THE CALUM R WILSON (Orcid ID : 0000-0001-5434-3816)

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Suberin deposition in potato periderm: a novel resistance mechanism against tuber greening

Sabine Tanios[1] Tamilarasan Thangavel[1] Alieta Eyles[1,2] Robert S. Tegg[1] David S. Nichols[3] Ross Corkrey[2] Calum R. Wilson[1]

[1] Tasmanian Institute of Agriculture, University of Tasmania, New Town Research Laboratories, 13 St. Johns Avenue, New Town, Tasmania 7008, Australia.

[2] Tasmanian Institute of Agriculture, University of Tasmania, Private Bag 98 Hobart, Tasmania 7001, Australia.

[3] Central Science Laboratory, University of Tasmania, Private Bag 74, Hobart, Tasmania 7001, Australia.

Author for correspondence:
Calum R. Wilson.
Tel: +61 3 6226 6381
Email: calum.wilson@utas.edu.au

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ORCID INFORMATION

Sabine Tanios	https://orcid.org/0000-0003-1420-412X
Tamilarasan Thangavel	https://orcid.org/0000-0002-8800-0990
Alieta Eyles	https://orcid.org/0000-0003-4432-6216
Robert Tegg	https://orcid.org/0000-0001-7668-7927
David Nichols	https://orcid.org/0000-0002-8066-3132
Ross Corkrey	https://orcid.org/0000-0002-2242-2891
Calum Wilson	https://orcid.org/0000-0001-5434-3816

Summary

- Light-induced tuber greening is one of the most important quality defects of potato. Although varietal and maturity factors are known to affect greening resistance, physiological mechanisms of resistance are poorly understood. We proposed that physiological and biochemical factors within the tuber periderm provide resistance and hypothesised that resistance is primarily related to suberin content.
- We investigated differences in the tuber periderm between genotypes and tuber maturities that varied in greening propensity. We examined suberin and light-induced pigment accumulation, and phellem cell development, and studied greening propensity in mutant and chemically-treated tubers with enhanced suberisation.
- Resistance to greening was strongly linked to increased suberin in the periderm, which varied with variety and tuber maturity. Furthermore, greening was reduced in mutant and chemically-treated tubers with enhanced suberisation. Increases in phellem cell layers and light-induced carotenoids and anthocyanins were identified as secondary resistance factors.
- Our work represents the first physiological mechanism of varietal and tuber maturity resistance to greening, expanding the known functionality of suberin and providing for the first time a biomarker that will aid producers and breeders in selection and improvement of potato varieties for greening resistance.

Keywords: anthocyanins, carotenoids, chlorophyll, phellem, potato, suberin, tuber greening resistance

Introduction

As underground-modified stems, potato tubers are non-photosynthetic tissues that lack photosynthetic machinery. However, following light exposure, potatoes can accumulate chlorophyll in their peripheral cell layers. These pigments reflect predominantly green light, which gives potato tubers a green colour. Greening, which can occur in the field or post-harvest (Bamberg *et al.,* 2015; Tanios *et al.,* 2018), is due to the conversion of amyloplasts into chloroplasts, which pre-empts chlorophyll formation in the tuber peripheral cell layers (Anstis & Northcote, 1973; Zhu *et al.,* 1984; Muraja-Fras *et al.,* 1994). Concurrent with tuber greening, although under independent genetic control, is the light-induced accumulation of glycoalkaloids that causes a bitter taint in potato tubers (Bamberg *et al.,* 2015; Friedman *et al.,* 1997; Friedman, 2006; Nema *et al.,* 2008; Ginzberg *et al.,* 2009; Omayio *et al.,* 2016). Glycoalkaloids pose a perceived health risk, although accumulation rarely reaches toxic levels and is restricted to the outer cell layers of the tuber (Burton, 1974; Smith *et al.,* 1996; Valkonen *et al.,* 1996). Tuber greening however, is an important indicator of tuber quality and a major cause of consumer rejection and therefore, of economic significance (French-Brooks, 2012).

Tuber greening is influenced by varietal genetics (Akeley *et al.,* 1962; Brown & Riley, 1976; Butcher, 1978; Reeves, 1988), tuber physiology (Griffiths *et al.,* 1994), and the environment (Tanios *et al.,* 2018). However, the possible mechanisms underlying variations in greening susceptibility have not been elucidated and further contemporary mechanistic investigations are needed.

The potato periderm consists of three different multilayered cell types; 1) the phellem which comprises 6-12 layers of dead cells with suberised walls; 2) the phellogen, a meristematic layer of cells that gives rise to neighbouring phellem and phelloderm cells; and 3) the phelloderm which consists of one or few cell layers, that are difficult to distinguish from the cortical parenchyma (Reeve et al., 1970; Lulai & Freeman, 2001; Lulai, 2002). The periderm of immature tubers has thin phellogen cells making it very fragile and susceptible to wounding. As the periderm matures, phellogen cell walls thicken and strengthen (Lulai & Freeman, 2001; Schreiber et al., 2005a), and the adjacent phellem cells develop suberin and wax deposits (Schreiber et al., 2005b), forming a constitutive physical barrier that reduces water loss and confers protection against pathogens (Lendzian, 2006).

Suberin is a complex layered heteropolymer that consists of a fatty acid-derived domain (or aliphatic suberin) cross-linked to a polyaromatic lignin-like domain (or aromatic suberin) (Bernards, 2002; Beisson *et al.,* 2012). Upon trans-esterification, the aliphatic domain releases alcohols, ω-hydroxyacids, α,ω-diacids, very-long-chain fatty acids, reticulated with glycerol and small amounts of hydroxycinnamic acids, mainly ferulic acid (Schreiber *et al.,* 2005b; Graça, 2010). The aromatic domain is a lignin-like polymer composed of cross-linked hydroxycinnamic acid monomers that are covalently bonded to the aliphatic suberin (Bernards, 2002; Kolattukudy, 2001; Graça, 2007). Suberin is also associated with waxes, accounting for 4% of the lipids present in the periderm (Serra *et al.,* 2010).

Suberin in the tuber periderm is constitutively synthetized and can also be induced by wounding, pathogen attack and stresses such as salinity (Schreiber *et al.,* 2005a; Reinhardt & Rost, 1995; Karahara *et al.,* 2004) and phytotoxins (Thangavel *et al.,* 2016). Suberin plays key roles in the adaptation of plants to biotic and abiotic stress, control of water exchange and resistance to desiccation (Andersen *et al.,* 2015; Graça, 2015). To our knowledge, the role of suberin in resistance to tuber greening has not been previously examined. Grunenfelder *et al.* (2006) suggested a role of periderm thickness in greening resistance, however, no documented evidence is available to confirm this.

The goal of this study was to develop a fundamental understanding of the physiological factors associated with tuber greening resistance. We assessed the relationship between the propensity for tuber greening, amongst a cohort of distinct potato genotypes at differing physiological age, and tuber periderm properties (suberin content, number of phellem cell layers and pigmentation). We hypothesised that resistance to greening is primarily related to the periderm suberin content.

Materials and Methods

Plant material

This study examined greening response within 104 potato clones comprised of 61 different varieties. When multiple clones of individual varieties were assessed (designated by numerals after the variety name), these had been maintained independently in tissue culture for several

decades and could have been subject to somaclonal drift (Dann & Wilson, 2011). Tissue-culture plants (two-nodes segments) of all clones were cultured in Murashige and Skoog medium supplemented with sucrose (30 g L^{-1}), ascorbic acid (0.04 g L^{-1}), casein hydrolysate (0.5 g L^{-1}), and agar (8 g L^{-1}), with a pH of 5.8, and grown at 22 °C with a 16 h photoperiod under cool white fluorescent lamps (65 μmol m^{-2} s^{-1}). Four-week-old plants were transferred to potting mix containing sand, peat, and composted pine bark (10:10:80; pH 6.0) premixed with Osmocote 16–3.5–10 NPK resin-coated fertiliser (Scotts Australia Pty Ltd. Baulkham Hills, Australia), and grown under controlled glasshouse conditions, between 18 and 24 °C. Soil was regularly topped up to protect growing tubers from light exposure. Each clone was harvested following natural senescence. Tubers that formed close to the soil surface were discarded while the rest were stored in the dark at room temperature for approximately 30 days to allow post-harvest maturation.

