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**Dissecting the molecular mechanism of russeting in sand pear ( *Pyrus pyrifolia* Nakai.) by metabolomics, transcriptomics and proteomics**

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# *the plant journal*

## **Dissecting the molecular mechanism of russeting in sand pear (*Pyrus pyrifolia* Nakai.) by metabolomics, transcriptomics and proteomics**

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**Dissecting the molecular mechanism of russetting in sand pear (*Pyrus pyrifolia* Nakai.) by metabolomics, transcriptomics and proteomics**

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## SUMMARY

The brown colouration and rough appearance as russet and semi-russet (partial russet) are features unique to the popular Asian sand pear (*Pyrus pyrifolia* Nakai.). The degree of russetting is different between different genotypes. Russetting is sensitive to water fluctuations, where excessive rainwater can trigger/elevate its development. However, the molecular mechanism of russetting is currently unclear. Here, we employed multi-omics, i.e., metabolomics, transcriptomics and proteomics and analyzed the effect of different sand pear genotypes and artificial rainfall on russetting of pear fruits. This led to the identification of 79, 64 and 29 differentially produced/expressed metabolites, transcripts and proteins that are involved in the biosynthesis of suberin, phenylpropane, cutin and waxes. Further analysis of these differentially expressed genes and their encoded proteins revealed that four of them exhibited high expression at both transcript and protein levels. Transient expression of one such gene, *PbHHT1* (accession number 103966555) that encodes for  $\omega$ -hydroxypalmitate-O-feruloyl transferase (HHT) in the young green non-russet fruits triggered premature suberization in the russetting pear genotypes. This coincided with the increased production of 16-feruloyloxypalmitic acid, a conjugated compound between phenols and esters during the polymerization for suberin formation. Collectively, our data from the combined three omics demonstrates that russetting in sand pear is a complex process involving the biosynthesis and transport of suberin and many other secondary metabolites.

**Keywords:** sand pear, russetting, multi-omics, suberin, rainfall, *PbHHT1*

11 INTRODUCTION

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5 Fruit colour, a key indicator for quality, is a major factor affecting consumer choice (Gamble *et al.*, 2006).  
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7 Russet and semi-russet (partial russet) color is unique to sand pear (*Pyrus pyrifolia* Nakai.) which is native to  
8  
9 Eastern Asia, including China, Japan and Korea. However, in recent years it has also gained popularity in  
10  
11 other countries, such as India, Australia, New Zealand and the United States.  
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15 Similar to many other fruits such as apple, the peel colour of pears are usually determined by anthocyanins.  
16  
17 However, the brown colour exhibited on the peel of sand pear fruits is thought to be due to the accumulation  
18  
19 of suberin lamellae (SL) caused by the defective cuticle developed on the otherwise green peel (Inoue *et al.*,  
20  
21 2006, Wang *et al.*, 2014a). Suberin, the main component of SL is a cutin analog and is primarily composed of  
22  
23 C<sub>16</sub>-C<sub>26</sub> long chain fatty acids (LCFAs) and phenolics linked with alkyl ferulate-waxes. It is polymerized from  
24  
25 a range of compounds, such as glycerol, fatty acids (FAs), phenols, suberin-related waxes, lignin, tannins and  
26  
27 other phenolics (Franke and Schreiber, 2007, Pollard *et al.*, 2008). Both suberin lamellae and stratum corneum  
28  
29 (SC) have similar functions in protecting the fruit from damage. However, SL is more plastic under  
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31 environmental stress (Riederer and Schreiber, 2001, Lara *et al.*, 2015). For example, in rice (*Oryza sativa* L.),  
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33 the deposition of SL in the root was induced by salt-stress where its accumulation was negatively correlated  
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35 with the transportation of salt to the shoot from the root (Krishnamurthy *et al.*, 2011). In Arabidopsis the  
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37 synthesis of suberin is mediated by stress hormones such as abscisic acid and ethylene in the root, indicating  
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39 a positive role of stress on russetting (Barberon *et al.*, 2016). Studies also showed that lignin, cellulose and  
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41 hemicellulose were significantly higher while pectin polysaccharides and long-chain fatty acids were lower in  
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43 russet compared to green non-russet pears, indicating that these compounds also play roles in pear russetting  
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45 (Heng *et al.*, 2014, Wang *et al.*, 2014a).  
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Many candidate genes involved in suberin biosynthesis in both russet pears and apples have been identified via transcriptional analysis, including genes encoding for BAHD family acyltransferase,  $\omega$ -hydroxypalmitate-O-feruloyl transferase (HHT), glycerol phosphate acyltransferase (GPAT), 3-ketoacyl-CoA synthase (KCS), ATP binding cassette (ABC) transporters, MYB93-like transcription factors and acyltransferases. Genes involved in lignin biosynthesis such as those encoding for Caffeic acid 3-O-methyltransferase (COMT), Caffeoyl CoA-O-methyltransferase (CCOAOMT), Cinnamoyl-CoA reductase (CCR), Cinnamyl alcohol dehydrogenase (CAD) and Peroxidase (POD) were also found in transcriptome datasets in both russet apple and pear fruits (Justin *et al.*, 2015, Legay *et al.*, 2015, Wang *et al.*, 2016, Hou *et al.*, 2018).

Studies by combined high-throughput omics, such as genomics, transcriptomics, proteomics, and most recently, metabolomics, have been employed to study complex signaling pathways including russetting in many plants in recent years (Cohen *et al.*, 2017, Jia *et al.*, 2019). For example, the cuticular membrane and the periderm membrane between the russet pear ‘Niitaka’ and its green non-russet mutant ‘Suisho’ were compared at both transcript and protein levels in order to dissect the russetting mechanisms. It was found that both the transcript and protein levels of *GLP* (Germin-like protein) gene were higher in the russet ‘Niitaka’ than the green ‘Suisho’ fruits, indicating that GLP, which is related to jasmonic acid (JA) signaling pathway could promote periderm membrane synthesis hence russetting in ‘Niitaka’ pear fruits (Wang *et al.*, 2020).

Previously, we showed that russetting in sand pear fruit was triggered and enhanced by suberin lamellae accumulation upon excess water application, mimicking heavy rainfall. A sharp increase in lignin content in the russet fruit peel was detected, which was coincided with the increased expression levels of proteins related to the biosynthesis of phenylalanine, the lignin monomer, as well as those related to defense (Shi *et al.*, 2019). In order to further understand the molecular regulatory network of russetting in sand pear fruit we took

11 advantage of the recently developed multi-omics techniques to compare the differences in russetting between:  
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42 (1) three different genotypes, i.e., the russet ‘Zaoshengxinshui’ pear (ZS), the semi-russet ‘Cuiguan’ pear (CG),  
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63 and the green non-russet ‘Cuiyu’ pear (CY); (2) rainfall- and non-rainfall treatment on the russet ZS and semi-  
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94 russet CG fruits. By combining the three datasets of metabolomics, transcriptomics and proteomics we created  
10  
11 a comprehensive molecular network showing changes in major metabolites, genes and proteins related to  
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14 russetting in sand pear fruit. Importantly, a number of putative genes and their encoded proteins involving in  
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16  
17 russetting were identified where one of them, *PbHHT1* was experimentally confirmed to be involved in  
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19  
20 russetting in sand pear fruit. This is expected to provide a rich resource for future in-depth study into the  
21  
22 mechanism of russetting in pear and perhaps other fruit, such as apple.

