Phase II represents the period after moisture termination (dry). The dotted line indicates the end of Phase I and the beginning of Phase II. * indicates a significant difference between dry/dry and wet/dry treatment at $p < 0.05$. Data represent means ± SE (n = 8 to 20).

3. Discussion

Our results establish two important findings—(1) Periderm formation in young 'Pinova' apple fruit is not induced during moisture exposure but after termination of moisture exposure and (2) decreased rate of cuticle deposition contributes to moisture-induced microcracking.

3.1. Periderm Formation in Young Fruit Is not Induced During Moisture Exposure but After Termination of Moisture Exposure

Our study is consistent with earlier observations [20]. First, microcracks traversing the cuticle are the first visible symptom in moisture-induced russeting. We have not found a single instance where russet formation was not preceded by microcracking. Second, the periderm formed in the hypodermis, beneath the cuticle and epidermis was as described by Meyer [22] and Pratt [25]. Third,

early stages of fruit development were most susceptible to russet [1,20,26,27]. Indeed, no russeting occurred following exposure to moisture at later stages of fruit development. Fourth, our experimental approach provides conclusive evidence that surface moisture is the cause of russeting. A role of surface moisture in russeting has been suggested previously [18,19,24,28].

Our results consistently show that periderm formation is triggered following termination of the moisture treatment—not during it. This conclusion is based on the observation that increased durations of exposure to moisture beyond a minimum of 4 d had no effect on periderm formation. Regardless of the duration of moisture exposure, a periderm always formed about 4 d after moisture termination. This implies (1) that it is not microcracking per se that triggers russet formation and (2) that some sort of signal must be involved that has its source at the site of microcracking (the cuticle) and travels through two or three cell layers to the subtending hypodermis where the periderm is initiated. Whatever the nature of this signal, it triggers the process involved in the formation of a periderm. This process involves the dedifferentiation of a layer of cells in the hypodermis and their subsequent differentiation into a phellogen which divides repeatedly to produce a stack of suberized phellem cells [7].

Candidates for this signal could include mechanical stimuli, such as the one associated with the release of reversible strain (i.e., elastic and viscoelastic strains) when a microcrack forms in the cuticle. However, several arguments suggest this is unlikely to be the stimulus. First, there was little strain release on excision of an ES and on the isolation of the CM, thus indicating the absence of significant elastic strain in the apple fruit cuticle. This observation is consistent with an earlier one of Lai et al. [29]. Second, the contribution of the cuticle to the overall mechanical properties of the skin is small [30]. It is the epidermis and the hypodermis that together represent the structural backbone of the skin of an apple fruit. Third, if strain relaxation were a factor, one would expect periderm formation to begin after microcrack formation, that is, during moisture exposure (Phase I), not after a fixed time following termination of moisture exposure. We conclude that a mechanical signal is unlikely to be the cause.

An alternative signal candidate may be the change in the barrier properties of the microcracked cuticle. This type of signal could account for a response induced after removal of the tube. Furthermore, the remote response would also be accounted for. Changes in the chemical potential of substances for which the cuticle forms a primary barrier are probably candidates for such a signal. Following the formation of a microcrack, these substances will now move more freely across the skin. Such substances include the chemical potential of both liquid and vapor-phase water (the water potential) and the chemical potentials (partial pressures, concentrations) of dissolved moieties such as O_2, CO_2 and C_2H_4. The consequences of a suddenly less-restricted movement of water would be a change in water potential and thus of turgor. For a change of the chemical potential of the respiratory gases, for example, a decrease in $[CO_2]$ or an increase in $[O_2]$, there would likely be a change in pH. Whether these are the changes that trigger periderm formation is not known.

3.2. Moisture Exposure Increases Microcracking by Decreasing Cuticle Deposition

A causal role for moisture in microcracking has been documented for a number of fruit crop species including sweet cherry [31], apple [18,24], grapes [32], mango [33]. Several factors are involved in formation of microcracks. First, a mismatch of surface expansion rate and cuticle deposition rate causes increased elastic strain [29,34] leading to failure of the cuticle [35]. Second, moisture may exacerbate microcracking by altering the mechanical properties of the cuticle [23,31]. Third, our results suggest that cuticle deposition is reduced as a consequence of moisture exposure and this will likely increase microcracking. The CMs isolated from moisture-exposed regions showed a higher elastic strain than CMs from the control surfaces that remained dry. This could well have been due to decreased deposition of cuticle (cutin and wax) due to moisture exposure. That wax plays an important role, is inferred from the marked differences in strain release on extraction between the moisture treatment and the control. Earlier studies established that depositions of wax in the expanding cutin network on a growing fruit surface substantially reduce build-up of elastic strain by converting the elastic strain into a plastic strain [36]. Further, deposition of new layers of cutin underneath the existing old layers

fixes the elastic strain of the CM [37]. Continuing cutin and wax deposition will therefore fix the elastic strain in the dry control skins but to a lesser extent in the skins exposed to moisture. This would result in greater strain release upon wax extraction in the control, as compared to the conditions found in the moisture treatment. Further molecular and biochemical evidence is needed to draw a firmer conclusion on this point.

3.3. Conclusions

The exposure of discrete patches of the fruit skin of an apple to moisture induces the formation of a periderm after termination of the moisture treatment and after the formation of microcracks. The search for a signal that links the formation of cuticular microcracks, on the fruit surface, to the initiation of dedifferentiation and redifferentiation in the hypodermis, several cell layers below, must focus on this time slot. Our results provide indirect evidence that reduced cuticle deposition and, in particular, reduced wax deposition, is the result of moisture exposure and contributes to the formation of microcracks.

4. Materials and Methods

4.1. Plant Materials

'Pinova' apple trees (*Malus* × *domestica*, Borkh.) grafted on M9 rootstocks were cultivated at the Horticultural Research Station of the Leibniz University Hanover at Ruthe, Germany (52°14′ N, 9°49′ E) according to current regulations for integrated crop production. The planting year was 1999, the experiments were conducted in the 2016, 2018 and 2019 growing seasons. Mean daily temperatures, mean daily precipitation and the daily radiation are provided as a supplemental file (Table S1). 'Pinova' was selected because it responded consistently to moisture exposure by russeting (Khanal, unpublished data). Vigorous flower clusters were selected randomly from a total of 125 trees at full bloom (0 days after full bloom; DAFB) and thinned to one flower, so that only the king flower remained. Fruitlets without visual defects and of uniform size and color were selected for the experiments.

4.2. General Experimental Procedures

4.2.1. Moisture Treatment

Moisture was applied locally to a defined patch on the fruit surface [24]. Briefly, a polyethylene tube (8 mm inside diameter; Sarstedt, Nümbrecht, Germany) was cut to a 17 mm length and mounted on the fruit surface in the equatorial region using a non-phytotoxic, fast-curing silicone rubber (Dowsil™ SE 9186 Clear Sealant, Dow Toray, Tokyo, Japan). Deionized water was introduced through the open end of the tube and this open end was then sealed with silicone rubber. In this way, the patch of skin exposed to liquid water was limited to that enclosed within the tube (ca. 50 mm^2). To avoid leakage, the silicone seal between tube and fruit was renewed every 2 d until the moisture treatment was terminated. An equivalent patch of skin was identified on the opposite face of the same fruit to serve as the control. Unless specified otherwise, no tube was mounted over the control patch. Earlier experiments established that russeting was due to moisture exposure and not to the mounting of the tube [24]. On the day moisture exposure was terminated, the tube was removed and the fruit surface dried with a soft paper tissue. The tube detached very easily from the epidermis, so that no significant physical force was needed and the fruit surface displayed no visible sign of injury. The footprints of the treated and control patches on each fruit were delineated using a permanent marker. A particular fruit was either sampled immediately or left on the tree for later evaluation. Following sampling, a fruit was transferred to the laboratory within 3 h. Intact fruit (21 or 31 DAFB) or sections of the fruit (66 or 93 DAFB) were stored in Karnovsky fixative [38] or immediately processed fresh, as described below.

4.2.2. Microcracks

Microcracks were quantified in both the 2018 and 2019 growing seasons following the procedure described earlier [24,35]. Briefly, whole fruit were dipped in a 0.1% (*w*/*v*) aqueous acridine orange solution (Carl Roth, Karlsruhe, Germany) for 10 min, rinsed with distilled water and carefully blotted dry using a soft paper tissue. The treated and the control patches of the skin were inspected using fluorescence microscopy (MZ10F; GFP-plus filter, 440–480 nm excitation, ≥510 nm emission wavelength; Leica Microsystems, Wetzlar, Germany) and imaged with a DP71 camera (Olympus Europa, Hamburg, Germany). Three or four images were recorded from different locations within each treated or control patch, on each of a total of six to ten fruit per sampling date. The areas (mm^2) infiltrated by acridine orange were quantified using image analysis ($Cell^P$, Olympus, Hamburg, Germany). The total fluorescing area within each treated (or control) patch, in each image, was calculated and was expressed as a percentage of the whole treated (or control) patch to which it referred.

4.2.3. Cross-Section of Fruit Skin

Tissue blocks (ca. 3 mm thick) comprising the fruit skin and some subtending parenchyma cells were excised from the treated or the control patches of the fixed fruit using a scalpel. The blocks were rinsed in distilled water and immersed in 70% (*v*/*v*) aqueous ethanol for 16 h. The blocks were then dehydrated in an ascending series of ethanol (80%, 90% and 96% *v*/*v*; 30 min each) under a partial vacuum (pressure 10.8 kPa). Subsequently, the blocks were transferred to 100% isopropanol for 40 min (twice) and a xylene substitute (AppliClear; AppliChem, Münster, Germany) for 40 min (twice) to displace the ethanol in the tissues, under the same partial vacuum. The dehydrated blocks were then infiltrated with a 1:1 (*v*/*v*) paraffin/xylene substitute mixture (Carl Roth) for 40 min (once) and paraffin alone for 40 min (twice). Finally, the blocks were embedded in paraffin. The paraffin blocks so obtained were cooled and stored at 4 °C pending later sectioning.

Thin sections (10 µm) were cut using a rotary microtome (Hyrax M 55, Zeiss, Germany). Sections were transferred to microscope slides, dried in an oven for 16 h at 38 °C and rehydrated as follows: xylene substitute (2 × 10 min); descending series of ethanol (96%, 80%, 70% and 60% for 10 min each) and finally for 2 × 5 min in distilled water.

4.2.4. Microscopy

Sections were stained for 1 h with 0.005% Fluorol Yellow 088 (Santa Cruz Biotechnology, Texas, USA) [39] dissolved in 90% glycerol and melted polyethylene glycol 4000 (SERVA Electrophoresis, Heidelberg, Germany). The sections were transferred to the stage of a fluorescence microscope (BX-60 equipped with a DP 73 digital camera; Olympus and viewed in transmitted white light or under incident fluorescent light (filter U-MWB; 450–480 nm excitation; ≥520 nm emission wavelength; Olympus, Hamburg, Germany). The minimum number of biological replicates was three. To confirm the occurrence of a periderm, a minimum of 50 sections through the whole block were examined.

4.2.5. Cuticle Thickness Measurement

Cross-sections of the skin from the moisture treated and the control patches were inspected at ×200 in white light using a fluorescence microscope (BX-60; Olympus, Hamburg, Germany). The thickness of the CM above the anticlinal cell walls (ridge) or above the periclinal cell walls (lamella) were measured in two sets of images using image analysis (CellSens; Olympus, Hamburg, Germany). The first set comprised images selected for the absence of cuticular cracks. The thickness of the lamella and ridge were measured in a 350 µm long transect. For this, four images per fruit from a total of six fruits were used. For the second set, images were selected which had a single cuticular crack. Here, the width of the crack and the thickness of the cuticular ridges were measured in a 275 µm (0 d and 4 d) or 125 µm (8 d) long transect from the center of the crack to either side. A total of 14 to 19 images on six fruits were used.

4.2.6. Russet Quantification

Mature fruit were harvested at 156 DAFB. Digital calibrated images (Canon EOS 550D, lens: EF-S 18-55 mm, Canon Germany, Krefeld, Germany) were taken from the moisture treated and control patches on the fruit surface. The areas (mm^2) of the russeted spots on the fruit surface (as indexed by their brownish, rough, corky appearance) were quantified (Cell^P; Olympus, Hamburg, Germany) and summed within each patch of skin enclosed by the tube. The area of russet is expressed as a percentage of the area of the patch. The number of replicates ranged from 9 to 31.

4.2.7. Cuticle Isolation and Strain Analysis

The ES were punched from the treated and control patches using a biopsy punch (8 mm diameter; Kai Europe, Solingen, Germany; 10 and 12 mm diameter; Acuderm, Terrace, FL, USA). The CMs were isolated enzymatically by incubating the ES in an isolation medium containing pectinase (9%, *v*/*v*; Panzym Super E flüssig; Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark) and cellulase (0.5% *v*/*v*; Cellubrix L.; Novozymes A/S) in a 50 mM citric acid buffer at pH 4.0 at ambient temperature [40]. NaN_3 was added at a final concentration of 30 mM to prevent microbial growth. Enzyme solutions were replaced periodically until CM separated from adhering cellular debris (about 4 weeks). The isolated CMs were carefully cleaned using a soft camel-hair brush. The CM were rinsed in distilled water, dried at 40 °C for a minimum period of 16 h and stored in multi-well cell culture plates held in polyethylene boxes above dry silica gel. For determination of the wax mass, the CM discs were extracted for 2 h using $CHCl_3$/MeOH (1:1, *v*/*v*; Carl Roth) in a Soxhlet apparatus. The dewaxed CMs are referred to as DCMs.

The elastic strain was quantified using the procedure described in Lai et al. [29] with minor modifications. The CMs were rehydrated, placed on a microscope slide, flattened by placing a coverslip on top and then imaged under a dissecting microscope (Wild M10; Leica Microsystems; camera DP71). For the DCMs, the discs were transferred from the $CHCl_3$/MeOH to MeOH and then directly to water, before being positioned on a microscope slide and flattened as described above. The areas of the CM and DCM discs were quantified by image analysis (Cell^P; Olympus, Hamburg, Germany).

The strains released following excision of the ES and isolation of the CM ($\varepsilon_{exci+isol}$) and following wax extraction (ε_{extr}) were calculated as follows:

$$\varepsilon_{exci+isol} = \frac{A - A_{CM}}{A_{DCM}} \times 100 \quad (1)$$

$$\varepsilon_{extr} = \frac{A_{CM} - A_{DCM}}{A_{DCM}} \times 100 \quad (2)$$

$$\varepsilon_{tot} = \varepsilon_{exci+isol} + \varepsilon_{extr}. \quad (3)$$

In this equation, A represents the area of the disc on the fruit surface before excision, that is, the cross-sectional area of the biopsy punch corrected for curvature of the disc. The A_{CM} and A_{DCM} represent the areas of the isolated CM and the extracted DCM. Because the $\varepsilon_{exci+isol}$ and the ε_{extr} are additive, the total strain ε_{tot} equals the sum of the two component strains. The number of replicates ranged from 8 to 20.

4.3. Experiments

All experiments were conducted in two phases: the moisture treatment was imposed during Phase I. The moisture treatment was then terminated, the tube removed and the treated patch now opened up to the natural atmosphere of the orchard—this second period was Phase II. The following experiments were conducted:

(1) The first experiment established that moisture exposure was the cause of periderm formation (and not the mounting of a polyethylene tube using silicone sealant). The experiment was conducted at 28 DAFB and comprised a control (without tube, without water) and the following two treatments:

(i) an empty 8.5 mm long tube (no added water) with its distal end left open to the atmosphere and (ii) a moisture treatment in which an attached 17 mm long tube was filled with water and its distal end sealed with silicone sealant. The tube in (i) was half length so as to minimise any increase in humidity in the tube—earlier experiments showed that microcracking can also result from exposure to high humidity [16,19]. This tube was also mounted in such a position that, although open to the atmosphere, rainwater could not enter it. All tubes were removed after 12 d and the fruit sampled for histological analysis after a further period of 8 d in the orchard.

(2) The time course of the duration of exposure to the atmosphere (Phase II) following removal of surface moisture was studied. The fruit surface was exposed to moisture at 31 DAFB (2019 season) for 6 or 12 d when the moisture treatment was terminated and the time course of exposure to the atmosphere began. Fruit were sampled for microcracking, CM strain and histology at 0, 1, 2, 3 or 4 d after termination of moisture exposure (Phase II) or at maturity (156 DAFB).

(3) The time course of the duration of moisture exposure (Phase I) was studied by exposing fruit surfaces from 21 DAFB (2018 season) or 31 DAFB (2019 season) onwards to moisture for 0, 2, 4, 6, 8, 12 or 16 d. Fruit were sampled either immediately after termination of the moisture treatment for microcracking, CM strain and histology or at maturity (156 DAFB) to quantify the frequency of fruit with russet and the percentage of russeted surface area.

(4) A developmental time course was established to identify any changes in periderm formation during fruit development. Moisture was applied to the surface of developing fruit, beginning at 31, 66 or 93 DAFB (2019 season) for 12 d (Phase I) and fruit were sampled 8 d after termination of the moisture treatment (Phase II). At this time, any periderm formed was clearly detectable by microscopy. Some fruit were left on the tree, sampled at maturity (156 DAFB) and used to quantify the frequency of fruit with russet and the percentage of russeted surface area.

4.4. Data Analyses and Presentation

Data are presented as means ± SE. Where error bars are not visible, they were smaller than data symbols. Data for strain relaxation analysis and cuticle thickness were subjected to one-way analysis of variance (ANOVA) using SAS (Version 9.1.3; SAS Institute, Cary, NC, USA). Means were compared using Tukey's studentized test at $p \leq 0.05$.

Supplementary Materials: The following is available online at http://www.mdpi.com/2223-7747/9/10/1293/s1, Supplementary Table S1: Meteorological data.

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2.3 Russeting in apple is initiated after exposure to moisture ends— Molecular and biochemical evidence

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Type of article	Research article
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Author contributions	Conceptualization: T.D., M.K. and B.P.K. Funding acquisition: T.D. and M.K. Project administration: T.D. and M.K. Methodology: M.K., T.D., L.S. and B.P.K. Investigation: J.S., Y.-H.C., A.S., V.Z.-D., K.S. and B.P.K Supervision: M.K., T.D., L.S. and B.P.K. Data curation: J.S., Y.-H.C., B.P.K., V.Z.-D. and K.S. Validation: J.S., Y.-H.C., B.P.K., V.Z.-D. and K.S. Visualization: J.S., Y.-H.C. and B.P.K. Formal analysis: J.S., Y.-H.C., B.P.K., V.Z.-D. and K.S. Writing—Original draft: J.S., T.D., M.K. and B.P.K. Writing—Review and editing: J.S., T.D., M.K., B.P.K. and L.S.
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Russeting in Apple Is Initiated After Exposure to Moisture Ends: Molecular and Biochemical Evidence

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Abstract: Exposure of the fruit surface to moisture during early development is causal in russeting of apple (*Malus* × *domestica* Borkh.). Moisture exposure results in formation of microcracks and decreased cuticle thickness. Periderm differentiation begins in the hypodermis, but only after discontinuation of moisture exposure. Expressions of selected genes involved in cutin, wax and suberin synthesis were quantified, as were the wax, cutin and suberin compositions. Experiments were conducted in two phases. In Phase I (31 days after full bloom) the fruit surface was exposed to moisture for 6 or 12 d. Phase II was after moisture exposure had been discontinued. Unexposed areas on the same fruit served as unexposed controls. During Phase I, cutin and wax synthesis genes were downregulated only in the moisture-exposed patches. During Phase II, suberin synthesis genes were upregulated only in the moisture-exposed patches. The expressions of cutin and wax genes in the moisture-exposed patches increased slightly during Phase II, but the levels of expression were much lower than in the control patches. Amounts and compositions of cutin, wax and suberin were consistent with the gene expressions. Thus, moisture-induced russet is a two-step process: moisture exposure reduces cutin and wax synthesis, moisture removal triggers suberin synthesis.

Keywords: russet; cuticle; periderm; *Malus* × *domestica*; cutin; wax; suberin

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1. Introduction

Russeting is a surface disorder of many fruitcrop species including of apple [1–5]. Russeting is characterized by the formation of rough, brownish patches on the fruit skin. The impaired appearance of the skin reduces the fruit's marketability and an associated increase in water vapor permeability compromises its postharvest performance [6]. In botanical terms, russet is the result of the formation of a periderm, the cell walls of the phellem being suberized. The periderm assumes the barrier functions of the epidermis and cuticle—the fractured epidermal cells soon drying and sloughing off. Despite of its economic importance, the sequence of processes that lead to russeting are not entirely clear.

Some progress has been made in genetic analyses. Using crosses of apple clones that differ in russet susceptibility Falginella [7] and Lashbrooke [8] identified several QTL (Quantitative Trait Locus) regions on chromosomes 2, 12 and 15 that affected russet susceptibility under field conditions [7,8]. Within these, SHN3 was located and identified as a candidate gene responsible for fruit skin development due to its differential expression in russeted and non-

russeted clones [8]. Legay [9] compared gene expressions in russeted and non-russeted mature fruit of a range of apple cultivars. A number of differentially-regulated genes were identified. Some of these were related to cutin, wax, suberin and lignin synthesis and others to the transport and transcriptional regulation of these moieties [9]. Unfortunately, all these studies focused on fruits at the mature stage. The only exception was Lashbrooke [8] who also investigated an early green stage. To our knowledge, there is no further information available on russeting during early fruit development of apple—when russeting susceptibility is at maximum [1,10–14]. Further, comparison of differential gene expressions in fruits from russeted and non-russeted genotypes may not be conclusive, since properties other than russet susceptibility may also differ.

Microscopic cracks ('microcracks') in the cuticle are the first visible symptom of russeting [10,15,16]. Microcracks form when the fruit skin is strained during periods of rapid surface expansion [17]. This period typically occurs during early fruit development [1,10–14,17]. Microcracking is exacerbated by surface moisture [18–20]. Recently, a system was developed that allows microcracks, and hence also russet, to be induced in the skins of developing apples by localized exposure to moisture [15]. The remaining unexposed skin of the same fruit may serve as the control. Briefly, a short length of tube is mounted on the fruit surface and filled with water. This procedure exposes a defined patch of the fruit surface to water, while the remaining fruit surface represents the unexposed control. A periderm forms in the skin area defined by the tube aperture due to the induction of microcracks by moisture. This experimental setup avoids a number of shortcomings associated with comparisons of fruits of different genotypes or fruits of the same genotype but collected from different sites, from different trees or even from different positions in the canopy of the same tree. It thus allows critical comparisons to be made by eliminating a range of potential sources of variability in russet formation, such as by the stage of fruit development, the microenvironment of the fruit in the canopy, etc. Using this system, the effect of moisture exposure on the histology of russet formation was investigated in greater detail [16]. Several findings were reported: (1) Microcracking of the cuticle occurred during moisture exposure, but there was no periderm formation during moisture exposure. (2) Cuticle deposition ceased during moisture exposure. (3) After removal of the moisture treatment a periderm formed within 4 d, regardless of the duration of moisture exposure. (4) The periderm formed in the hypodermis, several cell layers beneath a microcrack. (5) There was no difference in histology between natural and artificial moisture-induced russet. Unfortunately, the time resolution of such histological studies is limited. Moreover, changes at the transcriptional and biochemical levels will precede those detected at the histological level.

To develop a better understanding of the mechanism(s) of russet formation we (1) investigated the expressions of genes putatively involved in cutin, wax and suberin synthesis and (2) analyzed the compositions of the cuticle and the periderm during and after moisture exposure. To identify whether duration of moisture exposure was a factor in russeting, the fruit skin was exposed to continuous surface moisture for 6 or for 12 d periods. We focused on those genes that were found to be differentially expressed in russeted and non-russeted apple in previous studies [8,9,21].

2. Results

2.1. Changes in Gene Expression and Metabolism in Young Fruit During and after Moisture Exposure

During moisture exposure (Phase I) beginning at 31 days after full bloom (DAFB), genes involved in cutin (*ABCG11*, *GPAT6*) and wax (*KCS10*, *SHN3*, *WSD1* and *CER6*) syntheses were significantly down-regulated compared to in the un-exposed (dry) control (Figures 1a–f and 2a–f). The down-regulation occurred fairly consistently for all genes and after both the 6 d and the 12d moisture exposure (Figures 1a–f and 2a–f). The longer exposure duration generally resulted in a greater down-regulation. The down-regulation was consistent for *ABCG11*,

GPAT6, *KCS10* and *SHN3* in all three seasons of the experiment but down-regulation was less for *WSD1* and *CER6*, particularly in the 2018 season (Figures S1a–f and S2a–f).

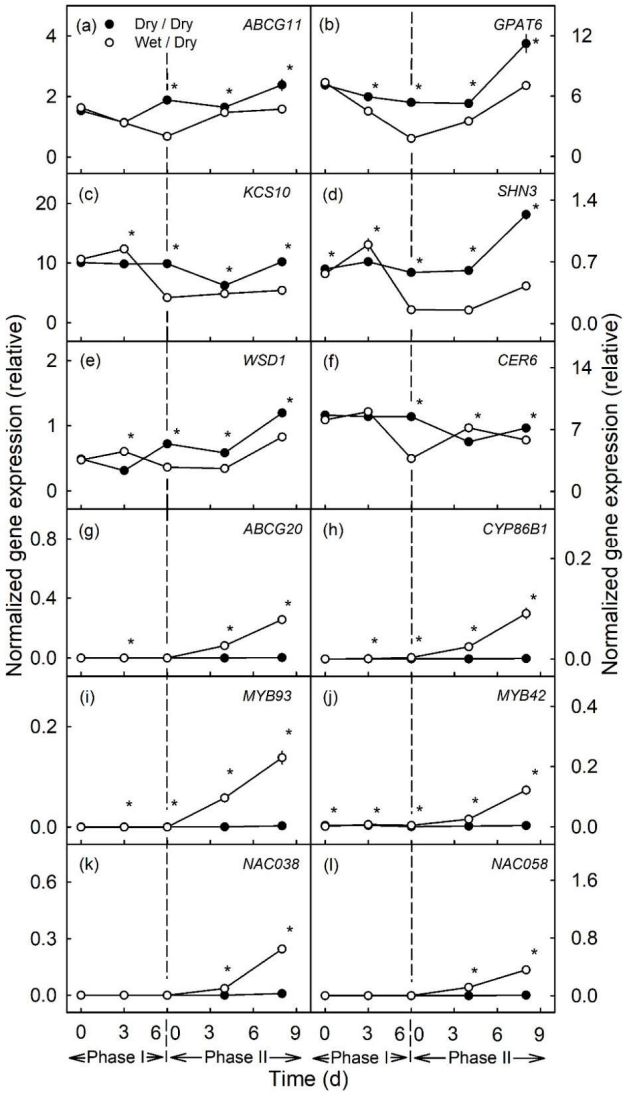

Figure 1. Time courses of expression of genes related to cutin and wax synthesis (**a**–**f**) and to suberin and lignin synthesis (**g**–**l**) of apple fruit skin during (Phase I) of exposure to moisture and after exposure was discontinued (Phase II). During Phase I, a patch of the fruit skin was exposed to moisture for 6 d beginning at 31 days after full bloom (DAFB) (wet). During the subsequent Phase II, moisture was removed, and the patch was exposed to the atmosphere (dry). Moisture-exposed patches of the fruit skin are referred to as wet/dry, unexposed control patches as dry/dry. The end of moisture exposure is indicated by the vertical dashed line. The expression values are means ± SE of three independent biological replicates comprising ten fruit each. The '*' indicates significant differences between dry/dry and wet/dry at $p \leq 0.05$ (Student's *t*-test).

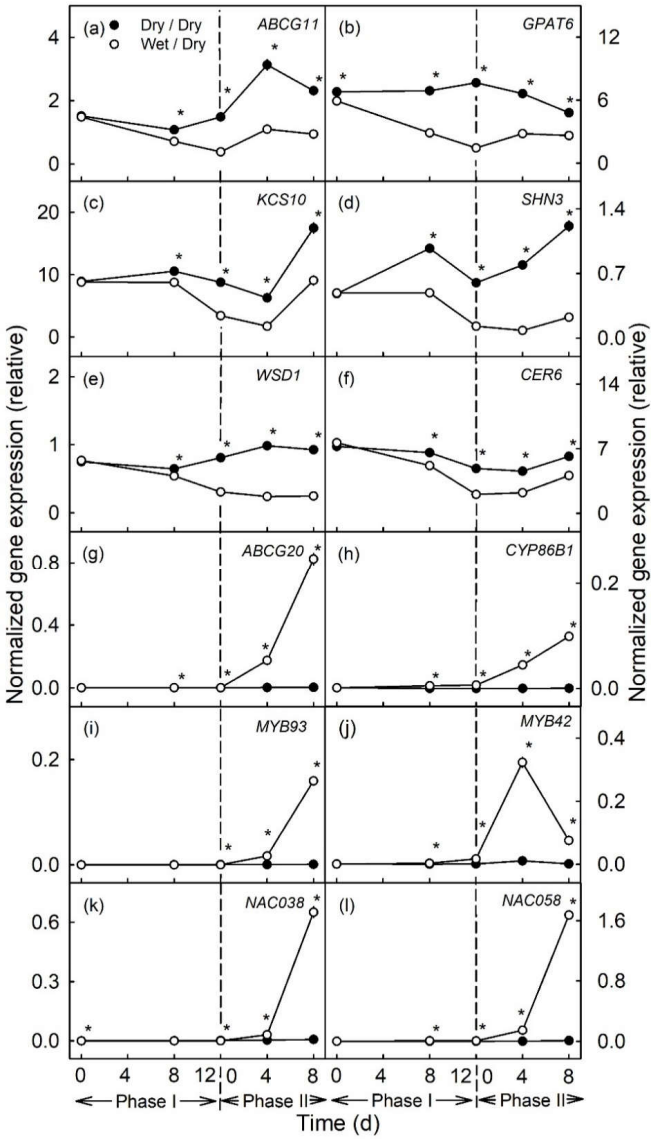

Figure 2. Time courses of expression of genes related to cutin and wax synthesis (**a**–**f**) and to suberin and lignin synthesis (**g**–**l**) of apple fruit skin during (Phase I) of exposure to moisture and after exposure to moisture was discontinued (Phase II). During Phase I, a patch of the fruit skin was exposed to moisture for 12 d beginning at 31 days after full bloom (DAFB) (wet). During the subsequent Phase II, moisture was removed, and the patch was exposed to the atmosphere (dry). Moisture-exposed patches of the fruit skin are referred to as wet/dry, unexposed control patches as dry/dry. The end of moisture exposure is indicated by the vertical dashed line. The expression values are means ± SE of three independent biological replicates comprising ten fruit each. The '*' indicates significant differences between dry/dry and wet/dry at $p \leq 0.05$ (Student's *t*-test).

In contrast, there was no change in expression of genes related to suberin synthesis (*ABCG20*, *CYP86B1*, *MYB93*) during moisture exposure (Phase I) (Figures 1g–i and 2g–i). *MYB42*, a regulator of lignin synthesis, was slightly but significantly up-regulated during moisture exposure (Figures 1j and 2j). Meanwhile, *NAC038* and *NAC058*, that do not yet have assigned functions, were not differentially expressed during Phase I (Figures 1k–l and 2k–l).

After discontinuation of moisture exposure (Phase II), the expression of cutin- and wax-related genes in the moisture-exposed patches increased again slightly but the relative expressions were still significantly lower than the expressions of these genes in the control patches of the same fruit. The relative expression of *CER6* in the 6 d moisture treatment, was generally similar in the moisture-exposed and control patches (Figures 1a–f and 2a–f).

In contrast, suberin- and lignin-related genes were consistently up-regulated, regardless of whether the moisture exposure during Phase I was for 6 or for 12 d (Figures 1g–l and 2g–l, Figure S1g–l and Figure S2g–l). The up-regulation of expression increased from 4 to 8 d after discontinuation of moisture exposure. Only for *MYB42* was a transient peak in expression observed at 4 d after moisture exposure (Figure 2j).

2.2. Changes in Gene Expression and Metabolism Caused by Moisture Exposure (Phases I and II) during Later Stages of Fruit Development

In the later stages of fruit development moisture exposure [from 66–78 DAFB (Figure 3a–f) and from 93–105 DAFB (Figure 4a–f)] also caused the down-regulation of the genes related to cutin and wax synthesis, as compared to the unexposed controls. However, the magnitudes of the down-regulations of expression were markedly less than for moisture exposure during the early stages of fruit development (moisture exposure from 31–43 DAFB). There were no changes in expressions of genes related to suberin or lignin synthesis, either during Phase I or Phase II (Figures 3g–l and 4g–l).

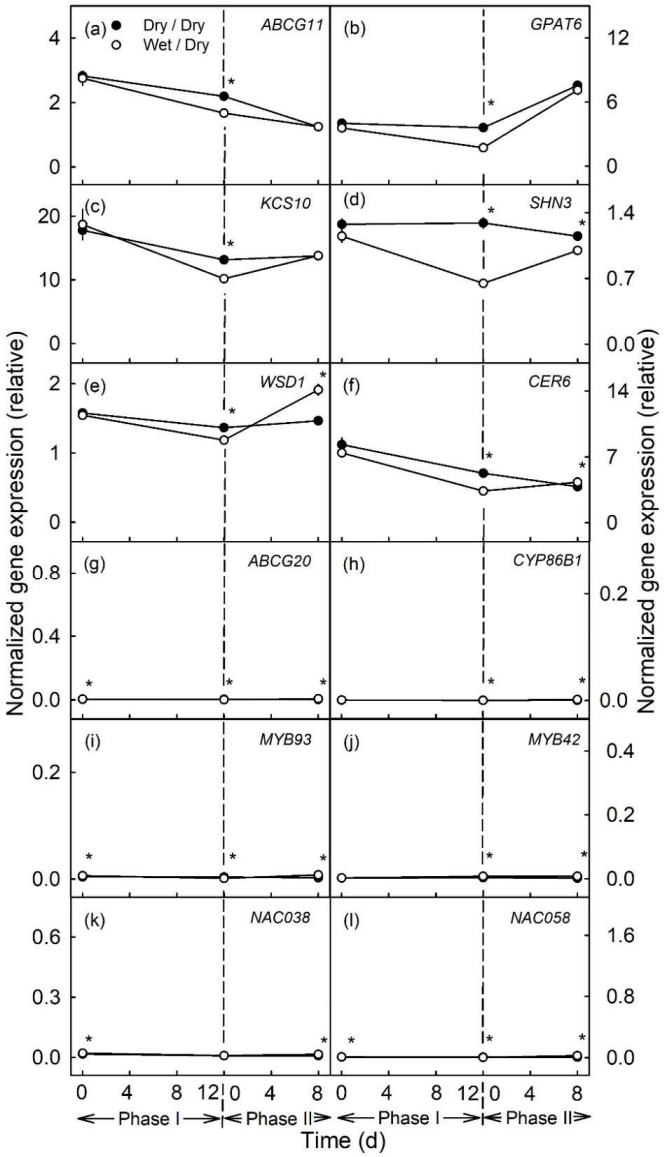

Figure 3. Time course of expression of genes related to cutin and wax synthesis (**a**–**f**) and to suberin and lignin synthesis (**g**–**l**) of apple fruit skin during moisture exposure (Phase I) and after exposure to moisture was discontinued (Phase II). During Phase I, a patch of the fruit skin was exposed to moisture for 12 d beginning at 66 days after full bloom (DAFB) (wet). During the subsequent Phase II, moisture was removed, and the patch was exposed to the atmosphere (dry). Moisture-exposed patches of the fruit skin are referred to as wet/dry, unexposed control patches as dry/dry. The end of moisture exposure is indicated by the vertical dashed line. The expression values are means ± SE of three to five independent biological replicates comprising ten fruit each. The '*' indicates significant differences between dry/dry and wet/dry at $p \leq 0.05$ (Student's *t*-test).