Light exposure treatment

In each experiment three tubers of each clone were exposed to a fluorescent light source (Supporting Information Figure S1) with an intensity of 12 μmol m^{-2} s^{-1}at the tuber surface for 120 hours at room temperature. The tubers were arranged in rows and their places within the row were repositioned daily, ensuring that the orientation of the tuber remained the same, to avoid any possible bias of positioning in relation to variation in light intensity. All selected tubers were of similar size and free of visible damage.

Varietal screening for greening resistance

Following light exposure, the increase in chlorophyll and change were assessed using a colourimeter and spectrophotometer respectively, as detailed below.

Colour assessment

Tuber colour was measured with a colourimeter (Konica Minolta CR-400), standardised against a white tile, using L* (lightness), a* (green-red colour axis), and b* (blue-yellow axis) parameters. Colour measurements were taken with three technical replicates, from the stem, the middle and the

bud end of each tuber, before and after five days light exposure treatment. Colour difference was calculated as follows:

$\Delta E^*ab = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$

Where ΔL^*, Δa^* and Δb^* represent the differences in L*, a* and b* values before and after light treatments.

Chlorophyll, carotenoid and anthocyanin analysis

Six periderm disks (1.5 mm thick and 1 cm diameter) were cut using a cork borer from the stem, the middle and the bud end of each tuber periderm. The disks were ground to powder in liquid nitrogen using a mortar and pestle. Half the samples were extracted with 5 mL of N, N-dimethylformamide for chlorophyll and carotenoid assessment, while the other half was extracted with 12 mL of 1% HCL in methanol for anthocyanin analysis. All samples were stored at 4 °C in the dark for 24 hours. After centrifugation for 15 min at 2500 × g, the absorbance was measured with a spectrophotometer at 647, 664 and 480 nm for chlorophyll and carotenoid, and at 530 and 657 nm for anthocyanin.

Total chlorophyll components (Porra *et al.,* 1989), carotenoid (Wellburn, 1994) and anthocyanin concentrations (Mancinelli *et al.,* 1975) were determined before and after light treatment using the following equations:

Total chlorophyll = 17.67 (A647) + 7.12 (A664)

Total carotenoids = (1000 A480 – 1.12 Chla – 34.07 Chlb)/245

Total anthocyanins = $(A \times MW \times V \times 10^3)/ (\varepsilon \times l \times W)$

The anthocyanin content was determined as cyanidin-3-glucoside equivalent, where A = (A530 - 1/3 A 657); MW is 484.83 g/mol (the molecular weight of cyanidin-3-glucoside); V is the volume of extraction solution, ε is 26900 L mol^{-1} cm^{-1} (the molar extinction coefficient for cyanidin-3-glucoside), l is the path length (cm) and W is fresh weight (g). Total pigments show the sum of chlorophyll, carotenoid and anthocyanin. All pigment concentrations are expressed in mg k^{-1} fresh weight (FW).

Maturity effect on greening

Based on the results of the clone screening trial, 22 varieties with differing greening tendency were selected to examine the effect of tuber maturity on chlorophyll concentration. Potato plants were grown under controlled glasshouse conditions as before. Three tubers from each variety at three different maturity stages were assessed 1) tubers harvested approximately 1 month before natural senescence (denoted as immature; I), 2) tubers harvested at senescence and used immediately (mature without storage; M-S) and 3) tubers harvested at senescence and stored at 4 °C for 4 months before testing (mature with storage; M+S). Tubers were subject to light exposure following which chlorophyll and ΔE*ab were assessed.

Histological analysis of tuber periderm

The number of phellem cell layers were measured for 14 varieties at two different maturity stages, immature (I), and mature with storage (M+S) as previously defined. Three biological replications were examined per variety with five measurements per tuber. Tuber periderm samples, taken from internode two, were hand-sectioned with a razor blade and stained with 0.1% Toluidine blue, which has been shown to clearly differentiate between suberised phellem layers that stained orthochromatically in blue and phellogen and phelloderm layers that stained metachromatically in violet (Sabba & Lulai, 2005). The number of phellem cell layers were examined using a light microscope (Leica DMLB, Type LB 30T; Leica Microsystems).

Extraction of suberised tissues

The chemical composition of periderm aliphatic suberin was tested in immature and mature tubers for the same 14 varieties of various periderm colour and with different greening susceptibility. The analysis was limited to the insoluble aliphatic fraction since it accounts for 96% of the total lipids in wild-type periderm (Serra *et al.,* 2009). For clarity in the terminology of this study, we refer to suberin as the aliphatic polyester. Periderm discs were peeled from tubers and immersed in a 25 ml mixture of cellulase (5 g L^{-1}) and pectinase (1 g L^{-1}) in acetate buffer (0.4 g L^{-1}, pH 4) for 5 days. The solution was changed twice until clear. Sodium azide (0.065 g L^{-1}) was added to prevent bacterial growth. The isolated periderm membranes were washed twice in 10^{-2} M borate buffer

(pH 9) for 24 h and then carefully washed with deionised water. Extraction of soluble materials from isolated periderm samples were performed in a mixture of 1:1 (v/v) of chloroform:methanol for 18 h. The resulting extractive-free suberin-rich membrane samples were washed with water and air-dried before depolymerisation. All incubations were carried out at room temperature with shaking at 80 rpm.

Chemical depolymerisation of suberin and GC-MS analysis

Dried suberin enriched samples (50 mg) were treated with 4 mL of 14% (w/w) boron trifluoride in methanol overnight at 80 °C. After resuspension with 1 mL H_2O, suberin monomers were extracted with 1.5 mL 4:1 (v/v) hexane:chloroform. Once the phases had separated, the organic phase containing the monomers was transferred to GC vials. Trimethylsilyl (TMS) derivatives were prepared after complete solvent evaporation under nitrogen by adding 50 µL chloroform and 50 µL N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and then incubated overnight at 50 °C. After evaporation, 150 µL of an internal standard, tricosanoate (0.1 g L^{-1} in chloroform), was added. Analysis of the composition of the suberin monomers was determined by a Varian CP-3800 gas chromatograph with CP-8400 autosampler coupled to a Brüker 300-MS triple quadrupole mass spectrometer fitted with an Agilent DB-5MS column (30 m × 0.25 mm, with 0.25 µm phase thickness). GC operating conditions were: injections 1 µL, injector and transfer line temperatures 290 °C, split injection (14:1); carrier gas He (linear velocity = 42 cm s^{-1}). The oven temperature was programmed from 100 °C to 220 °C at 10 °C /min, from 220 °C to 290 °C at 3 °C /min, and held for 15 min, then increased from 290 °C to 320 °C at 10 °C /min, and held for 5 min. Full scan mass spectra were acquired over the range (m/z) 40 to 550. Monomers were identified by interpretation of their full scan mass spectra and comparison to known mass spectra within the National Institute of Standard and Technology (NIST, USA) Mass Spectral Library (2017). All monomers were quantified by comparison to the internal standard and expressed in µg mg^{-1} dry weight. Monomers were grouped into different classes for further analysis.