## RESULTS

### Phenotypic analysis of russetting in the sand pear fruits

Russetting is a unique feature of sand pear fruit which is believed to be mainly due to the accumulation of suberin lamellae in its peel. Different degrees of russetting are found between different colored sand pear genotypes. Indeed, our observations show that on maturity the peel of the russet ZS fruit was fully covered by brown spots with a russet index of 100% (full russet), the semi-russet CG fruit were also largely covered and the russet index was 85% while no russet was present on the green non-russet CY fruit where the russet index was close to 0% (Fig. 1A&1D). It is noteworthy that there was still a localized green background remaining on the peel of both ZS and CG fruit despite their high russet indexes.

Scanning electron microscopy (SEM) observation of the ZS fruit peel revealed that the stratum corneum (SC) was cracked into small pieces, and these cracks extended to reach the epidermal cells. The cuticle cracks were also evident in the peel of the semi-russet CG fruit where they were filled with cork tissues. However, in the peel of the green non-russet CY fruit where no russet spots were formed, the cuticle was smooth with no obvious cracks shown (Fig. 1A, middle panel). Ultra-thin sections were prepared from the plastic embedded peels of these three genotypes of fruit and observed by transmission electron microscopy (TEM). This showed that the epidermal cells of the russet and semi-russet fruit peel were filled with flocculent tannin cells (TC). However, those of the green non-russet pear fruit were rich in liposomes (LAMB), which were surrounded by small lipid droplets which were scarce in the russet and semi-russet pear fruit (Fig. 1A, right panel).

The phenotype of the rainfall-treated fruit was also investigated. It was found that rainfall treatment aggravated russetting in the ZS and CG fruit (Fig. 1B). The SL in the peel of the semi-russet CG was enlarged and that of the russet ZS became even thicker compared to the non-rainfall-treated controls (Fig. 1E and 1F;

Fig. S1). The russet index of the rainfall treated semi-russet CG fruit reached 93% compared to 30% in the non-treated control fruit, i.e., three times higher than the controls. The thickness of SL was 87  $\mu\text{m}$  in the rainfall-treated russet ZS fruit, double that of the non-rainfall-treated fruit (43  $\mu\text{m}$ ) (Figs. 1E and 1F; Fig. S1). Extensive cracks were also apparent on the epidermal cuticle of the ZS fruit peel where the most severe cracks led to shedding, epidermal damage and SL formation. Although there were also cracks formed in the epidermal cuticle of the non-treated control fruit they were not filled by the suberized cells as observed in the rainfall-treated fruit (Fig. 1C).

**Analysis of differentially expressed metabolites (DEMs) in sand pear fruit**

In order to identify the macromolecules related to sand pear fruit russetting we first performed UPLC-Q-TOF/MS (Ultra-performance liquid chromatography/time-of-flight mass spectrometry) metabolomic analysis and found a total of 3,391 metabolites from the mature pear fruit peel. Principal Components Analysis (PCA) revealed clear differences between the three pear genotypes as well as between rainfall-treated and non-rainfall-treated controls from the same russet and semi-russet fruit (Fig. S2). After combining the results of Partial Least Squares Discriminant Analysis (PLS-DA) modeling and the student's *t*-test (Perez-Enciso *et al.*, 2003), 611 and 430 DEMs were identified between the peel of the russet ZS and the semi russet CG, and the green non-russet CY fruit, respectively. Three hundred and sixty-three (363) DEMs were identified between the rainfall treated ZS and 320 in CG pear fruit and the non-treated controls (Fig. 2B). Subsequent KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis showed that these DEMs were the main components for the biosynthesis of secondary metabolites, such as cutin, suberin, wax, long chain fatty acids and phenylpropanoid (Fig. 3A). Importantly, 70 DEMs were found to be common between all the russet samples (Fig. 2A and table. S1).

Further analysis clustered these DEMs into 7 functional groups, i.e., phenolics, fatty acids, lipids, terpenoids, ester, fatty alcohol and glycerol (Fig. 2A and table S1). Higher levels of phenolics were found in the russet ZS and semi-russet CG and their rainfall-treated fruit compared to the green non-russet CY and the non-rainfall-treated controls (Fig. 2A, 2C and table S1). The lignin, ( $\pm$ )-catechin, epicatechin and ferulic acid were all significantly higher in the russet and semi-russet, as well as in the rainfall-treated fruit compared to the non-russet and no-rainfall-treated fruit (Fig. 2A, 2C and Fig. S3). A similar trend was found with the  $\omega$ -6 polyunsaturated long chain fatty acids, such as 3E, 9Z, 12Z-octadecatrienoic acid and 5Z, 9Z, 12Z-octadecatrienoic acid, the  $\omega$ -9 unsaturated fatty acids oleic acid (9Z-octadecadienoic acid) (C18:1n-9),  $\alpha,\omega$ -diacids (eicosadienoic acid), and the  $\omega$ -OH fatty acids, hydroxy fatty acids (24-hydroxy-tetracosanoic acid) (Fig. 2C and Fig. S3). The levels of palmitic acid (C16:0) and its acylglycerides (2-palmitoylglycerol) were also higher in the rainfall-treated russet ZS and semi-russet CG than those in the non-treated control fruit (Fig. S3). On the contrary, very long chain alkanes such as triacontane were decreased remarkably ( $p < 0.05$ ) in the peel of russet ZS and semi-russet CG, as well as in their rainfall-treated than the non-russet CY and non-rainfall-treated fruit (Fig. 2C).

### Transcriptome profiling of the peels of sand pear fruit

To see changes in transcript levels of genes related to russeting, transcriptome analysis was performed using the same samples as for the metabolome analysis described above. After quality assessment and data filtering, a total of 7,630 transcripts were found differentially expressed between russet ZS and non-russet CY fruit (ZS vs CY), 5,171 between the semi russet CG and non-russet CY (CG vs CY), 803 between the rainfall-treated and non-treated control russet ZS (ZS-R vs ZS-C), and 1,718 between the rainfall-treated and non-treated semi-russet (CG-R vs CG-C) fruit. Using KEGG pathway analysis we found that the differentially expressed

genes (DEGs) that were common between the two russet genotypes as well as between the rainfall-treated samples were those involved in the four main biosynthesis pathways involved in (1) secondary metabolites; (2) cutin, suberin and waxes; (3) very long-chain fatty acids, and (4) phenylpropanoid. In addition, genes encoding for ABC transporters were also found among the common DEGs (Fig. S2).

We next carried out a comparative analysis of all the data generated from samples of the three genotypes as well as those from the rainfall-treated and non-rainfall-treated control fruit. This led to the identification of 64 common DEGs where 13 were related to the biosynthesis of cutin, suberin and waxes (Fig. S5 and Table S2). Further comparison and verification of data consistency resulted in the discovery of a few important genes that showed significant changes in their transcript levels (Table 1). Notably, the transcript level of *PbHHT1* (acc no. 103966555) was 2.7- and 3.7-fold higher in the russet ZS and the semi-russet CG compared to the green non-russet CY fruit, respectively (Table 1 and Fig. 4C). Rainfall treatment also enhanced the expression level of this gene where its transcript level increased by 0.2-fold in the russet ZS and 3.2-fold in the semi-russet CG fruit respectively, compared to the untreated controls. A second gene, *PbCYP749a22* (acc no. 103948102), one of the important regulatory genes of cytochrome P450 monooxygenase, was also significantly higher in the russet ZS (5.1-fold) and semi-russet CG (3.5-fold) compared to the green non-russet CY fruit. However, rainfall treatment increased transcript levels in the russet ZS (ZS-R) (1.1-fold), but not in the semi-russet CG fruit. Beta-keto ester CoA synthetase (KCS) is an integral part of the fatty acid elongase complex (FAE). It was found that *PbKCS10-like* (acc no. 103949661) showed markedly lower expression in the peel of the russet ZS and semi-russet CG fruit, as well as in its rainfall-treated fruit. Similarly, *PbCER1-like* (acc no. 103929753) and *PbWSD1-like* (acc no. 103928845) that possibly have roles in the biosynthesis of wax esters, were also significantly downregulated in the peel of both russet ZS and semi-russet CG and their rainfall-treated fruit.