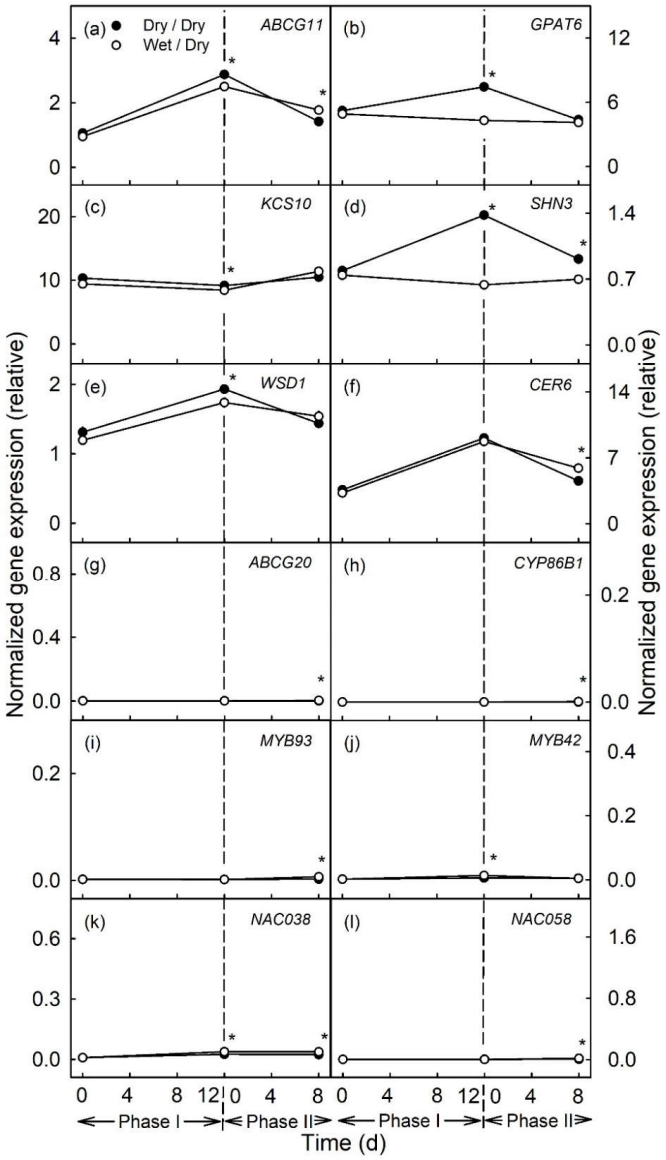

Figure 4. Time course of expression of genes related to cutin and wax synthesis (**a**–**f**) and to suberin and lignin synthesis (**g**–**l**) of apple fruit skin during exposure to moisture (Phase I) and after exposure to moisture was discontinued (Phase II). During Phase I, a patch of the fruit skin was exposed to moisture for 12 d beginning at 93 days after full bloom (DAFB) (wet). During the subsequent Phase II, moisture was removed, and the patch was exposed to the atmosphere (dry). Moisture exposed patches of fruit skin are referred to as wet/dry, unexposed control patches as dry/dry. The end of the moisture exposure is indicated by the vertical dashed line. The expression values are means ± SE of three independent biological replicates comprising ten fruit each. The '*' indicates significant differences between dry/dry and wet/dry at $p \leq 0.05$ (Student's *t*-test).

2.3. Histological and Metabolic Changes during and after Moisture Exposure

The skin patches with and without moisture exposure differed in both appearance and composition. The surfaces of skin samples of the unexposed controls comprised a cuticle, occasionally interrupted by lenticels (Figure 5a,g). There was no macroscopically or microscopically detectable periderm, except for that associated with the lenticels (Figure 5c,i). However, for the moisture-exposed skin patches, there were large areas of periderm (Figure 5b,h). A periderm had begun to develop in the underlying hypodermis by 8 d after moisture exposure was discontinued (Figure 5d). By 113 d after discontinuation of moisture exposure, both the periderm thickness and also the proportion of the area covered by periderm within the tube footprint had increased markedly (Figure 5h,j). At this stage, the periderm had reached the fruit surface and was visible macroscopically as irregular, brown patches.

When skin patches were subjected to enzymatic isolation using cellulase and pectinase, the isolated polymers obtained 8 d after moisture exposure had been discontinued in the exposed, and also in the unexposed control patches, comprised only cutin and wax, but no periderm (Figure 5e,f). The periderm that had begun to develop in the hypodermis of moisture-exposed patches and that was also plainly visible in cross-sections under the light microscope (Figure 5d) was probably lost during the isolation process. Thus, it is not surprising that suberin was detectable only in trace amounts in the GC-MS analyses at 8 d after moisture exposure had been discontinued. In contrast, by 113 d after moisture exposure had been discontinued, the periderm in the moisture-exposed patches had extended to the surface and 'connected' to the overlying cuticle. This periderm also remained connected during isolation (Figure 5l). There was no detectable periderm in the polymer membrane isolated from the moisture-unexposed (control) patches (Figure 5k).

Moisture exposure also altered the cutin and wax compositions. The most abundant constituents of the cutin were the hydroxy fatty acids, i.e., 16-hydroxy-C_{16} acid, 10,16-dihydroxy-C_{16} acid and 9,10,18-trihydroxy-C_{18} acid (Figure 6a). Compared with the unexposed controls, in the cuticles of the moisture-exposed patches these constituents were significantly reduced (Figure 6a,b). Moisture exposure also decreased the levels of transcoumaric acid, α,ω-dicarboxylic-C_{16} acid, 9,10-dihydroxy-α,ω-dicarboxylic-C_{16} acid and 9,10-dihydroxy-α,ω-dicarboxylic-C_{18} acid. Similarly, the content of carboxylic-C_{16} acid was reduced after 12 d of moisture exposure. The reductions were even more pronounced as the duration of moisture exposure increased from 6 to 12 d. After discontinuation of moisture exposure (Phase II) the amounts of ω-hydroxy-C_{20}, -C_{22} and -C_{24} acids and of carboxylic-C_{22} acid in the moisture-exposed patches all increased and were significantly higher than in the unexposed control patches. The amounts of α,ω-dicarboxylic acids, which decreased during Phase I, increased again during Phase II in the moisture-exposed patches (Figure 6c,d).

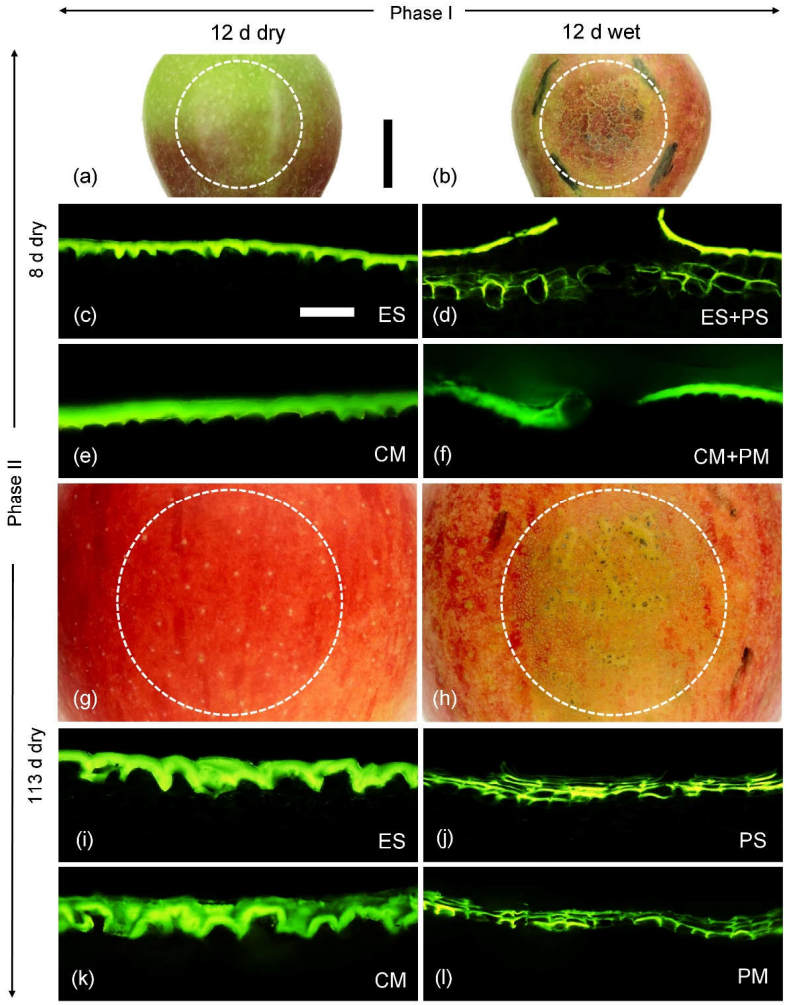

Figure 5. Macroscopic view of unexposed control patches (**a**,**g**) and moisture exposed (**b**,**h**) skin patches of apple fruit. Cross-sections of epidermal skin samples (ES) of control patches (**c**,**i**) and of the composite skins of moisture-exposed patches comprising epidermal plus peridermal sections (ES+PS) (**d**) or peridermal section only (PS) (**j**). Cross-sections of isolated cuticular membranes (CM) (**e**,**k**) and cuticular plus periderm membranes (CM+PM) (**f**) or periderm membranes only (PM) (**l**). The moisture treatment was applied as a two-phase experiment. During Phase I, a patch of the fruit skin was exposed to moisture for 12 d beginning at 31 days after full bloom (DAFB) (wet). During the subsequent Phase II moisture was removed, and the patch was exposed to the atmosphere (dry) (**b**,**d**,**f**,**h**,**j**,**l**). A portion of the unexposed surface on the same fruit served as control (**a**,**c**,**e**,**g**,**i**,**k**). Micrographs were taken 8 d (**a**–**f**) and 113 d (**g**–**l**) after moisture exposure was discontinued. Images in (**c**–**f**) and (**i**–**l**) were taken under incident fluorescent light (U-MWB) after staining with Fluorol Yellow 088. The scale bar in (**a**) equals 10 mm and is representative for all surface views (**a**,**b**,**g**,**h**). The scale bar in (**c**) equals 50 µm and is representative for all cross-sections of the composite (**c**–**f**, **i**–**l**). The dotted circles in (**b**) and (**h**) mark the original footprint of the tube that was mounted on the fruit surface to enable moisture exposure, the dotted circles in (**a**) and (**g**) are unexposed control patches on the same fruit. For details of the moisture treatment, see Materials and Methods.

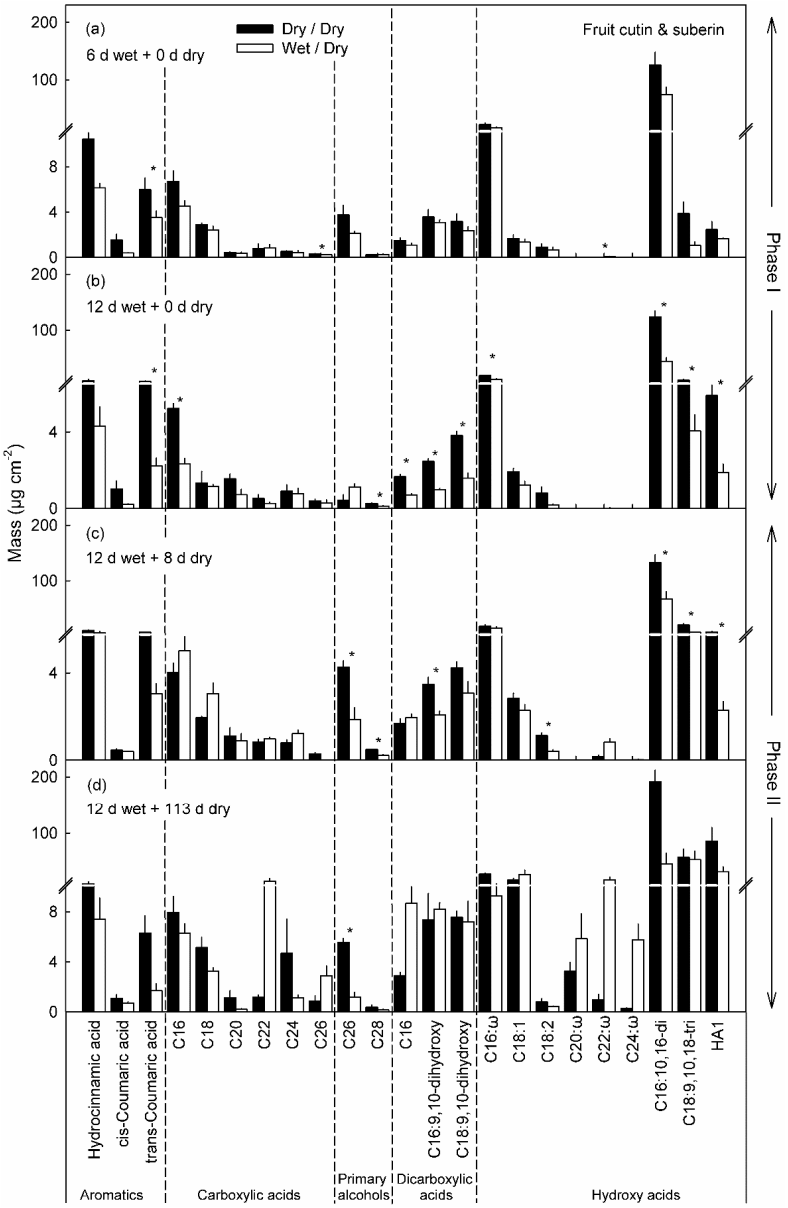

Figure 6. Cutin and suberin monomers in patches of apple fruit skin that were exposed to moisture for 6 d (**a**) and 12 d (**b**) (Phase I, wet). During the subsequent Phase II, the moisture exposure was discontinued (dry) and the cutin and suberin compositions of the patches analyzed after 8 d (**c**) and 113 d (**d**) after moisture exposure was discontinued. Unexposed patches of the fruit skin that remained dry throughout, served as controls (dry/dry). Data represent means ± SE of two to three replicates comprising cuticles of five fruit each. Significance of differences between dry/dry and wet/dry at $p \leq 0.05$ are indicated by '*' (Student's *t*-test).

The ω-hydroxy-C_{20}, -C_{22} and -C_{24} acids are characteristic and unique suberin monomers as indexed by the composition of the pure periderm (i.e., no cuticle) of the bark of the apple tree trunk (Figure 7a). For all other constituents of cutin, and for the wax, there was significant overlap in composition between the cutin and wax of fruit cuticle and of the bark periderm (Figure 7a,b). Normalizing for the three unique characteristic constituents allowed estimation of suberin mass per unit area of the mixed cuticle/periderm composites of the moisture-exposed fruit skin patches.

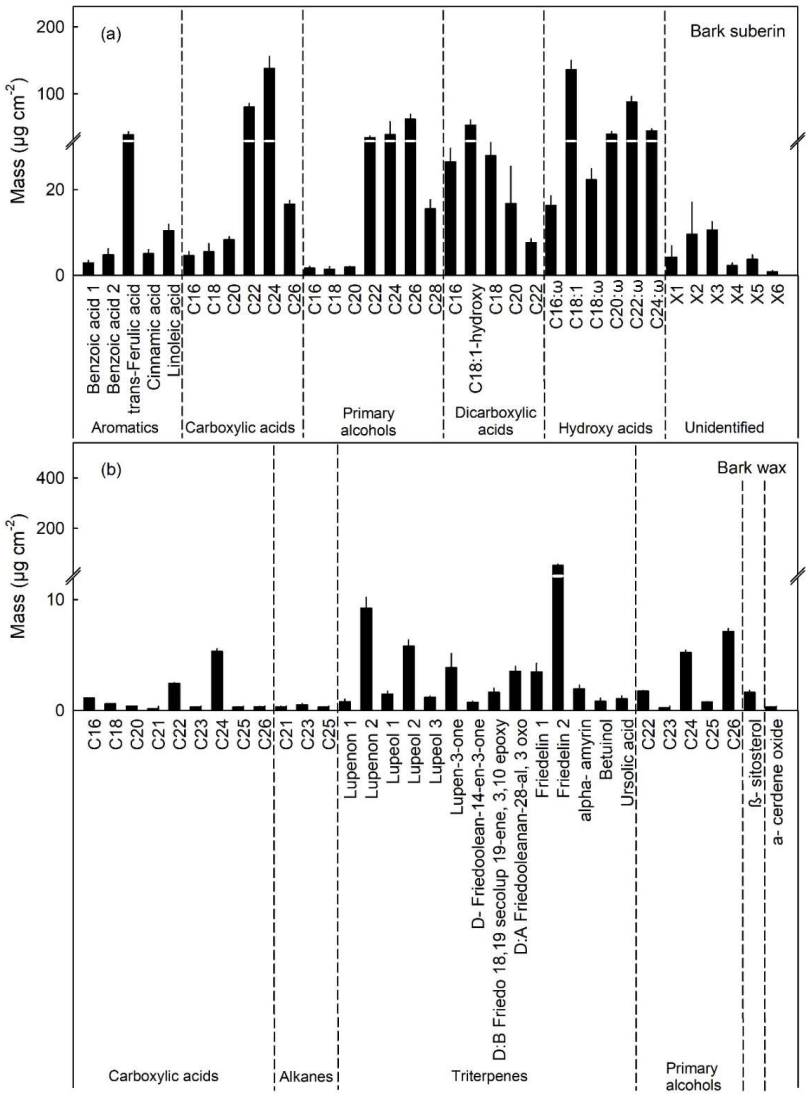

Figure 7. Composition of the periderm of the bark of the trunk (BP) of a 'Pinova' apple tree. (**a**) Constituents of the suberin and (**b**) constituents of the wax. The BP represents a pure periderm without any remnants of a cuticle.

The most abundant components of the wax were the triterpenes (oleanolic acid and ursolic acid), the sterols and C_{28} aldehyde. All of these were significantly lower in the moisture-exposed patches compared with the unexposed patches (Figure 8). The mass per area remained constant in the moisture-exposed patches but continued to increase in the unexposed control patches (Figure 8a,b). This pattern was particularly evident for the amounts of ursolic acid and C_{28} aldehyde that increased markedly up to maturity in the unexposed control patches—but not in the moisture-exposed patches (Figure 8).

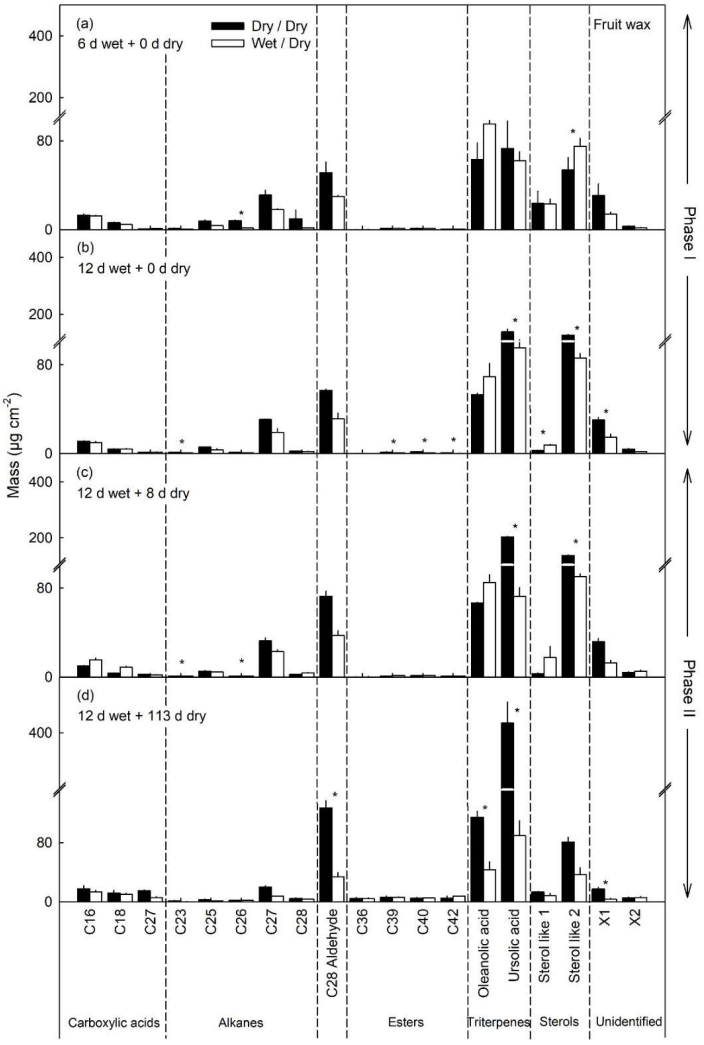

Figure 8. Wax constituents in patches of apple fruit skin that had been exposed to moisture for 6 d (**a**) and for 12 d (**b**) (Phase I, wet). During the subsequent Phase II, the moisture exposure was discontinued (dry) and the cutin and suberin compositions of the patches analyzed after 8 d (**c**) and 113 d (**d**). Unexposed patches of the fruit skin served as controls (dry/dry). Data represent means ± SE of two or three replicates comprising cuticles of five fruit each. Significance of differences between dry/dry and wet/dry at $p \leq 0.05$ is indicated by '*' (Student's *t*-test).

The compositional changes of individual constituents described above resulted in significant changes in the masses per unit area of cutin, wax and suberin. The masses of cutin and wax per unit area were lower in moisture-exposed patches, compared to the unexposed controls (Figure 9a–d). The decreases in mass occurred primarily during Phase I. They remained at about the same levels during the subsequent Phase II until 8 d after moisture exposure had been discontinued. The changes were qualitatively identical for 6 and 12 d of moisture exposure but were larger quantitatively for the longer exposure time (Figure 9a–d). During Phase I, suberin was essentially undetectable, regardless of the duration of moisture exposure. However, low levels of suberin were detectable 8 d after moisture exposure had been discontinued, while levels were markedly higher at 113 d (Figure 9e,f). It is interesting to note that some suberin deposition—albeit at low levels—was also recorded in the unexposed control patches. This last is not surprising because lenticels occur naturally in the unexposed control patches of an apple fruit skin and represent small areas of periderm usually associated with degenerate stomata (Figure 5g) [22].

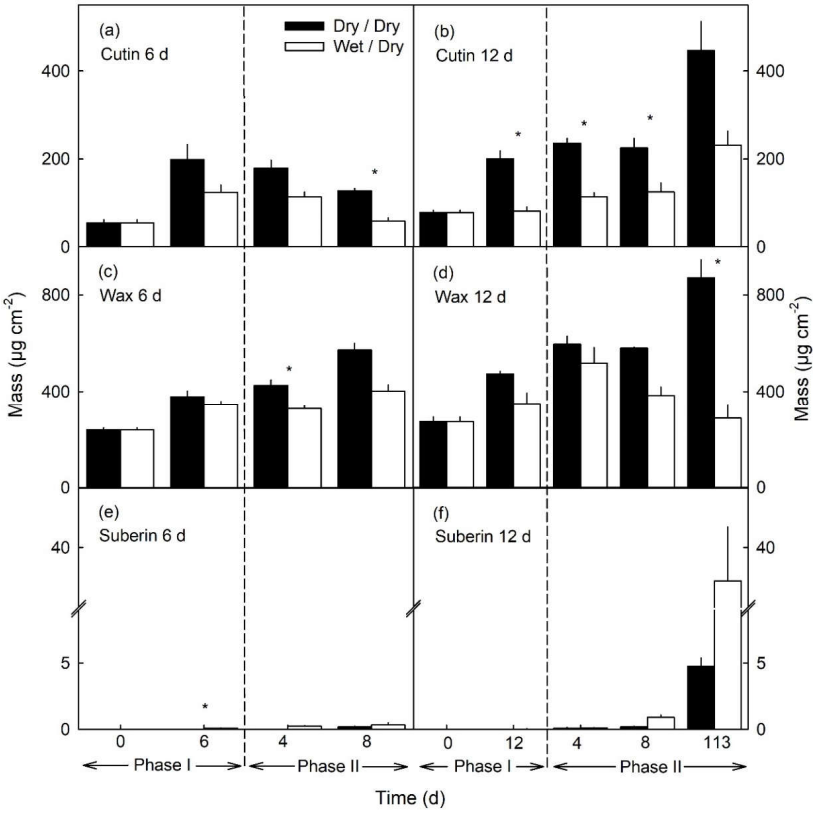

Figure 9. Total mass of cutin (**a**,**b**), wax (**c**,**d**) and suberin (**e**,**f**) in patches of the apple fruit skin during exposure to moisture (Phase I) and after exposure to moisture had been discontinued (Phase II). During Phase I, a patch of the skin was exposed to moisture for 6 d (**a**,**c**,**e**) or 12 d (**b**,**d**,**f**) beginning at 31 days after full bloom (DAFB) (wet). During the subsequent Phase II, the exposure to moisture was discontinued and the patch exposed to the atmosphere (dry). Moisture exposed patches of fruit skin are referred to as wet/dry, unexposed control patches as dry/dry. The end of the moisture exposure period is indicated by the vertical dashed line. The data represent the means ± SE of two or three samples comprising five fruits each. Significance of differences between dry/dry and wet/dry at $p \leq 0.05$ is indicated by '*' (Student's *t*-test).

3. Discussion

Our results establish that:

(1) Moisture exposure resulted in down-regulation of the genes involved in cutin and wax synthesis and deposition. The discontinuation of moisture exposure resulted in the up-regulation of genes involved in suberin synthesis.
(2) The early fruit development stage was more responsive to moisture than later stages when effects of moisture exposure on cutin and wax deposition were much less and those on suberin deposition essentially absent.

3.1. Gene Expression

Expressions of the genes involved in all steps of cuticle formation, account for the decrease in cuticle deposition during exposure to surface moisture. These included genes involved in the synthesis of monomers and constituents (*GPAT6*, *KCS10*, *SHN3*, *WSD1*, *CER6*) and their transport across the plasma membrane (*ABCG11*). The down-regulation occurred at the same time as microcracks formed [15,16], as cuticle thickness around microcracks decreased [16] and as the amounts of the amounts of the major constituents of cutin and wax decreased. These observations suggest a causal relation between moisture exposure, a reduction in the expressions of genes involved in cuticle synthesis, a decrease in cuticle mass and the subsequent formation of a periderm and the onset of suberin synthesis and deposition. Because moisture and its removal affected all levels of potential control (synthesis, transport and transcriptional regulation) it is most plausible that these associations are causal, rather than merely correlative.

3.1.1. Cutin, Wax and Suberin Synthesis

Moisture exposure during Phase I, down-regulated *GPAT6*. *GPAT6* and its orthologs have important functions in cuticle formation for example in the synthesis of 2-monoacylglycerols as shown for *Arabidopsis* [23]. A defect of an orthologous gene in tomato *SlGPAT6* led to reduced cutin content and decreased cuticle thickness compared to the wildtype [24]. Consistent with this is the observation by Legay [9] who reported decreased gene expression of *MdGPAT6* in russeted as compared to non-russeted apple cultivars. This is in line with our observation of decreased expression of *GPAT6* during moisture exposure.

Decreased expression during moisture exposure was also observed for *KCS10* in russeted fruit skins [9]. *KCS10* is involved in long-chain fatty acid synthesis in *Arabidopsis* [25]. Furthermore, Legay [9] also observed a down-regulation of genes involved in the synthesis of wax constituents such as *WSD1* and *CER6* in skins of russeted fruit. In *Arabidopsis*, *WSD1* is involved in the synthesis of wax esters. It also has diacylglycerol acyltransferase activity [26]. *CER6* is involved in the elongation of C_{24} very long chain fatty acids (VLCFAs). A loss of function in *Arabidopsis* led to an accumulation of the C_{24} wax component [27].

After moisture removal, genes related to the synthesis of suberin and, possibly, the formation of a periderm (*ABCG20*, *CYP86B1*, *MYB93*, *MYB42*, *NAC038* and *NAC058*) were subsequently up-regulated. This is consistent with an up-regulation of the expressions of *CYP86B1*, *MYB93*, *NAC038* and *NAC058* in skins of russeted apple fruit, but not in non-russeted mature fruit [9]. In *Arabidopsis*, *CYP86B1* is involved in the synthesis of ω-hydroxy-C_{22} and -C_{24} acids and α,ω-dicarboxylic acids. A knockout of this gene led to an accumulation of C_{22} and C_{24} fatty acids [28]. For *NAC038* and *NAC058* an involvement in the synthesis of suberin monomers is not unlikely. Experiments on overexpression of *MdMyb93* in *N. benthamiana* not only led to an increased suberin formation but also to an upregulation of *NAC038* and *NAC058* orthologues of *Nicotiana* [21].

3.1.2. Transport of Cutin Monomers, Wax Constituents and Suberin Monomers

During moisture exposure (Phase I) genes involved in the transport of cutin monomers or wax constituents across the plasma membrane were down-regulated. These included *ABCG11* that encodes an ATP binding cassette transporter essential for the transport of cuticular lipids in *Arabidopsis* (*AtABCG11*; [29]). The related orthologous gene *MdABCG11* (MDP0000200335) of apple was localized in a major QTL controlling russeting of 'Renetta Grigia di Torriana' [7]. Also, *MdABCG11* was down-regulated in russeted as compared to non-russeted cultivars in a bulk transcriptomic study [9].

The ABCG transporters *ABCG2*, *ABCG6* and *ABCG20* are involved in the transport of suberin monomers in *Arabidopsis* [30]. The up-regulation of *ABCG20* after termination of moisture exposure (Phase II) during the period of periderm formation in apple fruit skin therefore implies a requirement for transport of suberin monomers across the plasma membrane as would be needed for suberin incrustation of the phellem cell walls. At 8 d after moisture removal, ω-hydroxy-C_{22} acid had increased there and even more so at 113 d. This monomer is associated with russeted fruit skin at maturity [31].

3.1.3. Transcriptional Regulation of Cutin, Wax, and Suberin Synthesis

Moisture exposure also affected the transcriptional regulation of cuticle development by *SHN3*. The SHN transcription factor genes are known as positive regulators of cuticle formation and of patterning of epidermal cells in *Arabidopsis* and tomato [32–34]. The silencing of *SlSHN3* in tomato led to reduced amounts of cuticular lipids and alterations in cuticle morphology [34]. In apple fruit, markers linked to the *MdSHN3* gene co-segregate with decreased cuticle thickness, increased microcracking, decreased expression in russeted clones compared to non-russeted ones and increased potential for russet formation [8].

MYB93 is a key factor for the transcriptional regulation of suberin deposition in apple. It affects the synthesis and transport of suberin monomers, and their polymerization [21]. The transcription factor *MYB42* is involved in the synthesis of secondary cell wall, specifically in secondary cell wall thickening [35]. *MYB42* is also involved in the activation of genes for synthesis of lignin and phenylalanine, which serves as a precursor of many secondary metabolites in *Arabidopsis* [36]. We observed an up-regulation of the expression of *MYB42* during early formation of periderm whereas Legay [9] observed a down-regulation. The reason for this discrepancy is unknown. Increased expression of *MYB42* indicates concurrent lignin synthesis and secondary cell wall thickening during early phases of russeting. *NAC038* and *NAC058* also increased during Phase II of russet formation but their functions are not yet known.

3.2. Metabolites

The decreased expression of genes involved in cutin and wax synthesis resulted in decreased deposition in moisture-exposed skin patches. The 16-hydroxy-C_{16} acid, 10,16-dihydroxy-C_{16} acid and 9,10,18-trihydroxy-C_{18} acid are major constituents of cutin [37–39]. Furthermore, C_{16} acids are more abundant in the cutin of young and rapidly expanding organs and the amount of C_{18} acids increases as the organ develops and matures [38,39]. This was also observed in this study of apple fruit cutin. The mass of these three major constituents significantly decreases after 12 d moisture exposure. At maturity (113 d), the mass of the three major constituents was still lower in the moisture-exposed skin patches than in the control ones.

Within the wax fraction the C_{27} and C_{29} alkanes, the C_{26} and C_{28} primary alcohols, sterols and the triterpenes ursolic and oleanolic acid, are the dominant constituents in apple fruit wax [31,40–43]. These constituents are typical of the wax of *Rosaceae* species [44]. These constituents all decreased during moisture exposure indicating a decrease in the expressions of wax-related genes, paralleled by corresponding decreases in synthesis and

deposition. Similarly, Legay [31] reported decreased masses of ursolic acid and oleanolic acid in russeted apple skins at maturity, compared to non-russeted skins.

Deposition of wax in microcracks is an effective repair mechanism that re-establishes the cuticle's barrier function [15,45,46]. Furthermore, wax deposition in the cuticle of an expanding fruit surface converts elastic strain into plastic strain, thereby fixing both strain and stress [47]. Our observations suggest that decreased expression of genes involved in cutin and wax synthesis during moisture exposure led to decreased deposition. This may have contributed to, or even caused, the increased microcracking of the cuticle.

The increase in suberin content is less clear from the analysis of composition. First, most constituents of suberin also serve as monomers in cutin synthesis. Notable exceptions are the long chain (C_{20}, C_{22}, C_{24}) ω-hydroxy acids that are unique for suberin [31,48]. Second, despite a marked and consistent up-regulation of genes involved in synthesis of monomers for suberin, there was no clear corresponding increase in suberin monomers 8 d after discontinuation of moisture exposure. At this stage, a periderm had begun to develop in the hypodermal cell layers, in this and also our earlier study, as inferred from cross-sections of skin patches [16]. However, when skin patches were incubated in pectinase and cellulase, the cell layers separating the periderm from the epidermis were digested and, hence, the developing islands of periderm were lost to the isolation medium. This observation explains, why the periderm was detectable in cross-sections of the skin 8 d after discontinuation of moisture exposure but were not evident in the isolated cuticle polymer or as a major chemical constituent in the mass spectra of the moisture-exposed cuticles of fruit skins. By 113 d a complete periderm had developed, and this extended to the skin surface in the moisture-exposed fruit. This periderm remained attached to the cuticle during isolation at 113 d, but not at 8 d after moisture exposure was discontinued. Consequently, the characteristic constituents of suberin were clearly detectable. The slight increase in the un-exposed control patches does not conflict with the above conclusion. This suberin is accounted for by the presence of lenticels that form in the apple fruit skin during normal development.