Relative transcript expression of CYP86A33, a gene associated with aliphatic suberin biosynthesis

Total RNA was isolated from the tuber periderm of the same 14 potato varieties, harvested at senescence and stored at 4 °C for 4 months before testing, using the PowerPlant® RNA isolation Kit (MOBIO) according to the manufacturer's instructions. Following DNase treatment (DNase Max ™ kit, MOBIO), RNA yield was quantified using a Qubit® 2.0 Fluorometer (Life Technologies, Mulgrave, VIC, Australia). One microgram of RNA was reverse transcribed using the SuperScript™ IV VILO™ Master Mix (ThermoFisher) following manufacturer's protocol. Quantitative real-time PCR was performed using 1 µL of 10-fold-diluted cDNA in a 10 µL total volume reaction. The primers sequences for CYP86A33 (Gene ID: PGSC0003DMG400030349) and the reference gene EF1α were as follows: F: GGTGGGTAAACCGGACCATC; R: GCAACTCGACCGGGTTTTTT and F: ATTGGAAACGGATATGCTCCA; R: TCCTTACCTGAACGCCTGTCA, respectively. We note there are two genes within potato annotated CYP86A33, but only the cited gene has been demonstrated to have a role in suberin biosynthesis. The CYP86A33 gene was sequenced in four potato varieties that represent a range of greening propensity (Nicola, Maris Piper, Maranca and Kennebec). No SNP's were present in the primer binding sites or the amplified product within or between varieties that could affect the RT-qPCR efficiency (Supporting Information Fig. S2). RT- qPCR were carried out using iTaq Universal SYBR Green Supermix (BIO-RAD) in a Rotor Gene 6000 instrument (Qiagen) with a thermocycle of 95 °C for 15 mins, followed by 40 cycles of 95 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s. The mRNA levels were calculated relative to that of the reference gene.

To determine whether CYP86A33 expression was affected by light treatment, two potato varieties Nicola and Kennebec, with low and high greening propensity, respectively, were used after being stored for approximately 30 days following natural senescence. Gene expression of CYP86A33 was compared in tubers kept in the dark and after 168 hours of light treatment, using RT- qPCR, as detailed above.

Effect on greening of induced suberisation following treatment with the phytotoxin analogue Thaxtomin D

To explore the specific role of suberin in greening without the confounding nature of diverse genetic backgrounds, we examined the effect of chemically induced suberisation. Potato tubers of

variety Maranca, were treated with synthetically produced thaxtomin D (Molesworth *et al.,* 2010), diluted to 3.5 μM in distilled water, which has been shown to induce suberin deposition in the potato tuber periderm (Thangavel *et al.,* 2016). Control tubers were treated with water only. Tubers were treated every second day for 10 days using a misting spray and stored in the dark at room temperature. Tubers were exposed to light as before and suberin and chlorophyll concentrations were measured by GC-MS and spectrophotometry respectively.

Comparison of greening between a highly suberised potato mutant and its parent

The Russet Burbank mutant A380 (Wilson *et al.,* 2010) that had previously been shown to have enhanced constitutive and induced suberisation compared to the parental line (Thangavel *et al.,* 2016) was assessed for resistance to greening. Tubers of the mutant and parent were produced from tissue-cultured plantlets, exposed to light and assessed for change in chlorophyll concentration and ΔE*ab as previously described.

Statistical analysis

Unless otherwise stated, for each experiment, three biological replications were used per treatment. The effect of 1) maturity on Δchlorophyll for each variety, 2) thaxtomin treatment on suberin and Δchlorophyll, and 3) variety on CYP8633 relative expression were analysed using one-way analysis of variance. The effect of variety and maturity and their interaction on pigments accumulation (Δchlorophyll, Δcarotenoid, Δanthocyanin and Δtotal pigment), number of phellem cell layers, suberin monomers were examined using two-way analysis of variance. Means were separated using Fisher's protected least significant difference (LSD) test ($P < 0.05$). All statistical analyses were carried out using R statistical software version 3.5.

Given that our results showed that light induces the accumulation not just of chlorophyll, but also other pigments, we examined the relationship of each of Δchlorophyll, Δcarotenoid, Δanthocyanin, Δtotal pigments with phellem layers and suberin. Initially, varieties were classified depending on their skin colours in three different groups (white/yellow; russet and red/pink) and Pearson correlation analysis was conducted to examine the correlations between different datasets. A regression approach was used to assess the influence of suberin, phellem layers and maturity on

each of chlorophyll, carotenoids, anthocyanin and total pigments. All models were fitted using the 'lm' function in R statistical software version 4.3. After examination of residuals, only total pigments required a square root transformation. To examine whether increases in pigments could be best explained by 1) number of phellem layers only; 2) suberin concentration only; or 3) an interaction of phellem layers and suberin concentration, we fitted a multiple regression model using these terms as predictors with maturity as a categorical factor. Interactions of maturity with the continuous effects were also included. Starting with the full model consisting of continuous predictors and maturity as well as interactions of maturity with the predictors, the stepAIC function in the MASS library (Venables & Ripley, 2002) was used to obtain a simpler model. This approach made use of Akaike information criterion (Akaike, 1974) to select the best fitting model while minimizing model complexity. Modelling assumptions were checked using quantile-quantile plots and residual plots. The outcome was transformed if needed and the model assumptions checked again.

Results

Varietal screening for greening resistance

A significant increase in chlorophyll was noted in all 104 clones, ranging from 0.32 to 4.02 mg kg^{-1} FW (Fig. 1A). Similarly, colour difference (ΔE*ab) varied from 2.22 to 17.33 (Fig. 1A). For example, Nicola 2 accumulated 0.73 mg kg^{-1} FW chlorophyll with a periderm colour change of 5.06 (Fig. 1B), while Kennebec 5 accumulated 4.02 mg kg^{-1} FW with a colour change of 17.33 (Fig. 1C). A Pearson correlation analysis showed that chlorophyll increase, and colour change were highly positively correlated ($r=0.85$, $p<0.001$). Testing of multiple clones of the same variety revealed most clustered together showing a similar greening response with minor, but occasionally significant, differences between clones (Fig. 1A).

For white and yellow-skinned varieties, greening was clearly visible on the periderm surface, however for russet, red, pink and purple skin varieties the greening was less clear due to masking by the pigments. A prominent light-induced deep brown discolouration was observed in all clones of certain varieties (Wilwash, Pink Eye and Coliban) which interfered with the colorimetric assessment of these tubers (Fig. 1D). If we exclude these varieties, a correlation coefficient of $r=0.93$ ($p<0.001$) was obtained between chlorophyll content and colour change

across the studied clones. When varieties were classified into three groups based on their skin colour, significantly less change in both chlorophyll content and colour was observed for pink/purple/red compared to the white/yellow and Russet varieties, the latter two groups showing no statistical difference between them (Fig. 2).

Greening susceptibility influenced by tuber maturity

Change in chlorophyll concentration was influenced by tuber age, with the highest greening rates observed in tubers classified as immature (I), followed by mature without storage (M–S) and then mature and stored (M+S) (Table 1). Change in chlorophyll concentration varied depending on variety, from 0.73 to 4.59 mg kg^{-1} FW for (I) tubers, from 0.5 to 4.26 mg kg^{-1} FW for (M–S) and from 0.41 to 4.17 mg kg^{-1} FW for (M+S) (Table 1). Significantly greater change in chlorophyll concentration was found in (I) compared to (M–S) and (M+S) tubers in 15 and 18 out of 22 varieties, respectively. When (M–S) tubers were compared to (M+S), no significant differences were found in 19 varieties out of 22 for change in chlorophyll concentration.