Different expression profiles of many genes involved in the biosynthesis of lignin were found between the fruit of the two russet and the green non-russet genotypes. For example, the transcript level of *PbCAD1* (acc no. 103951782) was higher in the peel of both the russet ZS and semi-russet pear CG than the non-russet CY fruit. The expression levels of *PbCOMT1* (acc no. 103927980) and *PbCCoAOMT* (acc no. 103933993) were significantly higher in the peel of the russet ZS fruit, while that of *PbCOMT2* (acc no. 103951572) was significantly higher in the peel of the semi-russet CG fruit compared to the non-russet CY fruit. Suppressing the activity of CCR could lead to lignin-monomer loosening and promote the movement of phenolics, such as ferulic and sinapic acids to the cell wall (CW), causing russetting in pear (Wang et al., 2014a). In line with this we also found that the transcript level of *PbCCRSNL-like* (acc no. 103943280) was lower in the peel of the russet and semi-russet, also in their rainfall-treated fruit compared to the controls.

ABC transporters and lipid transfer proteins (LTPs) are the primary proteins that are involved in the transmembrane transport of cutin and wax to the cell wall. Notably, genes encoding for the G family members of the ABC transporters exhibited lower- (*PbABCG15*, acc no. 103942099), or higher- (*PbABCG10*, acc no. 103934278) expression levels in the russet ZS and semi-russet CG than in the green non-russet CY fruit. Treatment by rainfall resulted in similar trends in their expression profiles (Table S2). Further, the transcripts of both *PbnsLTP* (acc no. 103938613, 103961206) and *PbnsLTP-like* (acc no. 103961207) showed significantly lower levels in the russet and rainfall-treated than their controls, indicating that the transmembrane transport of cutin and wax to CW were less efficient in the russet and rainfall-treated fruit than the control (Fig. 4B and 4C). This may lead to the accumulation of these compounds, resulting in russetting in the russet and rainfall induced russet ZS and semi russet CG fruit.

Altered transcript levels of genes involved in fatty acid biosynthesis were also detected in the rainfall-treated russet and semi-russet fruit. For example, the transcript levels of both *PbGPAT5* (glycerol-3-phosphate acyltransferase 5, acc no. 103942961) and *PbFAR3* (fatty acyl-CoA reductase, acc no. 103931004) were

11 significantly higher than in the control fruit (C groups). *PbKCS19* (acc no. 108865656), *PbKCS10* (acc no.  
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4 103952998) and *PbKCS20* (acc no. 103940571), which all participate in fatty acid elongation, were all lower  
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7 in the rainfall treated fruit of the russet fruits compared to the untreated fruit. Genes encoding for cytochrome  
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9 P450 monooxygenase, oxidoreductase (*PbP45086A8*, acc no. 103957382) and fatty acid elongase (FAE)  
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11 (*PbP45086B1*, acc no. 103943216) in the semi-russet CG fruit were significantly upregulated compare to  
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13 those of the controls (Fig. 4C). Therefore, it seems that altered fatty acid biosynthesis is involved in the rainfall  
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15 induced russetting in sand pear fruit.  
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19       Expansins and aquaporins are both non-enzymatic proteins that are believed to play roles in water stress  
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21 by changing the CW structure and the water channels of the cell membrane (Li *et al.*, 2015, Secchi *et al.*,  
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23 2017). In line with this, genes encoding for expansin, a protein that relaxes plant cell walls, including *PbExpA4*  
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25 (acc no. 103954300) and *PbExpB15* (acc no. 103959804), showed higher expression levels in the peel of the  
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27 russet ZS and semi-russet GS fruit under rainfall-treatment (Fig. 4C). The transcript levels of the three  
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29 aquaporin genes, *PbTIP1-3* (acc no. 103930703), *PbPIP2-2* (acc no. 103956770) and *PbPIP2-5* (acc no.  
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31 103937541), as well as the Dehydrin 3 encoding gene *PbDHN3* (acc no.103937812) were all upregulated in  
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33 the peel of russet and semi-russet pears. Therefore, it appears that rainfall can cause cell wall relaxation by  
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35 enhancing the expression of these genes.  
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### 45 **Proteome profiling of the peels of sand pear fruits**

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48 We also profiled and analyzed the differentially expressed proteins (DEPs) by iTRAQ from the same samples  
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50 as described for the RNA-seq above. Two hundred and sixty-eight (268) DEPs were identified between the  
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52 russet ZS and non-russet CY (ZS vs CY) and 241 DEPs between the semi russet CG and non-russet CY (CG  
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54 vs CY). In the rainfall treated fruit, 121 and 85 DEPs were identified between the treated ZS and CG fruit and  
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56 the non-treated controls, respectively. Further comparison and analysis of these DEPs led to the discovery of  
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23 DEPs that could be related to russetting of sand pear fruit (Table S3). They were again compared against the common DEGs identified from the RNA-seq analysis from above. This resulted in the discovery of 4 candidates that were differentially expressed at both the protein and transcript levels: *PbHHT1/PbHHT1*, *PbKCS10 /PbKCS10*, *PbnsLTP3/PbnsLTP3* and *PbnsLTP4/PbnsLTP4*. Interestingly, these four DEPS are related to the biosynthesis of cutin, suberin and wax as well as to stress (Table 2). The relative expression levels of these four co-expressed DEPs and DEGs were analyzed by targeted proteomics by PRM (Parallel Reaction Monitoring) and showed that those of *PbHHT1*, *PbnsLTP3* and *PbnsLTP4* were consistent with their absolute expression levels where the expression of *PbHHT1* was enhanced, while *PbnsLTP3* and *PbnsLTP4* were significantly depressed in the peel of rainfall-treated russet and semi-russet pear fruit compared to the non-treated controls (Fig. 5).

### ***PbHHT1* plays essential roles in russetting in sand pear fruit**

Because both the expression levels of the transcript and protein of *PbHHT1* were significantly higher in the russet and semi-russet, as well as in the rainfall-treated compared to the non-russet and non-treated fruit, we next carried out experiments to verify the function of *PbHHT1* in pear russetting. This was done by transiently expressing *PbHHT1* in the young non russet green fruit of the semi-russet CG and the non-russet CY by injecting the 35S:*PbHHT1* containing transgenic *Agrobacterium* solution. Controls of the empty vector containing *Agrobacterium* (EV), infiltration buffer (MMA) and water were included for comparison.