Unfortunately, the overlap of many constituents between suberin and cutin made it impossible to calculate the amount of suberin deposited in moisture-exposed skin patches simply by summation. Further, moisture-treated skin patches are composite polymers comprising both cuticle and periderm to varying extents. For these a first estimate of the total amount of suberin present may be obtained by using pure suberin from the bark periderm of the trunk. In contrast to the moisture-treated fruit skin patches, the isolated periderm of the bark of the trunk is comprised of suberin only, there is no cuticle. Using the bark periderm of 'Pinova' apple trees as a reference, the masses of the suberin constituents relative to those of the three suberin-specific character constituents, i.e., the ω-hydroxy-C_{20}, -C_{22} and -C_{24} acids was calculated. This analysis revealed a marked increase in suberin deposition in line with that expected, based on the increases in gene expression.

3.3. Russet Susceptibility is Highest during Early Fruit Development

The histological, biochemical and molecular results demonstrate that moisture-induced russet is limited to the early stages of fruit development [16]. This is consistent with field observations where the first four weeks after full bloom are considered critical [1,10–14]. Moisture exposure occurring later in fruit development (for example between 66 and 78 DAFB or 93 and 105 DAFB) resulted in only slight decreases in expression of cutin- and wax-related genes and no increases in expression of suberin-related genes. This is consistent with the observed lack of periderm formation [16] and the lack of visual symptoms of russeting [15]. The higher susceptibility to russet during early fruit development results from the high relative area growth rates at this stage [49]. Unless matched by high rates of cutin and wax deposition [17], high relative area growth rates (high rates of strain) result in microcracking. Thus, growth strain, microcracking, macrocracking and russeting are interrelated [4,46,50–52].

3.4. Conclusion

The molecular and biochemical results presented here are consistent with the histological observations reported earlier [16]. Based on both studies, russeting must be viewed as a two-step process comprising the following sequence of events (Figure 10). A young fruit, that typically has a high growth rate and, hence, a strain rate of the skin [17], responds to surface moisture by decreasing cutin and wax synthesis and deposition due to the down-regulation of *ABCG11*, *GPAT6*, *KCS10*, *SHN3*, *WSD1* and *CER6*. As a consequence, the fixation of elastic strain by cutin and wax deposition is decreased and so, elastic strain builds up [47]. The increase in strain and (possibly) a change in the rheological properties of the cuticular membrane (CM) due to hydration [53] results in the formation of microcracks. These microcracks generally extend tangentially and so form a crack network on the fruit surface that continues to extend even after moisture exposure is discontinued. As a result, the cuticle's barrier function is impaired. A deposition of wax in developing microcracks may 'repair' the microcrack and so restore the cuticle's barrier function [45,54,55] and so avert the development of russeting. However, if this repair process lags too far behind, Phase II of the russeting cascade is initiated [46]. Following drying of the fruit surface, a yet unknown signal triggers the formation of a periderm. This signal must be transmitted from the microcrack (or the immediate vicinity thereof) deeper down to the hypodermal cell layers where a periderm begins to differentiate. Genes involved in suberin and lignin synthesis including *ABCG20*, *CYP86B1*, *MYB93*, *MYB42*, *NAC038* and *NAC058* are all up-regulated. Suberin is deposited in the cell walls of the phellem. The process continues until the cuticle and epidermis and the outer hypodermis dry and are sloughed off and the phellem becomes exposed at the skin surface. The suberized phellem now appears as the typical rough, dull brown of a russeted fruit skin.

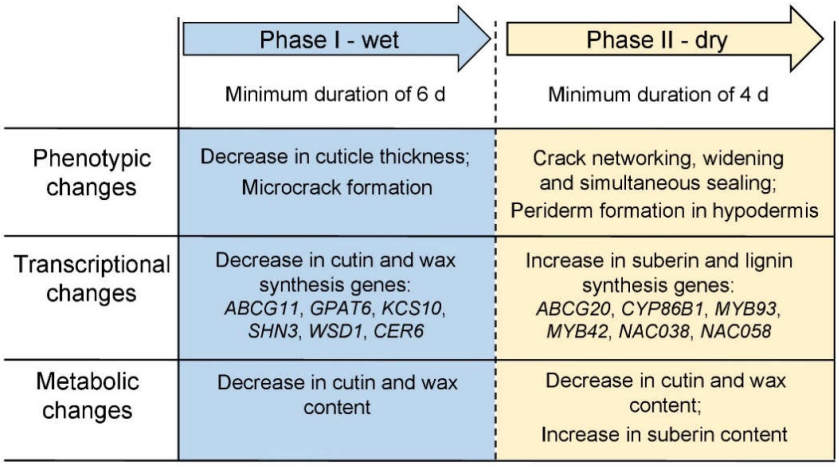

Figure 10. Schematic of the process of russeting at the phenotypic, transcriptional and metabolic level during exposure of apple fruit skin patches to moisture (Phase I) and following discontinuation of exposure (Phase II).

The triggers have not yet been identified that lead to the differential expression of both cutin- and wax-related genes during moisture exposure, nor those of the suberin-related genes after moisture exposure is discontinued. It is speculated that the expression of suberin-related genes is triggered by the impaired barrier properties of the cuticle. Potential candidates for this trigger are a high (O_2), a low (CO_2) or a more negative water potential in the tissues immediately subtending a microcrack. Interestingly, in potato, a low (O_2) inhibited suberization of the tuber following wounding [56]. Further experiments

employing techniques such as transcriptomic analysis would be helpful in identifying the potential triggers for the down-regulation of expression of the genes associated with cutin and wax synthesis during moisture exposure, as well as for the up-regulation of suberin-synthesis genes after moisture exposure has been discontinued.

The model of periderm formation presented here will apply equally to other fruitcrop species that develop microcracks in the cuticle during the early phase of development and subsequently russeting (e.g., pear). However, fruitcrop species that bear fleshy fruit and that are susceptible to cracking, usually are not susceptible to russet. In these, a comparable mechanism for fixing the impaired barrier properties of the fruit skin at that stage of development is absent.

4. Materials and Methods

4.1. Plant Materials

`Pinova` apple trees (*Malus* × *domestica*, Borkh.) grafted on M9 rootstocks were cultivated in the experimental orchards of the horticultural research station of the Leibniz University Hanover at Ruthe (52°14′ N, 9°49′ E) according to current regulations for integrated fruit production. Developing fruit were sampled randomly over three growing seasons from a total of 125 trees. For comparison, bark sections were excised from the base of the trunks of 21-year-old 'Pinova' trees about 10 cm above the graft union.

4.2. Moisture Treatment

Flowering spurs were randomly selected, and the clusters thinned at full bloom to one flower per cluster—usually the king flower. The moisture treatments were started when fruits had reached 10–12 mm diameter (usually about 21–31 DAFB). Experiments were carried out in two consecutive Phases. During Phase I a skin patch was exposed to moisture. For the subsequent Phase II, exposure to moisture was discontinued. For the moisture treatment, a 2 mL polyethylene tube (8 mm diameter; Eppendorf, Hamburg, Germany) was cut to 17 mm length and a hole (1.4 mm diameter) drilled into the tip. The tube was fixed in the equatorial plane of the fruit using a non-phytotoxic silicone rubber (Dowsil™ SE 9186 Clear Sealant; Dow Toray, Japan) [15]. Following curing, the tube was filled through the hole in the tip with deionized water using a syringe. The hole in the tip was then sealed with silicone rubber. The silicone was inspected for leakage and resealed every second day. The opposite, un-treated side of the same fruit served as the control [15]. The duration of moisture exposure (Phase I) was either 6 or 12 d. Thereafter, the tube was carefully removed. Unless specified otherwise, formation of a periderm was monitored up to 113 d after termination of the moisture treatment (Phase II).

4.3. RNA Extraction

Apple fruit skin from moisture-exposed and unexposed (control) areas were excised using a razor blade and immediately frozen in liquid N_2. Fruit skins were stored at −80 °C till processing. The skin tissue was ground to a powder with pestle and mortar in liquid N_2. RNA extraction was done using the InviTrap Spin Plant RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's protocol. To remove genomic DNA, total RNA was treated with DNase using the DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity and quantity were determined by measuring the absorbance at 230, 260 and 280 nm using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was determined on a 1.5% agarose gel. cDNA synthesis was carried out with the LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA) using 600 ng of RNA in a 40 µL reaction volume following the manufacturer's protocol. The number of biological replicates was from three to five. Each biological replicate comprised the skin from six to ten fruits.

4.4. Quantitative Real-Time PCR

Gene expression was determined by quantitative real-time PCR using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Genes observed in this study are listed in Table 1 and the corresponding specific primers in Table S1. Primer design was done using the Primer3 software (Primer3, http://primer3.ut.ee/). Gene expression values each represent three to five biological replicates and two to three technical replicates. To normalize gene expression, the reference genes *PROTEIN DISULFIDE ISOMERASE* (*PDI*) (MDP0000233444) and *MdeF-1alpha* (AJ223969.1) were used. Reactions were carried out using 1 µL undiluted cDNA in 8 µL volume of the Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) following manufacture`s guidelines. The final concentration was 200 nM for each specific primer. PCR cycle conditions were: one cycle of 95 °C for 60 s, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. After amplification melting curve analysis (95 °C for 15 s, 60 °C for 60 s, 60 to 95 °C in 0.5 °C increments) was used. Primer efficiency was determined in a five-fold dilution series of a cDNA pool covering five dilution points, each using the QuantStudio™ Real-Time PCR Software v1.3 (Applied Biosystems, Waltham, MA, USA).

Relative gene expression was calculated according to Pfaffl [57]. Modifications were according to Chen [58].

Table 1. List of genes analyzed in the gene expression study.

Gene Name	Accession	AGI Locus Code	Description	Reference
Cuticle-related				
ABCG11	MDP0000200335	AT1G17840.1	ABCG11, white-brown complex homolog protein 11, cuticular lipid transport to the extracellular matrix	[29]
CER6	MDP0000392495	AT1G68530.1	3-Ketoacyl-CoA synthase 6, involved in the synthesis of VLCFAs	[27]
FDH, KCS10	MDP0000235280	AT2G26250.1	FIDDLEHEAD,3-Ketoacyl-CoA synthase 10, probably involved in synthesis of long-chain lipids	[25]
GPAT6	MDP0000479163	AT2G38110.1	Glycerol-3-phosphate acyl transferase 6, synthesis of cutin monomers	[24]
SHN3	MDP0000178263	AT5G25390	Positive transcriptional regulator of cuticle synthesis	[32]
WSD1	MDP0000701887	AT5G37300.1	Wax Ester Synthase/Acyl-Coenzyme A:Diacylglycerol Acyltransferase, Wax ester synthesis and diacylglycerol acyltransfer	[26]
Periderm-related				
ABCG20	MDP0000265619	AT3G53510	ATP-binding cassette G20, involved in transport of aliphatic suberin polymer precursors	[30]
CYP86B1	MDP0000306273	AT5G23190.1	Cytochrome P450, family 86, subfamily B, polypetide 1, synthesis of very long chain ω-hydroxyacid and α,ω-dicarboxylic acid in suberin polyester	[28]
MYB42	MDP0000787808	AT4G12350.1	MYB domain protein 42, involved in secondary cell wall biosynthesis and regulation of lignin synthesis	[35,36]
MYB93	MDP0000320772	AT1G34670.1	MYB domain protein 93, positive regulator of suberin synthesis	[21]
NAC038	MDP0000232008	AT2G24430.1	NAC domain containing protein 38	uncharacterized
NAC058	MDP0000130785	AT3G18400.1	NAC domain containing protein 58	uncharacterized

4.5. Isolation of Fruit Cuticular Membranes and Periderm Membranes and Bark Periderm Membrane

Cuticular membranes and periderm membranes (PM) of developing apple fruit and periderm membranes from the bark (BP) of the trunk were isolated enzymatically [59]. Moisture exposed and unexposed skin samples were excised using biopsy punches (8 mm diameter, Kai Europe, Solingen, Germany; or 10 or 12 mm diameter, Acuderm, Terrace, FL, USA). The sections of the trunk bark were excised using a scalpel. Skin discs or bark sections were incubated at room temperature in 50 mM citric acid buffer at pH 4.0 containing pectinase (90 mL L^{-1}; Panzym Super E flüssig, Novozymes A/S, Krogshoejvej, Bagsvaerd, Denmark), cellulase (5 mL L^{-1}; Cellubrix L; Novozymes A/S) and 30 mM NaN_3 [59]. The enzyme solution was periodically replaced until CMs and PMs separated from their adhering cellular debris. Isolated CMs, PMs and BPs were rinsed in deionized water, dried at 40 °C for 20 h and stored at room temperature.

4.6. Cross-Sections of Skin Segments and Isolated Cuticular Membranes/Periderm Membranes and Microscopy

Tissue blocks were cut from moisture exposed and unexposed control patches of the fruit skin, transferred into Karnovsky fixative [60] and stored at 4 °C. The blocks were rinsed in distilled water, transferred to 70% (*v*/*v*) aqueous ethanol (EtOH) for 16 h and dehydrated in an increasing series of aqueous EtOH solutions (80%, 90% and 96% EtOH (*v*/*v*) for 30 min each). Subsequently, blocks were transferred to 100% isopropanol (twice for 40 min each) and then in a xylene substitute (AppiClear AppliChem, Münster, Germany; twice for 40 min each). The dehydrated blocks were infiltrated with a 1:1 (*v*:*v*) paraffin/xylene-substitute mixture (Carl Roth, Karlsruhe, Germany) for 40 min once, followed by two infiltrations with pure paraffin for 40 min each supported by a mild vacuum (absolute pressure 10.8 kPa).The embedded ES were stored at 4 °C. Sections of 10 µm thickness were cut using a rotatory microtome (Hydrax M 55, Zeiss, Oberkochen, Germany), collected on glass microscope slides and dried for 16 h at 37 °C. The paraffin was removed using xylene substitute (twice for 10 min each). Sections were rehydrated in a decreasing series of aqueous EtOH (96%, 80%, 70%, and 60%, all for 10 min each), followed by two final incubations in distilled water for of 5 min each. Cross-sections of isolated CM or PM were obtained by hand, using a razor blade.

Sections were stained for 1 h with 0.005% Fluorol Yellow 088 (Santa Cruz Biotechnology, TX, USA) [61] dissolved in a 1:1 mixture (*v*:*v*) of melted polyethylene glycol 4000 (SERVA Electrophoresis, Heidelberg, Germany) and 90% glycerol. Sections were inspected under incident fluorescent light (filter U-MWB, 450–480 nm excitation; ≥520 nm emission wavelength) using a fluorescence microscope (BX-60, Olympus, Hamburg, Germany). Three biological replicates each were observed for the ES, CM and PM.

4.7. Quantification of Wax Constituents by GC/FID and GC/MS

Isolated CM/PM discs and BP sections were cut into small pieces. An equal number of CM/PM pieces from five individual CM/PM discs (each represents a fruit) or of BP from samples of the trunk were pooled to make about 0.5 to 1 mg of material which represents a sample/replication. Samples were extracted in 5 mL chloroform overnight at room temperature on a horizontal rolling bench (CAT RM. 5–30 V, Staufen, Germany). The wax extract was immediately spiked with an adequate amount of internal standard (100 µL tetracosane of a chloroform solution of 10 mg tetracosane in 50 mL) later enabling the quantification of the single wax compounds. The chloroform volume was reduced under a gentle stream of N_2 at 60 °C in a heating block. The extracted CM, PM and BP pieces were dried on Teflon discs for further cutin/suberin analysis. Since some wax molecules contain polar hydroxyl- and carboxyl groups which negatively interfere with the GC column, all samples were derivatized by silylation yielding the corresponding trimethylsilyl ethers and -esters. For sylilation 20 µL of BSTFA (N, O-bis(trimethylsilyl)-trifluoracetamid, Machery-Nagel, Düren, Germany) and 20 µL of pyridine (Sigma Aldrich, Deisenhofen, Germany) were added to each sample. Derivatization took place for 45 min at 70 °C in a heating block. Of each sample 1 µL was injected on-column to a gas chromatograph coupled to 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 i.d. 0.32 mm, film 0.2 µm; J&W Scientific, Folsom, CA, USA). For identification of wax constituents, the extracted wax was analyzed by GC-MS (Quadrupole mass selective detector HP 5971, Hewlett-Packard, Palo Alto, CA, USA) by injecting 1 µL on-column. The constituents were quantified using the internal standard. Identification of the molecules was carried out by comparing fragmentation patterns with literature data and with our own data library. Data are expressed as mass per unit fruit surface area or trunk surface area. The number of replications was two to three, where each replicate comprised a subsample of five pooled CM, PM discs from five different fruit. The number of replications for the BP was three, each representing a different tree.

4.8. Quantification of Apple Cutin and Suberin Monomers by GC/FID and GC/MS

The extracted and dried CM/PM and BP were transesterified in glass vials by incubation in 1 mL boron trifluoride-methanol solution (BF_3/MeOH) for 16 h at 70 °C. After cooling of the samples, 20 µg of internal standard (100 µL dotriacontane of a chloroform solution of 10 mg dotriacontane in 50 mL) was added to each sample. Saturated $NaHCO_3$ (2 mL) was added to stop the depolymerization reaction. Cutin/suberin monomers were extracted three times by adding 2 mL chloroform. The chloroform phase was collected, washed by adding 1 mL HPLC grade water and then dried using $NaSO_4$. The water phase was discarded. The chloroform solution containing the cutin/suberin monomers was concentrated under a gentle stream of N_2 at 60 °C. Samples were derivatized as described above by adding 20 µL of BSTFA and 20 µL of pyridine. Monomers were quantified by injecting 1 µL of each sample on-column on a gas chromatograph coupled to a FID (GC-FID; CG-Hewlett Packard 5890 series H, Hewlett-Packard, Palo Alto, CA, USA, 307 column-type: 30 m DB-1 i.d. 0.32 mm, film 0.2 µm; J&W Scientific, Folsom, CA, USA). The individual constituents were identified on a gas chromatograph coupled to a mass spectrometer (Quadrupole mass selective detector HP 5971, Hewlett-Packard, Palo Alto, CA, USA) relative to the internal standard in each sample. Monomers were identified by comparing the fragmentation patterns with known standards from the literature or from our own library. Data are expressed as mass per unit fruit surface area. The number of replications was two or three, where each replicate comprised pooled CM/PM from five individual CM/PM discs obtained from five different fruit. The number of replications for the BP was three, each representing a different tree.

4.9. Data Analyses

Because moisture exposure of a patch of fruit skin results in formation of a periderm only in parts of the moisture treated area, the polymer obtained following enzymatic isolation from such surfaces is a mixed polymer comprising cuticle (cutin and wax) and periderm (suberized phellem and wax) of varying amounts. Furthermore, cutin and suberin and their waxes share common monomers and constituents. This makes it impossible to quantify the amounts of cutin and suberin or the amounts of cuticular and periderm wax deposited per unit surface area of moisture-treated patches of fruit skin. However, for the dewaxed suberin fraction, the ω-hydroxy-C_{20}, -C_{22} and -C_{24} acids are major and unique constituents of suberin that together account for 17.6% of a pure suberin of bark periderm of apple tree. As a first approximation, we assumed the composition of the suberin of a composite cuticle with periderm of moisture-treated apple fruit skin and that of the bark of a trunk of the same apple cultivar to be identical. Hence, the total amount of suberin in the cuticle may be calculated relative to the amounts of the ω-hydroxy-C_{20}, -C_{22} and -C_{24} acids. In contrast to the cuticle, with periderm of the moisture-treated fruit surface, the bark periderm of a trunk is comprised of suberin only—a cuticle is absent. We therefore used the suberin of the bark periderm as a standard. The periderm from the bark of the trunk were extracted, depolymerized and analyzed by GC-MS. Using the three hydroxy acids, a normalized suberin composition of the moisture-treated fruit was then calculated. This procedure allowed quantification of the time course of cutin and suberin deposition of the mixed polymer of a moisture-treated fruit surface. Due to the lack of unique constituents, the same calculation could not be carried out for the wax of cuticles with periderm.

Data are presented as means ± standard errors. When error bars are not shown, they were smaller than the data symbols. Paired sample Student's *t*-tests were run. Significant differences between dry/dry and wet/dry at $p \leq 0.05$ is indicated by '*'.

Supplementary Materials: The following are available online at www.mdpi.com/2223-7747/10/1/65/s1, Figure S1: Time course of expressions of genes related to cutin, wax (a–f) and suberin and lignin synthesis (g–l) of apple fruit skin during exposure to moisture (Phase I) and after exposure to moisture ceased (Phase II). Figure S2: Time course of expressions of genes related to cutin, wax (a–f) and suberin and lignin synthesis (g–l) of apple fruit skin during exposure to moisture (Phase I) and after exposure to moisture was discontinued (Phase II)., Table S1 Primers for genes analyzed used in the present study [62], Table S2: Effect of moisture exposure on the composition of the cuticle/periderm polymer.

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

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Author contributions	Conceptualization: B. P. K., T. D., M. K. Data curation: Y.-H. C., J. S., B. P. K. Formal analysis: Y.-H. C., J. S., B. P. K. Funding acquisition: T. D., M. K. Investigation: Y.-H. C., J. S., B. P. K., V. Z.-D., K. S. Methodology: B. P. K., T. D., M. K. Project administration: T. D., M. K. Supervision: B. P. K., L. S., T. D., M. K. Validation: Y.-H.C., J. S., B. P. K. Visualization: Y.-H. C., J.S., B. P. K. Writing – original draft: Y.-H. C., B. P. K., M. K. Writing – review & editing: Y.-H. C., B. P. K., L. S., T. D., M. K.
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RESEARCH ARTICLE

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 (*ABCG20*) 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-C_{20}, -C_{22} and -C_{24} 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, russeting 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

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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 (Dowsil™ 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 russeting [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 NaN_3 (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 $CHCl_3$ (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 $CHCl_3$) was added to the wax extract as an internal standard. The volume of the extract was reduced under a gentle stream of N_2 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 BF_3/MeOH for 16 h at 70 ˚C. Thereafter, 20 μg of dotriacontane (100 μl of 10 mg dotriacontane in 50 ml $CHCl_3$) was added as an internal standard. Depolymerization was stopped and 2 ml of saturated $NaHCO_3$ was added.

The cutin and suberin monomers were extracted using $CHCl_3$ (×3, 2 ml each). The $CHCl_3$ phase was separated, washed with 1 ml HPLC grade water, dried with Na_2SO_4 and concentrated under a gentle stream of N_2 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_{20}, -C_{22} and -C_{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 analysis of variance, regression analysis or t-tests using the statistical software SAS® 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

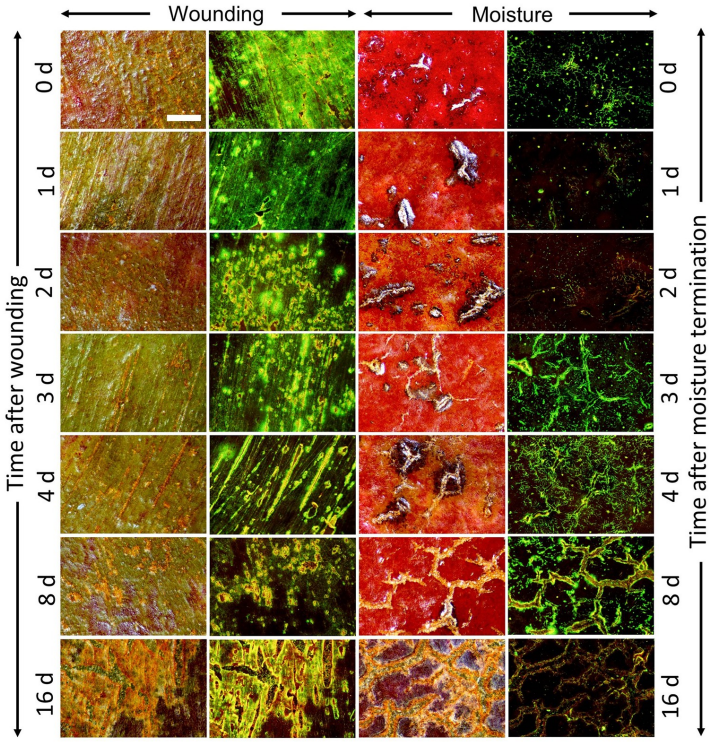

Fig 1. Time course of change in infiltration of 'Pinova' fruit skin patches following wounding by abrasion of the cuticle at 40 days after full bloom (DAFB) using fine sandpaper ('Wounding') or by exposure of the fruit skin to moisture for 12 d ('Moisture'). Moisture exposure began at 28 DAFB. At 40 DAFB, moisture exposure was terminated and the time-course of change in infiltrated fruit surface area was established. Micrographs from the same surface area were taken under incident white light or incident fluorescent light. The green/yellow fluorescence resulted from localized penetration of the tracer acridine orange through microcracks in the cuticle into the underlying tissues. Scale bar equals 400 μm and is representative for all the images of the composite.

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(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

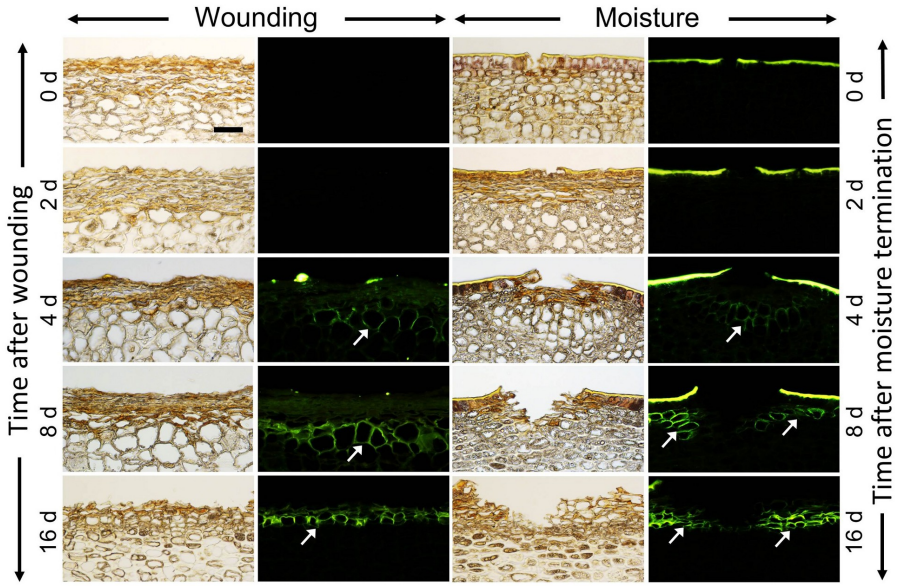

Fig 2. Time course of periderm development following wounding (left panel) and moisture exposure (right panel) of skin patches of 'Pinova' apple. Patches of skin were abraded 40 days after full bloom (DAFB) using fine sandpaper. Microcracks induced by surface moisture served as control ('Moisture'). Here, the fruit surface was exposed to surface moisture for 12 d from 28 to 40 DAFB. Pairs of micrographs were taken under transmitted white light or incident fluorescent light (filter module U-MWB) after staining with Fluorol Yellow. Fluorol Yellow stains the cuticle and suberized cell walls. Scale bar equals 50 μm and is representative for all images of the composite.

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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

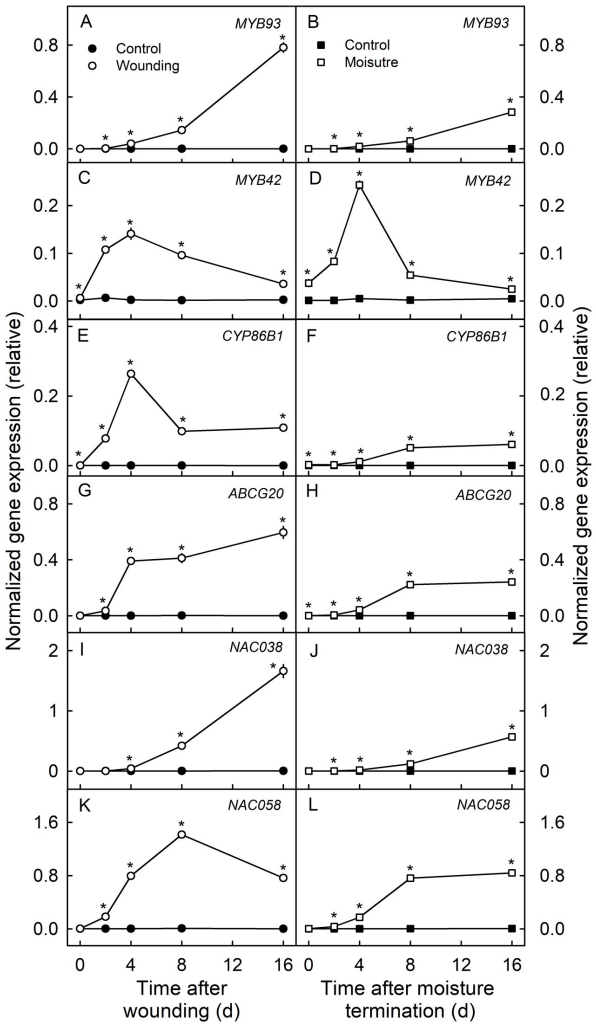

Fig 3. Time courses of change in the expressions of two transcription factors involved in the regulation of the synthesis of suberin (*MYB93*) and lignin (*MYB42*), a gene involved directly in the synthesis of suberin monomers (*CYP86B1*) and a gene involved in the transport of suberin monomers (*ABCG20*) 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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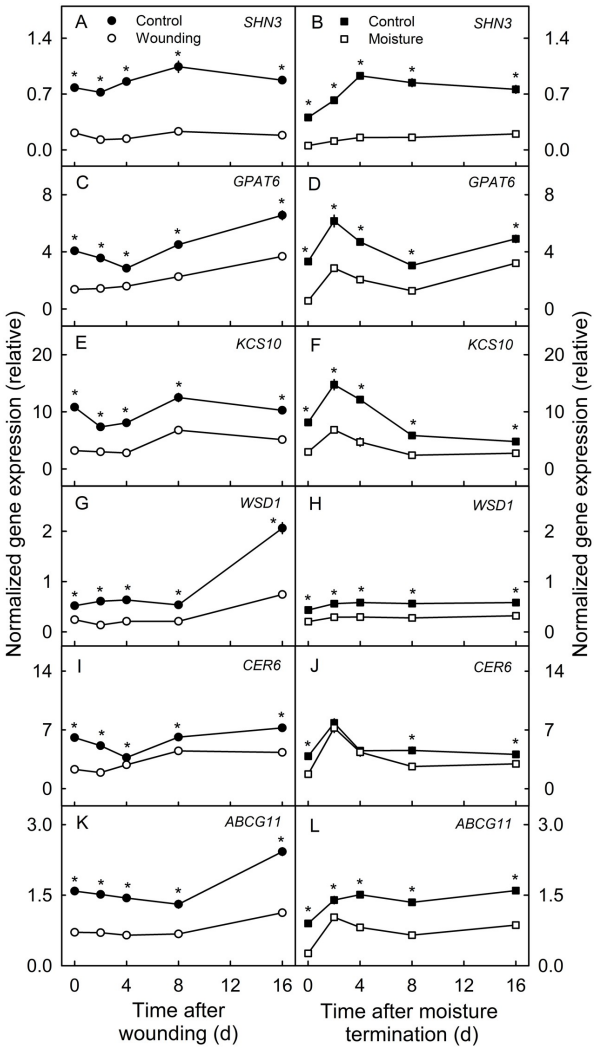

Fig 4. Time courses of change in the expression of genes involved in the synthesis of cutin monomers (*SHN3*, *GPAT6*) and wax constituents (*KCS10*, *WSD1*, *CER6*) and their transport (*ABCG11*) in the skin 'Pinova' apple fruit following wounding or following exposure of the fruit surface to moisture. 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, $P \leq 0.05$ (Student's t-test).

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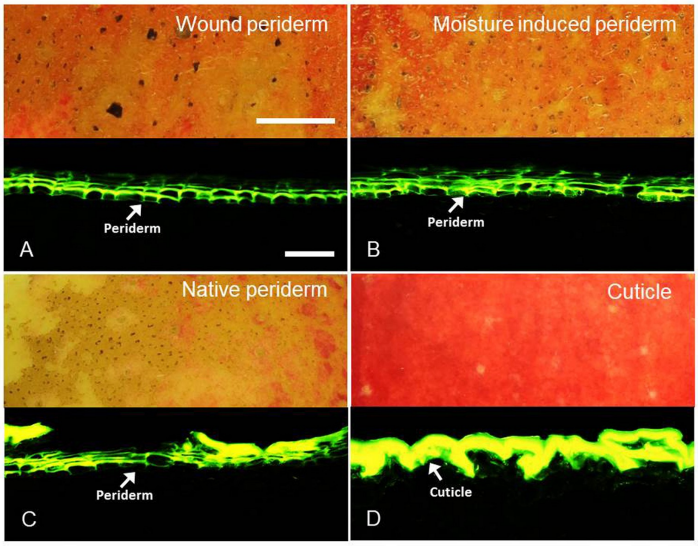

Fig 5. Cross-sections through patches of 'Pinova' apple fruit skin at the mature stage (156 days after full bloom (DAFB)) that had been wounded or exposed to surface moisture during early fruit development. Patches of fruit skin were wounded at 32 DAFB by abrading the cuticle using abrasive paper ('Wound 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.

https://doi.org/10.1371/journal.pone.0274733.g005

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).