Light exposure increased carotenoid and anthocyanin concentration

Light induced the synthesis not just of chlorophyll but also of carotenoids and anthocyanins in tubers. While all 14 studied varieties showed an increase in carotenoids, the increase in anthocyanins were noted in red-skinned varieties only (Table 2). The increase in both carotenoids and anthocyanins significantly varied between variety ($p<0.0001$; Supporting Information Table S1) and tuber maturity with a higher accumulation in immature (I) compared to mature tubers (M+S) ($p<0.0001$; Supporting Information Table S2) and a significant interaction between varieties and their maturity stage ($p<0.0001$; Table 2).

The number of phellem layers and suberin concentration in tuber periderm influenced by variety and maturity stage

The average number of phellem cell layers ranged from 4.33 to 9.00 for immature Kennebec and Ruby Lou, respectively and from 5.50 to 12.33 for mature King Edward and Ruby Lou,

respectively (Table 2). The number of phellem layers was significantly influenced by variety (p<0.0001; Supporting Information Table S1). Immature tubers had a significantly lower number of phellem cell layers than mature ones (p<0.001; Supporting Information Table S2), with a significant interaction was found between variety and tuber maturity (P<0.001; Table 2).

The depolymerisation of tuber aliphatic suberin yielded four major classes consisting of alcohols, α,ω-diacids, ω-hydroxyacids, and long-chain fatty acids. The ω-hydroxyacids accounted for the highest proportion of the polymer while there were very low levels of dihydroxy acids and ferulic acid (Table 2). For immature tubers (I), total aliphatic suberin concentration ranged from 304.81 ± 21.44 μg mg^{-1} for Maris Piper to 187.72 ± 17.33 μg mg^{-1} for Kennebec (Table 2). For mature tubers (M+S), it varied from a maximum of 389.46 ± 39.53 μg mg^{-1} for Maris Piper to a minimum of 242.87 ± 4.2 μg mg^{-1} for Red Ruby (Table 2). Significant differences were found between varieties for all classes of suberin compounds (Supporting Information Table S1). Suberin concentration was significantly higher in mature than immature tubers (Supporting Information Table S2). A significant interaction was found between varieties and their maturity stage for α,ω-diacids (p<0.0001), ω-hydroxyacids (p<0.05), dihydroxy acids (p<0.05) and total suberin (p<0.0001), but not for alcohols (p=0.84), fatty acids (p=0.31) and ferulic acid (p=0.3) (Table 2).

The relationship between the number of phellem layers, suberin and pigments concentration

For each colour group, positive correlations were found between the different pigments, as well as between the number of phellem layers and suberin concentration, while negative correlations were found between the different pigments and phellem layers and suberin concentration (Fig. 3). None of the fitted regression models included a significant effect for the interaction of maturity with the continuous predictors, although it was retained in some cases as an additive effect. The final fitted model for chlorophyll decline showed total suberin as the best predictor (p<0.001) followed by phellem cell layers (p=0.003) (Fig. 4A; Supporting Information Table S3). At any specific suberin level, mature tubers have higher chlorophyll than immature tubers (Fig. 4A; Supporting Information Table S3). Carotenoid similarly declined with increasing suberin (p<0.001) and phellem cell layers (p<0.001) while maturity was not a significant predictor (Figure 4B; Supporting Information Table S3). Anthocyanin also decreased with increasing suberin (p<0.001),

which was the only significant predictor (Fig. 4C; Supporting Information Table S3). Total pigments were also found to decline with increasing suberin (p<0.001) with mature tubers having higher pigments that immature tubers (Fig. 4D; Supporting Information Table S3).

Enhanced suberisation limits chlorophyll accumulation

Suberin concentration of tubers treated with thaxtomin D were significantly increased by 24% compared to the untreated control (Supporting Information Table S4A). Conversely, chlorophyll concentration of the treated tubers significantly decreased by 16% compared to the untreated control. Similarly, following light exposure, tubers of the Russet Burbank mutant A380 with an enhanced suberisation phenotype, accumulated 32% less chlorophyll than the parent variety (Supporting Information Table S4B).

CYP86A33 expression pattern varied between varieties and correlates with suberin amount

CYP86A33, important for aliphatic suberin biosynthesis, mRNA levels significantly varied between the 14 varieties with a 5.8-fold change between the lowest expression in Kennebec, and Maris Piper, with the highest expression. The expression pattern for the different varieties showed a positive correlation with the total amount of aliphatic suberin (r=0.95; p<0.001; Fig. 5). CYP86A33 expression levels in tubers, prior to and after light exposure, were similar for both Nicola and Kennebec (Supporting Information Fig. S3).

Discussion

Our study provides new and substantive evidence that varietal and tuber maturity resistance to greening is strongly associated with higher suberin deposition in the tuber periderm, while the number of phellem cell layers and varietal periderm colour were identified as secondary resistance mechanisms.

Several lines of evidence are reported to support the main finding, that suberin deposition in tuber periderm improves resistance to greening. Firstly, this study clearly showed that increased suberin content in tuber periderm was strongly associated with reduced greening propensity,

demonstrated by a strong negative correlation between suberin content and chlorophyll across different varieties (Figs. 3 and 4). The amount and composition of suberin of the varieties investigated here fall within the range described in previous studies (Schreiber *et al.,* 2005; Yang & Bernards, 2006). Further to this, the transcript profiles of CYP86A33, a strong candidate gene for suberin biosynthesis, in potato tubers (Serra *et al.,* 2009) and other plants (Li *et al.,* 2007; Soler *et al.,* 2007; Höfer *et al.,* 2008), which appeared to be independent of light, were consistent with the suberin amount found in the tested varieties, implying a difference in suberin biosynthesis between varieties, and suggesting that the decrease in suberin in some varieties may be due to the downregulation of suberin biosynthesis. Secondly, regardless of variety, physiologically mature tubers had higher suberin concentration than immature ones, which was consistent with a reduction in chlorophyll concentration in more mature tubers, as previously suggested (Griffiths *et al.,* 1994), but also previously challenged (Buck & Akeley, 1967). Thirdly, the specific role of periderm suberin in reducing greening without the confounding nature of a diverse genetic background between different varieties, was confirmed from the observed reduction in chlorophyll concentration in tubers with enhanced suberisation from chemical induction (Supporting Information Table S4A) and a potato mutant with enhanced periderm suberisation (Supporting Information Table S4B). Taken together, our results provide the first evidence of a physiological mechanism for resistance to tuber greening, indicating a central role of periderm suberisation.

A secondary resistance mechanism to greening was associated with increased number of phellem cell layers. This was demonstrated by a negative correlation between chlorophyll and the number of phellem cell layers, suggesting that a thin periderm provides less protection against pigment accumulation than a thicker one. A previous study found that after 10–15 days from harvest, the periderm continues to differentiate and the phellem becomes more tightly attached to the tuber via the connecting radial phellogen cell walls (Lulai & Freeman, 2001), giving rise to the multilayered mature periderm, which has been shown to protect tubers against biotic and abiotic stresses including pathogen attack, dehydration, wounding (Graça, 2015) and as shown in this study, to greening.

Varietal skin colour was also an important determinant of greening susceptibility, which we propose is a complementary mechanism of resistance to tuber greening. Based on tuber skin colour, varieties with red, purple and pink hue accumulated less chlorophyll than the white, yellow and russet skinned varieties, as previously observed (Reeves, 1988). This could be explained by

the presence of anthocyanin pigments in the periderm of these varieties, which can act as light attenuators, absorbing high-energy blue-green quanta, potentially competing with chlorophyll (Chalker-Scott, 1999) and limiting its accumulation in underlying cells.