As shown in Figure 6A, after injection of 35S:*PbHHT1* containing transgenic *agrobacterium* russetting of the peel (SL accumulation) and lenticel suberization were clearly observed on the peel of the young semi russet CG fruit. The lenticel suberization was also intensified and the filamentous-like russet around the

11 injection site was observed in the CY peel. However, no SL accumulation and/or lenticel subrinization were  
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3 present in the peel of other controls (Fig 6A, top panels). To visualize the presence of suberin in the peel, they  
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5 were stained with berberine-aniline blue and observed under a fluorescence microscope. A large area of strong  
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7 blue fluorescent signal was observed showing the suberin layer in the peel of CG fruits that was injected with  
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94 the *PbHHT1* containing agrobacteria. However, in the peel of the control fruit only a weak and scattered blue  
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11 light staining was observed showing the lenticels (Fig 6A, bottom panel, arrows).  
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17 In order to determine whether the observed accumulation of suberin was indeed due to the overexpression  
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19 of *PbHHT1* in the peel of injected fruit, we carried out qRT-PCR on RNAs isolated from the tissue covering  
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21 the injection sites (arrows in Fig 6A, top panel). The results showed that the transcript levels of *PbHHT1* was  
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23 up-regulated by 6.7- and 2.5-fold in the CG and CY fruit respectively, injected with the *PbHHT1* containing  
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25 transgenic agrobacteria, while no such changes were observed in the control fruit injected with the EV, MMA  
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27 and water (Figure 6B).  
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33 Next, we analyzed the metabolites in the transgenic peel expressing *PbHHT1* and found that the content  
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35 of the suberin monomer was increased significantly in both the CG and CY fruit peel. However, the amount  
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37 of  $\omega$ -feruloyloxypalmitic acid was 2.63 times higher in the *PbHHT1* expressing CG peel than in the CY peel  
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39 (2.16 $\mu$ g/g in CG compared to 0.57 $\mu$ g/g in CY fruit) (Fig. 6B). Therefore, the combined results above clearly  
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41 demonstrated that russetting in sand pear fruit was closely related to the high expression levels of *PbHHT1*.  
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43 This led to the increased production of  $\omega$ -feruloyloxypalmitic acid, the suberin monomer hence russetting in  
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45 the fruit.  
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51 To understand the effect of *PbHHT1* on triggering russetting in young green fruit, RNA-seq was carried  
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53 out to monitor the altered expression profiles of relevant genes. It was found that in addition to the significantly  
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55 up-regulated transcript level of *PbHHT1* in both CG and CY fruit, the *PbCYP86A8* (acc no.103957382), a  
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gene upstream of *PbHHT1* in the suberin biosynthesis pathway and *PbGPAT5* were also significantly up-regulated in the *PbHHT1* overexpressing CG and CY fruit peel. However, the expression of some russetting related genes was only significantly changed in the CG but not in the CY fruit peel (Figure. 6D). For example, lignin synthesis related gene *PbCOMT1* was upregulated, while *PbCCRSNL-6* was downregulated in the CG but not the CY fruit. Genes related to fatty acid elongation and wax biosynthesis, such as *PbKCS19*, *PbKCS10*, *PbKCS20*, *PbCER1-like*, *PbWSD1-like*, *PbnsLTP* and *PbnsLTP-like* were all down-regulated in the semi-russet CG whilst they showed no significant changes in the non-russet CY fruit. Genes encoding for expansins such as *PbExpA4* and *PbPIP2-2* were upregulated in the peel of the semi-russet CG but not the CY fruit.

Therefore, it seems that the gene expression profiles in the premature russetting of CG fruit peel caused by transient expression of *PbHHT1* were very similar to those observed in the rainfall triggered russetting in the sand pear fruit, as described earlier.

### Comparative analysis of all the data generated from metabolome, proteome and transcriptome

Because russetting occurred in the russet ZS and semi-russet CG fruit and this was enhanced/triggered by rainfall, we integrated all the data obtained from the metabolome, transcriptome and proteome by converting and mapping the genes, proteins and metabolites into the KEGG database. This resulted in the identification of 103 DEGs, 18 DEPs and 8 DEMs that are common between all the russet and non-russet control samples. Further analysis indicated that they are all involved in the biosynthetic pathways of secondary metabolites (Fig. 7). These DEGs, DEPs and DEMs were further grouped into 5 clusters (I–V) according to the specific metabolites that they are associated with. Cluster I includes genes and proteins mainly involved in the biosynthesis of phenylpropanoid (CAD1, PER53, COMT1, BGLU17), cluster II for cutin, suberine and wax

(HHT1, KCS4, CER1), cluster III for fatty acids (ADH2, FAD2), cluster IV for flavonoid (LDOX, CHS) and cluster V for fatty acid elongation (CUT1, KCS4, KCS2, KCS12) (Table S5 and S6). Interestingly, higher expression levels of DEGs, DEPs and DEMs in clusters I–IV (such as CAD1, BGLU17, HHT1, and COMT1) while lower expression levels of those in cluster V (such as CUT1, KCS4) were detected in the russet and semi-russet, as well as in their rainfall-treated fruit compared to their relevant controls (Fig. 7).

Taken together, our data from the combined 3 omics demonstrate that russetting in sand pear fruit is regulated by the biosynthesis of phenylpropanoid, suberin, wax, fatty acids, flavonoid and their polymerization components. Furthermore, heavy rainfall can aggravate russetting by up- or down-regulating the expression of relevant genes and proteins, resulting in the accumulation and polymerization of these secondary metabolites.

DISCUSSION

The russet colour is a special feature of sand pear fruit and is thought to be due to suberin lamellae coverage on the fruit peel. Indeed, we demonstrated here that in the russet fruit of sand pear, the cracks of the stratum corneum on the peel were filled by the suberized cells while this was not observed in the green non-russet fruit (Fig 1). As the major structural compound of SL suberin is a complex heteropolymer formed from aromatic substances including phenols, hydroxycinnamic acids, ferulic acid, cinnamic acid and lignin, it also contains a large amount of aliphatic polymers produced from  $\omega$ -hydroxy fatty acids. These aliphatic compounds have been previously identified in pears and apples (Franke and Schreiber, 2007, Pollard *et al.*, 2008, Legay *et al.*, 2015, Vishwanath *et al.*, 2015). Suberin lamellae not only affects the coloration of the pear fruit but it is also evolved to protect plants from damage caused by harsh environment, such as heavy rainfall (Lara *et al.*, 2015, Shi *et al.*, 2019).

In this study, we first carried out metabolome profiling of sand pear fruit and found that phenols, lignin,  $\omega$ -unsaturated fatty acids,  $\alpha$ ,  $\omega$ -diacids,  $\omega$ -OH fatty acids and glycerides were enriched in the peel of the russet pear fruit (Fig 3C and Fig S3). These compounds were confirmed to be the major components of suberin in *Arabidopsis* and potatoes (Franke and Schreiber, 2007, Li *et al.*, 2007, Serra *et al.*, 2014). The aliphatic compounds of suberin are mainly  $C_{16-20}$   $\omega$ -hydroxy fatty acids (Bernards and Mark, 2002, Krishnamurthy *et al.*, 2011). Interestingly, while the components of suberin were variable between different species of *Quercus variabilis* Blume, *Picea fortunei* (*Pseudotsuga sinensis* Dode) and potato (*Solanum tuberosum* L.) the same  $C_{18}$  unsaturated fatty acids were always found in their suberin-rich tissues (Graça and Santos, 2010). Consistent with this we also detected high content of  $C_{18}$  unsaturated fatty acids in the russet peel of the artificial rainfall treated russet ZS and semi-russet CG pear fruit (Fig 3 and Table S1). Therefore, we propose that these  $C_{18}$  unsaturated fatty acids could be used as good indicators of the presence of suberin hence russet index in pear and perhaps other plant tissues/fruits in the future.