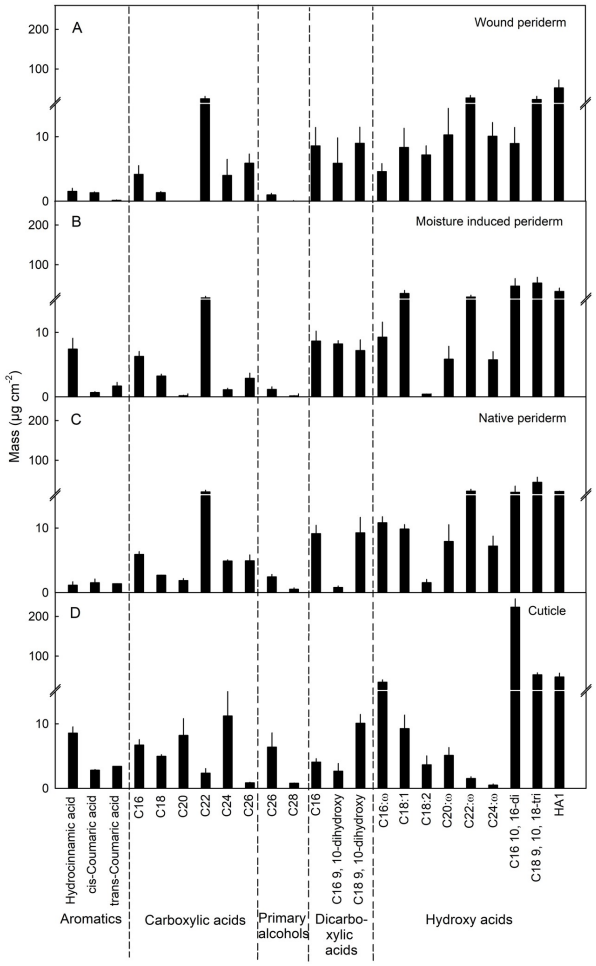

Fig 6. Composition of cutin and suberin of skins of mature apple fruit. Periderm formation in the fruit skin was induced during early development by abrading the cuticle using abrasive paper ('Wound periderm') (A) or by exposing the fruit skin to surface moisture for 12 d between 31 and 43 days after 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].

https://doi.org/10.1371/journal.pone.0274733.g006

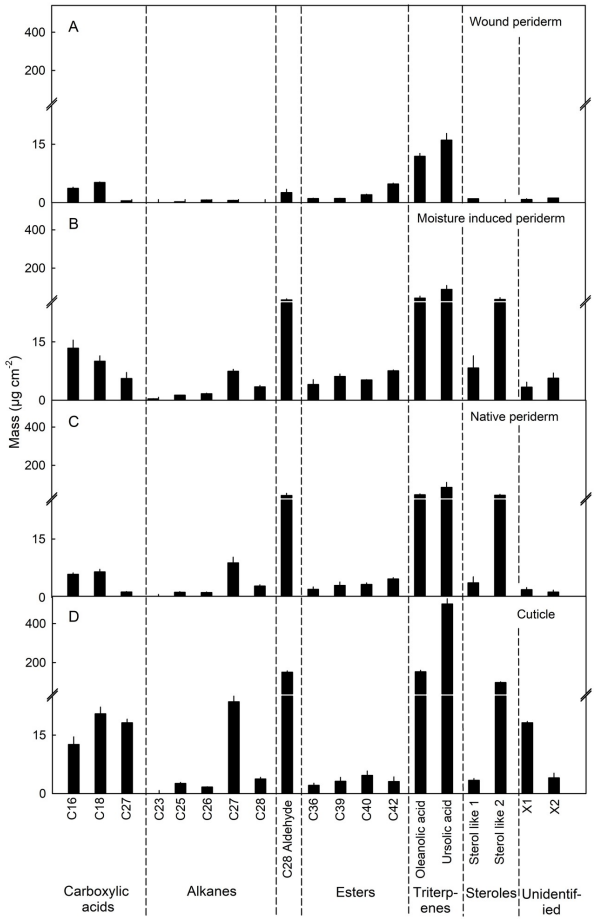

Fig 7. Wax constituents of the skins of mature apple fruit. Periderm formation in the fruit skin was induced during early development by abrading the cuticle using fine sandpaper ('Wound periderm') (A) or by exposing the fruit skin to surface moisture for 12 d between 31 and 43 days after full bloom (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].

https://doi.org/10.1371/journal.pone.0274733.g007

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.

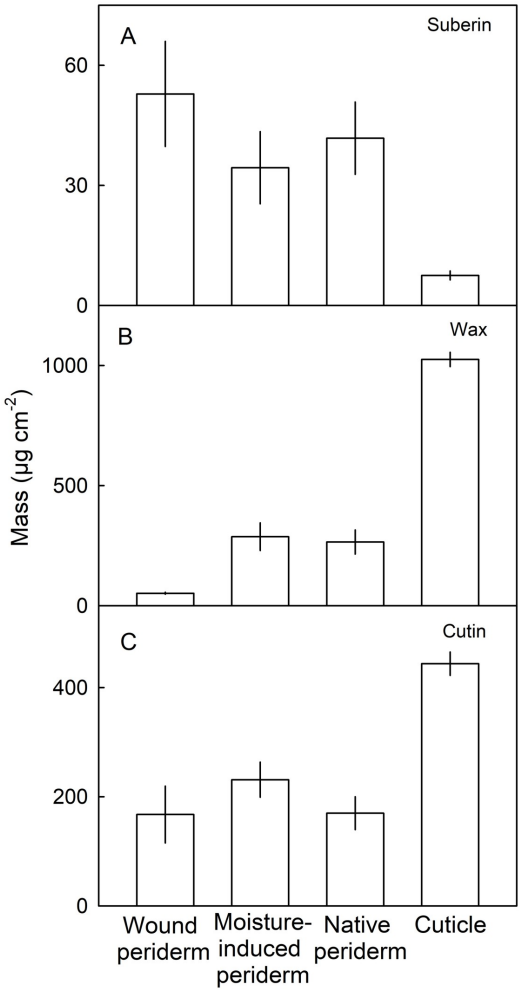

Fig 8. Total masses of suberin (A), wax (B) and cutin (C) in patches of skin of mature apple fruit. Periderm formation in the fruit skin was induced during early fruit development by abrading the cuticle using fine sandpaper ('Wound periderm') or by exposing the fruit skin to surface moisture for 12 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.

https://doi.org/10.1371/journal.pone.0274733.g008

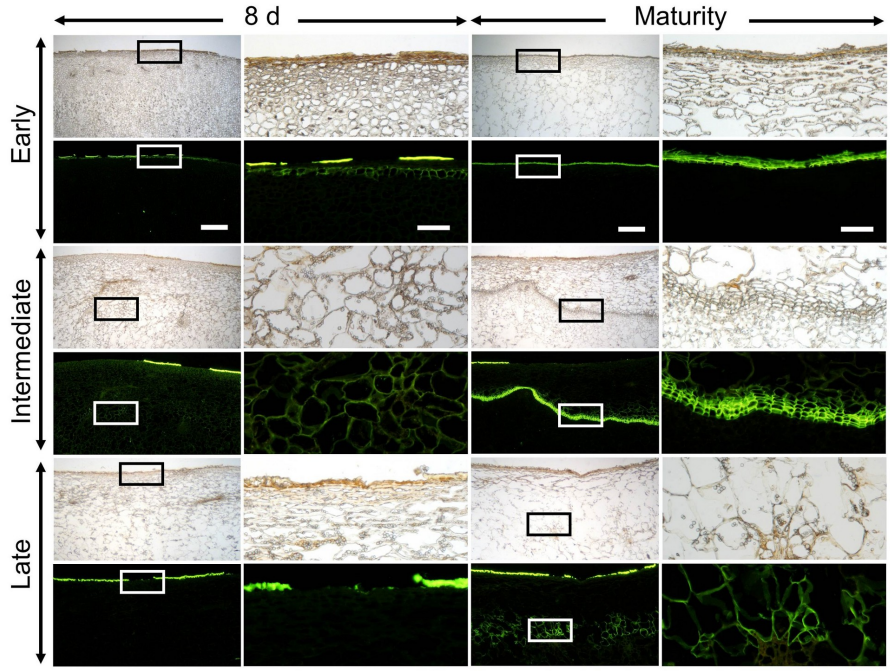

Fig 9. Developmental time course of periderm formation following wounding of 'Pinova' apple at 32 days after full bloom (DAFB) ('early'), 66 DAFB ('intermediate') and 93 DAFB ('late'). For wounding, the cuticle was abraded using fine sandpaper. Cross-sections were prepared 8 d after wounding (left panel) or at maturity (156 DAFB) (right panel). Pairs of micrographs were taken under transmitted white light or incident fluorescent light (filter module U-MWB) after staining with Fluorol Yellow. Fluorol Yellow stains the cuticle and suberized cell walls. Sections were viewed at 20× (scale bar 500 μm, left column) or at 100× (scale bar 100 μm, right column).

https://doi.org/10.1371/journal.pone.0274733.g009

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 α,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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Funding acquisition: Thomas Debener, Moritz Knoche.

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Writing – original draft: Yun-Hao Chen, Bishnu P. Khanal, Moritz Knoche.

Writing – review & editing: Yun-Hao Chen, Bishnu P. Khanal, Lukas Schreiber, Thomas Debener, Moritz Knoche.

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2.5 Time course of changes in the transcriptome during russet induction in apple fruit

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Type of authorship	Third author
Type of article	Research article
Contribution to the article	Y.H.C was involved in the sampling the fruits in the field for all the experiments. In addition, he participated in the histological examination and the russeting assessment at maturity (in the supplementary data).
Author contributions	Conceptualization, J.S., B.P.K., M.K. and T.D. Funding acquisition, M.K. and T.D. Project administration, M.K. and T.D. Methodology, J.S., B.P.K., M.K. and T.D. Investigation, J.S., S.S., Y.C. and B.P.K. Supervision, B.P.K., M.K. and T.D. Data curation, J.S. Validation, J.S. Visualization, J.S. Formal analysis, J.S. Writing—original draft, J.S. and T.D. Writing—review and editing, J.S., B.P.K., M.K. and T.D.
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Time course of changes in the transcriptome during russet induction in apple fruit

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Abstract

Background Russeting is a major problem in many fruit crops. Russeting is caused by environmental factors such as wounding or moisture exposure of the fruit surface. Despite extensive research, the molecular sequence that triggers russet initiation remains unclear. Here, we present high-resolution transcriptomic data by controlled russet induction at very early stages of fruit development. During Phase I, a patch of the fruit surface is exposed to surface moisture. For Phase II, moisture exposure is terminated, and the formerly exposed surface remains dry. We targeted differentially expressed transcripts as soon as 24 h after russet induction.

Results During moisture exposure (Phase I) of 'Pinova' apple, transcripts associated with the cell cycle, cell wall, and cuticle synthesis (*SHN3*) decrease, while those related to abiotic stress increase. *NAC35* and *MYB17* were the earliest induced genes during Phase I. They are therefore linked to the initial processes of cuticle microcracking. After moisture removal (Phase II), the expression of genes related to meristematic activity increased (*WOX4* within 24 h, *MYB84* within 48 h). Genes related to lignin synthesis (*MYB52*) and suberin synthesis (*MYB93*, *WRKY56*) were upregulated within 3 d after moisture removal. *WOX4* and *AP2B3* are the earliest differentially expressed genes induced in Phase II. They are therefore linked to early events in periderm formation. The expression profiles were consistent between two different seasons and mirrored differences in russet susceptibility in a comparison of cultivars. Furthermore, expression profiles during Phase II of moisture induction were largely identical to those following wounding.

Conclusions The combination of a unique controlled russet induction technique with high-resolution transcriptomic data allowed for the very first time to analyse the formation of cuticular microcracks and periderm in apple fruit immediately after the onset of triggering factors. This data provides valuable insights into the spatial-temporal dynamics of russeting, including the synthesis of cuticles, dedifferentiation of cells, and impregnation of cell walls with suberin and lignin.

Keywords Russeting, *Malus x domestica*, Fruit skin, Periderm, Cuticle, Transcriptome, Suberin, Lignin, Wounding

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Background

Russeting is a skin disorder in many fruit crop species, including apple [1–5]. In russeting, the cuticle and the epidermis are replaced by periderm. In many apple cultivars, russeting compromises the visual appearance of the fruit, thereby reducing market value. Furthermore, postharvest performance is impaired by the increased permeance of the skin to water vapor, which may result in increased mass loss and shriveling [6–8].

Periderm formation begins in the hypodermis in the vicinity of microcracks in the cuticle [9–11]. Microcracks are minute microscopic cracks that are limited to the cuticle and not visible to the naked eye [12–14]. They are the first visible symptoms in russeting [12, 15] and result from a mismatch between cuticle deposition on the one hand and growth stress during periods of rapid surface expansion on the other hand [16, 17]. Russeting is influenced by both environmental and genetic factors. Environmental factors include the exposure of fruit surfaces to moisture or high humidity during periods of high strain or mechanical damage [13, 14, 18–21]. There are genetic differences in the susceptibility of cultivars to russeting. Generally, cultivars with high variability in the cell sizes of the epidermis and hypodermis are most susceptible [22].

Molecular studies indicate that the downregulation of cuticle synthesis is an important factor in russeting. QTLs (quantitative trait loci) for russeting on chromosomes 2, 12 and 15 were identified in populations segregating for russet susceptibility [23, 24]. Within these QTLs, the major cuticle regulator *MdSHN3* [24] as well as the cutin/wax transporter *MdABCG11* [23] were associated with russet susceptibility in apple under field conditions. Comparisons between a russet-resistant and a fully russeted sport of 'Golden Delicious' demonstrated downregulation of two oxidosqualene cyclases (*MdOSC1* and *MdOSC*3) during microcracking of the cuticle. A change in triterpene content from ursan-type to lupane-type triterpenes was observed in russeted skins, together with an increase in *MdOSC5*, which is activated by MdMYB66 and to a lesser extent by MdMYB52 [25]. Furthermore, a bulk transcriptomic study on russeted and nonrusseted fruits revealed a large number of cuticle-related genes to be downregulated in russeted apple fruit skins at maturity [26]. Additionally, suberin-associated genes were highly expressed, together with a dense network of possible transcriptional regulators of the later processes of russeting (e.g., maturation of the periderm, impregnation of cell walls with suberin) [26]. The transcription factor *MdMYB93* was later identified as a major regulator of suberin synthesis [27], and *MdMYB52* was identified as a regulator of lignin synthesis [28]. In addition, investigations at the multispecies level revealed MYB9 and MYB107 to be major regulators of suberin formation in angiosperms [29]. The majority of transcription factors associated with the later processes of russeting belong to the R2R3-MYB family and, to a minor extent, to the AP2/EREBP, bHLH, C2H2, WRKY, and NAC-domain transcription factor families [25–27, 30–32]. Unfortunately, all of the above analyses were conducted on fruit at the mature stage, while russeting typically occurs during early development. In apple, russet susceptibility peaks during the first four weeks after full bloom [1, 12, 20, 33–36]. Unfortunately, only a few studies focused on this time period. We therefore used moisture treatment to induce russeting at defined developmental stages, including the period of highest russet susceptibility [19]. Khanal and coworkers [14] refined the system to specifically target early events in russet formation. This modification allowed a patch of fruit skin to be exposed to moisture (Phase I), while the remaining fruit surface stays dry and serves as a control. The moisture is then removed (Phase II), and the events occurring after moisture removal can be monitored [13, 21].

Here, we present a high-resolution transcriptomic study performed during the onset of russeting in apple. The objectives of this research were to analyze genes representative of Phase I and Phase II of moisture-induced russeting and to identify candidates with putative functions in russeting.

Results

We induced russeting on fruits of the cultivar 'Pinova' in the 2018 and 2019 growing seasons by exposing fruits 21 or 31 days after full bloom (DAFB) to moisture for 12 d (Phase I, '12 d wet+0 d dry'). During Phase I, the control fruit remained dry ('12 d dry+0 d dry'). For the subsequent Phase II, moisture was removed, and samples were taken at 1 d ('12 d wet+1 d dry') to 8 d after moisture removal ('12 d dry+8 d dry'). The number of read pairs obtained after quality filtering and trimming of raw reads ranged from 56.6 M to 70.5 M for independent replicates. Reads mapped uniquely to the HFTH1 genome with a frequency between 82.9 and 95.5% (Table S1).

Transcriptomic data obtained during the 2018 and 2019 seasons displayed low variability between replicates as indexed by principal component analysis (PCA) (Fig. 1A, B). Control samples clustered closely together for both seasons. In contrast, moisture-treated samples compared to untreated controls showed a pronounced diverging pattern, with distances between treatments increasing with time after moisture removal (Phase II). This corresponded to the observed progress of microcrack formation within Phase I and periderm development during the consecutive Phase II (Figure S1). Clusters observed in PCA for the 2018 season (Fig. 1A) were less compact than those in the 2019 season (Fig. 1B).

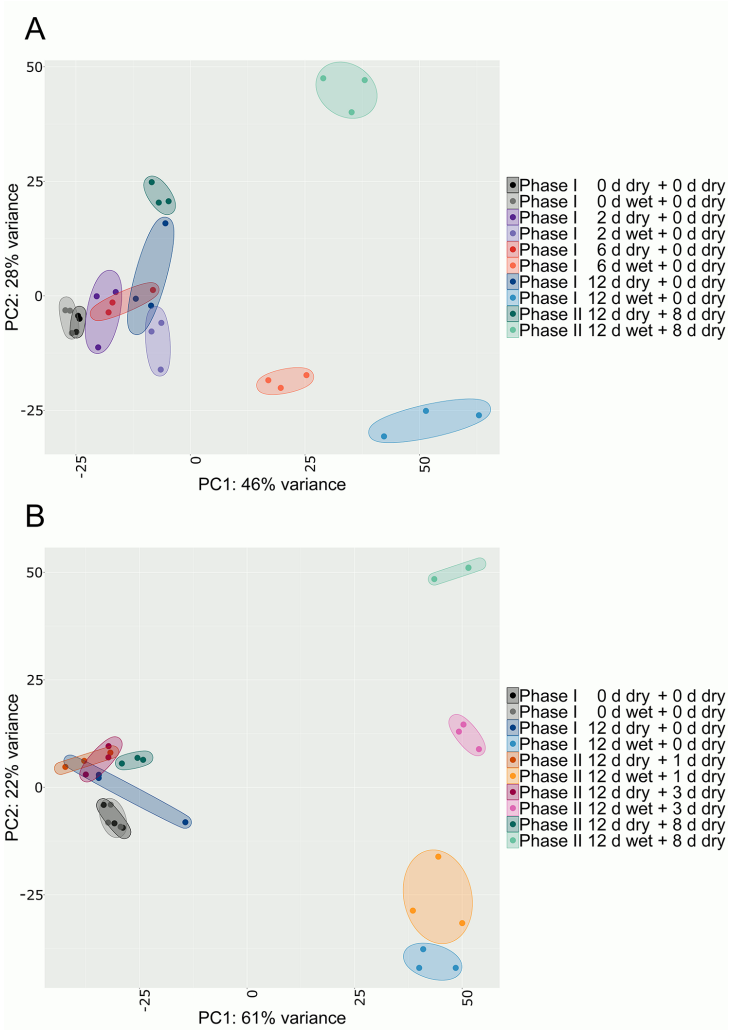

Fig. 1 Variability between biological replicates in the RNA-Seq datasets. Apple fruit skin patches of 'Pinova' apples were induced to russet by exposed to surface moisture for 12 d (Phase I). After termination of moisture exposure, the treated skin patch was exposed to ambient atmosphere (Phase II). Non-treated control surfaces remained dry during Phase I and Phase II. The distribution of the transcriptome during the 2018 **(A)** and 2019 **(B)** seasons was determined by principal component analysis (PCA) based on variance stabilization transformation in 'DESeq2'. PCA revealed clear separation of clusters between moisture-exposed ('x d wet + y d dry') and control ('x d dry + y d dry') samples, whereas the biological replicates within each treatment were consistent (indicated by ellipses)

Transcripts differ between phases I and II of russet induction

Stringent filtering of the differentially expressed genes (DEGs) obtained from the various datasets revealed a total of 3533 DEGs. The number of DEGs was higher in 2019 than in 2018 (Table S2). Four times more genes were downregulated and two times more genes were upregulated in 2019 than in 2018 between the corresponding time points at '12 d wet + 0 d dry' (Phase I) and '12 d wet + 8 d dry' (Phase II) (Table S2). Consistent with

this observation, there were more russeted fruits in the 2019 season than in the 2018 season (Figure S2A, B).

For both seasons, we found DEGs putatively involved in microcracking in Phase I as well as in periderm formation in Phase II.

In Phase I samples, in the 2018 season, 242 genes were downregulated compared to 22 in Phase II. Of the 242 genes, 54 genes were downregulated at '6 d wet+0 d dry' as well as '12 d wet+0 d dry' (Fig. 2A). In contrast, 700 genes were upregulated exclusively during Phase I and 310 during Phase II. Of the 700 genes specific to Phase I, 14 were already differentially expressed after 2 d of surface moisture ('2 d wet+0 d dry') (Fig. 2B).

In 2019, 421 genes were downregulated during Phase I and 335 during Phase II, whereas 32 genes were already downregulated at '12 d wet+1 d dry' in Phase II (Fig. 2C). The number of upregulated genes was 375 in Phase I and 959 in Phase II. Of the 959 genes, 103 were already upregulated at '12 d wet+1 d dry' during Phase II (Fig. 2D).

Three sampling dates were common in both seasons: '0 d wet+0 d dry' (Phase I), '12 d wet+0 d dry' (Phase I), and '12 d wet+8 d dry' (Phase II). The last two sampling times revealed a large number of season-specific DEGs. To avoid artifacts from confounding factors unrelated to russeting but differing between seasons, further analysis was restricted to DEGs consistent between seasons. These comprised 106 genes at '12 d wet+0 d dry' (Phase I) and one gene at '12 d wet+8 d dry' (Phase II) (Data S1). Eight genes were downregulated on both sampling dates (Fig. 2E). In both seasons, the number of upregulated genes was 414 at '12 d wet+0 d dry' (Phase I) and 238 at '12 d wet+8 d dry' (Phase II) (Fig. 2F; Data S1).

The DEGs downregulated in Phase I ('12 d wet+0 d dry') were characterized by gene ontology (GO) terms related to cellular processes (e.g., cell division, cell wall associated or cytoskeleton). Cuticle-related GO terms (e.g., lipid metabolic process, fatty acid metabolic process, fatty acid synthetic process, and cellular lipid metabolic process) were downregulated in 2019 and to a lesser extent (log_2-fold change (log_2FC) ≤ -1) in 2018 (Fig. 3A, Data S2, S3, S4, S5). The DEGs upregulated in Phase I due to moisture exposure comprised stress-related genes (e.g., oxidative stress and osmotic stress) (Fig. 3B, Data S2, S3, S4).

The DEGs during early Phase II ('12 d wet+1 d dry') were similar to those at '12 d wet+0 d dry' (Phase I). The number of GO terms for downregulated DEGs decreased over time during Phase II. Beginning at '12 d wet+3 d dry', DEGs associated with suberin and lignin formation and cell wall metabolism were upregulated (Fig. 3C, Data S2, S3, S4). After '12 d wet+8 d dry' (Phase II), only one gene was consistently downregulated in both years (Fig. 2E). The upregulated genes at '12 d wet+8 d dry' (Phase II) included genes responsive to hormones, including abscisic acid (ABA), and a range of transcription factors (Fig. 3C; Data S4). At '12 d wet+8 d dry' (Phase II), processes associated with the metabolism of phenylpropanoids, suberin, and secondary metabolites, as well as response to lipids and apoplasts, were activated (Fig. 3C).

Cluster analysis revealed four clusters of DEGs with highly correlated expression patterns, suggesting a close relation to the onset of russeting (Fig. 4).

The first cluster contained nine genes that were downregulated in Phase I as early as '2 d wet+0 d dry', which remained so until '12 d wet+8 dry' in Phase II. *SHN3* [37–39] and *MYB94* [40, 41] were identified within this cluster, where orthologous genes had major regulatory functions in cuticle synthesis. The second cluster contained putative regulators for russeting, e.g., *MYB93*. This gene is a major regulator of suberin formation in apple [27]. This cluster was characterized by strong upregulation ($log_2FC \geq 2$) several days after moisture removal ('12 d wet+3 d dry') in Phase II. The third cluster contained transcriptional regulators that were activated immediately or shortly after termination of the moisture treatment ('12 d wet+0 d dry' and '12 d wet+1 d dry') on the fruit skin patches. Within this cluster, *Wuschel-related homeobox 4* (*WOX4*) and *MYB84* were observed, which are orthologous genes of major regulators during periderm initiation. Many genes in the fourth cluster were already slightly upregulated at the timepoint of moisture removal ('12 d wet+0 d dry'), especially in season 2019 (Fig. 4). This cluster contains *MYB36*, the orthologous gene of which in *Arabidopsis thaliana* regulates developmental transitions from proliferation to differentiation of cells in the root endodermis [42].

Validation of selected DEGs by qPCR

Earlier studies established that (1) microcracking of the cuticle occurs within 48 h of moisture exposure and (2) periderm initiation begins within 24 h of moisture removal [13, 14, 21, 43]. Therefore, putative regulators must be expressed early during Phase I and at the beginning of Phase II, i.e., at '12 d wet+1 d dry'. Based on these findings, a set of 12 DEGs was selected that represented candidate genes for early regulation during Phase I and Phase II (Fig. 5A) and/or are related to either cuticle (Phase I) or periderm formation (Phase II). These comprised transcription factors derived from clusters specific to Phase I (*MYB17*, *NAC35*) or Phase II (*AP2/B3-like transcription factor family protein* (*AP2B3*), *WOX4*, *MYB84*, *MYB-like 102* (*MYB102*), *MYB52*, *WRKY56*, *MYB67*, *MYB93*), one *late embryogenesis abundant hydroxyproline-rich glycoprotein* (*LEA*) and one *SGNH hydrolase* (*SGNH*). The genes were analyzed by quantitative real-time PCR (qPCR) to validate their expression patterns (Fig. 5B). The expression patterns of the selected

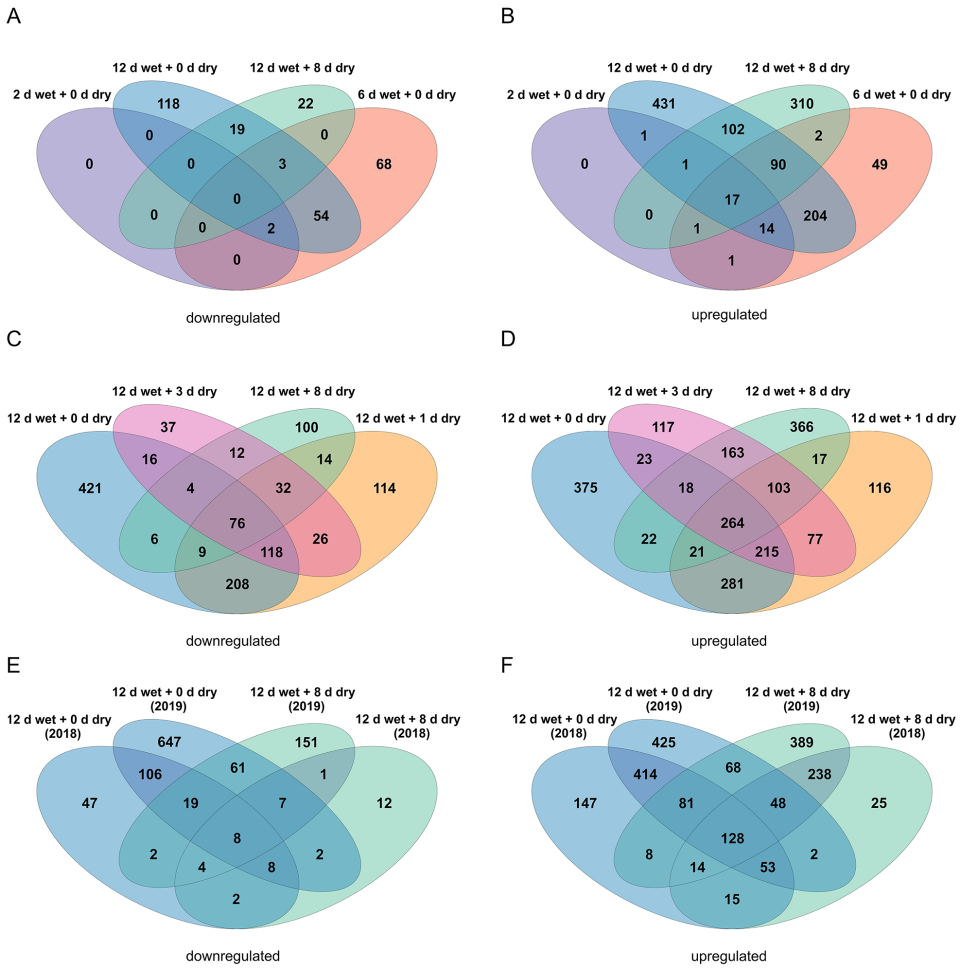

Fig. 2 Effect of the growing season on gene expression patterns in moisture-induced russeting in 'Pinova' apples. Venn diagrams of differentially expressed genes during the 2018 **(A, B)** and 2019 growing seasons **(C, D)**. Comparison of common treatments and their respective controls between the two seasons **(E, F)**. Only genes with a $\log_2$-fold change ($\log_2$FC) ≥ 2 or ≤ -2, a false discovery rate (FDR) ≤ 0.05 and a mean of at least five transcripts per million (TPM) for moisture-exposed ('x d wet + y d dry') or control ('x d dry + y d dry') samples are illustrated

genes were similar for RNA-Seq and qPCR. *MYB93* was used to trace the early processes of suberin synthesis [21, 27, 43] during Phase II and thus was representative of periderm formation as indexed by the occurrence of phellem cells.

NAC35 and *MYB17* were downregulated during early Phase I within both seasons at time points when microcracking occurred (Fig. 5A, B; Figure S1). The genes *AP2B3*, *WOX4* and *LEA* showed increased expression at '12 d wet + 1 d dry' (Fig. 5A, B). At '12 d wet + 2 d dry', the expression of *MYB84*, *MYB102*, *MYB52* and *WRKY56* increased as indexed by qPCR. Upregulation of *MYB93* and *SGNH* started one day later at '12 d wet + 3 d dry'. The increase continued until '12 d wet + 8 dry' (Fig. 5B). The expression pattern was consistent between the two seasons (Fig. 5A, B). The transcriptional regulator *MYB67* was only differentially expressed at '12 d wet + 8 d dry',

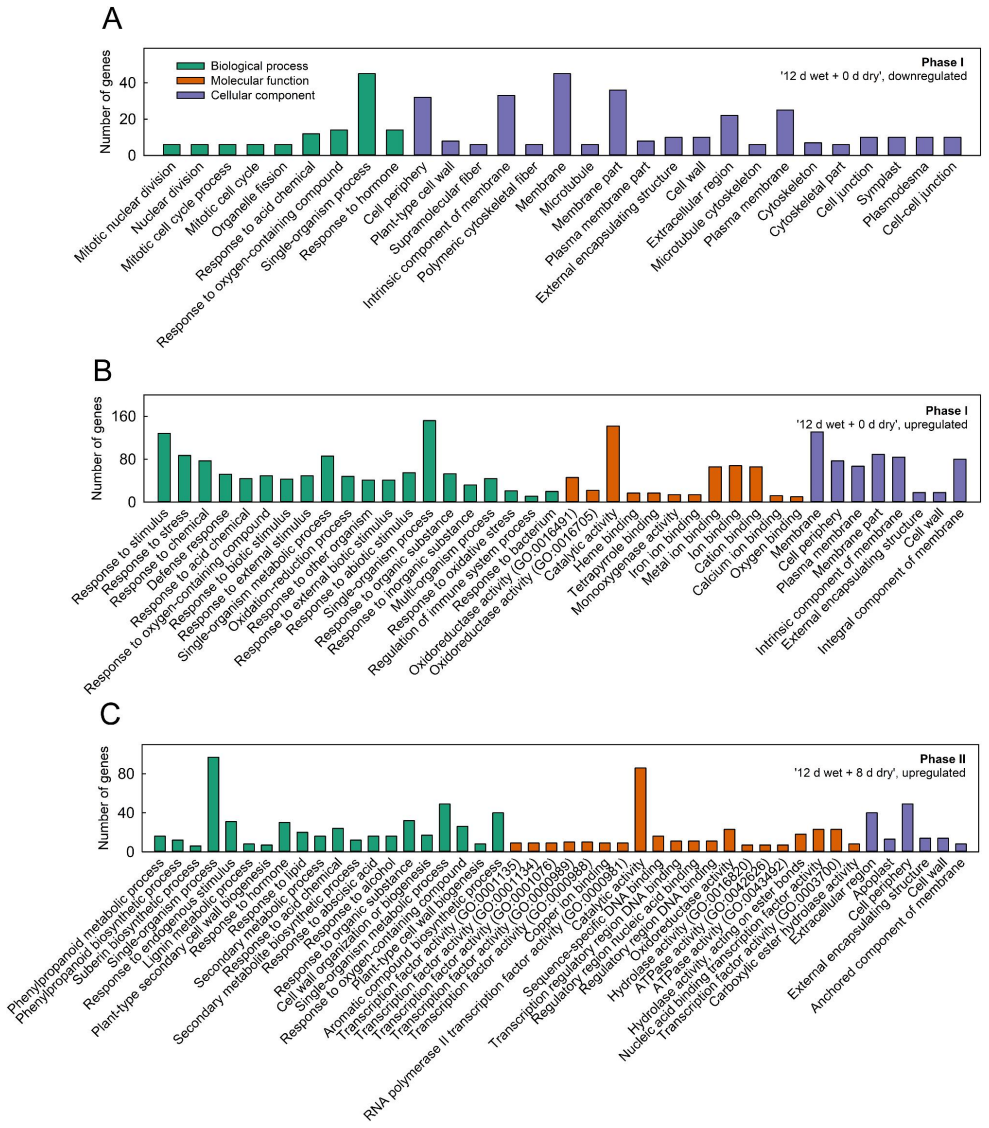

Fig. 3 GO term analysis following moisture-induced russeting of apple. Russeting on 'Pinova' apple fruits was induced by moisture in a two-phase experiment. During Phase I, a patch of fruit skin was exposed to surface moisture for 12 d (Phase I). After termination of moisture exposure (Phase II), the treated skin patch was exposed to the ambient atmosphere. The nontreated controls remained dry during Phase I and Phase II. Treatments and respective controls are listed in Table S3. Moisture exposure began at 21 or 31 days after full bloom (DAFB) during the 2018 and 2019 seasons. The GO term analysis indicated weakening of the cell structure during Phase I and hormone-regulated repair mechanisms of microcracks during Phase II. Common DEGs at '12 d wet + 0 d dry' **(A, B)** and '12 d wet + 8 d dry' **(C)** identified by the Venn diagrams (see Fig. 2) were subjected to singular enrichment analysis (SEA) to obtain GO terms associated specifically with Phase I or Phase II. The top 20 GO terms for biological process, molecular process and cellular component are shown, which were derived from the orthologous genes found in the TAIR10 database. Only GO terms with a minimum of five genes and an FDR ≤ 0.01 were selected

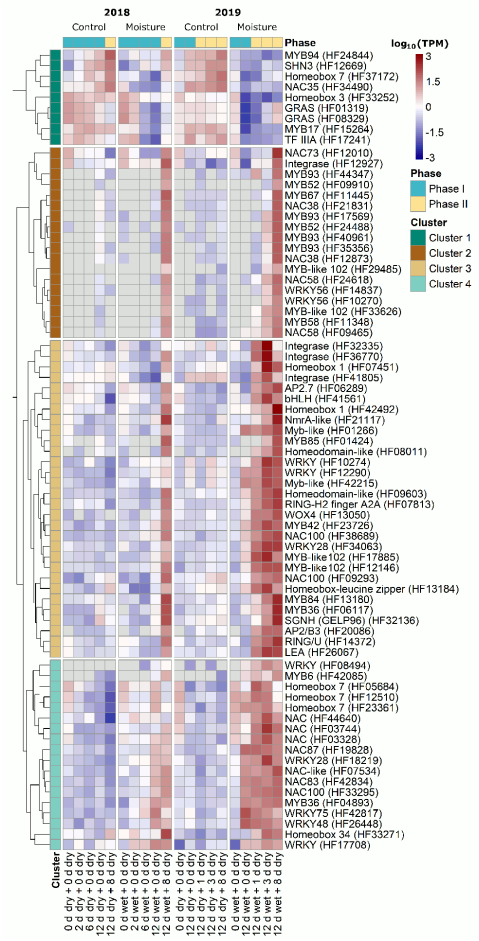

Fig. 4 Heatmap illustrating distinct expression patterns of transcriptional regulators during moisture-induced russeting. Russeting in 'Pinova' apples was induced in a two-phase experiment: During Phase I, a patch of fruit skin was exposed to surface moisture for 12 d (Phase I, '12 d wet'). After termination of moisture exposure (Phase II), the treated skin patch was exposed to the ambient atmosphere ('y d dry'). The nontreated control ('Control') remained dry during Phase I and Phase II ('x d dry + y d dry'). The heatmap revealed a dense network of transcriptional regulators that were differentially expressed during the early phase of russet formation. Cluster 1 contains Phase I-related genes, and Clusters 2 to 4 contain Phase II-related genes. Expression values are the mean $\log_{10}$(TPM) values of three independent biological replicates comprising six (season 2018) or ten (season 2019) fruits each. Genes with a $\log_2$FC ≥ 2 or ≤ -2, an FDR ≤ 0.05 and a mean of at least five TPM in 'Moisture' or 'Control' at any time during the two seasons are illustrated. Gene clusters were obtained via hierarchical clustering with the R package 'pheatmap'

when the first phellem cells had formed (Fig. 5, Figure S1).