In conclusion, the suberin barrier plays a central role in the adaptation of plants to terrestrial life, particularly by improving resistance to biotic and abiotic stress such as controlling water, solutes and gases movement and imparting resistance to many pathogens (Graça, 2015, Pollard & Beisson, 2008). In this work, multiple lines of evidence indicate a new function for suberin, conferring resistance to potato tuber greening. Therefore, breeding and agronomic approaches that increase suberin deposition in tubers could be valuable in improving resistance to greening, increasing product shelf life and reducing food waste.

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Author Contributions

CRW, ST, AE, TT, RST and DSN designed the study and developed the methodology. ST, TT and AE performed the experiments. RC, TT and ST analysed the data. DSN performed the GC-MS analysis. ST wrote the manuscript with input from all authors.

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Figure Legends

Fig. 1. Response of potato clones to 120 hours of light exposure. (A) Change in chlorophyll concentration and colour (ΔE*ab) within 104 potato clones, data represents the mean ± SE. The r value is the Pearson's correlation coefficients, showing the correlation between ΔChlorophyll concentration and ΔE*ab data. Potato varieties (B) Nicola, (C) Kennebec and (D) Coliban before and after light exposure. $\Delta E^*ab = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$.

Fig. 2. Change in chlorophyll concentration and colour (ΔE*ab) for potato tubers of three periderm colour classes after 120 hours of light exposure. The three different classes represent the means ± SE of 18, 66 and 16 clones for pink/purple/red, white/yellow and Russet, respectively. Capital and small different letters indicate significant differences between groups by Fisher's protected least significant difference (LSD) test for ΔChlorophyll and ΔE*ab, respectively. $\Delta E^*ab = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$.

Fig. 3. Correlation matrix for change in chlorophyll, carotenoid, anthocyanin, and total pigments, total suberin and number of phellem layers in white/yellow (A), russet (B) and red/pink (C) potato varieties. The Pearson correlation coefficient between a pair of variables is presented by a corresponding colour as indicated in the colour key.

Fig. 4. Associations between change in tuber pigments, phellem cell layers and periderm suberin content in potato tubers. Observed data and fitted model for chlorophyll (A), carotenoid (B), anthocyanin (C) and total pigments (D). Fitted lines are shown for selected values of phellem layers in (A) and (B). Red and blue dots refer to immature and mature tubers, respectively. The significant regressions shown are described by the following equations: (A) Chlorophyll = 8.30 − 0.133 · Phellem Layers − 0.0197 · Total Suberin + (0 if Maturity=I; 0.777 if Maturity=M), (B) Carotenoid = 1.98 − 0.069 · Phellem Layers − 0.003 · Total Suberin, (C) Anthocyanin = 4.73−0.011 · Total Suberin, (D) √Total pigments = 4.15 − 0.009 · Total Suberin + (0 if Maturity=I; 0.196 if Maturity=M), with I and M referring to immature and mature, respectively.

Fig. 5. Differential expression of suberin biosynthesis gene CYP86A33 and relationship to suberin content within select potato varieties. (A) Expression profile of CYP86A33 gene for 14 potato varieties. Gene transcription was monitored using quantitative RT-PCR and the expression levels were determined relative to the reference gene EF1α. Mean values of three biological replicates are shown. Different letters indicate that means are significantly different at $P<0.05$ using Fisher's protected least significant difference (LSD) test. (B) Relationship between CYP86A33 relative expression and total suberin. *R* value represent Pearson correlation coefficient.

Supporting Information

Fig. S1. Spectra of the fluorescent light source used in this study to induce greening of potato tubers.

Fig. S2. CYP86A33 gene sequences in four potato varieties and the reference genome.

Fig. S3. Expression levels of suberin biosynthesis gene CYP86A33 pre- and post- light treatment of potato tubers.

Table S1. Change in pigments, phellem cell layers and suberin components after light exposure in potato varieties.

Table S2. Change in pigments, phellem cell layers and suberin components after light exposure in immature and mature potatoes.

Table S3. Regression models for influence of tuber suberin, phellem layers and maturity on potato tuber pigment.

Table S4. Change in chlorophyll concentrations and colour after light exposure in potato tubers with enhanced suberization.

Table 1. Change in chlorophyll concentrations after 120 hours of light exposure in potato tubers assessed at three different maturities.

Variety	ΔChlorophyll (mg kg^{-1} FW)				
	I	M – S	M + S	*LSD*	*P*
Atlantic 3	3.69 ^{a}	3.24 ^{b}	3.15 ^{b}	*0.34*	*0.01*
Bintje 3	2.57	1.95	1.93	-	*ns*
Coliban 2	2.17	1.87	1.82	-	*ns*
Desiree 1	2.05 ^{a}	1.51 ^{b}	1.42 ^{b}	*0.35*	*0.009*
Kennebec 5	4.59 ^{a}	4.26 ^{ab}	4.17 ^{b}	*0.33*	*0.04*
King Edward 2	3.37 ^{a}	2.67 ^{b}	2.55 ^{b}	*0.31*	*< 0.001*
Kipfler 2	4.05 ^{a}	3.43 ^{b}	3.38 ^{b}	*0.47*	*0.02*
Maranca	3.51	3.29	3.05	-	*ns*
Maris Piper	1.29 ^{a}	0.98 ^{b}	0.86 ^{b}	*0.29*	*0.03*
Nampa	4.35 ^{a}	3.85 ^{b}	3.76 ^{b}	*0.43*	*0.03*
Nicola 2	1.03 ^{a}	0.89 ^{ab}	0.74 ^{b}	*0.22*	*0.05*
Pink eye 2	3.44 ^{a}	2.78 ^{b}	2.53 ^{b}	*0.51*	*0.01*
Pontiac	1.36 ^{a}	0.83 ^{b}	0.81 ^{b}	*0.45*	*0.04*
Ranger Russet 2	0.96 ^{a}	0.58 ^{b}	0.38 ^{c}	*0.11*	*<0.001*
Red Ruby	3.24 ^{a}	2.97 ^{b}	2.89 ^{b}	*0.21*	*0.01*
Ruby Lou	0.73 ^{a}	0.50 ^{ab}	0.41 ^{b}	*0.24*	*0.03*
Russet Burbank 4	3.14 ^{a}	2.71 ^{b}	2.45 ^{c}	*0.28*	*<0.001*
Russet Nugget	2.29 ^{a}	1.92 ^{b}	1.91 ^{b}	*0.20*	*0.005*
Shepody 1	3.38 ^{a}	2.58 ^{b}	2.47 ^{b}	*0.18*	*<0.001*
Southern Cross 1	2.29 ^{a}	1.83 ^{b}	1.64 ^{b}	*0.33*	*0.007*
Sunrise	4.24	4.01	3.97	-	*ns*
Toolangi Delight	1.84 ^{a}	1.52 ^{b}	1.10 ^{c}	*0.31*	*0.002*
Mean	2.64^{a}	2.20^{b}	2.10^{b}	*0.39*	*0.015*