Consistent with the high level of  $C_{16-20}$  fatty acids (palmitic acid and dodecanoic acid) found in russet fruit peel, the expression levels of genes related to their biosynthesis, such as *PbGPAT5*, *PbCYP86A1* and *PbCYP86B1* were significantly upregulated (Figs 3&4A, Table S2). GPAT5 has specificity towards acyl-CoA substrates of  $C_{16-20}$  fatty acids and their  $\omega$ -oxidized derivatives, the major determinants of suberin components (Yang *et al.*, 2012), while the CYP86 subfamily, including CYP86A4, CYP86A8, CYP86A1, and CYP77A6 catalyzes the  $\omega$ -hydroxylation of  $C_{16}$  and  $C_{18}$  fatty acids in plants (Duan and Schuler, 2005; Wang *et al.*, 2019). Therefore, the increase of oleic acid ( $C_{18:1}$ ) and palmitic acid ( $C_{16:0}$ ) in the suberin tissue we detected in the russetting sand pear fruit further confirms the role of CYP86 in suberin biosynthesis (Fig. S3). On the other hand, the very long chain alkane triacontane,  $C_{30}$  alkanes, the component of cutin and waxes in pear fruit (Norris and Bukovac, 2010, Wang *et al.*, 2014b) was extremely reduced in the rainfall-

11 treated russet sand pear fruit (Fig 3C). Coincided with this the expression of *PbCER1*, a core component of  
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3 the complex that controls the biosynthesis of C30 alkanes, was significantly downregulated (Fig 4B). In  
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5 addition, genes involved in fatty acid elongation, such as *PbKCS19*, *PbKCS10* and *PbKCS20* were also  
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7 downregulated in the russet fruit (Fig 4).  
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12 Therefore, the combined metabolome and transcriptome data analysis indicates that as russetting was  
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14 intensified in sand pear fruit suberin and related genes were upregulated while the accumulation of long  
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16 chain fatty acids, waxes and their metabolic genes were suppressed.  
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28 As russetting aggravated the primary components of lignin and phenolics in the SL, ferulic acid,  
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23 epicatechin, and (±)-catechin significantly increased (Table S1). In line with this several recent studies  
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25 reported that the lignin content was much higher in the peel of the russet than in the green non-russet pear  
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27 and apple fruit (Wang *et al.*, 2014b, Legay *et al.*, 2015, Shi *et al.*, 2019). Interestingly, we discovered that  
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29 in the rainfall-treated fruit, higher expression levels were detected for *PbHCT*, *PbCAD1*, *PbCOMT1*,  
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31 *PbCOMT2*, *PbCCoAOMT* and *PbLAC*, genes encoding for key enzymes in the biosynthesis of lignin  
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33 monomer phenylpropane. Further, genes encoding for PODs (Q9FLC0, Q42578), the key enzymes for the  
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35 final step of lignin biosynthesis were also significantly upregulated whilst the expression of *PbCAD6* and  
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37 *PbCCRSNL-6-like* isoform X2 were severely suppressed in the russet and semi-russet pears (Fig 4B).  
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39 Suppressing CAD and CCR may cause loosening of the lignin monomer (Bernards and Mark (2002).  
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41 Therefore, we speculate that there may be two metabolic branches in lignin biosynthesis in russetting in sand  
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43 pear fruit where *PbCAD1* attributes to the biosynthesis of lignin monomers, whilst *PbCAD6* and  
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45 *PbCCRSNL-6-like* isoform X2 participate in loosening the lignin structure (Kaur *et al.* (2012). Together  
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47 they may be beneficial for the fatty substances and lignin monomers biosynthesis and polymerization into  
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49 suberin, hence promoting russetting in sand pear fruit.  
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Damage caused by long-term rainfall induces an anoxic environment in plants and causes rapid expansion and rupture of the outer epidermal cells. The loosening of cell walls is the pre-requisition for cell expansion which in turn can cause russetting. Our transcriptomics analysis revealed that genes encoding for expansin, including *PbExpA7*, *PbExpA4*, *PbExpA6* and *PbExpB15* all showed much higher expression levels in the russet and semi-russet fruit. Further, rainfall treatment resulted in the rupture of the SL structure of the peel, and induction of increase expression levels of those genes encoding for expansin (Fig 4 and Table S2). Therefore, these results imply that expansin is involved in cell wall loosening hence russetting in sand pear fruit which is enhanced by rainfall.

Studies on aquaporins such as PIPs and TIPs indicated that these proteins may regulate the transmembrane transport of water, thereby maintaining water balance in plants especially under stress conditions (Shao *et al.*, 2008). A study showed that under dry conditions, the expression level of *Aquaporin-1* was reduced in the peel of the sand pear fruit, and this was coincided with reduced transpiration and water loss (Wang *et al.*, 2014a). Up-regulation of aquaporin genes such as *PbTIP1-3*, *PbTIP2-1* and *PbPIP2-5* was found in the rainfall-treated russet and semi-russet sand pear fruit (Fig 4 and Table S2). Therefore, it appears that both expansins and aquaporins were upregulated and involved in rainfall aggravated russetting by loosening cell walls and transmembrane transporting water in sand pear fruit.

Transmembrane transport of cutin and wax to the cell wall is important for the synthesis and deposition of SC. Non-specific lipid transfer proteins (nsLTPs) in Arabidopsis and rice were thought to be involved in cutin and wax synthesis processes (Edstam *et al.*, 2013). The results showed that the expression of nsLTPs protein and *PbnsLTPs* genes were higher in the no-russet peel than it in the russet- and semi-russet peel from the transcriptome and proteome analyses (Fig 5). It was speculated that nsLTPs can promote the transport of small lipophilic molecules that make up wax and cutin to the outside of the cell membrane so

that the integrity of the SC was maintained. Therefore, it seems that russetting is negatively regulated by genes involved in the biosynthesis of long chain fatty acids and waxes in sand pear fruit.

To verify the reliability of our data we tested the relationship between russetting of sand pear fruit and one of the highly expressed genes, *PbHHT1* identified from our RNAseq. High level of its encoded protein, PbHHT1 was also detected in the iTRAQ analysis of the russet fruit (Table 2). The homologous gene of *PbHHT1* was first detected in tobacco and subsequently isolated from suberized potato tuber (Lotfy *et al.*, 1996, Wang *et al* 2020 ). It plays important roles in russetting by catalyzing the conjugation of ferulic acid and palmitic acid to  $\omega$ -hydroxypalmitic acid, one of the key steps in the biosynthesis of suberin (Schomburg, D. and Schomburg I 2013, Wei *et al* 2019). Our metabolomics analysis showed that the contents of ferulic acid and palmitic acid as well as 16-feruloyloxypalmitic acid were all much higher in the russet and semi russet pears than the control non-russet fruit (Fig 6). Together, these data imply that *PbHHT1* and its encoded protein PbHHT1 most likely play positive roles in the production of 16-feruloyloxypalmitic acid hence russetting in sand pear fruit.