Expression patterns of DEGs match russet susceptibility in cultivars differing in russet susceptibility

To confirm a role in russeting, the expression pattern of the selected DEGs was studied in four cultivars differing in russet susceptibility. Russet susceptibility decreased from 'Karmijn'>'Pinova'>'Idared'>'Gala', as indexed by the portion of russeted surface area within the moisture-exposed skin patch [44] (Figs. S3, S4). Downregulation of the Phase I-related gene *MYB17* correlated with the degree of russet susceptibility (Fig. 6). Only for *NAC35* was there no relationship to russet susceptibility (Fig. 6).

Generally, the expression patterns of Phase II-related genes (*LEA*, *WOX4*, *AP2B3*, *MYB52*, *MYB67*, *MYB84*, *MYB93*, *MYB102*, *WRKY56* and *SGNH*) corresponded to the extent of microcracking during Phase I (Figure S5) and matched the degree of russet susceptibility of the four cultivars during Phase II (Fig. 6, Figure S3).

Phase II genes display similar expression patterns in samples where russeting is induced by moisture or by mechanical wounding

Mechanical wounding of apple fruit skins induced russeting. Hence, the expression patterns of the DEGs were also analyzed following wounding [43].

The Phase I-specific transcription factors *MYB17* and *NAC35* were downregulated immediately after wounding (Fig. 7).

AP2B3, *WOX4*, *MYB84*, *MYB102*, *MYB52*, *WRKY56* and *LEA* were upregulated 2 d after wounding, and *SGNH*, *MYB67* and the suberin-specific gene *MYB93* were upregulated after 4 d (Fig. 7). The expression of *AP2B3*, *LEA* and *MYB102* peaked at 2 d and decreased to a constant level thereafter. The expression of *MYB67* was similar to that of *MYB93* and *SGNH*, although the increase was somewhat smaller. Similar DEGs were identified in the cultivar comparison following wounding. There was no relationship between the russet susceptibility of the cultivars and the DEGs (Figure S6). In contrast to moisture-induced russeting, which induced russeting only in a fraction of the exposed skin patch, russeting following wounding covered the entire area of the wounded patch in all four cultivars (Table S4).

Discussion

Our discussion focuses on (1) the suitability of moisture-induced microcracking for studying russeting in apples, (2) the changes in the transcriptome occurring during Phase I and (3) those occurring during Phase II of moisture-induced russeting.

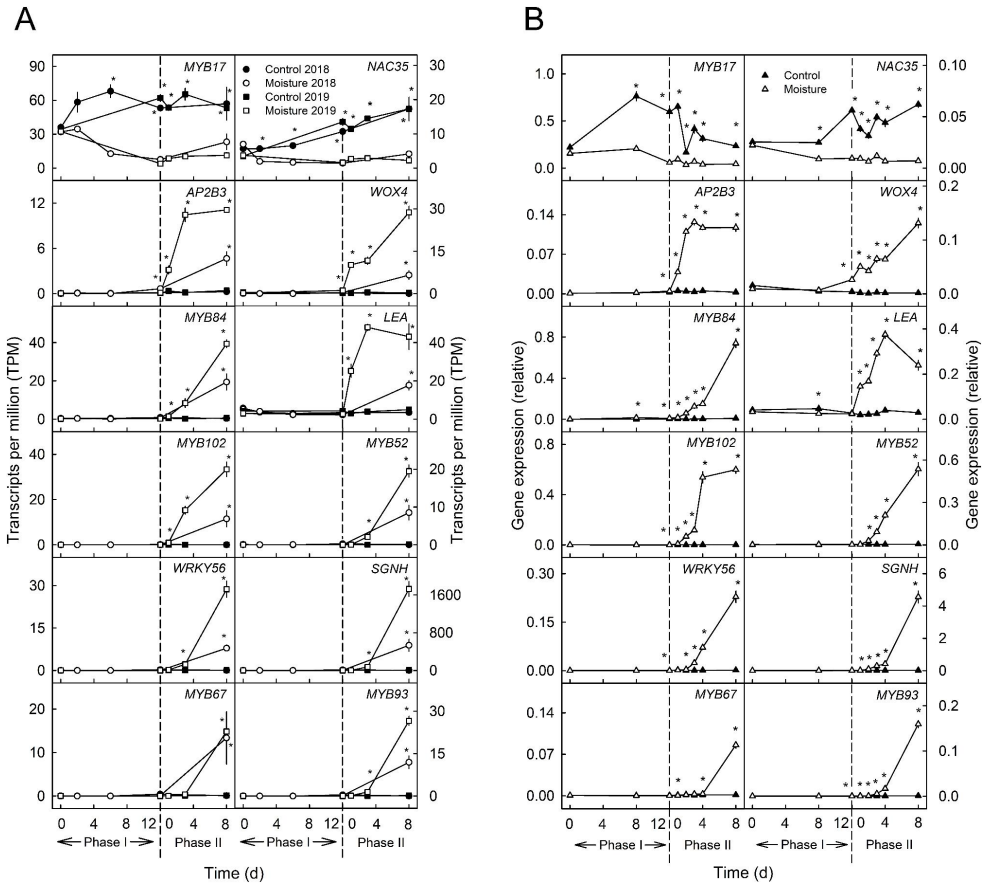

Fig. 5 Comparison of gene expression results obtained by RNA-Seq **(A)** and qPCR **(B)**. The data obtained by the two methods reveal consistent gene expression. Russeting in 'Pinova' apples was induced in a two-phase experiment: During Phase I, a patch of fruit skin was exposed to surface moisture for 12 d (Phase I, '12 d wet'). After termination of moisture exposure (Phase II), the treated skin patch was exposed to the ambient atmosphere ('y d dry'). The nontreated control ('Control') remained dry during Phase I and Phase II ('x d dry + y d dry'). Moisture exposure began at 21 or 31 days after full bloom (DAFB) during the 2018 and 2019 seasons. The dashed line indicates the termination of moisture exposure. Genes with specific patterns for Phase I and Phase II were analyzed. Expression values obtained from RNA-Seq data **(A)** represent means ± SEs of TPM of three independent biological replicates comprising six (season 2018) or ten (season 2019) fruits each. * indicates a significant difference between 'Moisture" and 'Control' at FDR ≤ 0.05. Expression values derived from qPCR **(B)** represent means ± SEs of three independent biological replicates comprising ten fruits each. '*' indicates a significant difference between 'Moisture' and 'Control' at $p \leq 0.05$ (Student's t test)

Moisture-induced russeting

Russeting in susceptible apple cultivars is triggered by a number of environmental factors [13, 14, 18–21, 36, 45–53]. Under field conditions, these factors are impossible to control, resulting in high variability of russeting within a tree, between trees, and between orchards, regions and seasons.

This makes systematic studies on russeting and the identification of triggers of russeting at a molecular level difficult. Moisture-induced russeting is a promising system that offers several advantages. First, surface moisture is a common factor in the natural russeting of apples [15, 18, 36, 54]. Second, experimental induction of russeting using moisture may be performed at the developmental stage where fruit is most susceptible to russeting. This is the first 40 days after full bloom [1, 12, 20, 33–36]. However, most studies of transcriptomes of russeted apple fruit are based on natural russeting assessed at the

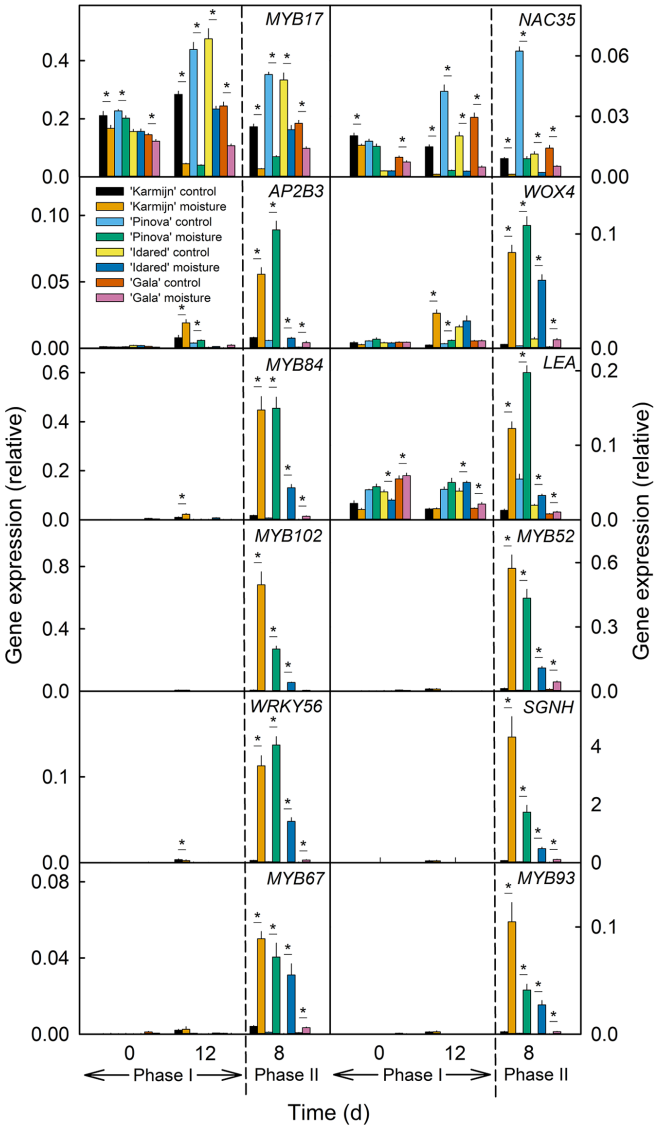

Fig. 6 Expression pattern of putative candidate genes during moisture-induced russeting of four apple cultivars. A two phase experiment was conducted to induce russeting in four apple cultivars ('Karmijn', 'Pinova', 'Idared', and 'Gala') that vary in their susceptibility to russet: During Phase I, a patch of fruit skin was exposed to surface moisture for 12 d (Phase I, '12 d wet'). After termination of moisture exposure (Phase II), the treated skin patch was exposed to the ambient atmosphere ('y d dry'). The nontreated control ('Control') remained dry during Phase I and Phase II ('x d dry + y d dry'). The dashed line indicates the termination of moisture exposure. The expression of genes associated with Phase I *(MYB17, NAC35)* as well as Phase II (*AP2B3*, *WOX4*, *MYB84*, *LEA*, *MYB102*, *MYB52*, *WRKY56*, *SGNH*, *MYB67*, *MYB93*) was analyzed. Expression values represent the means ± SEs of three independent biological replicates comprising six fruits each. '*' indicates a significant difference between 'Moisture' and 'Control' in each cultivar at $p \leq 0.05$ (Student's t test)

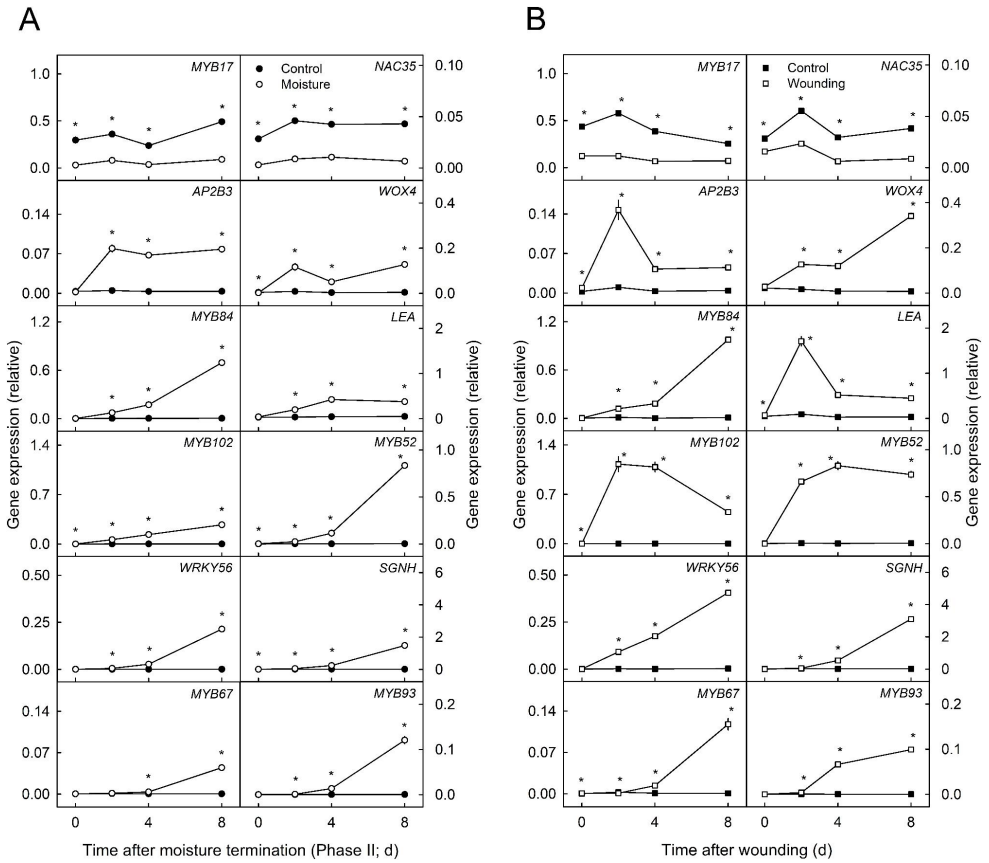

Fig. 7 Expression of putative candidate genes involved in russeting during moisture-induced **(A)** or wound-induced **(B)** russeting. Russeting in 'Pinova' apples was induced by surface moisture in a two-phase experiment: During Phase I, a patch of fruit skin was exposed to surface moisture for 12 d (Phase I, '12 d wet'). After termination of moisture exposure (Phase II), the treated skin patch was exposed to the ambient atmosphere ('y d dry'). The nontreated control ('Control') remained dry during Phase I and Phase II ('x d dry + y d dry'). Russeting was also induced by mechanical wounding using sandpaper ('Wounding'). The nontreated fruit skin served as a control ('Control'). The data revealed similar expression patterns between the two types of russet induction. Gene expression of candidate genes for the onset of periderm formation was determined at 0, 2, 4 and 8 d after moisture termination **(A)** or after wounding **(B)**. Expression values represent the means ± SEs of three independent biological replicates comprising six fruits each. '*' indicates a significant difference between 'Moisture' and 'Control' or 'Wounding' and 'Control' at $p \leq 0.05$ (Student's t test)

mature stage [24–26, 29–32]. Furthermore, the beginning of russet induction is precisely defined in moisture-induced russeting. In contrast, when assessing russeting at the mature stage, the time of the onset of russeting is unknown. This makes it impossible to establish causal relationships between potential trigger(s) of russeting. Third, our system of moisture-induced russeting allows us to compare the transcriptomes of the nonrusseted control and the russet-induced skin patch on an individual fruit basis. Thus, differential gene expression is standardized for differences between cultivars, stages of fruit development and environmental factors to which the fruit is exposed in the tree canopy. This is not the case when susceptible and resistant cultivars are compared. In the latter case, differences between cultivars and the specific environment of the fruit cannot be separated from genetic differences in russet susceptibility. These arguments demonstrate that moisture-induced russeting offers a high degree of control. The system was successfully used previously [13, 14, 21, 43]. The data obtained

demonstrate that during Phase I within 48 h of moisture exposure, cuticle synthesis decreases, and microcracks are formed. The microcracks represent the first visible symptoms of russeting [10, 12, 15]. During the subsequent Phase II after moisture removal, microcracks are exposed to the ambient atmosphere, and the periderm begins to differentiate [13, 21, 43]. Stringent filtering of DEGs combined with high sequencing depth (59.8 to 75.1 million read pairs per biological replicate) allowed us to also identify lowly expressed genes such as transcription factors relevant to russeting. Furthermore, performing the experiment in different growing seasons allowed us to identify consistent changes between the two seasons. This comparison demonstrated a remarkable degree of overlap that was further confirmed by qPCR of selected genes in subsequent seasons. In addition, Phase II processes were consistently altered in the wounding treatments. Like exposure to surface moisture, the developmental stage of the wounding treatment is well defined, and the treatment is performed during the phase of maximum susceptibility to russeting. Based on these arguments, the induction of russeting by moisture exposure or wounding is a helpful tool in identifying triggers of russeting.

Genes differentially expressed in phase I

Phase I of russet induction was characterized by a large number of downregulated genes related to either cutin and wax synthesis (*SHN3* [37], *GPAT6* [55], *WSD1* [56], *ABCG11* [57], *LTP3* [30]), transcriptional regulation (*MYB17*, *NAC35*) or cell cycle and microtubule formation (*Tubulin/FTsZ family protein* (HF08104) and *ATP binding microtubule motor family protein* (HF30539)). These data are consistent between RNA-seq and qPCR. They also confirm the findings of earlier studies on cutin and wax deposition in relation to moisture-induced russeting [21, 43].

The downregulation of genes involved in cuticle formation is considered to be an early factor associated with microcracking during Phase I [23–26, 30]. We therefore compared the transcriptional dynamics of these genes to those of our new set of candidate genes in our RNA-Seq dataset (Figure S7). Cuticle-related genes decreased during Phase I after 6 d of moisture exposure ('6 d wet+0 d dry') in 2018 and after 12 d ('12 d wet+0 d dry') in both seasons (Figure S7). Several regulators in Cluster 1 (Fig. 4) were downregulated, including *homeobox 7*, *NAC35*, two *GRAS transcription factors*, *MYB17*, and *TF IIIA*. These were downregulated before the cuticle-related genes *SHN3*, *ABCG11*, *GPAT6*, *KCS10*, *WSD1* and *CER6* (Figure S7) were downregulated during Phase I.

The transcription factors *MYB17* and *NAC35* were the earliest genes downregulated after the beginning of the moisture treatments. The expression pattern of *MYB17* correlated closely with that of *SHN3* [24, 38, 39]. However, the downregulation of *MYB17* occurred slightly earlier during Phase I as well as to a greater extent. *MYB17* is highly similar to *AtMYB16* and *AtMYB106*. The last two are involved in the regulation of epidermal cell growth and cuticle formation [58, 59]. A putative role of *MYB17* in cuticle formation is also consistent with the cultivar comparison of moisture-induced russeting (Fig. 6) and the experiment on wound-induced russeting (Figure S6). Here, the expression of *MYB17* was much lower in susceptible cultivars than in resistant cultivars. Wounding resulted in decreased expression of *MYB17*.

The second transcription factor, *NAC35*, was chosen because overexpression of *AtLOV1*, an ortholog of *NAC35* in *Arabidopsis thaliana*, changed epidermal cell organization and increased lignin content in cell walls when overexpressed in switchgrass [60]. The *MYB17* expression patterns of NAC35 were consistent between the qPCR experiments and the RNA-Seq analysis.

The expression of *MYB17* and *NAC35* after moisture treatment was also confirmed in a fourth experiment in which russet induction by wounding and moisture was compared. In both treatments, *MYB17* and *NAC35* were downregulated. This downregulation is in line with that of other cuticle-specific genes, such as *SHN3*, *GPAT6*, *KCS10*, *WSD1*, *CER6* and *ABCG11*, described in earlier studies [43]. However, their differential regulation after mechanical wounding indicates that the downregulation is not related to microcracking typical of Phase I of russet induction but rather to the tissue damage that accompanies skin cracking.

Genes upregulated during Phase I were stress response genes such as *1-aminocyclopropane-1-carboxylic acid (acc) synthase 6* (HF20852), *peroxidase superfamily protein* (HF39739), and *heat shock protein 70* (HF0032) and genes related to oxidative stress, osmotic stress and salt stress. There were no upregulated genes that are involved in periderm formation.

Interestingly, the regulation of genes as indexed by the $\log_2$FC in expression was larger in the 2019 than in the 2018 growing season. This was consistent with more severe russeting in 2019 than in 2018, probably as a result of seasonal differences in temperature and rainfall (Supplementary data, [13]) (Figure S2).

The mechanism of moisture-induced microcracking of the cuticle is probably related to failure of the hydrated cuticle when exposed to growth stress and strain. Cuticle hydration decreases the fracture force, which facilitates microcracking [61]. Additionally, the growth strain is particularly high during early fruit development, when the growth rate in surface area is high relative to the surface area present at that time (Figure S8). Importantly, the mechanical properties of the cuticle do not differ between russet-susceptible and nonsusceptible cultivars

[44]. In apple, the epidermis and hypodermal cell layers form the structural backbone of the fruit skin [62]. Russet-susceptible cultivars differ from nonsusceptible cultivars in that they have a higher variability of cell sizes in the epidermis and hypodermis [22]. Variable and larger cell sizes of the fruit skin cause stress concentration and failure when the skin is strained. During this process, the cuticle is dragged along and fails in response to the underlying cells [63]. Based on the above arguments, the change in mechanical properties of the cuticle and the decrease in cuticle deposition as a result of the down-regulation of genes involved in cuticle formation during a phase of high growth stress are causal in failure. Variable cell sizes predispose fruit skins to russeting.

Genes differentially regulated during phase II

Periderm formation occurs during Phase II. It requires the differentiation of a periderm in hypodermal cell layers underneath an epidermis with a microcracked cuticle. Periderm formation is a three-step process comprising (1) the formation of a meristem, the phellogen, that (2) then begins to divide to produce stacks of phellem cells. The final step in periderm formation (3) is the incrustation of the phellem cell walls with suberin and lignin. Earlier studies established that in moisture-induced russeting, this three-step process begins in Phase II only after removal of moisture when the treated skin patch is exposed to the ambient atmosphere [13, 21], irrespective of the duration of moisture exposure. Our findings are consistent with this conclusion.

Based on the above arguments, during **the early Phase II, differentially expressed genes should comprise genes characteristic of meristematic tissue.** This was indeed the case. Differentially expressed genes included various MYB, NAC, WRKY, and homeobox transcription factors, *WOX4*, *AP2/B3* and several LEAs, expansins, laccases and peroxidases (Figure S9, Data S6, S7). The expression of these genes increased immediately after moisture removal and exposure of the skin patch to the ambient atmosphere. Many of these genes are related to periderm formation (Figs. 4, [25, 30, 64–68]).

Recently published studies suggested that genes encoding proteins with acyltransferase or esterase/lipase activity, cell wall metabolism, pentacyclic triterpene synthesis, the phenylpropanoid pathway, suberin synthesis and transport of lipids are possible candidates in russeting [23, 25, 26, 30]. The cell wall-associated genes xyloglucan endotransglucosylases/hydrolases (XTH), expansins (EXP), peroxidases (PRX) and *laccase 7* (*LAC7*) increased in moisture-exposed patches during the transition from Phase I to Phase II at '12 d wet+0 d dry'. Three acyltransferases associated with triterpene-hydroxycinnamates as well as several genes associated with esterases/lipases (GDSL), pentacyclic triterpene synthesis, suberin synthesis, phenylpropanoid synthesis and lipid transport increased in gene expression at '12 d wet+3 d dry' or afterward (Figure S9). Transcriptional regulators found within Clusters 3 and 4 (Fig. 4) were upregulated earlier than most of the genes associated with phenylpropanoid or suberin synthesis (Figure S9), while genes found in Cluster 2 showed expression patterns similar to those of suberin-associated genes.

A total of four genes (*WOX4, AP2B3, LEA*, and *MYB84*) were validated by qPCR. The increase in expression was consistent between qPCR and RNA-Seq and occurred within 24 h (*WOX4, AP2B3, LEA*) and 48 h (*MYB84*) after exposure to the ambient atmosphere. Furthermore, the expression of all four genes was markedly higher in russet-susceptible cultivars than in nonsusceptible cultivars, implying a role in russeting.

The ortholog of *WOX4* in *Arabidopsis* [69–72] and poplar [73] is related to the formation of the vascular cambium. In moisture-induced russeting in apple, *WOX4* was among the earliest expressed genes in Phase II. In the 2019 and 2020 seasons (cultivar comparison), it was already expressed to some extent late in Phase I (Figs. 5 and 6). The upregulation, however, was restricted to cultivars of high susceptibility in Phase I (Fig. 6). In Phase II, *WOX4* was more regulated in susceptible than in resistant cultivars. Interestingly, *WOX4* was also expressed after mechanical wounding (Fig. 7, Figure S6). These arguments suggest that *WOX4* is a candidate gene for phellogen formation.

An ortholog of *AP2B3* in *Arabidopsis*, *AtNGA1*, regulates 9-cis-epoxycarotenoid dioxygenase 3 (AtNCED3), which is involved in ABA formation upon drought stress [74]. *AP2B3* was induced even earlier than *WOX4* (Fig. 5). The function of *AP2B3* is consistent with its expression during early Phase II. Moisture removal after Phase I increased water loss from the microcracked cuticle – the microcracks shunted the barrier properties of the cuticle [63]. The water loss, in turn, induced drought stress. In line with this, we found an ortholog of *NCED3* in apple (HF22773) that was differentially expressed in Phase II. *AP2B3* expression was also reported in russeted fruit at later developmental stages [25].

The early induction of LEA is consistent with the above arguments (Figs. 5 and 7). LEA proteins are known to be responsive to ABA and are enriched in response to abiotic stress, including drought [75]. The *LEA* gene in our study is an ortholog of *AtNHL26*, which is active within the phloem [76].

The DEG *MYB84* is an ortholog of *MYB1* of *Quercus suber*, where it is specific to phellem cells [66, 67]. Additionally, in *Arabidopsis* hypocotyls and roots, *MYB84*/*RAX3* are expressed in the periderm [65]. These arguments are consistent with a role of *MYB84* in the formation of the phellogen.

During the **later Phase II of periderm formation, we expect differential expression of genes related to the incrustation of cell walls with suberin and lignin.** This was confirmed in our experiment. The GO term analysis of the differentially expressed genes identified genes involved in suberin, phenylpropanoid and lignin metabolism and synthesis, genes involved in ABA metabolism and genes related to cell wall synthesis (Fig. 3). In addition, a number of transcription factors belonging to the MYB, WRKY and NAC families were found to be solely expressed during late Phase II (Fig. 4).

We selected six genes (*MYB93, MYB102*, *MYB52*, *WRKY56*, *SGNH*, and *MYB67*) with putative functions in suberin formation for further validation by qPCR. Again, the expression patterns obtained by qPCR and RNA-Seq were consistent. The increased expression of *MYB93* was consistent with that obtained in earlier studies [21, 43]. Its expression pattern perfectly mirrored the differential russet susceptibility in the cultivar comparison (Fig. 6). Additionally, the expression of *MYB93* after wounding further supported a role in russeting. In response to mechanical wounding, a periderm was induced after four days, which then began to divide to produce phellem [43]. The suberization of the cell wall is consistent with the expression of *MYB93*. *MYB93* has been reported to be involved in suberization of russet periderm [27]. *MYB93* was also reported to interact with other genes. When overexpressed in *N. benthamiana* leaves, *MYB93* induced the expression of *MYB52*, *MYB67*, *WRKY56* and *MYB84*, the last to a slightly lower extent.

Similar to *MYB93, MYB102* is another interesting candidate for periderm formation during late Phase II. The expression of its ortholog *AtMYB102* in *Arabidopsis* is directly induced by ABA. In *Arabidopsis thaliana*, ABA increased the suberization of roots [77]. Furthermore, *MYB102* responded to wounding [78], which is consistent with its role in the late phase of periderm formation.

Similarly, *AtGELP96*, an ortholog of *SGNH*, has key functions in the polymerization of suberin together with four other GELPs (*GELP22*, *GELP38*, *GELP49*, and *GELP51*) in *A. thaliana* roots [79]. This finding supports the putative functions of these genes in the accumulation of suberin in phellem cells after the phellogen has developed. Both the expression pattern and the annotations of *MYB52*, *MYB67*, *MYB102* and *WRKY56* indicated that these genes also contributed to the differentiation of the developing periderm during late Phase II rather than the development of the phellogen. *MYB52*, *MYB67*, *MYB102* and *WRKY56* were all induced at later stages of periderm formation. Their expression patterns were highly correlated with the extent of russeting in the cultivar comparison (Fig. 6).

Conclusion

The analysis of the transcriptome during periderm formation revealed a distinct pattern of gene expression. Based on the expression profiles and the supposed functions in heterologous plant systems, the following sequence of events results in periderm formation and, hence, russeting (Fig. 8). The downregulation of genes involved in cutin and wax synthesis and deposition and the simultaneous change in the mechanical properties of the cuticle due to hydration result in microcrack formation during moisture exposure. After moisture removal, the tissue underneath the microcracks comes into contact with the ambient atmosphere. A cascade of transcriptional regulatory events is now initiated. The increase in transpiration caused by the impaired barrier properties of the cuticle locally induces water stress as indexed by the expression of stress-related genes. At the same time, a yet unknown trigger induces the differentiation of the phellogen, as indexed by the expression of genes related to meristematic activity during early Phase II. The subsequent incrustation of the phellem with suberin and lignin (late Phase II) is consistent with the expression of genes involved in suberin and lignin synthesis and the regulation thereof. Notably, the differentially expressed genes identified in the transcriptomic analysis of the developmental time course during Phase II were also observed in the comparison of cultivars varying in russet susceptibility and the response to mechanical wounding.

This study provides transcriptomic resources for early events of artificially induced russeting in apple and further data on the comparison of mechanically induced versus moisture-induced russeting in terms of the expression of selected genes, which may help finally identify the molecular triggers of russet induction.

Materials and methods

Plant materials

Apple fruits (*Malus x domestica* Borkh.) of 'Karmijn', 'Pinova', 'Idared' and 'Gala', all grafted on M9 rootstocks, were cultivated in experimental orchards of the horticultural research station of the Leibniz University Hanover at Ruthe (52° 14' N, 9° 49' E). These cultivars differ in susceptibility to russeting in the order 'Karmijn'>'Pinova'>'Idared'>'Gala' [44] (Figs. S3, S4).

A total of four experiments were conducted. First, the time course of change in the transcriptome was investigated in moisture-induced russeting in 'Pinova' using RNA-Seq and validated via qPCR. Samples were taken from a total of 125 trees. Second, gene expression in moisture-induced russeting was investigated in four cultivars differing in russet susceptibility using qPCR. The number of trees sampled was 30 per cultivar. Third, gene expression in wounding-induced russeting was investigated in four cultivars differing in russet susceptibility

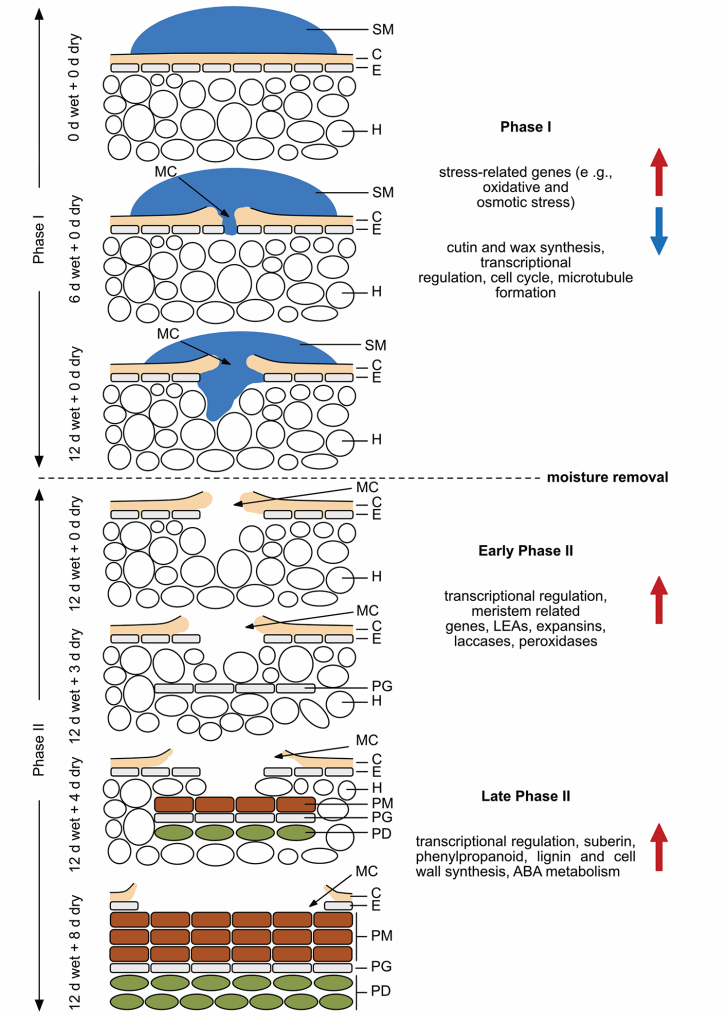

Fig. 8 Sketch of sequence of events in moisture-induced russeting of apple fruit skins. In Phase I, the skin patch is exposed to moisture for 12 d during early fruit development (21–31 days after full bloom (DAFB)). In Phase II, the moisture is removed and the fruit surface exposed to atmospheric conditions. In Phase I microcracks in the cuticle are detected as early as 2 d of moisture exposure. Over time, these microcracks expand tangentially and radially. They traverse the cuticle radially by day 6 of moisture exposure. As the fruit enters Phase II, meristem-related genes are activated indicating the formation of a phellogen in the hypodermis underneath a microcrack (0–3 d after moisture removal). During the late stage of Phase II (starting 3–4 d after moisture removal), the phellogen differentiates a phelloderm and produces suberized phellem cells. By 8 d after moisture exposure, a continuous periderm has developed. Gene groups that are up-regulated during each phase (Phase I, early Phase II, and late Phase II) are marked by a red arrow on the right side of the panel. Conversely, gene groups that are downregulated during these phases are indicated by a blue arrow. SM = surface moisture, C = cuticle, E = epidermis, H = hypodermis, MC = microcrack, PG = phellogen, PM = phellem, PD = phelloderm.

using qPCR. The number of trees sampled was 20 per cultivar. Fourth, gene expression was compared between moisture- and wounding-induced russeting in 'Pinova' using qPCR. Here, the number of trees was 125. Experiments were performed in four different growing seasons (Table S3, Figure S10).