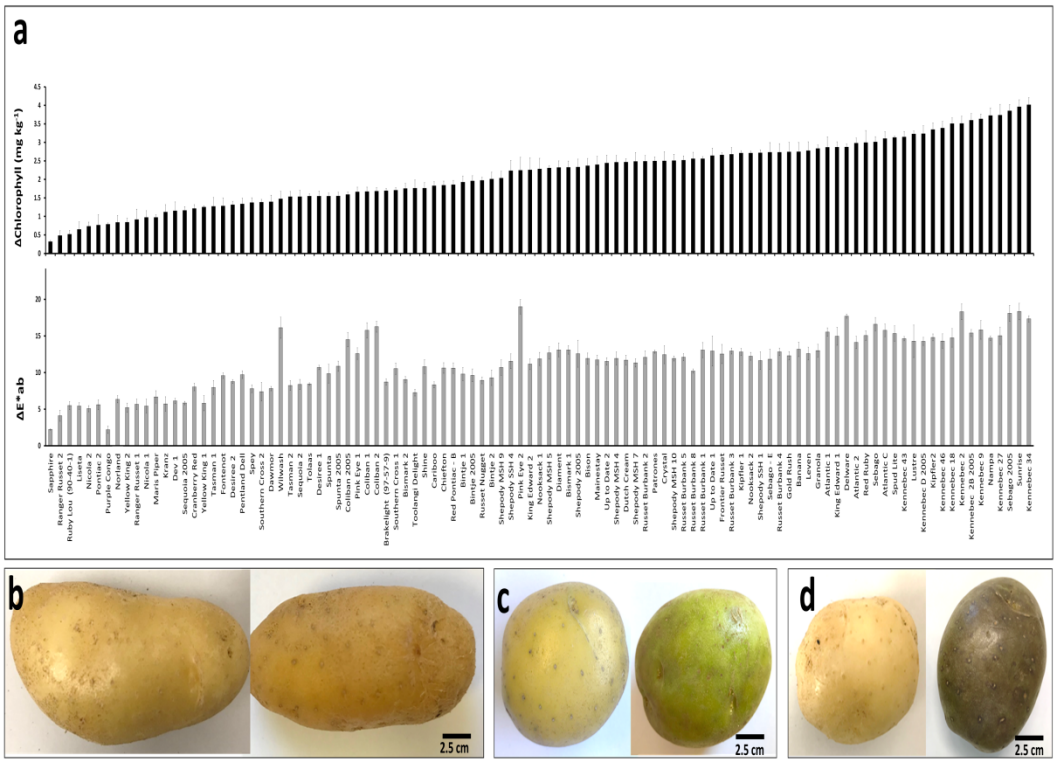
(I) immature tubers harvested approximately 1 month before natural senescence, (M-S) mature tubers harvested at senescence without storage and (M+S) mature tubers harvested at senescence and stored at 4 °C for 4 months. Data represents the mean of three replicates; different letters indicate that means across rows for each variety are significantly different at P<0.05 using Fisher's protected least significant difference (LSD) test; ns indicates non-significant difference.

Table 2. Change in pigment, number of phellem cell layers and suberin monomers concentration after 120 hours of light exposure in potato tubers tested at two different maturities.

Variety	Tuber maturity	ΔPigments			Phellem layers (No.)	Suberin constituents							
		Carotenoid (mg kg^{-1} FW)	Anthocyanin (mg kg^{-1} FW)	Total (mg kg^{-1} FW)		Alcohols (µg mg^{-1})	α,ω-diacids (µg mg^{-1})	ω-hydroxy acids (µg mg^{-1})	Fatty acids (µg mg^{-1})	Dihydroxy acids (µg mg^{-1})	Ferulic acid (µg mg^{-1})	Others (µg mg^{-1})	Aliphatic Suberin (µg mg^{-1})
Atlantic 3	I	0.57 ^{fg}	0.00 ^{f}	4.26 ^{def}	7.00 ^{gh}	19.66	118.20 ^{efghi}	43.74 ^{lm}	44.41	0.13 ^{ijk}	0.19	5.06 ^{fghij}	231.37 ^{k}
	M+S	0.44 ^{ghi}	0.00 ^{f}	3.59 ^{hi}	7.00 ^{gh}	29.32	105.64 ^{ijklm}	57.14 ^{ghij}	65.69	0.31 ^{bcdef}	0.19	4.81 ^{ghij}	263.09 ^{ij}
Desiree 1	I	0.74 ^{de}	1.68 ^{c}	4.47 ^{de}	8.67 ^{cdef}	21.21	113.72 ^{ghij}	53.4 ^{jkl}	44.56	0.08 ^{jk}	0.32	5.09 ^{fghij}	238.36 ^{jk}
	M+S	0.56 ^{fgh}	1.26 ^{d}	3.23 ^{ij}	9.53 ^{bcd}	32.32	124.09 ^{efgh}	66.17 ^{efghi}	52.17	0.17 ^{ghijk}	0.70	5.60 ^{efghi}	281.21 ^{ghi}
Kennebec 5	I	1.39 ^{a}	0.00 ^{f}	5.98 b	4.33 ^{j}	14.09	91.75 ^{m}	37.36 ^{m}	39.8	0.17 ^{ghijk}	0.25	4.35 ^{ij}	187.75 ^{m}
	M+S	1.03 ^{c}	0.00 ^{f}	5.2 ^{c}	6.37 ^{hi}	29.1	98.82 ^{klm}	52.33 ^{jkl}	57.8	0.31 ^{bcdef}	0.34	4.12 ^{j}	242.80 ^{jk}
King	I	0.86 ^{d}	0.00 ^{f}	4.23 ^{efg}	6.33 ^{hi}	19.35	112.74 ^{ghijk}	44.83 ^{klm}	47.09	0.17 ^{ghij}	0.27	6.62 ^{de}	231.06 ^{k}
Edward 2	M+S	0.57 ^{fg}	0.00 ^{f}	3.23 ^{ij}	5.50 ^{ij}	33.07	116.44 ^{efghi}	71.31 ^{cdef}	56.51	0.37 ^{bc}	0.41	6.85 ^{cde}	284.93 ^{fghi}
Maranca	I	1.1 bc	0.00 ^{f}	4.61 ^{d}	5.33 ^{ij}	20.88	100.65 ^{jklm}	43.69 ^{lm}	46.16	0.06 ^{k}	0.27	5.71 ^{efgh}	217.40 ^{kl}
	M+S	0.83 d	0.00 ^{f}	3.88 ^{gh}	7.33 ^{fgh}	33.82	107.74 ^{ijkl}	68.84 ^{defg}	59.96	0.25 ^{defgh}	0.33	7.12 ^{cd}	278.04 ^{hi}
Maris Piper	I	0.32 ^{ijk}	0.00 ^{f}	1.60 ^{lm}	8.50 ^{def}	26.35	149.56 ^{bc}	68.89 ^{defg}	52.28	0.16 ^{hijk}	0.26	7.04 ^{cd}	304.53 ^{efg}
	M+S	0.24 ^{kl}	0.00 ^{f}	1.10 ^{n}	9.50 ^{bcd}	38.65	160.36 ^{b}	97.43 ^{a}	78.77	0.27 ^{cdefg}	0.4	9.83 ^{a}	385.70 ^{a}
Nicola 2	I	0.64 ^{ef}	0.00 ^{f}	1.67 ^{l}	8.00 ^{efg}	29.19	139.48 ^{cd}	67.60 ^{efgh}	65.16	0.16 ^{ghijk}	0.3	6.05 ^{defgh}	307.92 ^{ef}
	M+S	0.41 ^{hi}	0.00 ^{f}	1.15 ^{n}	10.00 ^{bc}	40.93	148.75 ^{bc}	90.91 ^{ab}	77.42	0.48 ^{a}	0.32	9.65 ^{a}	368.45 ^{ab}
Pontiac	I	0.26 ^{jk}	1.70 ^{c}	3.31 ^{ij}	6.50 ^{hi}	21.06	108.61 ^{ijkl}	55.42 ^{ijkl}	52.02	0.23 ^{efghi}	0.27	4.15 ^{j}	241.74 ^{jk}