To test this hypothesis PbHHT1 was transiently overexpressed in the young immature semi-russet CG pear fruit before russetting developed, as well as in the same aged non-russet CY fruit. This resulted in the premature russetting covering the entire area where the bacteria solution spread, which corresponded to the large aniline blue stained suberin observed in the CG peel (Fig 6A). Transient expression of PbHHT1 in the CG fruit caused increased expression of genes related to phenylpropanoid-suberin biosynthesis as revealed by the RNAseq analysis. These include *PbCOMT1* and *Pb4CL* which encodes for the key enzymes caffeic acid 3-O-methyltransferase (COMT) and 4-coumarate-CoA ligase (4CL) for the biosynthesis of ferulic acid and feruloyl-CoA, the intermediates for the rapid biosynthesis of 16-feruloyloxypalmitic acid by HHT1 (Graça *et al*, 2010). Conversely, downregulation of *PbKCS19*, *PbKCS10*, *PbKCS20*, *PbCER1-like* and

*PbWSDI-like* was detected in the PbHHT1 expressing semi-russet CG fruit whilst no changes were found in the CY fruits. While their upregulation was shown and suggested to promote the integrity of the SC structure and therefore inhibit russetting (Wang et al, 2016), lower activities of these genes could lead to a less intact SC structure, hence russetting as found in the PbHHT1 triggered russetting in the young semi-russet CG fruit (Fig 6).

It is noteworthy that the CG and CY fruit exhibited different degrees of russetting after transiently expressing PbHHT1 where the extent was much higher in the CG than the CY fruit (Fig 6A). Although the content of its catalytic product 16-ferulyloxypalmitate was significantly increased in the peel harbouring the *PbHHT1* construct compared to the empty vector in the non-russet CY fruit it was still much lower than in the EV control CG fruit (Fig 6B). Therefore, it seems that there is a threshold amount of 16-ferulyloxypalmitic acid where once this amount is reached genes in the phenylalanine synthesis pathway can be activated, leading to suberin accumulation and russetting. In addition, it also has the potential to negatively regulate cutin and wax biosynthesis, transport and accumulation hence integrity of SC. Together, this can trigger the formation of suberin hence russetting in sand pear fruit.

Through the multi-omics analysis and transient expression of *PbHHT1* in the peel of russet and non-russet pear fruit we show here that russetting in sand pear is a complex process, involving changes in the expression of multiple genes and proteins participating in the biosynthesis of polyphenolics, fatty acids and other secondary metabolites. This leads to the alteration of the biochemical composition as well as physical appearance (texture and colour) of the fruit. By combining all the data obtained in this study we generated a tentative mechanistic model of russetting in sand pear fruit and presented in Figure 8. It shows that the russetting related metabolites are first synthesized in the cytoplasm, transported through the plasma membrane and finally assembled at the cell walls of the cells of the fruit peel. Both the phenylpropanoid

biosynthesis pathway and the LCFAs-monoacylglyceride (16-feruloyloxypalmitic acid) for surberin  
 biosynthesis pathway are involved. In the phenylpropanoid biosynthesis pathway, L-phenylalanine  
 ammonia-lyase (PAL) catalyzes the removal of ammonia from L-phenylalanine to generate trans-cinnamic  
 acid. This is followed by the action of cinnamate-4-hydroxylase (C4H), 4-coumaric acid:CoA A ligase  
 (4CL), the lignin synthase (HCT), p-coumaryl coenzyme A3-hydroxylase (C3H) and caffeoyl-CoA O-  
 methyltransferase (CCoAOMT) which results in the production of feruloyl-CoA, the substrate for PbHHT1  
 ( $\omega$ -hydroxypalmitate-O-feruloyl transferase). PbHHT1 catalyzes the conjugation of ferulic acid and  
 $\omega$ -hydroxypalmitic acid to form 16-feruloyloxypalmitic acid. On the other hand, feruloyl-CoA can be further  
 converted by cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD) and LAC to create  
 the lignin monomer. Higher transcript levels of *PbHCT*, *PbCAD1*, *PbCOMT1*, *PbCOMT2*, *PbCCoAOMT*  
 and *PbLAC*, as well as high protein level of PbPAL1 were detected in the russet ZS and semi-russet CG and  
 in their rainfall treated fruit than the controls (in red, Fig 8). This is also consistent with the increased content  
 of lignin and p-cinnamic acid detected in our study (Table 1). Upregulation of *BGLU44* ( $\beta$ -Glucosidase)  
 and related metabolites such as coumarine and 3-O-p-trans-coumaroylaliphitolic acid in the russetting peel  
 were also detected. On the other hand, in the LCFAs-monoacylglyceride for surberin biosynthesis pathway,  
 the biosynthesis and transporter of the very-long chain alkanes such as triacontane (C30) for stratum  
 corneum-related waxes were suppressed, leading to the SC to form on the cell wall. Long-chain fatty acids  
 such as palmitic acid is converted by long-chain acyl-CoA synthetase (LACS) to produce fatty acyl CoA  
 which is further catalyzed by cytochrome P450 to produce  $\omega$ -hydroxylated fatty acids ( $\omega$ -OHs). They are  
 further esterified into the suberin monomer 16-feruloyloxypalmitic acid by the action of HHT1.  
 Subsequently, the suberin monomer /oligomer, lignin monomer and small phenolic molecules are  
 transported out of the PM through the active ABC transporters such as ABCG7, ABCG6 and ABCG15.  
 These molecules are finally assembled into a macromolecular suberin polymer on the cell walls and their

accumulation can lead to the russet appearance on the sand pear fruit peel.

High expression levels of *PbP450 86B*, *PbHHT1*, *PbLACS4* and *PbFAR3* were detected in the rainfall enhanced russet peel of the CG and ZS fruit. Further functional verification of *PbHHT1* confirmed its important role in triggering premature russetting in the young semi russet CG pear fruit, demonstrating the positive role of *PbHHT1* in suberization of the russetting pear genotypes. This is perhaps because it can 'bridge' the phenylpropanoid biosynthesis pathway and the surberin biosynthesis pathway, and therefore, can alter and coordinate the expression of various genes related to russetting in sand pear fruit.

## EXPERIMENTAL PROCEDURES

### Plant materials

The pear trees of the russet 'Zaoshengxinshui' (ZS), semi-russet 'Cui guan' (CG) and green non-russet 'Cuiyu' (CY) were grown in the Jinshan Fruit Experiment Station of the Shanghai Academy of Agricultural Sciences (30.7875 N, 121.1438 E), Shanghai county, China. Ten days after full bloom (DAFB) the control non rainfall-treated fruit were wrapped with transparent nano-pore bags to avoid contact with rain. For artificial rainfall treatment (rainfall-treated), fruit was sprayed with water to saturation, once a week. Six fruits from each of the 5 selected pear trees were treated for each genotype. The microenvironments within the bags for the non-rainfall and artificial rainfall-treated fruit were monitored throughout the growth season. No significant difference between them in terms of temperature, humidity and light quality were found (Table S7 & Fig. S10). At 80 DAFB, all the fruit from untreated controls ZS-C, CG-C and CY-C, and rainfall treated ZS-R, CG-R and CY-R were collected, the peel was removed, sliced and immediately frozen in liquid nitrogen in the field. These samples were then transported back to the laboratory in liquid nitrogen and stored at  $-80^{\circ}\text{C}$

11 until further analysis.

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**Scanning electron microscopy**

To observe the suberin layer formation, the peel (5 mm x 5 mm) was excised from freshly harvested fruit and frozen in a pre-cooled tissue holder with frozen glue in a cryogenic liquid nitrogen slurry at a temperature lower than  $-143.15^{\circ}\text{C}$  for rapid freezing. Subsequently, the samples were fixed, observed and images were captured by the quorum 2100 scanning electron microscope cryoscopy (Quorum, England).