Russet induction

Russeting was induced either by moisture exposure or by mechanical wounding [13, 14, 21, 43]. For moisture exposure, two-phase experiments were conducted (Figure S11). Apple fruits 10–12 mm in diameter (21–32 DAFB) were selected (Table S3, [13, 14, 21, 43]). The tip of a 2.0 ml polyethylene tube (Eppendorf, Hamburg, Germany) was mounted in the equatorial plain of the apple fruit using nontoxic silicone rubber (Silicone RTV; Dow Toray, Japan). After curing (approximately 1 h), the tubes were filled with 1 ml deionized water for moisture exposure (Phase I) through a hole in the tip. The hole was then sealed, and the tube was checked for leakage on a daily basis. The opposite side of the fruit served as a control and remained dry [13, 14, 21]. The fruit skin was exposed to moisture ('Moisture') for 12 d ('12 d wet+0 dry', ('Phase I+Phase II')) during Phase I. For termination of moisture exposure, the tube was removed, and the treated skin patch was exposed to the atmosphere (Phase II). At this point, the treatments were terminated. During the subsequent Phase II, changes in the treated fruit skin patches were observed for up to 136 d ('12 d wet+136 d dry') after termination of the moisture treatment (Phase II).

For wounding-induced periderms, the fruit skin was gently abraded in the equatorial plane using sandpaper (grit size 1000; Bauhaus, Mannheim, Germany) ('Wounding'). The opposite surface of the same fruit served as the control. Wounding was performed at 38–40 DAFB (Table S3). This time point corresponded to the time of moisture termination in the moisture-induced russeting experiment.

RNA extraction and quality assessment

Patches of treated, i.e., moisture-exposed or wounded, or nontreated, i.e., control, skins were excised using a razorblade, immediately frozen in liquid N_2 and held at -80 °C until further analysis. Each replicate comprised skin patches of a minimum of six fruits (approximately 60–80 mg). The tissue was ground in liquid N_2 to a fine powder using a mortar and pestle. Total RNA was extracted using the InviTrap Spin Plant RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) and lysis buffer RP according to the manufacturer's protocol. Total RNA was treated with DNase using the DNA-free™ Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove remaining DNA. The quantity and purity of RNA were determined photometrically at 230, 260 and 280 nm on a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The integrity and purity of the RNA were checked on a 1.5% agarose gel. Before RNA-Seq, the RNA integrity number (RIN) was determined using the Agilent RNA 6000 Nano Kit on a Bioanalyzer 2100 and the Plant RNA Nano parameters (Agilent Technologies, Santa Clara, CA, USA). The RIN ranged from 8.4 to 10.0 (Table S1).

RNA-Seq library preparation and sequencing

For each replicate, 1 µg of total RNA was sequenced (Novogene, Cambridge, UK). The library was prepared with the NEBNext® Ultra™ RNA Library Prep Kit (Ipswich, Massachusetts, USA) according to the manufacturer's instructions. For sequencing, 2×150 bp *paired-end* cDNA libraries were prepared. Sequencing was performed on an Illumina® NovaSeq™ 6000. A minimum of 59.8 million read pairs were generated for each sample (Table S1).

Mapping and counting of reads

Reads obtained from Illumina sequencing were trimmed and filtered with Trimmomatic (v0.39) [80] with the following parameters: TRAILING: 20 AVQUAL: 20 SLIDINGWINDOW: 5:20 MINLEN: 75. The quality of trimmed reads was checked by FastQC (v0.11.9) [81]. Afterward, reads were aligned to the *Malus x domestica HFTH1* v1.0 genome using STAR (v2.5.4b) followed by read count quantification with the "--quantMode Gene Counts" function [82, 83]. Annotations of transcripts were obtained by blastp against the *Arabidopsis thaliana* genome (TAIR10, www.arabidopsis.org, 31.01.2023) as described by Zhang and coworkers [83].

Differential gene expression and enrichment analysis

Differential gene expression analysis was conducted with DESeq2 (v1.32.0) [84]. Genes with a $\log_2$FC≥2, ≤ -2 ('Moisture' vs. 'Control') and a false discovery rate (FDR)≤0.05 were considered to be differentially expressed and used for downstream analysis. Gene abundance was obtained through transcripts per million (TPM) calculation with StringTie (v2.1.3) [85]. Singular enrichment analysis (SEA) was performed with DEGs having a mean of at least five TPM for 'Moisture' or 'Control' samples. Orthologous genes from *Arabidopsis thaliana* were investigated using the webtool AgriGO (v2.0) and the parameters selected species: *Arabidopsis thaliana*; reference: TAIR genome locus (TAIR10_2017), user defined; statistical test method: hypergeometric; multitest adjustment method: Hochberg (FDR); significance level: 0.01; and minimum number of mapping entries:

5 [86]. Heatmaps of differentially expressed genes were generated with the R package 'pheatmap' (1.0.12) [87].

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was conducted on a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Primer design, cDNA synthesis, primer efficiency testing and qPCR were performed as described earlier [21] (Table S5). Gene expression values were determined according to Pfaffl [88] with slight modifications described by Chen and coworkers [89]. Gene expression data were normalized using *PROTEIN DISULFIDE ISOMERASE* (PDI) (MDP0000233444) [90] and *MdeF-1alpha* (AJ223969.1) [26] as reference genes. Each data point comprised three independent replicates of two to three technical replicates each.

List of abbreviations

ABA	Abscisic acid
AP2B3	*AP2/B3-like transcription factor family protein*
AtNCED3	9-cis-epoxycarotenoid dioxygenase 3
C	Cuticle
DAFB	Days after full bloom
DEGs	Differentially expressed genes
E	Epidermis
EXP	Expansins
FDR	False discovery rate
GDSL	Esterases/lipases
GO	Gene ontology
H	Hypodermis
LAC7	Laccase 7
LEA	*Late embryogenesis abundant hydroxyproline-rich glycoprotein*
log_2FC	Log_2-fold change
MC	Microcrack
MYB102	*MYB-like 102*
PCA	Principal component analysis
PD	Phelloderm
PDI	*Protein disulfide isomerase*
PG	Phellogen
PM	Phellem
PRX	Peroxidases
qPCR	Quantitative real-time PCR
QTLs	Quantitative trait loci
SEA	Singular enrichment analysis
SGNH	*SGNH hydrolase*
SM	Surface moisture
TPM	Transcripts per million
>WOX4	*Wuschel-related homeobox 4*
XTH	Xyloglucan endotransglucosylases/hydrolases

Supplementary Information

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Supplementary Material 1
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Data Availability
The datasets supporting the conclusions of this article are included within the article and its additional files. Raw Illumina sequencing data are available at the NCBI Sequence Read Archive (SRA) under the BioProject number PRJNA935373.

Declarations

Ethics approval and consent to participate
We declare that the experimental research and field study conducted on *Malus x domestica* Borkh. plants in this manuscript, comply with all relevant institutional, and international guidelines and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Links for review
SRA BioProject (publicly available upon publication): https://dataview.ncbi.nlm.nih.gov/object/PRJNA935373?reviewer=efbmo3ejavqu9s1aeom137tpni.

Data access for review:
https://my.hidrive.com/share/wymikjrtwa. Password: Russeting.

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2.6 Establishing a detached fruit system for russeting study in apple

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Establishing a detached fruit system for russeting studies in apple

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Abstract

Russeting is an important surface disorder that causes economic loss in apples. The etiology of russeting is associated with cuticular microcracking which leads to an impaired barrier. To date, some of the sequence of events following barrier impairment has been characterized, but the signal(s) linking microcracking to the onset of russeting remain unknown. Atmospheric O_2 has been considered as a potential trigger in this process based on its promoting role in periderm formation in other plant species. The objectives of the present study were (1) to establish a detached fruit system in young apples and (2) to use this system under anoxia to investigate the role of partial pressure of atmospheric O_2 on wound-induced periderm formation. The results showed that (1) the detached fruit did not perform as well as the attached fruit in regard to the response to wounding and (2) anoxia suppressed six selected genes involved in lignin and suberin metabolism, but did not completely prevent periderm formation. The attribute of these problems may be the sensitivity of the young fruit to the detachment. In conclusion, the established detached fruit system in young apple is not suitable for russeting studies such as investigating the potential trigger, e.g. O_2, using controlled environments.

Keywords anoxia · apple · cuticle · detached fruit · *malus* × *domestica* · microcrack · periderm · russeting · suberin · wounding

Abbreviations:

ABCG20: ATP Binding Cassette Transporter G family member 20

CO_2: Carbon dioxide

CYP86B1: Cytochrome P450, family 86, subfamily B, polypeptide 1

DAFB: Days after fullbloom

GPAT5: Glycerol-3-phosphate acyl transferase 5

MYB42: myb (myeloblastosis) domain protein 42

MYB93: myb (myeloblastosis) domain protein 93

O_2: atmospheric oxygen

SGNH: SGNH hydrolase-type esterase superfamily protein

Introduction

Russeting is an important surface disorder that occurs in many fruit crops (Winkler et al., 2022). In apple (*Malus* x *domestica*, Borkh.), russeting causes higher water loss rate, resulting in fruit shrinkage and consequent economic loss (Khanal et al., 2019; Skene, 1982a,b).

The typical dull and brownish appearance of russeting is attributed to the phellem cells, which are the outermost layer divided by the phellogen (i.e., cork cambium) of a periderm. Previous studies have shown that (1) russeting is associated with cuticular microcracks (Faust and Shear, 1972); and (2) a periderm initiates near the location of microcracks on the apple fruit surface (Meyer 1944; Pratt 1972). Both moisture and wounding induce russeting in apple (Chen et al. 2020; Khanal et al. 2021; Simons and Aubertin 1959; Skene 1981; Straube et al., 2021). Both result in similar initial events at histological and molecular levels and similar chemical constituents of the mature periderms by generating an impaired barrier through microcracking in young apples (Chen et al., 2022). Although the sequence of events following a barrier impairment has been progressively characterized, the signal(s) that link cuticular microcracking to the differentiation of a phellogen in the underlying cell layers remains unknown. The following three potential factors could be locally altered after a barrier impairment: (1) an increase in the internal partial pressure of oxygen (O_2) and/or (2) a decrease in the internal partial pressure of carbon dioxide (CO_2) and/or (3) a decrease in the water potential of the flesh due to water loss.

The objective of this study was to investigate the role of partial pressure of atmospheric O_2 on wound-induced periderm formation in young apples. To accomplish this technically, a detached fruit system was first established so that the fruit could be incubated in a controlled environment for a few days. O_2 was chosen as a factor because higher O_2 partial pressures are known to accelerate periderm development and russeting in other crops (Lipton, 1967; Wei et al., 2018; Wigginton, 1974). Wounding was adopted as the induction method for russeting because of its convenience and its role as a substitute for surface moisture in causing an impaired barrier (Chen et al., 2022).

Material and methods

Plant material

'Pinova' apple trees (*Malus* × *domestica* Borkh.; grafted on 'M9' rootstocks) grown in an orchard of the horticultural station of Leibniz University Hannover (LUH) at Ruthe (lat. 52˚14’N, long. 9˚49’E) were used for the experiments. The horticultural practices were in accordance

with the current regulations for integrated fruit production. Fruits of uniform size and free from damage were randomly selected and sampled from 80 trees in two adjacent rows.

Treatments

To induce wound periderm, abrasive sandpaper (grit size 1000; Bauhaus, Mannheim, Germany) was used to wound the fruit skin. The opposite side of the fruit served as a control in all experiments. The effectiveness of the wounding at the early fruit development (38 days after full bloom; DAFB) was demonstrated in the russeting on mature attached 'Pinova' fruit (109 d after wounding; **Fig. S1).**

Methods

Microscopy

The fruits were preserved in Karnovsky fixative (Karnovsky, 1965). After fixation, the fruits were cut into tissue blocks and subsequently embedded in paraffin following the procedure described by Chen et al. (2020). In brief, the blocks were first rinsed with deionized water to remove any residual fixative and then incubated in 70% (v/v) aqueous ethanol overnight. The following day, the tissue blocks were dehydrated using a series of ethanol concentrations (70%, 80%, 90%, and 96% v/v), with each concentration applied for 30 min. The blocks were then immersed twice (40 min each) in 100% isopropanol to displace any remaining ethanol. A xylene substitute (AppliClear; AppliChem, Münster, Germany) was then used to replace the isopropanol. To facilitate paraffin infiltration into the tissue, the blocks were incubated with a mixture of paraffin and the xylene substitute (1:1, v/v; Carl Roth, Karlsruhe, Germany) for 40 min and then treated twice (40 min each) with fresh paraffin at 60 °C. All incubations were performed at a reduced pressure within a vacuum desiccator (10.8 kPa) to facilitate the infiltration process. The infiltrated tissue blocks were embedded in paraffin using a metal mold. Specimens were stored at 4 ˚C until microscopic examination.

A rotary microtome (Hyrax M 55; Carl Zeiss, Oberkochen, Germany) was used to obtain thin sections from the tissue blocks. These sections were then transferred onto glass slides and left to dry overnight at 38 ˚C. Paraffin was then removed using the xylene substitute (two 10-min immersions; AppliChem) and the sections were rehydrated through a descending ethanol series (96%, 80%, 70%, and 60% in a v/v ratio, each for 10 min), followed by two 5-minute immersions in fresh deionized water. To visualize the suberization of the phellem cell walls, the sections were stained with Fluorol Yellow 088 (Santa Cruz Biotechnology, TX, USA) at a final concentration of 0.005% (w/v). The stain was prepared by mixing it with a combination of glycerol (90% v/v; Carl Roth) and melted polyethylene glycol 4000 (PEG 4000; w/v; Carl Roth) in a 1:1 ratio. Sections were incubated in this staining solution for 1 hour. After rinsing with water, the sections were examined under both bright light and incident fluorescent light (U-

MWB; 450-480 nm excitation; ≥520 nm emission wavelength; Olympus Europa SE & Co. KG, Hamburg, Germany) using a fluorescence microscope (BX-60 equipped with a DP 73 digital camera; Olympus Europa).

Two indices were used to evaluate and quantify periderm development: (1) sections with periderm and (2) the number of suberized phellem layers. For (1) and (2), a minimum of 72 sections (as technical replicates) were examined per biological replicate (one fruit), and a minimum of seven biological replicates were performed for each treatment at each sampling time. For (1), the criterion for counting the presence of periderm ("with periderm") was the presence of one or more suberized phellem layers. The percentage (%) was obtained by dividing the sections with periderm by the total number of sections examined. For (2), counts were made in three selected and representative images per biological replicate and at three locations within the image (700 µm wide).

RNA Isolation

Skin patches from the wounded or unwounded control area of the fruit were excised with a razor blade and immediately frozen in liquid nitrogen. Frozen patches from six fruits were pooled to form one biological replicate. The frozen patches were later ground to powder and extracted for RNA using the InviTrap Spin Plant RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions. The DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to eliminate potential genomic DNA (gDNA) contamination. The quality and quantity of RNA samples were determined using a spectrophotometer measuring absorbance at 230 nm, 260 nm, and 280 nm (Nanodrop 2000c; Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed by running samples on a 1.5% agarose gel. All RNA samples were stored at -80°C prior to further analysis.

Bioinformatics and gene expression

Six putative genes involved in lignin and suberin metabolism in apples were identified based on previous studies (Straube et al., 2021, 2023) (**Table S1**). The specific primer details are listed in **Table S2.**

The procedure of gene expression analysis followed Straube et al. (2021). In brief, a single RNA sample (900 ng) was first converted into cDNA using the LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA). For quantitative real-time PCR (qPCR), the cDNA, primers (final concentration: 200 nM) and the Luna® Universal qPCR Master Mix (New England Biolabs) were performed on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA). The qPCR conditions included an initial denaturation step at 95 ˚C for 60 s, followed by 40 cycles of at 95 ˚C for 15 s and at 60 ˚C for 60 s. Upon completion of the amplification cycles, a melting curve analysis was performed.

The melting curve conditions included heating the samples to 95 ˚C for 15 s followed by 60 ˚C for 60 s. The temperature was increased from 60 to 95 ˚C in 0.5 ˚C increments. Expression values were calculated using the method described by Pfaffl et al. (2001) with modifications according to Chen et al. (2019). Expression was normalized against the two reference genes *Protein disulfide isomerase* (*MdPDI*; Storch et al., 2015) and *MdeF-1 alpha* (Legay et al., 2015).

Experiments

Experiment 1: Comparison of attached and detached fruit

To investigate the similarity in performance and response to wounding between attached and detached fruits, a comparison was made under ambient conditions (21% O_2). Detached apple fruits were carefully collected by cutting them directly from the tree and placing the pedicel through the hole of a 15 ml tube filled with autoclaved deionized water. The pedicel was then re-cut under water and the base (cavity) of the fruit was sealed to the lid of the tube using a non-phytotoxic silicone rubber (Dowsil™ SE 9186 Clear Sealant, Dow Toray, Tokyo, Japan). Since the large number of samples made it impractical to start the experiments on the same day as the fruit collection, detached apple fruits were prepared at 37 DAFB and kept in the field or transported to the laboratory on the second day (one day after detachment). Attached apple fruits, which remained on the trees in the orchard, were used for comparison. Both detached and attached fruits were then wounded on the same day (38 DAFB) and sampled at 0 and 8 days after wounding for histology and gene expression analysis. To maintain longevity, the detached fruits were transferred daily to new tubes with fresh autoclaved deionized water.

Experiment 2: Comparison of 0% and 21% of oxygen atmospheres

To investigate the effect of partial pressure of O_2 on wound periderm formation, a comparative study was performed using two different atmospheric conditions: 0% and 21% O_2, was performed. In this experiment, only detached fruits were used. The apple fruits used to assemble the detached fruits were obtained from the same sources as described in **Experiment 1**. The detachment of fruit followed the same procedure.

The detached fruits were either placed in an anaerobic chamber (Plas-Labs, Inc., Lansing, MI, USA; 0% O_2) or kept in the normal laboratory atmosphere with 21% O_2. To achieve an anoxic (0% O_2) environment for the wounding procedure, the following steps were taken: First, the detached fruits were placed in a vacuum chamber connected to the anaerobic chamber. Vacuuming reduced the pressure in the chamber to 61 kPa. The vacuum chamber was then flushed with forming gas, a mixture of 95% nitrogen and 5% hydrogen, for three cycles. The fruits were then transferred from the vacuum chamber to the main chamber and allowed to incubate for a minimum of 30 minutes prior to wounding. Two catalyst heaters were used to eliminate O_2 throughout the experiment. For daily water replacement of the fruits incubated in

the anaerobic chamber (0% O_2), degassed autoclaved water was prepared at 50 mbar for 1 hour in a vacuum desiccator chamber.

To ensure that anoxic conditions were maintained during sampling, the fruits were carefully transferred into mouth vials. These vials were chosen to maintain the anoxic state. Sampling was performed within 30 min of fruit removal from the chamber. Sampling times for histology and gene expression analysis were 0 and 8 days after wounding.

Data analysis

Data were subjected to either Student's t-test or one-way analysis of variance (ANOVA) following mean comparison with Tukey's studentized test (HSD). All the statistical analyses were performed on the web-based SAS OnDemand for Academics (SAS Institute, Cary, NC, USA). The significance of P-values was set to 0.05 and indicated by an asterisk ('*'; t-test) or the different letters (ANOVA). All data in the present study are presented as means ± standard error (SE) of the means. Percentage data (%) were converted by arcsine transformation before analysis of variance.

Results

Sandpaper wounding of 'Pinova' apple fruit surfaces successfully induced microcracks and wound periderm after 8 d, as observed microscopically (**Fig. 1a,b**). Although both attached and detached fruits were inducible for wound periderm, the detached fruits had less extent of periderm development in the field or laboratory, as indexed by the number of microtome sections with periderm and suberized phellem cell layers (**Fig. 1c,d**).

Anoxia delayed wound-induced periderm formation on wounded apple fruit skin patches at 8 d of wounding (**Fig. 2a,b**). Compared to the ambient atmosphere (21% O_2), detached apple fruit wounded under the anoxia (0% O_2) had significantly slower periderm development in both microscopic sections and suberized phellem cell layers (**Fig. 2c,d)**.

The expression of putative genes involved in the regulation of lignin (*MdMYB42*) and suberin (*MdMYB93*) synthesis, synthesis (*MdCYP86B1* and *MdGPAT5*), transport (*MdABCG20*) and polymerization (*MdSGNH*) of suberin monomers was analyzed in both experiments (**Fig. 3**). Comparing attached and detached fruits at 0 d, *MdMYB42* was already up-regulated in both the wounded and unwounded skin patches of the latter. In the former, all genes except *MdMYB42* were more highly expressed in the wounded skin patch at 8 d (**Fig. 3a,c,e,g,i,k**). As expected, anoxia delayed the expression of these six genes at 8 d (**Fig. 3b,d,f,h,j,l**).

Discussion

The main findings of the present study were: (1) the detachment of the apple fruit did not respond to wounding as the attached apple fruit and (2) anoxia suppressed genes that are involved in lignin and suberin- metabolism but did not completely prevent the formation of a wound periderm.

The detachment of young fruits is not suitable for russeting studies because of the following problems: First, a significantly less extent of periderm development is found after 8 d of wounding compared to the attached fruit. Second, a less extent of inducibility of the select genes that are involved in: the regulation of suberin synthesis (*MdMYB93*) (Legay et al. 2016), suberin synthesis (*MdCYP86B1* and *MdGPAT5*) (Compagnon et al. 2009, Beisson et al. 2007), suberin transport (*MdABCG20*) (Yadav et al. 2014), and suberin monomer polymerization (*MdSGNH*) (Ursache et al., 2021) in the detached wounded apple fruit after 8 d of wounding. It is worth noting that the gene *MdMYB42*, which is involved in lignin synthesis (Geng et al., 2020), has an unexpected expression pattern from the day of wounding (i.e., 1d after detachment), as it is already induced in the control (unwounded) detached fruits. Third, many detached fruits showed surface rot after a few days (data not shown). Loss of fruits due to surface rot would increase the total number of fruits prepared, especially if longer sampling times and larger replicates are required.

It can be then concluded that the young apple fruit, at least that of ‘Pinova’, is very sensitive to the detachment from the tree, resulting in a reduced healing ability to seal the wounded surface (i.e. less suberization and corresponding gene expression). The reason for this could be the sum of a series of consequences of the detachment. First, the expansion of the young fruit is a basis for microcracking and the consequences. After the detachment, the fruit does not grow and expand as quick as the attached ones (data not shown) due to the disconnection from the support and transport from the tree through the vascular system. This leads to a reduced gaping of the wound and hence, a less extent of periderm development (i.e. the expansion of the periderm and the number of suberized phellem layers). Second, it is shown that detachment of mature fruits of ‘Golden Delicious’, the maternal parent of ‘Pinova’ (Fischer and Fischer, 2002), releases significantly higher levels of ethylene than attached fruits. (Lau et al., 1986; Suzuki et al. 1997). Similarly, in ‘Gala’, whose maternal parent is also 'Golden Delicious' (National Fruit Collection, 2023), the fruit detachment at stages close to or at commercial maturity result in ethylene production, and the earliest two stages (green and near mature) have higher production in 'Gala' (Lin and Walsh, 2008). This suggests that during early development, when the apple fruit is more susceptible to russeting (<40 DAFB; Chen et

al., 2020, 2022), more rapid ethylene production may occur. Third, although there is no evidence in the case of young apple fruit detachment, induction of several genes involved in lignin, ABA, and ethylene synthesis can be caused by cutting the lily flower (Wu et al., 2019). Therefore, the resulting less expanded fruit surface and accelerated senescence by ethylene and ABA may contribute to the reduced healing ability.

Another effect of detachment is the initial and rapid induction of *MdMYB42* on the day of wounding (i.e. 1 d after detachment). However, the possibility of signal transduction from the cut site on the pedicle to the fruit surface within one day remains questionable. In addition, the remaining periderm development below wound and the expression of *MdMYB42* can not be suppressed by anoxia at 8 d of wounding. This may be attributed to: (1) the initial induction of *MdMYB42* by the detachment, due to its involvement in lignin synthesis, which contributes to the building of suberin (e.g., ferulic acid; Bernards et al., 2004; Woolfson et al., 2022); (2) the initial induction of ABA by the detachment, due to its role in mediating suberization in russeting and wound periderm formation in other species [Chinese white pear (*Pyrus bretschneideri* Rehd.), tomato, and kiwifruit (Han et al., 2018; Tao et al., 2016; Wang et al., 2022; Wei et al., 2020a,b)]; (3) the induction of some degree of meristem activity and other parallel suberization-related events by wounding itself; (4) any combination of these three possibilities.

To the best of the author's knowledge, this is the first report in which the young apple fruit was detached in order to establish a system that could last for several days. Due to the above-mentioned difficulties of this system, the role of O_2 on the wound periderm in apple fruit could not be examined here. However, to achieve this, two options are proposed for future studies that could help to achieve the objective of studying the role of O_2 on wound periderm: (1) Fruit attached to a part of the cut branch with a phyto-nontoxic seal on its two cut sides could be a solution to prolong the longevity of this detached fruit system to exclude the localized and rapid reactions to detachment from the pedicel. This approach can be used in an anaerobic chamber; or (2) a fruit chamber such as that designed by Jones and Higgs (1982) with continuous nitrogen gas can be applied to potted apple trees (attached fruit).

Author contribution statement

Author contribution MK and TD initiated the study. MK, TD, and BPK designed the experiments. YHC and JS performed the experiments and analyzed the data. YHC, JS and MK wrote the original manuscript and YHC and MK revised the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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494 **Legends**

495

496 **Fig. 1. Effect of detaching apple fruit on wound induced periderm formation under**
497 **ambient condition (21% O_2)**. Apple fruits were detached at 37 days after full bloom (DAFB)
498 and held either in the laboratory or in the field next to the fruits that remained attached to the
499 tree under ambient conditions (21% O_2). The fruit skin of attached and detached fruits was
500 wounded at 38 DAFB and observed for periderm formation up to 8 d after wounding. Eight
501 days after wounding, skin segments of both attached (a) and detached (b) fruits were stained
502 with Fluorol Yellow 088 to visualize the development of suberized phellem cell layers. Skin
503 sections were observed under bright and fluorescent light. Periderm formation was indexed
504 by counting the number of microtome sections with periderm (c) and the number of phellem
505 layers in a section (d). Data in (c) and (d) represent means ± SE (n = 8). Means followed by
506 the same letter are not different according to Tukey's studentized range test (HSD) at $P \leq 0.05$.
507 The black scale bar in (a) equals to 100 µm.

508

509 **Fig. 2. Effect of O_2 deficiency on wound induced periderm formation in detached apple**
510 **fruit.** Apple fruits were detached at 37 days after full bloom (DAFB) and transferred either in
511 an anaerobic chamber (0% O_2) or in the adjacent laboratory (21% O_2) the next day. The skins
512 of detached fruits under anoxic or ambient conditions were wounded at 38 DAFB and
513 observed for periderm formation up to 8 d after wounding. Skin segments of both attached (a)
514 and detached (b) fruits were stained with Fluorol Yellow 088 eight days after being wounded.
515 This staining technique was used to visualize the formation and progression of suberized
516 phellem cell layers. Skin sections were observed under bright and fluorescent light. Periderm
517 formation was assessed by counting the number of sections with periderm (c) and the number
518 of phellem layers per section (d). Data in (c) and (d) represent means ± SE (n = 8). Significance
519 of differences is indicated by an '*', Student's t-test at $P \leq 0.05$. The black scale bar in (a)
520 equals to 100 µm.

521

522 **Fig. 3. Effect of detaching fruit and O_2 deficiency and sealing on the expression of genes**
523 **involved in periderm formation in the apple fruit skin.** Two experiments were conducted
524 to compare different factors affecting wound-induced periderm formation in apple fruits. The
525 first experiment compared attached and detached fruits (a,c,e,g,i,k; "Attached vs. detached").
526 Fruits were detached 37 days after full bloom (DAFB) and either stored in the laboratory or left
527 attached to the tree under ambient conditions (21% O_2). In the second experiment, the
528 comparison was between fruits stored in an anaerobic chamber (0% O_2) and those kept in the
529 adjacent laboratory (21% O_2) (b,d,f,h,j,l; "21% O_2 vs. 0% O_2"). Fruits were detached at 37
530 DAFB and transferred the next day. For both experiments, fruit skins were wounded at 38

DAFB, and periderm formation was observed up to 8 days after wounding. The non-wounded skin on the opposite side of the wound served as a control. Gene expression profiles of periderm-related genes were observed 0 and 8 days after wounding in the two experiments. The selected genes were involved in the regulation of lignin (*MdMYB42*) and suberin (*MdMYB93*) synthesis, suberin monomer synthesis (*MdCYP86B1*, *MdGPAT5*), transport (*MdABCG20*), and polymerization (*MdSGNH*). Data represent means ± SE (n = 3). Means followed by the same letter are not different according to Tukey's studentized range test (HSD) at $P \leq 0.05$.

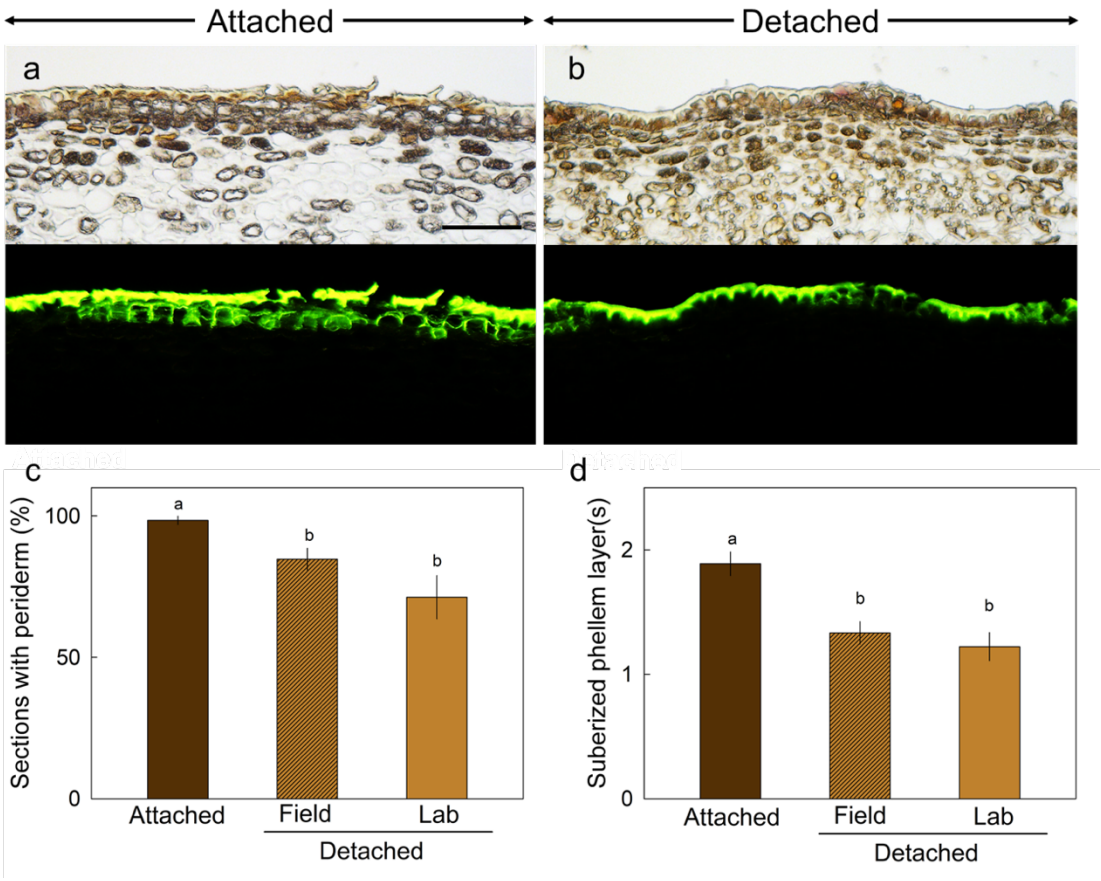
Attached
Detached
a
b
c
d
Sections with periderm (%)
100
50
0
a
b
b
Attached
Field
Lab
Detached
Suberized phellem layer(s)
2
1
0
a
b
b
Attached
Field
Lab
Detached

Fig. 1.