	M+S	0.2 kl	1.28 d	2.28 k	8.93 bcde	28.28	150.80bc	78.01 cde	71.4	0.36 bcd	0.37	5.9 defgh	335.09 cd
Ranger	I	1.19 b	0.00 f	2.14 k	6.50 hi	17.82	130.33 de	58.92 ghij	63.26	0.27 cdefg	0.35	6.17 def	277.10 hi
Russet 2	M+S	0.09 lm	0.00 f	0.47 o	9.01 bcde	32.89	153.42bc	80.37 bcd	86.7	0.32 bcdef	0.44	6.1 defg	360.23 abc
Red Ruby	I	0.74 de	3.23 a	7.21 b	5.50 ij	18.58	97.73 lm	37.70 m	45.93	0.10 jk	0.31	3.97 j	204.3 lm
	M+S	0.55 fgh	2.43 b	5.87 b	10.37 b	30.05	97.23 lm	56.53 hijk	52.73	0.36 bc	0.31	4.15 j	241.35 jk
Ruby Lou	I	0.05 m	1.71 c	2.49 k	9.00 bcde	23.22	192.63 a	37.19 m	52.13	0.33 bcde	0.34	8.11 bc	313.95 de
	M+S	0.05 m	0.71 e	1.25 mn	12.33 a	35.12	153.65 b	80.92 bc	72.78	0.39 ab	0.39	8.8 ab	352.04 bc
Russet	I	1.07 bc	0.00 f	4.21 efg	7.33 fgh	14.84	114.61 fghij	53.21 jkl	50.92	0.22 fghi	0.23	4.27 j	238.30 jk
Burbank 4	M+S	0.8 d	0.00 f	3.25 ij	7.33 fgh	29.96	118.68 efghi	60.89 fghij	60.16	0.37 bc	0.39	4.73 hij	275.16 i
Russet	I	0.74 de	0.00 f	3.03 j	-	-	-	-		-		-	-
Nugget	M+S	0.56 fgh	0.00 f	2.47 k	7.00 gh	23.02	125.04 efg	58.96 ghij	75.05	0.33 bcde	0.37	4.12 j	286.88 fghi
Shepody 1	I	0.63 ef	0.00 f	4.01 fg	8.00 efg	18.34	110.82 hijkl	56.70 hijk	43.28	0.09 jk	0.24	6.41 de	235.86 k
	M+S	0.41 ij	0.00 f	2.98 j	8.75 cdef	35.33	128.32 def	71.80cdef	60.74	0.40 ab	0.46	6.16 def	303.19 efgh
LSD		*0.14*	*0.19*	*0.36*	*1.44*	*6.79*	*14.11*	*11.99*	*11.77*	*0.11*	*0.19*	*1.32*	*26.42*
P		*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.8*	*<0.001*	*0.02*	*0.31*	*0.01*	*0.29*	*<0.001*	*<0.001*

(I) immature tubers harvested approximately 1 month before natural senescence and (M+S) mature tubers harvested at senescence and stored at 4 °C for 4 months. – indicates not tested. Others refer to unidentified compounds. Data represents the mean of three replicates; different letters indicate that means within columns are significantly different at $P<0.05$ using Fisher's protected least significant difference (LSD) test.



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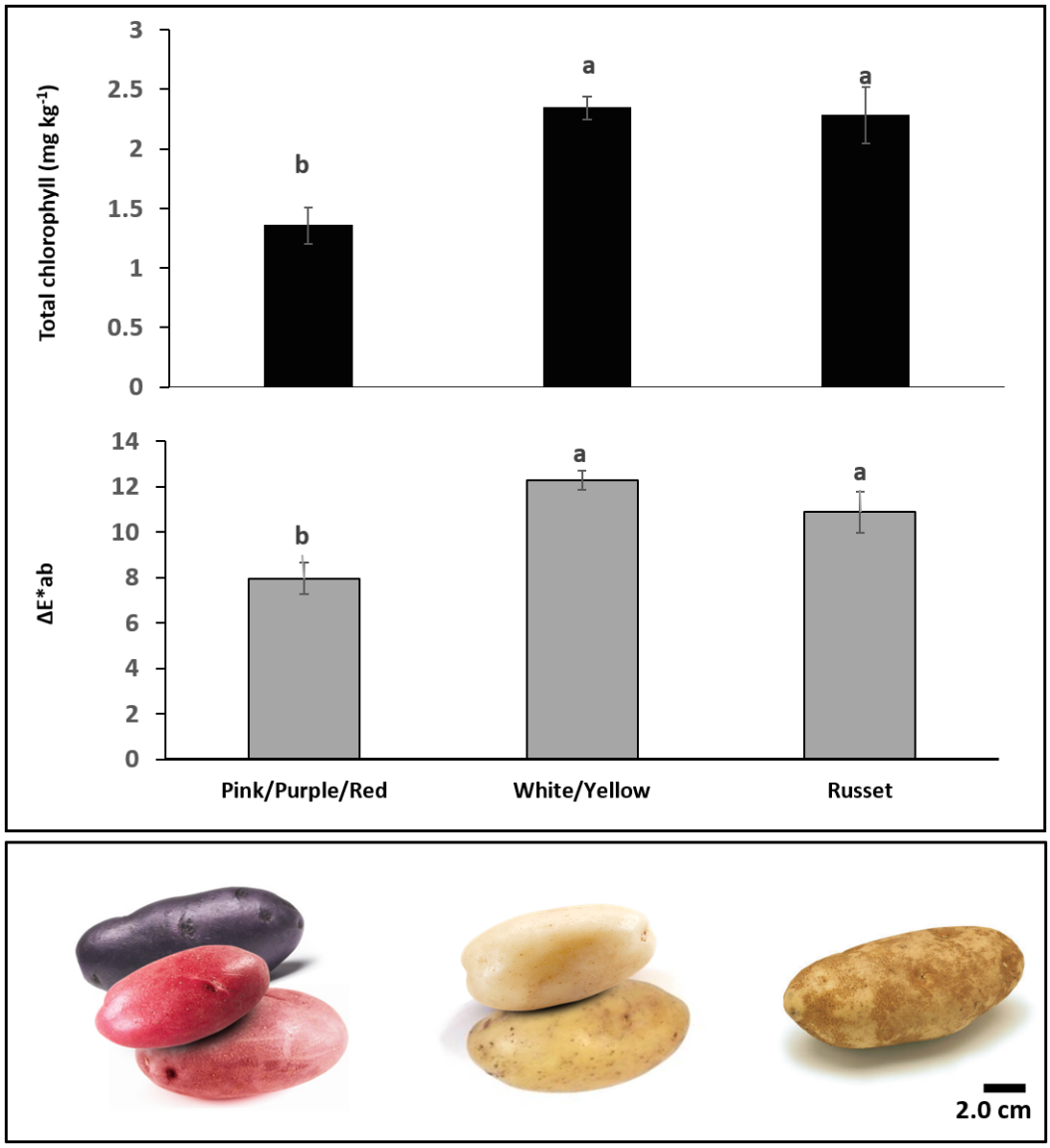

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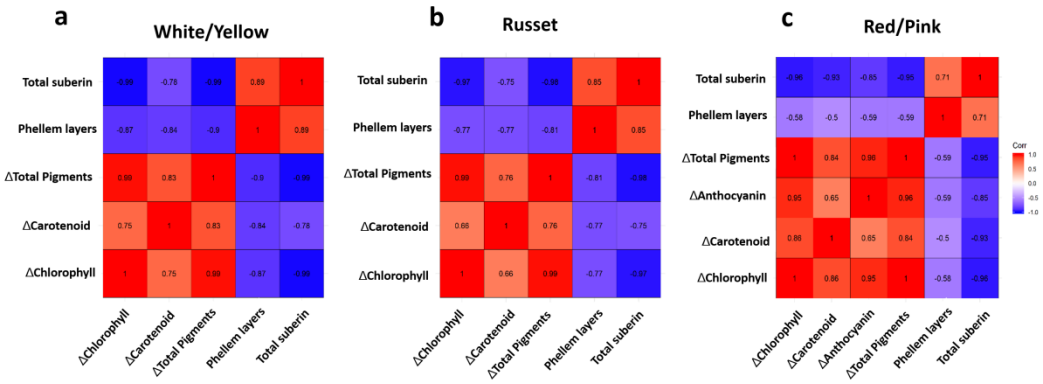