**Fluorescence microscopy observation of the suberinized fruit peel**

Peel slices were placed on the glass slides and soaked in 0.1% berberine hydrochloride solution for 60 minutes. After rinsing with distilled water 0.5% aniline blue was added and the samples were stained for 30 minutes. This was followed by the addition of a mixture of 0.1%  $\text{FeCl}_3$  and glycerol (volume ratio 1:1). After 5 minutes the samples were observed under UV using a fluorescence microscope (Brundrett et al 1988).

**Untargeted metabolomics analysis by LC-MS and GC-MS**

To analyze the metabolites the stored frozen peel slices were ground and the metabolites were extracted according to Shi et al (2019). For LC-MS analysis, 60 mg of each sample was used. An ACQUITY UHPLC system (Waters Corporation, Milford, USA) fitted with an ACQUITY UPLC BEH C18 column ( $1.7\text{ }\mu\text{m}$ ,  $2.1\text{ x }100\text{ mm}$ ) coupled with an AB SCIEX Triple TOF 5600 System (AB SCIEX, Framingham, MA) was used in both electrospray ion (ESI) positive and negative modes. To assess the repeatability, the QC sample (a mixture of an equal volume of each sample) was injected at regular intervals of every 10 samples throughout

the analytical run. Metabolite labeling was accomplished using the Human Metabolome Database and LIPID MAPS® Structure Database (LMSD) (Scandiani *et al.*, 2015).

For GC-MS analysis, the 7890B-5977A GC/MSD GC/MSD (Agilent Technologies Inc. CA, USA) fitted with a DB-5MS capillary column (30 m x 0.25 mm x 0.25  $\mu$ m, Agilent J & W Scientific, Folsom, CA, USA) was used. The carrier gas was high-purity helium, and the flow rate was 1.0 mL/min. The inlet temperature was maintained at 260°C. Metabolites were quantified using the NIST and Fiehn database.

For the identification of differentially expressed metabolites, unsupervised principal component analysis was used to observe the overall distribution among samples and the stability of the analytical process. Supervised projection to latent structure-discriminant analysis (PLS-DA) was carried out to distinguish the overall differences between different samples. A variable importance for the projection (VIP) >1, *p* value <0.05 were considered as threshold values for the differentially expressed metabolites.

### Metabolite targeted detection

The target compound (phenolic substances and long-chain/very long fatty acids) in the samples were detected qualitatively and quantitatively by the UPLC-Q-TOF/MS mass spectrometry system (Waters, USA) equipped with an electrospray ion (ESI) source and UNIFI 1.8.1 workstation.

### RNA-seq analysis

Total RNA was extracted from the pear peel slices and a library was prepared according to the Illumina standard instructions (TruSeq Stranded RNA LT Guide). The raw reads were filtered by Seqtk before mapping

11 to the pear genome using TopHat (version: 2.0.9). The gene fragments were counted using HTSeq followed  
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42 by trimmed mean of M (TMM) values normalization (Anders *et al.*, 2014). Significantly differentially  
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63 expressed genes (DEGs) were identified as those with a false discovery rate (FDR) value above the threshold  
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94 ( $Q < 0.05$ ) and fold change  $> 2$  calculated using the edgeR software. The identities of these DEGs were  
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11 confirmed by gene ontology (GO) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment  
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14 (Ashburner *et al.*, Ogata *et al.*, 1999).

28 **Proteomics by iTRAQ and LC-MS/MS**

24 Total proteins were extracted from the stored fruit peel and digested with trypsin (protein:trypsin, 30:1) at  
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26 37°C for 16 hours, as described by Zhao *et al.* (2015). The peptides were desalted and labeled with the 8-plex  
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28 iTRAQ reagents according to the manufacturer's protocols (AB SCIEX, Framingham, MA, USA). The  
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30 iTRAQ reagents had molecular masses of 113 (CG-R) and 114 (CG-C) for the rainfall-treated and non-treated  
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32 iTRAQ reagents had molecular masses of 113 (CG-R) and 114 (CG-C) for the rainfall-treated and non-treated  
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34 control semi-russet CG fruit; 115 (ZS-R) and 116 (ZS-C) for the rainfall-treated and non-treated control russet  
35  
36 ZS fruit; and 119 Daltons for the QC (QC mixture), respectively.

40 For LC-MS/MS analysis, the parameters and methods were carried out as described by Fu *et al* (2015).  
41  
42 Subsequently, the data was analyzed for protein identification and quantification using Protein Pilot Software  
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44 v.5.0 (Sciex Inc., USA). Those proteins identified at global  $FDR \leq 1\%$  and unique peptides  $\geq 1$  were selected  
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46 for further downstream analysis. The differentially expressed proteins (DEPs) were identified as those with  $p$ -  
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48 values  $< 0.05$  and fold change  $\geq 1.2$  between different treatments. They were further analyzed using Proteome  
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50 Discoverer TM 2.2 software (Thermo Corporation, USA) and searched against the pear genome database in  
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52 NCBI (LM07171185NCBI-Pyrus pear. fasta). Finally, the identified DEPs were annotated with GO terms and  
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KEGG enrichment analysis.

## Targeted analysis by parallel reaction monitoring (PRM)

The sample preparation for PRM was carried out using the method described by Martinez *et al* (2017). Proteins were digested and desalted by a C18-Reverse-Phase SPE Column and the iRT standard peptides were dissolved in a relative buffer according to the manufacturer's protocol (2xiRT Kit Quick Reference Card). PRM analysis was performed with a Q-Executive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The original offline files were imported into Proteome Discover 2.3 software to search the pear genome sequence database (LM07171185NCBI-Pyrus pear. fasta). Using Skyline software, the target peptide lists were selected from the data dependent acquisition (DDA) results and imported into the inclusion list of the Xcalibur PRM method editing module. The results were imported into the Skyline software. The actual and the relative retention time, i.e., iRT of the standard peptides were derived from the combined data from statistics (Excel), the Graphpad Prism 8.0 software, the retention time distribution and the correlation with the theoretical retention time (Table S4) (MacLean et al., 2010). Very small deviations between all the standard peptides (11/11) were detected (Fig. S6). The correlation coefficient ( $R^2$ ) between the actual and theoretical retention time was 0.9986, indicating data were reliable (Fig. S7).

## Integration analysis of transcriptome, proteome, and metabolome profiles

The data from the transcriptome, proteome and metabolome were integrated and analyzed in order to identify the key genes, proteins and metabolites that are involved in the common or unique pathways. ID conversion was used to convert genes, proteins and metabolite IDs into the KEGG format (Tables S5, and S6). The IDs were then simultaneously mapped to the KEGG database through GENMAPP. Protein-pathway and protein-

protein correlations were obtained, and the network maps were drawn using Cytoscape V3.6.1 bioinformatics analysis software.

**Expression analysis by quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from the pear peel using the Plant RNA Preparation Kit (Omega Bio-Tek, Guangzhou, China). The first strand cDNA was synthesized using a Revert Aid First Strand cDNA Synthesis kit (Takara RRO47) and used as templates for real-time PCR as described previously (Shi *et al*, 2019).