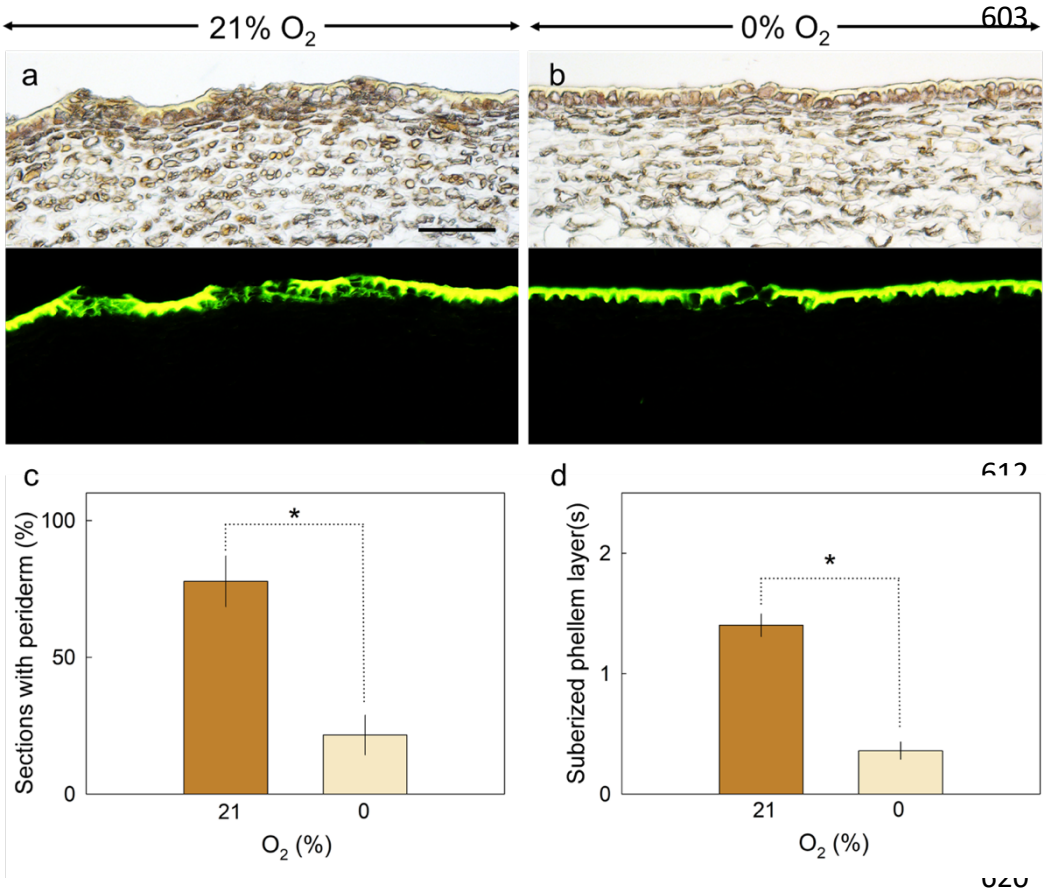
21% O_2
0% O_2
a
b
c
d
Sections with periderm (%)
100
50
0
*
21
0
O_2 (%)
Suberized phellem layer(s)
2
1
0
*
21
0
O_2 (%)

Fig. 2.

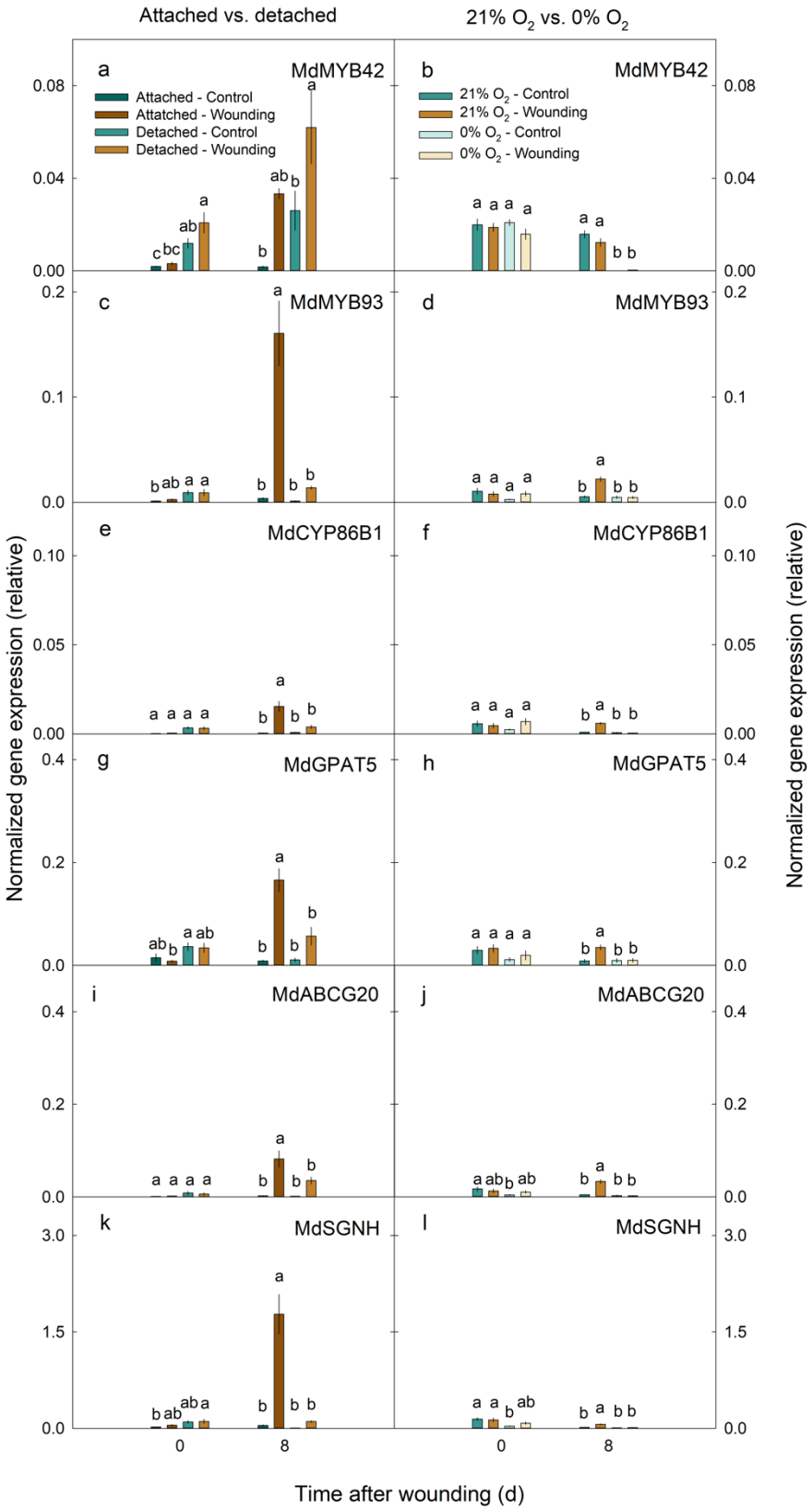
Attached vs. detached
21% O2 vs. 0% O2
a MdMYB42
b MdMYB42
c MdMYB93
d MdMYB93
e MdCYP86B1
f MdCYP86B1
g MdGPAT5
h MdGPAT5
i MdABCG20
j MdABCG20
k MdSGNH
l MdSGNH
Attached - Control
Attatched - Wounding
Detached - Control
Detached - Wounding
21% O2 - Control
21% O2 - Wounding
0% O2 - Control
0% O2 - Wounding
Normalized gene expression (relative)
Time after wounding (d)

Fig. 3.

Supplementary Information

Establishing a detached fruit system for russeting study in apple

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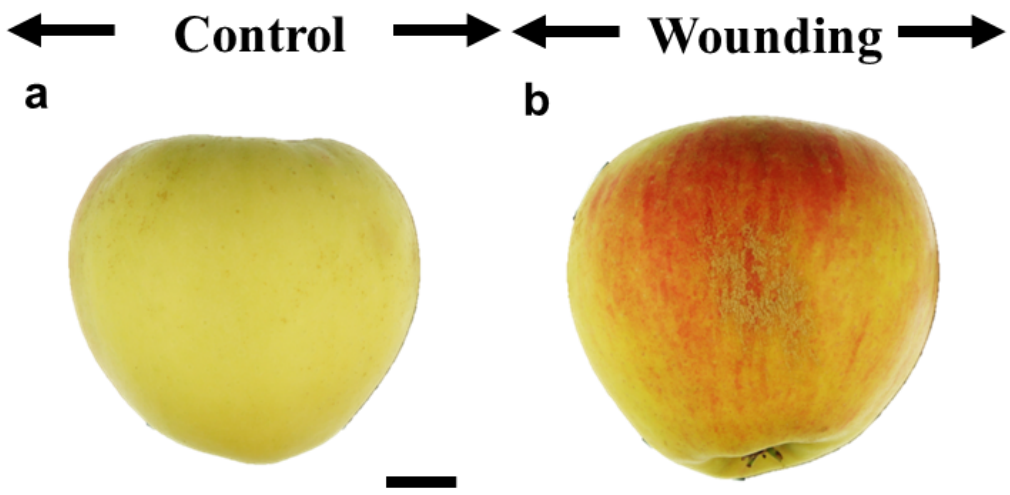

Fig. S1. Appearance of wound-induced periderms (russeting) on the fruit surface of 'Pinova' apple. The fruit skin of attached apple was wounded at 38 days after full bloom (DAFB) and left on tree till the assessment for periderm formation at 109 d after wounding **(a)**. The opposite side of the fruit served as (unwounded) control **(b)**. Black scale bar = 1 cm.

Table S1. Selected genes analyzed in the present study.

Gene name	Accession no.	AGI locus code	Description	Reference
MdMYB42	MDP0000787808	AT4G12350	MYB domain protein 42, involved in secondary cell wall synthesis and regulation of lignin synthesis	Zhong et al (2008); Geng et al. (2020)
MdMYB93	MDP0000320772	AT1G34670	MYB domain protein 93, positive regulator of suberin synthesis	Legay et al. (2016)
MdCYP86B1	MDP0000306273	AT5G23190	Cytochrome P450, family 86, subfamily B, polypeptide 1, involved in the synthesis of very long chain *ω*-hydroxyacid and *α*,*ω*-dicarboxylic acid in suberin polyester	Compagnon et al. (2009)
MdGPAT5	MDP0000150502	AT3G11430	Glycerol-3-phosphate acyltransferase 5, synthesis of suberin polyester	Beisson et al. 2007
MdABCG20	MDP0000265619	AT3G53510	ATP-binding cassette G20, involved in transport of aliphatic suberin polymer precursors	Yadav et al. (2014)
MdSGNH	MDP0000123818	AT5G37690	SGNH hydrolase-type esterase superfamily protein	Straube et al., 2023

The table is adopted and modified from Straube et al. (2021).

Table S2. List of primers utilized for gene expression analysis in the present study.

Gene name	Accession	Primer sequence (5' to 3')[z]		Primer efficiency (%)	Source of primer
		Forward Primer	Reverse Primer		
MdMYB42	MDP0000787808	CCTTGGCAATAGGTGGTCGA	TGATGTGCGTGTTCCAGTGA	94.2	Straube et al. (2021)
MdMYB93	MDP0000320772	TGGACAAACTATCTTAGGCCGG	GTTGCCGAGGATGGAATGGA	102.5	Straube et al. (2021)
MdCYP86B1	MDP0000306273	CGCTTTGTGACCCCATCC	AATGACGTCTTCCGCAAACT	109.3	Legay et al. (2015)
MdGPAT5	MDP0000150502	GAACAAATCCACCCACCACT	ATTAAGAGGGCGGTTGAAGG	88.9	Legay et al. (2015)
MdABCG20	MDP0000265619	ACTGGGCATGGACAACAACA	ATTTTCCCGACCCACTTGCT	102.9	Straube et al. (2021)
MdSGNH	MDP0000123818	CCCAGGGGCAAAACTCTCAT	TGCAACACGGAAGGTTCGAA	97.2	Straube et al. (2023)
MdeF-1alpha	AJ223969.1	ACTGTTCCTGTTGGACGTGTTG	TGGAGTTGGAAGCAACGTACCC	93.0	Legay et al. (2015)
MdPDI	MDP0000233444	TGCTGTACACAGCCAACGAT	CATCTTTAGCGGCGTTATCC	100.6	Storch et al. (2015)

[z]Primers were designed using the Primer3 software (http://primer3.ut.ee/) and the efficiency of the primers was tested according to Straube et al. (2021).

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2.7 Anoxia prevents wound periderm formation in tomato

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Anoxia prevents wound periderm formation in tomato

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Abstract

Russeting is an economically important surface disorder in many fruit crop species including in apple and tomato. Russeting represents the formation of a periderm, typically in response to microcracking of the cuticle. The microcracked cuticle's impaired barrier properties likely trigger periderm formation deeper down in the skin. Possible trigger signals include an increase in the O_2 partial pressure in the tissues immediately subtending a microcrack. The objective of our study was to establish the effects of O_2 partial pressure on periderm formation under controlled conditions. As a model system we employed wound-induced periderm formation in detached tomato fruit. Preliminary experiments established that the histology and expression of six putative genes involved in periderm formation and suberin metabolism (*MYB42*, *MYB93*, *CYP86B1*, *GPAT5*, *ABCG20* and *SGNH*) of the periderms after wounding were similar in still-attached and in detached tomato fruit. Anoxia delayed periderm formation in detached fruit after 8 d of wounding. At this time, the same six genes were downregulated under anoxia (zero O_2) compared with ambient conditions (21% O_2). The results indicate that anoxia inhibits periderm formation and, hence, russeting compared to ambient O_2 conditions.

Keywords **Cuticle · Microcrack · Russeting · *Solanum lycopersicum* · Suberin · Wounding**

Abbreviations:

ABCG20: ATP Binding Cassette Transporter G family member 20

BetaTub: Beta Tubulin

BLAST: Basic Local Alignment Search Tool

BLASTn: Search nucleotide databases using a nucleotide query

CO_2: Carbon dioxide

CYP77A6: Cytochrome P450, family 77, subfamily A, polypeptide 6

CYP86B1: Cytochrome P450, family 86, subfamily B, polypeptide 1

CYP88A8: Cytochrome P450, family 88, subfamily A, polypeptide 8

DAA: Days after anthesis

GPAT5: Glycerol-3-phosphate acyl transferase 5

H_2O_2: Hydrogen peroxide

MYB42: myb (myeloblastosis) domain protein 42

MYB93: myb (myeloblastosis) domain protein 93

O_2: atmospheric oxygen

PCD: Programmed cell death

ROS: Reactive oxygen species

SGNH: SGNH hydrolase-type esterase superfamily protein

SPPD: Suberin polyphenolic domains

tBLASTn: Search translated nucleotide databases using a protein query

Introduction

Russeting is an economically important surface disorder that affects both the pre- and postharvest performance of fruit. Russeting occurs in several fruit crop species, including apple (*Malus* x *domestica*, Borkh.) (Faust and Shear 1972; Skene 1981) and tomato (*Solanum lycopersicum*) (Bakker 1988).

Anatomically, russeting involves the formation of a periderm comprising a phellem, a phelloderm and a phellogen. Cell division in the phellogen produces stacks of loose phellem cells that are responsible for the dull, brownish appearance of the russeted fruit surface. Periderm formation begins in the hypodermis, in close proximity to the microcracks in the overlying cuticle (Meyer 1944; Pratt 1972). Microcracking is usually the result of prolonged exposure to surface moisture (Khanal et al. 2021; Knoche and Grimm 2008; Chen et al. 2020) or to a mechanical injury of some sort (Simons and Aubertin 1959; Skene 1981). The immediate result of microcracking is that the cuticle's barrier properties are impaired locally, and this impairment induces the formation of a local area of periderm. Compared with the surrounding skin, a periderm is dull, reddish brown in color and rough to the touch (Chen et al. 2022). The signal(s) that link cuticular microcracking to the differentiation of a phellogen, several cell layers deeper into the tissue, is/are unknown. Based on the hypothesis that the signal(s) is/are related to the cuticle's impaired barrier properties and the effect of this change on the cells immediately underlying the microcrack, obvious candidates must include (1) a local decrease in the partial pressure of internal carbon dioxide (CO_2) and/or (2) an increase in the partial pressure of internal oxygen (O_2) and/or (3) a reduction (more negative) in the water potential.

The objective of this study was to focus on the second possibility, by investigating the effects of O_2 partial pressure on wound-induced periderm formation in developing tomato fruit. We chose this possibility because it is known that higher O_2 partial pressures promote the suberization of wounded surfaces in kiwifruit and potato tubers (Lipton 1967; Wei et al. 2018; Wigginton 1974). We used tomato as a convenient model system because it can be produced out of season and it is easily accessible for molecular studies. We induced russeting by imposing a mild mechanical wound on the fruit surface using abrasive paper.

Materials and methods

Plant material

'Micro-Tom' tomato plants (*Solanum lycopersicum*) were cultivated in the greenhouses (lat. 52˚39'N, long. 9˚70'E). To accurately identify the stage of fruit development, flowers were date-tagged on the day of anthesis. Later, individual fruits were collected at random from 80 plants. To impose a slight wounding of the skin, it was abraded on one side in the equatorial region using a fine abrasive paper (grit 1000; Bauhaus, Mannheim, Germany). The area of skin on the opposite side of the fruit served as the unwounded control surface. This treatment induced russeting of the wounded area at maturity (**Fig. S1**).

Histology

Whole fruits were preserved in Karnovsky fixative (Karnovsky 1965), cut into blocks, embedded in paraffin wax and stored at 4˚C until later microscopic examination (for detail see Chen et al., 2020). Thin sections were prepared using a rotary microtome (Hyrax M 55; Carl Zeiss, Oberkochen, Germany) and stained for 1 h with Fluorol Yellow 088 (Santa Cruz Biotechnology, TX, USA) at a final concentration of 0.005% (w/v). The stain was prepared by mixing with a combination of glycerol (90% v/v; Carl Roth) and melted polyethylene glycol 4000 (PEG 4000; w/v; Carl Roth) in a 1:1 ratio. After rinsing with water, the sections were examined under both bright light and incident fluorescent light (U-MWB; 450-480 nm excitation; ≥520 nm emission wavelength; Olympus Europa SE & Co. KG, Hamburg, Germany) using a fluorescence microscope (BX-60 equipped with a DP 73 digital camera; Olympus Europa). A minimum of 72 sections (technical replicates) per biological replicate and a minimum of seven biological replicates per treatment and sampling time were inspected. The number of sections with periderm was recorded, and the number of phellem layers was also recorded. A section was counted as being 'with periderm' when a single phellem layer, or more, was present. The % incidence of periderm was calculated as the ratio of the incidence value to the total number of sections inspected ×100. Where a phellem layer was present, the number of suberized phellem layers was counted in three representative images per biological replicate and in five locations within the image (700 µm diam.).

RNA Isolation

Skin patches from wounded or control areas were excised using a razor blade and frozen in liquid nitrogen. To extract RNA, the frozen patches were ground to a powder and processed with the InviTrap Spin Plant RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions. To eliminate potential genomic DNA

contamination, the DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used. The purity and quantity of RNA were determined at 230 nm, 260 nm and 280 nm using a spectrophotometer (Nanodrop 2000c; Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the RNA was assessed by running samples on a 1.5% agarose gel. All samples were stored at -80°C. The number of replicates was three, where one replicate represented the pooled skin patches from the fruits of six plants, with one fruit per plant.

Gene expression

Six putative genes associated with periderm formation and suberin metabolism were selected based on previous studies in apple (Straube et al. 2021, 2023). To identify the corresponding putative genes in tomato, tBLASTn and BLASTn searches were conducted on the Sol Genomics Network (https://solgenomics.net; Fernandez-Pozo et al. 2015; **Table S1**). Primers were designed using the Primer3 software (http://primer3.ut.ee/) and tested for efficiency according to Straube et al. (2021) (**Table S2**).

The cDNA synthesis was performed with the LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. For quantitative real-time PCR (qPCR), the Luna® Universal qPCR Master Mix (New England Biolabs) was used. The qPCR was performed on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA). The qPCR conditions included an initial denaturation step at 95˚C for 60 s, followed by 40 cycles of 15 s at 95˚C and 60 s at 60˚C. Upon completion of the amplification cycles, a melting curve analysis was performed. The melting curve conditions included heating the samples to 95˚C for 15 s followed by 60˚C for 60 s. The temperature was increased from 60 to 95˚C in 0.5˚C increments. *SlActin* and *SlBeta-Tubulin* were used as reference genes (Girard et al. 2012; **Table S2**) to normalize expression values (Pfaffl et al. 2001; Chen et al. 2019).

Experiments

The effects of detaching fruit on periderm formation after wounding were studied. Briefly, a fruit was detached, the pedicel recut under autoclaved deionized water and the pedicel ‘extended’ using a length of Tygon® tubing (TYGON® S3™ E-3603, Saint-Gobain, Paris, France). The extended pedicel was then inserted through a hole in the lid of a 15 ml Falcon tube that was filled with autoclaved deionized water. The fruit was left overnight in the greenhouse and moved to the laboratory the next day. Wounding was performed as described above. Fruits that were wounded and remained attached to the plants in the greenhouse served as controls. Samples were collected for histology 8 d after wounding and for gene expression analysis at 0 and 8 d after wounding.

The effects of O_2 partial pressure on periderm formation were investigated using detached fruit. A fruit was placed on a Falcon tube filled with degassed and autoclaved water in an anaerobic chamber (partial pressure of O_2 = zero) (Plas-Labs, Inc., Lansing, MI, USA) or held in the laboratory under ambient conditions. After 30 min of equilibration, fruits were wounded and incubation continued for 8 d. The Falcon tubes were replaced daily. For histology, fruits were sampled 8 d after wounding and for gene expression analysis, after 0 and 8 d after wounding. Wounded detached fruits held under ambient conditions served as controls.

Data analysis and presentation

Data were analyzed by Student's t-test or one-way analysis of variance (ANOVA). Means were compared with Tukey's studentized range test (HSD). Statistical analyses were performed using SAS Studio (SAS OnDemand for Academics; SAS Institute Inc., Cary, NC, USA). Data are presented as means ± standard errors (SE). Percentage data (%) were converted by arcsine transformation before analysis of variance.

Results

Wounding the fruit surface of still-attached and detached tomatoes using abrasive paper ruptured the cuticle **(Fig. 1a,b, insets),** caused some damage to the epidermis and induced periderm formation within 8 d (**Fig. 1a,b**). In both cases (still attached and detached) the periderm formed deeper in the hypodermal cell layers, well below the ruptured cuticle and wounded epidermis. There were no visual differences between the periderms of detached and attached fruits. Statistical analysis revealed that the number of phellem layers was slightly but significantly reduced when the detached fruit were held in the greenhouse (fewer) as compared to in the laboratory (more) (**Fig. 1d**).

Wounding detached fruit resulted in both cuticle rupture **(Fig. 2a,b, insets)** and periderm formation under ambient atmospheric conditions, but not under anoxic conditions (**Fig. 2a,b**). Under anoxic conditions, none of the cross-sections examined showed any evidence of periderm formation (**Fig. 2c,d**).

The expression of six orthologous genes involved in periderm formation (regulation of lignin and suberin synthesis - *MYB42* and *MYB93,* respectively; suberin monomer synthesis - *CYP86B1* and *GPAT5*; monomer transport - *ABCG20* and polymerization- *SGNH*) was significantly upregulated in the wounded surfaces of still-attached and detached fruit **(Fig. 3a,c,e,g,i,k)**. The expression levels of *MYB42* and *MYB93, CYP86B1, GPAT5, ABCG20* and

SGNH were similar in still-attached and detached fruit. These genes were not upregulated in the intact surfaces of either the still-attached or detached fruit.

Anoxic conditions (0% O_2) significantly reduced the expressions of *MYB42* and *MYB93, CYP86B1, GPAT5, ABCG20* and *SGNH* as compared to wounded fruit held under ambient O_2 conditions (21% O_2) **(Fig. 3b,d,f,h,j,l)**.

Discussion

Anoxia inhibits wound-induced periderm formation in tomato fruit.

First, there was no evidence of any phellem or phellogen in wounded tomato fruit held in an anoxic atmosphere. This observation is consistent with the literature for other plant species. In potato and kiwifruit O_2 promotes suberization following wounding (Lipton 1967; Wigginton 1974; Wei et al. 2018). Also, in *Eucalyptus camaldulensis*, flushing stems with elevated O_2 accelerated the induction of a phellogen (Liphschitz and Waisel 1970).

Second, genes involved in the regulation of lignin (*MYB42*) (Geng et al. 2020) and suberin synthesis (*MYB93*; Legay et al. 2016), the synthesis of suberin monomer (*CYP86B1* and *GPAT5*) (Compagnon et al. 2009; Beisson et al. 2007), the transport of suberin monomer (*ABCG20*) (Yadav et al. 2014) and the polymerization of the monomers (*SGNH*) (Ursache et al. 2021) were not upregulated in an anoxic atmosphere in response to wounding. These genes are associated with periderm formation and are typically induced during early wound healing (Chen et al. 2022; Han et al. 2018; Straube et al. 2023; Woolfson et al. 2023). Interestingly, genes involved in the synthesis and transport of cutin monomers (including *CYP77A6* and *CYP88A8*) are downregulated by low O_2 partial pressures in *Arabidopsis thaliana* stems and leaves (Kim et al. 2017). The chemical compositions and synthetic pathways of cutin and suberin are similar (Phillipe et al. 2020).

Third, our result with tomato is consistent with the role of microcracking in russeting. In apple, not all microcracks trigger russet formation. Only those microcracks that traverse the cuticle and hence, impair the cuticle's barrier properties induce russeting. Furthermore, the cell layers underlying the microcracks must be exposed to elevated O_2 partial pressures (similar to those in the atmosphere) through the impaired cuticular barrier. This is consistent with the inhibition of russeting (pear) or cracking (tomato) following partial coating of the fruit surface using petroleum jelly (Lashbrooke et al. 2016; Wang et al. 2022a). The coated areas of the fruit surface were smooth, the un-coated areas were russeted.

The effect of O_2 on periderm formation may be explained by the relationship between O_2 partial pressure and the reactive oxygen species (ROS) produced by that O_2. The role of ROS in russeting may thus be twofold: (1) Russeting involves the formation of a phellogen which divides and produces the phellem. ROS are involved in regulating cell cycles and meristematic activity (Considine and Foyer 2021; Huang et al. 2019). (2) One of the ROS, hydrogen peroxide (H_2O_2) mediates the production of the polyphenolic domains of suberin (SPPD) (Bernards et al. 2004). In *Pyrus*, a russeted mutant of this species accumulates more H_2O_2 than a non-russeted cultivar (Heng et al. 2016). Recently, the gene Periderm Programmed Cell Death (*PyPPCD1*) was shown to be correlated with russeting in pear fruit (Wang et al. 2022a, b). Since wounding of plant tissues typically leads to programmed cell death (PCD) and ROS production (Iakimova and Woltering 2018), links between the enhanced penetration of atmospheric O_2 to the inner tissues through an impaired cuticular barrier, and PCD, ROS and russeting are highly likely. Given the economic importance of russeting to the fruit industry, these aspects merit further study.

Author contribution statement

Author contribution MK and TD initiated the study. MK, TD, and BPK designed the experiments. YHC and JS performed the experiments and analyzed the data. YHC, JS, MK, TD, and BPK wrote and revised the manuscript.

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Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends

Fig. 1 Wound induced periderm formation in detached and still-attached tomato fruit. Tomato fruit were wounded using abrasive paper, held at ambient O_2 concentrations and observed for periderm formation 8 d after wounding. **a,b**. Cross-sections viewed under incident bright light (top row) and under fluorescent light following staining with Fluorol Yellow 088 (center row). Periderm formation was indexed by the number of microtome sections with a visible periderm **(c)** and the number of phellem layers **(d)**. Data in **(c)** and **(d)** represent means ± SE (*n* = 7-8). Means followed by the same letter are not different according to Tukey's studentized range test (HSD) $P \leq 0.05$. Scale bar in **(a)** 100 µm.

Fig. 2 Effect of O_2 deficiency on wound-induced periderm formation in detached tomato fruit. Tomato fruit were wounded using abrasive paper and held under anoxic (0% O_2) or ambient O_2 concentration (21% O_2) and observed for periderm formation 8 d after wounding. **a,b**. Cross-sections were viewed under incident bright light (top row) and under fluorescent light following staining with Fluorol Yellow 088 (center row). Periderm formation was indexed by the number of microtome cross-sections with periderm **(c)** and the number of phellem layers **(d)**. Data in **(c)** and **(d)** represent means ± SE (*n* = 10). Significance of differences are indicated by an '*', Student's t-test at $P \leq 0.05$. Scale bar in **(a)** 100 µm.

Fig. 3 Expressions of genes involved in periderm formation in still-attached and detached tomato fruit (a,c,e,g,i,k) and in detached tomato fruit held either under anoxic (0% O_2) or ambient (21% O_2) conditions (b,d,f,h,j,l). Tomato fruit were wounded using abrasive paper and observed for periderm formation 8 d after wounding. In the "Attached vs. Detached" experiment, the detached fruit were held in the laboratory while the fruit that remained attached to the plant in the greenhouse were under ambient conditions (21% O_2) (**a**,**c**,**e**,**g**,**j,k**; left panel). In the "21% O_2 vs. 0% O_2" experiment, the comparison was between fruits stored in an anaerobic chamber (0% O_2) and those kept in the adjacent laboratory at ambient O_2 concentration (21% O_2) (**b**,**d**,**f**,**h**,**j,l**; right panel). Expression profiles of genes involved in the regulation of lignin (*SlMYB42*) and suberin (*SlMYB93*) syntheses, suberin monomer synthesis (*SlCYP86B1, SlGPAT5*), transport (*SlABCG20*), and polymerization (*SlSGNH*) were analyzed by qPCR. Data represent means ± SE (*n* = 3). Means followed by the same letter are not different according to Tukey's studentized range test (HSD) at $P \leq 0.05$.

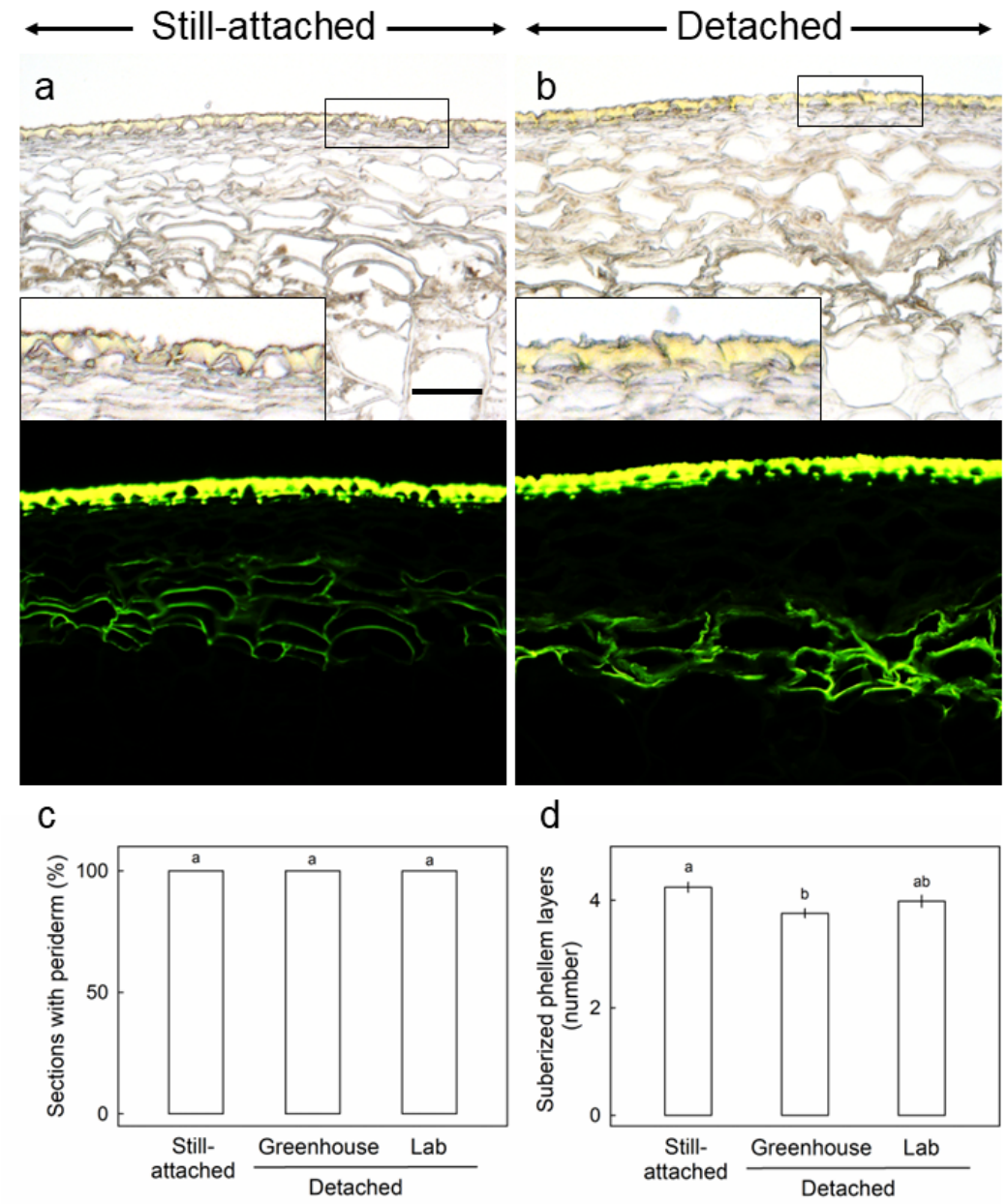
Still-attached
Detached
a
b
c
d
Sections with periderm (%)
100
50
0
a
a
a
Still-attached
Greenhouse
Lab
Detached
Suberized phellem layers (number)
4
2
0
a
b
ab
Still-attached
Greenhouse
Lab
Detached