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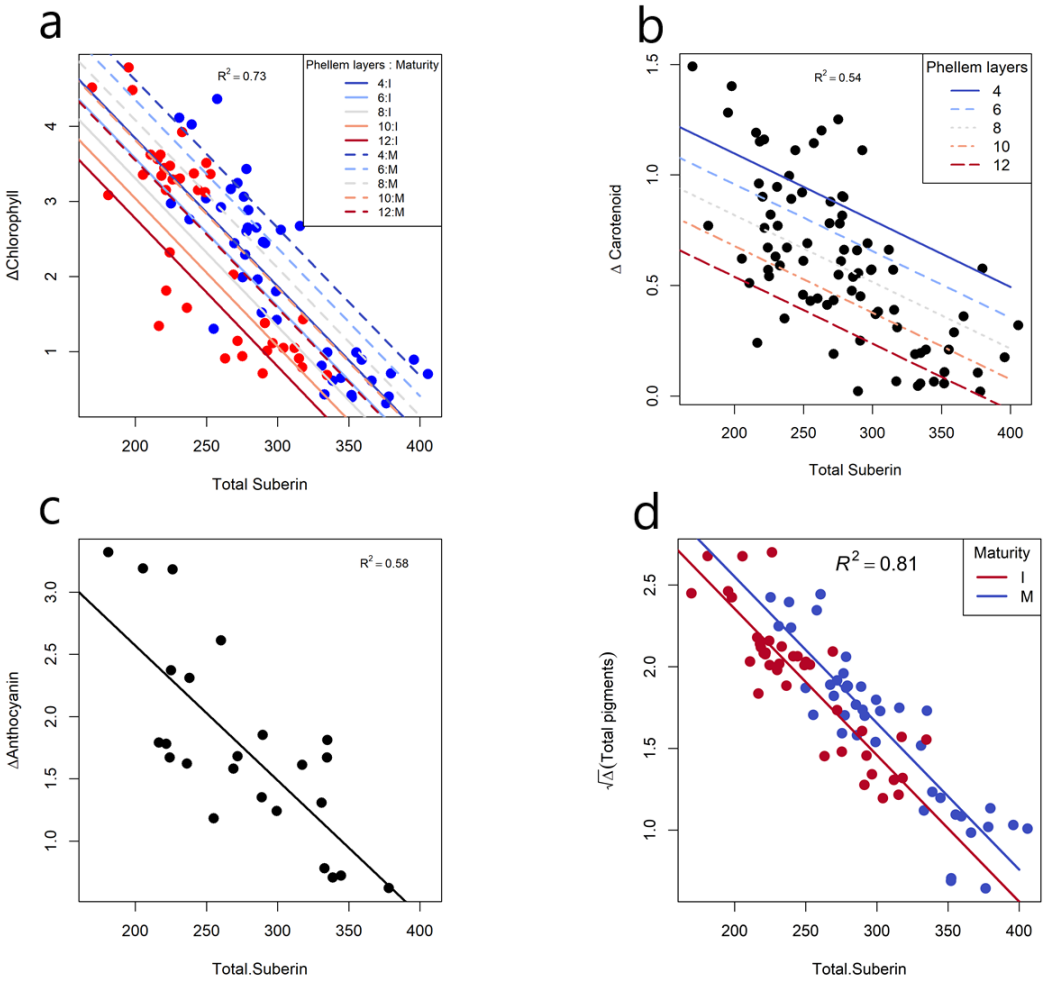

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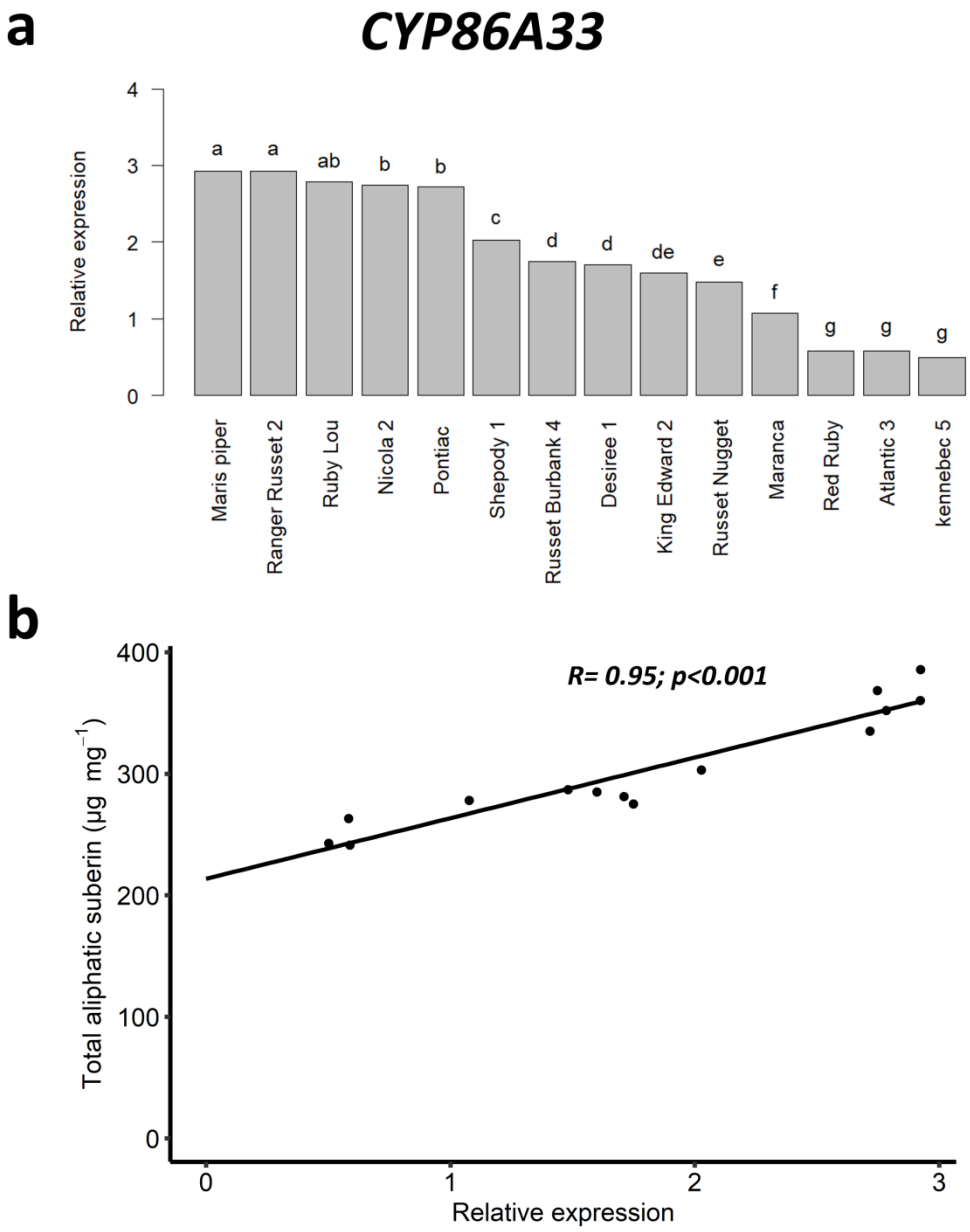

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