Fig. 1

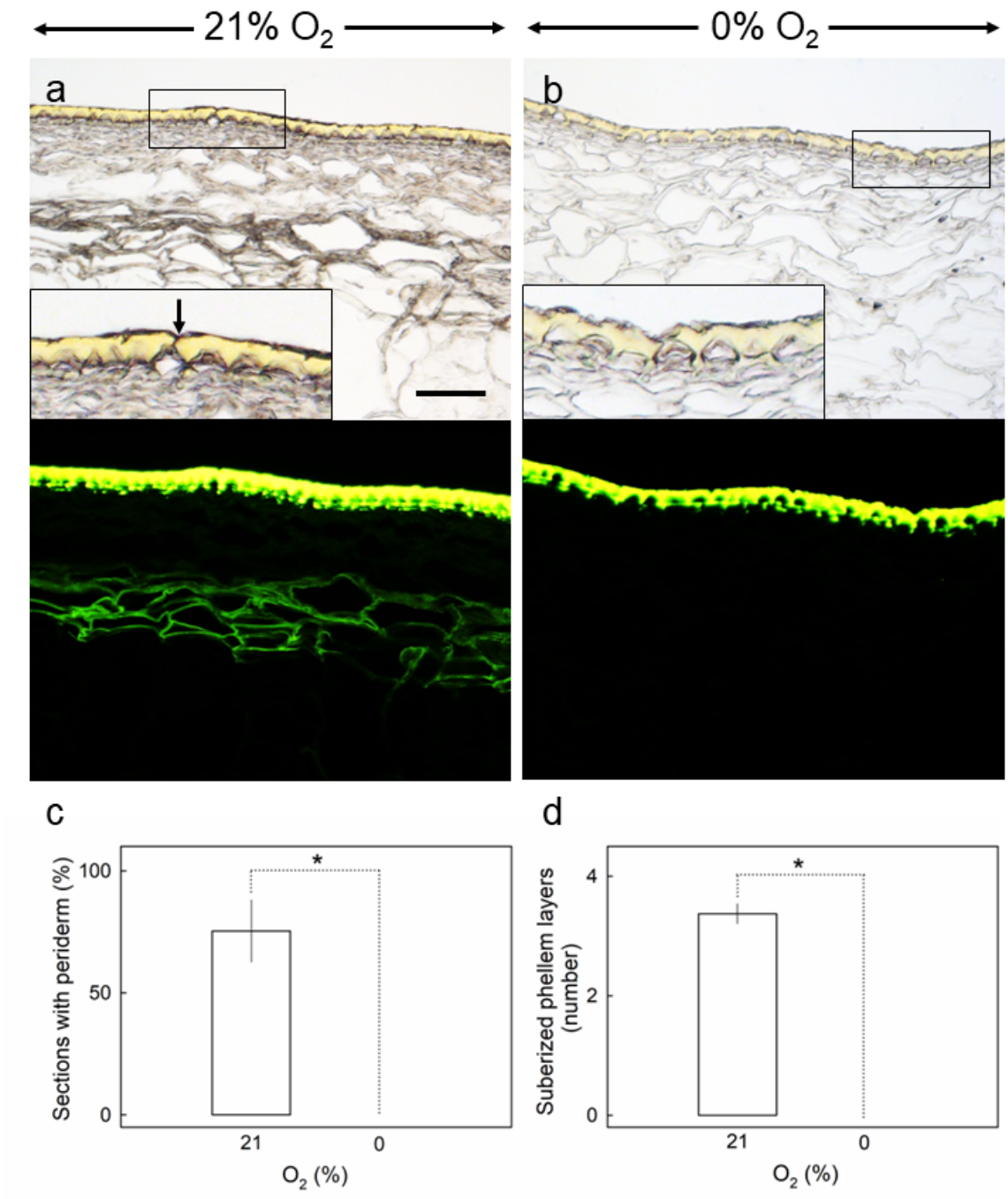
21% O2
0% O2
a
b
c
d
Sections with periderm (%)
100
50
0
21
0
O2 (%)
*
Suberized phellem layers (number)
4
2
0
21
0
O2 (%)
*

Fig. 2

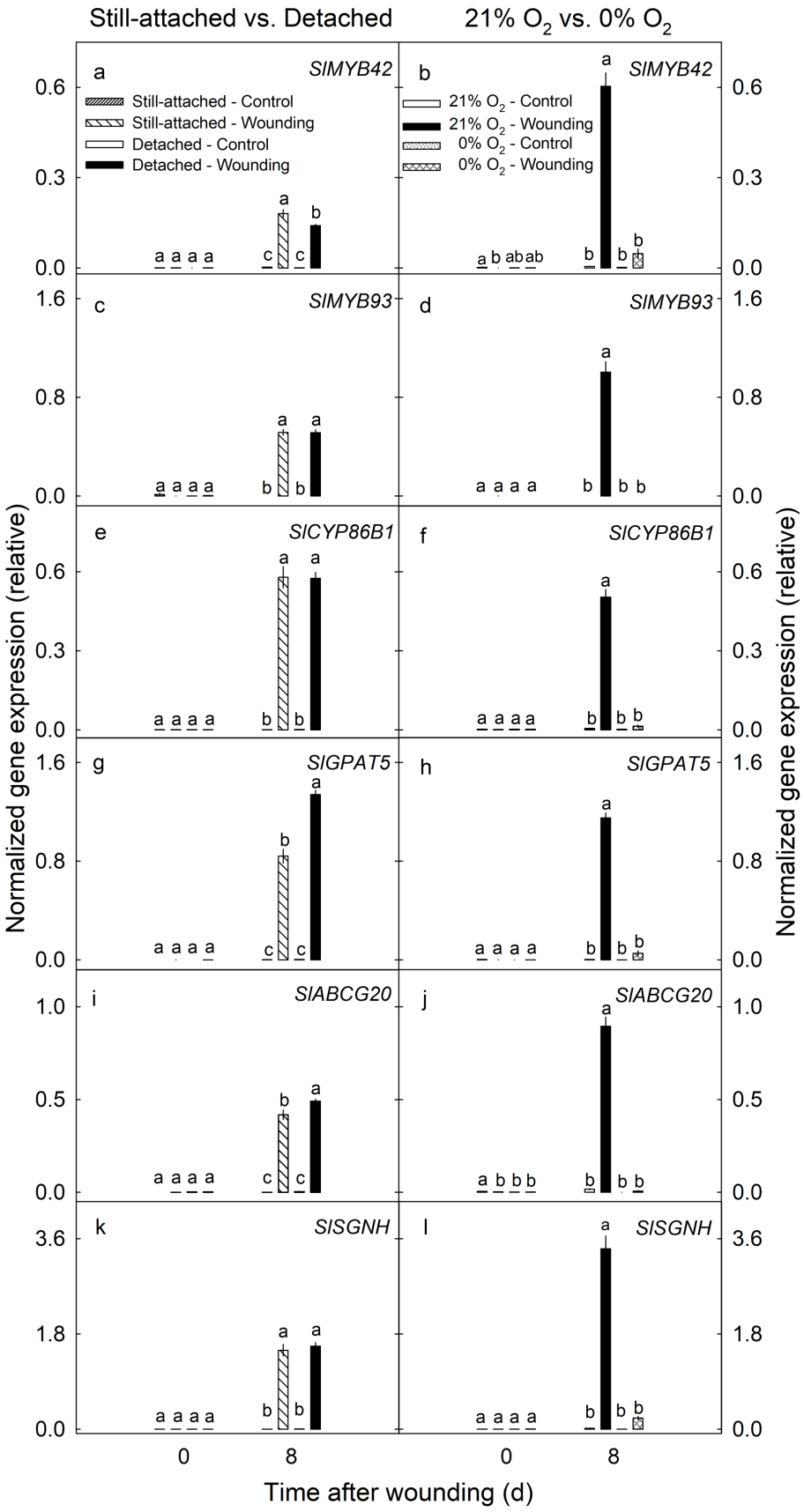
Still-attached vs. Detached
21% O2 vs. 0% O2
a SlMYB42
b SlMYB42
c SlMYB93
d SlMYB93
e SlCYP86B1
f SlCYP86B1
g SlGPAT5
h SlGPAT5
i SlABCG20
j SlABCG20
k SlSGNH
l SlSGNH
Still-attached - Control
Still-attached - Wounding
Detached - Control
Detached - Wounding
21% O2 - Control
21% O2 - Wounding
0% O2 - Control
0% O2 - Wounding
Normalized gene expression (relative)
Time after wounding (d)
0
8

Fig. 3.

Supplementary Information

Anoxia prevents wound periderm formation in tomato

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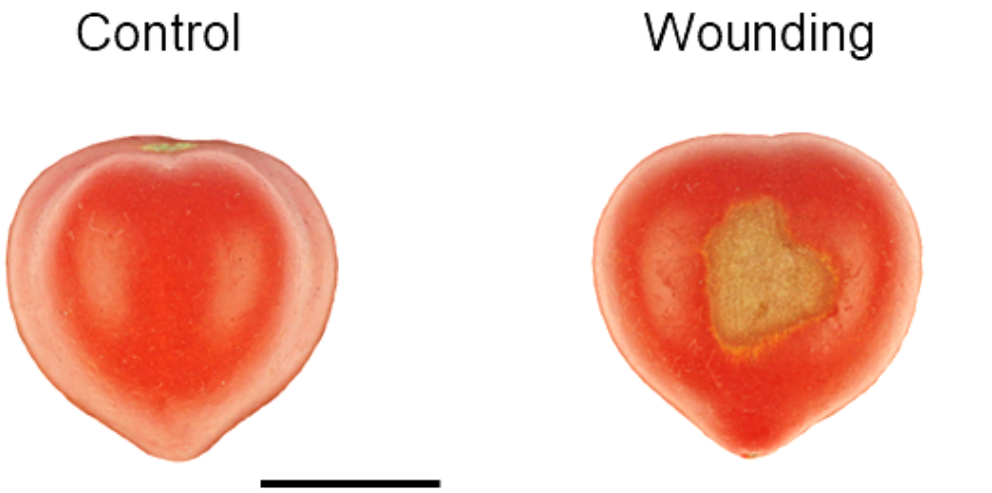

Fig. S1 Appearance of wound-induced periderm (russeting) on mature 'Micro-Tom' tomato fruit. The skin of still-attached tomato fruit was wounded at the immature green stage (18-22 days after anthesis; DAA) and observed for periderm formation up to 32 d after wounding. The skin area on the opposite side of the fruit served as the control surface. Black scale bar represents 1 cm.

Table S1. Identification of selected putative target genes and reference genes analyzed in tomato

Putative Gene	Query	Reference of sequence	Homologue[z]	Description[y]	E-Value	Identities	Positives
SlMYB42	AT4G12350.1	-	Solyc08g079270	SlMYB42 is a member of SlR2R3MYB gene family, there are 121 members in this gene family. SlR2R3MYBs plays major roles in the plant response to abiotic conditions and involved in signal transduction pathways.	2×10^{-91}	156/288 (54%)	199/288 (69%)
SlMYB93	AT1G34670.1	-	Solyc04g074170	MYB transcription factor (AHRD V1 *--* Q9SBF3_ARATH); contains Interpro domain(s) IPR015495 Myb transcription factor	5×10^{-110}	195/378 (52%)	242/378 (64%)
SlCYP86B1	LOC101264266	Han et al. (2018)	Solyc02g014730	Cytochrome P450 (AHRD V1 ***- B9HGL1_POPTR)	0	1818/1818 (100%)	-
SlGPAT5	AT3G11430.1	-	Solyc04g011600	ER glycerol-phosphate acyltransferase (AHRD V1 **** B9T011_RICCO); contains Interpro domain(s) IPR002123 Phospholipid/glycerol acyltransferase	0	360/495 (73%)	412/495 (83%)
SlABCG20	AT3G53510.1	-	Solyc05g054890	ABC transporter G family member 1 (AHRD V1 **** AB1G_ARATH); contains Interpro domain(s) IPR013525 ABC-2 type transporter	0	495/666 (74%)	563/666 (85%)
SlSGNH hydrolase	-	Lashbrooke et al. (2016)	Solyc11g011110	GDSL esterase/lipase At5g37690 (AHRD V1 ***- GDL80_ARATH); contains Interpro domain(s) IPR001087 Lipase, GDSL	-	-	-
SlActin	SGN-U213132	Girard et al. (2012)	Solyc03g078400	Actin (AHRD V1 ***- Q7XZJ2_GOSHI); contains Interpro domain(s) IPR004000 Actin/actin-like	0	591/591 (100%)	-
SlBeta-tubulin	DQ205342	Girard et al. (2012)	Solyc04g081490	Tubulin beta-1 chain (AHRD V1 ***- D7KT68_ARALY); contains Interpro domain(s) IPR002453 Beta tubulin	0	1475/1481 (99%)	-

[z]The tomato database ITAG4.0 cDNA on the Sol Genomics Network (Fernandez-Pozo et al., 2015) was used for the identification.

[y]Source of information: Sol Genomics Network (https://solgenomics.net/).

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614 **Table S2.** List of primers used for gene expression analysis

Gene name	Accession[z]	Sequence (5' to 3')		Primer efficiency (%)[y]	Source of primers
		Forward primer	Reverse primer		
SlMYB42	Solyc08g079270.2.1	ATGGACAGCTGAGGAAGACAA	CAACGCCTAAGACCAGCAAG	89.0	This study
SlMYB93	Solyc04g074170.3.1	GGACCATGGACTCCTGAAGAA	GCAGCTCTTCCCACACCTAT	82.0	This study
SlCYP86B1	Solyc02g014730.3.1	GCCATTTGTAGGGATGTTGCC	AACCATGGACCTCGGAATGT	89.6	This study
SlGPAT5	Solyc04g011600.2.1	CAATCACTAACTCGGGCGTG	GGCCGGTATCCTACGTTGAA	81.8	This study
SlABCG20	Solyc05g054890.4.1	CTGGGCCGGGAATTCATTTG	AAGTAGGCTAGGATCGCGAC	92.0	This study
SlSGNH	Solyc11g011110.1.1	TGGACAAGAGGCTGCAGAAA	TGTGTGTATTGTTGCCCAGC	83.1	This study
SlActin	Solyc03g078400.2.1	AAGTGCGAGTGTCCTGTCTG	TACCGTGCATTCATAGCCCC	88.4	This study
SlBetaTub	Solyc04g081490.3.1	GGCGCTGAGTTGATTGATGC	CATGCCAGATCCAGTCCCTC	86.1	This study

[z]Source of accession numbers: Sol Genomics Network (Fernandez-Pozo et al., 2015).

[y]The determination of primer efficiency was done with a five series dilution according to Straube et al. (2021).

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Straube J, Chen Y-H, Khanal BP, Shumbusho A, Zeisler-Diehl V, Suresh K, Schreiber L, Knoche M, Debener T (2021) Russeting in apple is initiated after exposure to moisture ends: Molecular and biochemical evidence. Plants 10:65. https://doi.org/10.3390/plants10010065

3. General discussion

The main findings of the present study were:

(1) Exposure of the surface of young apple fruit to long-term moisture (12 d) resulted in microcracking, increased water loss, and russeted fruits. This established exposure technique provided the basis for the later investigations in the present study. **[Chapter 2.1; i.e. Khanal et al. (2021)].**

(2) A biphasic behavior of the moisture-induced periderm is revealed: The initiation of a moisture-induced periderm occurred only when the fruit surface is exposed to moisture for a certain period of time (minimum: 6 d; Phase I) and in the absence of the moisture (Phase II). The mechanism behind this is a weakened cuticle that prone to microcracking caused by the down-regulation of genes that are involved in cutin and wax synthesis. **[Ch. 2.2 and 2.3; i.e. Chen et al. (2020) and Straube et al. (2021)]**.

(3) There were similarities between wound and moisture-induced periderm on the apple fruit surface in terms of their initiation, development and resulting chemical constituents. The time frame of wounding aligned perfectly with that of the Phase II. Both types of periderm were triggered by an impaired barrier **[Ch. 2.4; i.e. Chen et al. (2022)]**.

(4) By transcriptomic analysis, the biphasic behaviour of moisture-induced periderm is revealed by distinct sets of differentially expressed genes (DEGs) in each phase. Phase I is characterized by the down-regulation of genes involved in cuticle formation, cell wall and cell division and the up-regulation of genes involved in abiotic stress. Several meristem, abscisic acid (ABA)- and suberin-related genes were up-regulated during Phase II. Furthermore, the expression patterns of the DEGs of both phases were further verified in apple cultivars differing in their susceptibility to russeting. It was shown that the consequences of wounding are similar to those of Phase II due to an impaired barrier. **[Ch. 2.5; i.e. Straube et al. (2023)].**

(5) Detachment of young apple fruit resulted in a reduced response to wounding compared to attached fruit. Accompanying problems, including induction of the *MdMYB42* gene by detachment, and residual periderm development and suberization under anoxia, made this system unsuitable for experiments in controlled environments. Fortunately, a better system was later established in tomato. The critical role of atmospheric O_2 in wound-induced periderm in the tomato fruit surface was confirmed and demonstrated by the absence of

periderm and the suppression of several suberin and lignin-related genes under anoxia. **(Ch. 2.6 and 2.7)**.

For detailed discussions with respect to the above-mentioned publications and manuscripts the reader is referred to the individual chapters of the thesis. The general discussion below will focus on the detached fruit systems (**Ch. 3.1**), the implications of this study for future research (**Ch. 3.2**), the comparison of the present study to recent periderm studies in other plant species (**Ch. 3.3**) and the implications of this study for horticultural practice (**Ch. 3.4**).

3.1 The detached fruit systems

In the present study, two fruit systems have been established: apple and tomato. The original intention was to focus on the apple crop because of (1) its importance in research and production and (2) the foundation: a series of russeting studies were conducted by our group and provide updated knowledge on russeting (**Ch. 2.1-2.5**). Unfortunately, all the problems that made the system unsuitable for russeting studies point to the detachment itself. Possible solutions to improve this detached fruit system are discussed in **Ch. 2.6**.

Research later turned to establishing a similar system in the model plant tomato to study the role of O_2 in periderm formation **(Ch. 2.7)**. The results showed that it is a better system compared to the one in apple because of: (1) longevity and similarity: the detached tomato fruit has a much closer performance to the attached one regarding wound periderm formation after 8 d of wounding, both in histological and gene expression analyses, showing that the effect of the detachment is very marginal; and (2) suppression: anoxia successfully and completely suppressed formation of a periderm at the same time point (8 d), with supporting results of the corresponding suppressed gene expressions. In addition, the choice of 'Micro-Tom' tomato has several advantages: (1) flexibility: The cultivation of tomato is not limited by time; (2) convivence: this cultivar has a shorter developmental time (70-90 days; Meissner et al., 1997) compared to other large fruit cultivars, allowing the researcher to conduct more rounds of experiments in a given time; (3) productivity: It also has a smaller plant size and bears more fruits. The investment of time and space would be worthwhile.

As the first report on the role of O_2 on wound periderm formation in the tomato fruit, **Chapter 2.6** was designed to simply clarify the consequences of anoxia at the time point where a wound periderm can be identified (8 d). To answer questions regarding other effects of excluding O_2 in addition to suppressed periderm formation, it is necessary to look at the initial events after the impaired barrier forms. These would include: (1) whether the tomato fruit has an adaptive response to low O_2 by inhibiting wound-induced changes or switching to other alternative metabolic pathways, as occurs in wounded potato tubers (Butler et al., 1990; Geigenberger et al., 2000), and (2) whether these responses would interact with the suberin and lignin pathways.

Overall, the tomato detached fruit system allows the young fruit to be incubated under certain constant environments for a few days and is suitable for studying the effects of other biotic and abiotic factors on russeting, for example, and may even be useful for surface research in fruit and vegetable crops.

3.2 Potential implications for future research

3.2.1 Investigation on wound periderm

Because of the important role of an impaired barrier in periderm formation is discovered in the present study by two induction methods – wounding and surface moisture, its results may now be sufficient to answer a key question: “Does a reduction in cuticle integrity lead to periderm formation or does the initiation of periderm formation lead to reduced cuticle?” (Macnee et al., 2021). First, an impaired barrier and periderm initiation are interrelated - there was no periderm initiation prior to barrier impairment. This is verified not by a single induction method, but by both wounding and surface moisture, which have different mechanisms for causing loss of cuticle integrity (i.e., impaired barrier): the former by mechanical damage and the latter by reduced cutin and wax deposition. In addition, the selected lignin and suberin-related genes are upregulated only after the onset of barrier impairment (**Ch. 2.4**). Second, as an impaired barrier forms, the periderm gradually replaces the original cuticle and the cuticular deposition gradually decreases. This is evidenced by the immediate and continuous down-regulation of several cutin- and waxes-related genes after the onset of an impaired barrier (**Ch. 2.3-2.5**), by the less similar chemical constituents of a periderm to those of a cuticle at maturity (**Ch. 2.3-2.4**) and by the few remaining cuticle layers above the periderm on the mature fruit surface in histology (**Ch. 2.2**). Therefore, in a causal relationship, loss of cuticle integrity (i.e. an impaired barrier) is the cause and both periderm initiation/formation and reduced cuticle are the effect. The substitutive role to surface moisture and the effectiveness in causing an impaired barrier makes wounding to be the best method for periderm induction for future researchers.

From the results, the necessity of O_2 for the formation of a wound periderm is crystal clear. In fact, the results presented in the present study are a long-term effect (8 d after wounding) in which the selected suberin and lignin-related genes are suppressed by anoxia **(Ch. 2.7)**. To answer the question of the exact role of O_2 in the triggering of russeting, three aspects must be considered. First, to support the role of O_2 in the initiation of a phellogen, there must be evidence for a continuous suppression of meristem- and periderm- related genes between the onset of a barrier impairment and the time of a clear sign of periderm formation (8 d) caused by anoxia. Genes identified by our recent transcriptomic work (**Ch. 2.5**) that are associated with meristem activity and up-regulated in phase II: *MdWOX4* (*WUSCHEL-related homebox*) and *MdMYB84*, would be good candidates. Second, evidence that O_2 regulates those TFs involved in suberin and lignin metabolism (e.g., Xin and Herburger, 2021b) is also critical to understanding its role in russeting. The priority list would be those TFs that have been shown to be inducible and up-regulated in Phase II: *MdMYB42*, *52*, *67*, *93*, 1*02*, *NAC038*, *058* and *WRKY56* (**Ch. 2.3-2.5**) or those whose functions have been analyzed and linked to suberin

and lignin metabolism: *MdMYB52*, *68* and *93* (Legay et 2016; Xu et al., 2022, 2023). Among them, *MdMYB42* and *93* have been shown to be partially suppressed under anoxia in the wounded tomato fruit (**Ch. 2.7**) and it would be necessary to study the roles and regulation of the remaining TFs by O_2. Third, ABA has been reported to be involved in the suberization of russeting in Chinese white pear (*Pyrus bretschneideri* Rehd.) and wound-induced suberization in tomato and kiwifruit (Han et al., 2018; Tao et al., 2016; Wang et al., 2022; Wei et al., 2020a,b). In apple, two genes regulated in early Phase II, *AP2B3* (*AP2/B3-like transcription factor family protein*) and *LEA* (*Late embryogenesis abundant hydroxyproline-rich glycoprotein*) are ABA responsive (**Ch. 2.5**). This suggests a possible role for ABA in russeting in apple. How O_2 is linked to ABA and whether anoxia can also suppress ABA and its downstream consequences, requires further research.

3.2.2 Investigation on moisture-induced periderm

Although Phase II of the moisture-induced periderm has been used primarily to study periderm initiation **(Ch. 2.4-2.7)**, the importance and value of Phase I cannot be ignored because (1) moisture is the most natural way of russeting induction in apple (Creasy, 1980; Creasy and Swartz, 1981; Faust and Shear, 1972a; Tukey, 1959); (2) Phase I mimics the natural retention of moisture on the surface, and the resulting chemical constituents are in some respects closer to the native periderm than to the wound periderm (e.g. the wax constituents; **Ch. 2.4**); (3) its complexity: compared to the immediate damage to the cuticle and underlying cell layers by wounding, the mechanism to cause microcracking and an impaired barrier by Phase I is different. With prolonged exposure, surface moisture (Phase I) gradually alters the properties of the fruit surface and many underlying molecular, cellular, and metabolic processes, including reduction of cutin and wax deposition, induction of abiotic stress, and modification of the cell walls of underlying cells (**Ch. 2.2-2.3, 2.5**).

A unique feature of Phase I is that the microcracking induced during Phase I does not lead to periderm initiation. This can be explained by an unexposed surface in a low O_2 state covered by the surface moisture. First, the O_2 concentration dissolved in the normal river water ranges from 4 to 12 mg L^{-1} (Government of Northwest Territories, 2020) which corresponds to 4 to 12 ppm. This can be regarded anoxic. Second, a study on Arabidopsis leaves and stems showed that cutin and waxes synthesis in the tissues are down-regulated by low O_2 (Kim et al., 2017), which is similar to the observation in Phase I (**Ch. 2.3 to 2.5**). Third, in addition to the reduction of cutin and wax synthesis, oxidative and osmotic stresses and other stress-related genes such as the peroxidase superfamily (*MdPRX/POX*) and *Heat shock protein 70* (*MdHSP 70*) are upregulated during phase I (**Ch. 2.5**). This suggests that the retention of moisture may

block/limit the exchange of molecules (e.g. O_2) across the surface, causing a variety of stresses and affecting metabolism in the underlying cell layers. Therefore, it can be concluded that moisture exposure is anoxic and stressful to the fruit surface and acts as a double-edged sword for russeting – (1) its retention on the surface negatively regulates the synthesis of cutin and waxes and (2) its covering probably protects the microcracks from the atmospheric exposure, thus, triggering of russeting.

Although, on the basis of the above statements, it is likely that Phase I of the moisture exposure is a low O_2 (theoretically anoxic) state, proof is still lacking. In addition, the following questions regarding russeting need to be answered to uncover the unknown regulatory mechanism(s) that generate and repair the barrier impaired by surface moisture: (1) How is this low O_2 state related to the regulation of reduced cutin and wax synthesis? (2) Why the later developmental stages (66 and 93 DAFB; **Ch. 2.2 and 2.3**) do not respond to such a low O_2 state and form an impaired barrier and russeting? The uniqueness of Phase I deserves further investigation.

3.3 Comparison of the present study with the recent studies on periderm formation in other plant species

The findings from the present study are similar with to those of the recent studies on periderm studies in other plant species and organs. Cases of russeting (pear, grapevine, kiwifruit and mango), wound periderm (potato, Chinese yam, tomato, melon and kiwifruit), and reticulation (melon and cucumber) are included here.

First, the etiology is in common in the following aspects: (1) microcracking triggers russeting; (2) surface moisture induces microcracking and russeting by weakening cuticle properties; (3) preventing the fruit from surface moisture reduces russeting. In apple's relatives, the pears (*Pyrus* spp.), higher surface growth rate leads to greater strain and is correlated with russeting occurrence on certain portion of the fruit (calyx and cheek of European pear; *P. communis* L; Scharwies et al., 2014) and likely to be a cause for microcracking (Winkler et al., 2022). Further, preventing young Asian sand pear fruit (*P. pyrifolia* Nakai; 20 to 40 DAFB) from the exposure to water reduces russeting severity, expression of several lignin and suberin-related genes (shelter; Shi et al., 2019) and the typically accumulated suberin monomers (bagging; Zhang et al., 2021). Similarly in mangoes, the coincidence of low cuticle deposition and higher elastic strain and surface moisture induce both microcracking and russeting which initiate around the lenticel (Athoo et al., 2020, 2021, 2022, 2023) and bagging the fruit reversely reduces russeting at maturity (Athoo et al., 2024).

Second, the molecular mechanism behind periderm formation is similar and includes: (1) the up-regulation of genes that are involved in lignin and suberin metabolism and meristem regulation and (2) the down-regulation of cutin and waxes-related genes. In the Chinese yam (*Dioscorea opposita*) tuber, wounding-caused impaired barrier induces of genes such as *PAL* (*Phenylalanine ammonia lyase*), *CYP86A1*, *CYP86B1*, *GPAT5*, *GPAT8*, *ABCG2* and *ABCG6* within a similar time frame as **Chapter 2.7** (7 d after wounding; Liu et al. 2023). Similar observations can be found in the cases of wounding on potato tubers (Lulai and Neubauer, 2014; Wahrenburg et al. 2021), tomato fruits (Han et al., 2018) and melon fruits (Xue et al., 2023). An identified gene *WOX4* (**Ch. 2.5)** whose homologues in native periderms of other species have a putative cambial activity [Cork oak (*Quercus suber*; Fernández-Piñán et al., 2021); poplar (Kucukoglu et al., 2017); Arabidopsis (Leal et al., 2022)]. A gene with similar function, *VAS* (*Vascular Tissue Size*), was identified by a phellogen-specific transcriptome of the native periderm and possibly functions in pro-cambial regulation and is induced within 3 d after wounding in the potato tuber (Vulavala et al., 2019). In addition, wounding also induces gene groups of chromatin remodeling-, histone-, DNA replication- and cell division-related

within 1 d in the wounded potato tuber (Vulavala et al., 2019; Wang et al., 2023; Woolfson et al., 2023), showing a link between an impaired barrier, meristem regulation and periderm initiation. Compared to its smooth-skinned mutant, the down-regulation of cutin and wax-related genes, including *WSD1* (*wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase*), *CER3* (*ECERIFERUM*) and *CYP86A22*, occurs at the 'russet begin stage' (100 DAFB) in an Asian sand pear cultivar (*P. pyrifolia* Nakai; Wu et al., 2023). In grapevines (*Vitis* spp.), a group of cutin- and wax-related genes, including the cuticular wax-associated *CER* protein family, is down-regulated at maturity (90 DAFB) in a russet cultivar compared to the other two smooth-skinned cultivars, and this down-regulation is more pronounced at the mature stage compared to the veraison stage (65 DAFB; Niederauer et al., 2024). In fact, microcracking is considered by the authors to be a cause of russeting and can be identified as early as the veraison stage (Niederauer et al., 2024). The onset of microcracking, however, can occur at least 16 d earlier than the veraison stage (Goffinet and Pearson, 1991).

Third, similar suberin monomers accumulate in the russeted or reticulated skin at fruit maturity. In the russeted or reticulated skin, the accumulation of the typical suberin monomers such as C20, -C22 -C24 and -C26 ω-hydroxy acids and ferulic acid, are significantly enriched than the non-russeted or non-reticulated skin [pear russeting (Zhang et al., 2021), cucumber reticulation (Arya et al. 2022; Nomberg et al., 2022) and melon reticulation (Cohen et al., 2019; Manasherova and Cohen, 2022)]. It is worth noting that the beginning of switch of accumulated chemical constituents coincides with the first occurrence of microcracks and reticulation at early fruit development. Both the Skkim cucumber (*Cucumis sativus* var. *sikkimensis*) and melon (*C. melo*) have this switch about 30 days after fertilization (DAF) (Arya et al. 2022; Cohen et al., 2019).

Fourth, atmospheric O_2 plays a critical role in periderm formation, and eliminating an impaired barrier to it from the outset may halt the consequences. A study on kiwifruit, where suberization of the wounded surface is O_2-dependent, shows that O_2 concentration affects the expression of several genes involved in ROS (reactive oxygen species) production and lignin synthesis (Wei et al., 2018), which contribute to the building of suberin (Woolfson et al., 2022). However, how these and related genes in suberin and lignin metabolism are regulated by O_2 is still largely unknown.

Overall, all the consequences that are caused by an impaired barrier and link to periderm formation seems to be universal across plant species.

3.4 Potential implications for horticultural practice – with focus on the apple crop

Since the present study suggests that russeting is associated with atmospheric exposure via an impaired barrier, it can be concluded that all horticultural practices that empirically and experimentally cause russeting in apple, including foliar fertilizers, thinning agents and pesticides, are likely to cause barrier impairment and thus atmospheric exposure of the underlying cell layers and russeting. However, in early fruit development these cultural practices are essential to strengthen the tree, maintain good orchard ecology and improve fruit quality. For thinning agents, there may be a reliable solution that maintains fruit quality and does not induce russeting (Metamintron; Penzel and Kröling, 2020). Unfortunately, it is still difficult to strike a balance between choosing an environmentally friendly biological material for plant protection and reducing russeting, as the use of *A. pullulans* to control fire blight still carries a risk of inducing russeting under wet conditions (Kunz et al., 2023). Therefore, moisture is still the most common and unavoidable challenge for the grower, when it comes to russeting.

With the updated information from the present study and other related studies, it is known that surface moisture retention directly and continuously affects the surface of young fruit by regulating cutin, wax deposition and even the cell wall of the underlying cell layers (**Ch. 2.2-2.5**). As a result, the protective function of the developing apple cuticle is impaired. To prevent this, the primary fruit surface must be protected from moisture exposure or strengthened by the application of agrochemicals.

First, reduced russeting is observed on the fruit surface protected by external coverings such as bags or shelters (Creasy and Swartz, 1981; Yuan et al., 2019). This could protect the fruit from direct exposure to moisture. However, several aspects need to be considered: (1) there must be no residual moisture retention on the fruit surface: a suitable material must not generate higher humidity inside the covering, and air circulation with heating in the early morning (Lee et al., 2022) may be worth testing; (2) appropriate timing of removal of the covering to avoid overheating in early summer and to minimize labor inputs. This could be limited to early development as this is the most critical period (before 44 DAFB; Creasy, 1980; Creasy and Swartz, 1981; Knoche et al., 2011; Winkler et al., 2014; **Ch. 2.2**); (3) some side effects of the covering: e.g. the color of the bag may affect fruit quality (Sharma et al., 2014) or the covering may reduce light intensity (Creasy and Swartz, 1981).

Second, the application of certain plant growth regulators or agrochemicals to the fruit surface would help to build a more robust epidermis or increase cuticle deposition. The application of

gibberellin A_{4+7} in early fruit development results in smaller and more uniform cells (Curry, 2012) and reduces microcracking and russeting that induced by 2-d moisture immersion but does not affect cuticle deposition or characteristics during development (Knoche et al., 2011). Another potential method to address the problem of russeting could be the application of agrochemicals. Although exotic coating of the fruit surface has been suggested (Skae and Farcuh, 2023; Winkler et al., 2022), the author is not aware of any reports of commercial coating products applied to young apples with evaluation of mature fruit for russeting. In addition to that, stimulation of cutin synthesis would be an alternative to strengthen the cuticle. Recent advances have shown that feeding the fruit surface with fatty acid precursors, such as oleic acid, increases cuticle deposition by incorporation (Si et al., 2021a, b). This method looks promising to researchers. However, it has only been tested on a russeting non-susceptible cultivar 'Idared' and relies on contact with the fruit surface using a dosage vial, so the smaller fruits (<50 DAFB) could not be fixed with the vial and tested (Si, 2022; Si et al., 2021b). In the field, this method will need to be modified as a spray method to be practical. A lower incorporation efficiency and a less homogeneous distribution and coverage of the solution on the surface can be expected (Si, 2022). Whether the modified method will also work on (1) russeting-susceptible cultivars with uneven cuticle thickness and (2) the smaller fruits (<50 DAFB) which are more susceptible to russeting than the more developed fruits, deserves further investigation.

3.5 Conclusion

The present study reveals the nature of russeting. It is shown that russeting is triggered by an impaired barrier via microcracking. In addition, the impaired barrier that triggers russeting must be exposed to the normal atmosphere where atmospheric O_2 is abundant (21%). This is supported not only by the need for O_2 in wound periderm formation, but also by the different responses to russeting (periderm initiation) of a microcrack covered (Phase I) or not covered (Phase II) by surface moisture. Therefore, the involvement of O_2 in the triggering of russeting is clear. The underlying molecular and cellular mechanisms regulated by O_2 during russeting open an area of research to be explored.

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Curriculum Vitae

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09/2009 – 08/2012	Master of Science – Department of Horticulture and Landscape Architecture, National Taiwan University (Taiwan) • Thesis: Studies on inoculation methods of guava (*Psidium guajava* L.) seedlings with *Nalanthamala psidii* (syn. *Myxosporium psidii*) and the screening for resistance.
09/2005 – 06/2009	Bachelor of Science – Department of Horticulture, National Chung-Hsing University (Taiwan) • Thesis: Molecular marker-assisted selection of EMS induced pineapple (*Ananas comosus* (L.) Merr.) mutants.
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