***PbHHT1* cloning, vector construction and transient expression in pear fruit**

The coding region of *PbHHT1* (GenBank accession 103966555) was amplified using the primer pair CZ-HHT1-BamHI-F 5'-**ATGTCTAGACTCGAGATGGTTGCTGAATTCGGAG**-3' and CZ-HHT1-BamHI-R 5'-**AGCCTGCAGCCATGGCTAGACGTTTCATCAGTTCTTG**-3'(adaptor sequences for seamless cloning are in bold). The PCR product was verified by gel electrophoresis and purified (Fig. S8). This was followed by recombination into the plant expression vector pCambia 2301 using the ClonExpress II One Step Cloning Kit (<https://www.vazymebiotech.cn/>) (Fig. S9). The success of cloning was subsequently confirmed by sequencing.

The recombinant pCambia2301-PbHHT1 (PbHHT1) and the pCambia2301 empty vectors (EV) were independently transformed into the Agrobacterium strain GV3101 and a single transgenic colony was inoculated and cultured in LB medium containing kanamycin (50µg/ml), ampicillin (100µg/ml), rifampicin (50µg/ml) and gentamycin (25µg/ml) with shaking at 28°C for 24 hours. The cells were collected by

centrifugation and resuspended in the infiltration buffer (10 mM MgCl<sub>2</sub>, 10 mM MES at pH 5.5 and 20 mM acetosyringone) (MMA). The final OD<sub>600</sub> of the bacterial solution was adjusted to ~1.0 and injected into 1-5 mm below the peel of the young green pear fruit as described by Zhai *et al.* (2016). Those fruit injected with empty vector containing bacteria solution (EV), MMA and water were used as negative controls. The fruit was harvested thirteen days after injection when russet spots appeared. The peel around the injection sites covering the entire bacteria spreading areas was excised, frozen in liquid N<sub>2</sub> and stored in -80°C for the analysis of metabolites and RNAseq (Wu *et al.*, 2019).

## Statistical analyses

The content of various metabolites and the quantitative expression of DEGs, DEPs and DEMs were analyzed by univariate statistical analysis and presented as the mean ± standard deviation (SD). Significant differences were analyzed using a one-way analysis of variance (ANOVA) and the least significant difference (LSD) multiple range tests was carried out using SPSS 17.0 software (IBM, Armonk, NY, USA). A  $p < 0.01$  indicates a statistically significant difference between treatments.

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11 **AUTHOR CONTRIBUTIONS**

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5 YZ, JL, CS and BQ initiated and designed the project. CS, XW and JX performed and acquired experiments.  
6  
7 CS and BQ analyzed and interpreted the data. CS and BQ wrote the manuscript. All authors read and approved  
8  
9 the final manuscript for submission.  
10

16 **CONFLICT OF INTEREST**

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20 All authors declare that they have no competing interests.  
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26 **SUPPORTING INFORMATION**

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30 Additional Supporting Information may be found in the online version of this article.  
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34 **Table S1.** Overlapped differentially expressed metabolites identified from the rainfall treated and non-treated  
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36 russet ‘Zaoshengxinshui’ (ZS), semi-russet ‘Cuiguan’ (CG) and green non-russet ‘Cuiyu’ (CY) pear fruit.  
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40 **Table S2.** Overlapped differentially expressed genes identified from the rainfall treated and non-treated russet  
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42 ‘Zaoshengxinshui’ (ZS), semi-russet ‘Cuiguan’ (CG) and green non-russet ‘Cuiyu’ (CY) pear fruit.  
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46 **Table S3.** Differentially expressed proteins related to russetting by iTRAQ analysis.  
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49 **Table S4.** The retention time (iRT) of the internal peptide standards used in data dependent acquisition (DDA).  
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53 **Table S5.** ID conversion to genes and proteins identified from the rainfall treated and non-treated semi-russet  
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55 ‘Cuiguan’ CG.  
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**Table S6.** ID conversion to genes and proteins identified from the rainfall-treated and non-treated russet ‘Zaoshengxinshui’ ZS fruit peel.

**Table S7.** Measurements of the photosynthetically active radiation and illuminance (PAR) in the nanopore bags of the control and non-bagged rainfall-treated pear.

**Fig. S1.** The russet index and cork layer thickness of the russet ‘Zaoshengxinshui’ (ZS), semi-russet ‘Cuiguan’ (CG) and green non-russet ‘Cuiyu’ (CY) (control). R, rainfall-treated fruit, C, non-rainfall-treated control fruit, DAFB, day after full bloom. The data are represented as the mean  $\pm$  standard deviation ( $n = 9$ ). \*\*  $p < 0.01$ .

**Fig. S2.** Principal Component Analysis (PCA) of differentially expressed metabolites (DEMs) in the peel of russet ‘Zaoshengxinshui’ (ZS), semi-russet ‘Cuiguan’ (CG) and green non-russet ‘Cuiyu’ (CY) fruit.

(a) PCA of DEMs of ZS, CG and CY fruit by Liquid chromatography-tandem mass spectrometry (LC-MS).

(b) PCA of DEMs of ZS, CG and CY fruit by Gas chromatography–mass spectrometry (GC-MS).

R, rainfall-treated fruit, C, non-rainfall-treated control fruit.

**Fig. S3.** Absolute quantification of DEMs related to the suberin lamellae in the peel of rainfall-treated (R) and non-treated control (C) ZS, CG and CY fruit.

**Fig. S4.** KEGG enrichment of DEGs in the peel of ZS, CG and CY, as well as in the rainfall-treated (R) and non-treated control (C) of the russet ZS and semi-russet CG fruit.

**Fig. S5.** Venn diagrams to show DEGs that are common between ZS, CG and CY as well as between the rainfall-treated (R) and non-treated control (C) ZS and CG fruit.

11 **Fig. S6.** The eleven standard peptides and their retention time.  
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5 **Fig. S7.** The correlation between the measured and the theoretical values of the retention time (iRT) of the  
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73 internal standard peptides by regression analysis.  
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10 **Fig. S8.** PCR amplification of the coding region of *PbHHT1* from the semi-russet ‘Cuiguan’ (CG) pear fruit.  
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14 **Fig. S9.** Map of the *PbHHT1* plant expression vector used for transient expression in the young green pear  
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16 fruit.  
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20 **Fig. S10.** Temperature and humidity in in the nanopore bags of the control and non-bagged rainfall-treated  
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## REFERENCES

- Anders, S., Pyl, P.T. and Huber, W. (2014) HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics*, **31**, 166-169.
- Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S. and Eppig, J.T. Gene Ontology: tool for the unification of biology. *Nat Genet*, **25**, 25-29.
- Barberon, M., Vermeer, J.E.M., DeBellis, D., Wang, P., Naseer, S., Andersen, T.G., Humbel, B.M., Nawrath, C., Takano, J. and Salt, D.E. (2016) Adaptation of Root Function by Nutrient-Induced Plasticity of Endodermal Differentiation. *Cell*, **164**, 447-459.
- Bernards and Mark, A. (2002) Demystifying suberin. *Can.j.bot*, **80**, 227-240.
- Brundrett, M.C., Enstone, D.E., and Peterson, C.A. (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin, and callose in plant tissue. *Protoplasma*, **146**, 133-142.
- Cohen, H., Szymanski, J., Aharoni, A. and Dominguez, E. (2017) Assimilation of 'omics' strategies to study the cuticle layer and suberin lamellae in plants. *Journal of Experimental Botany*, **68**, 5389-5400.
- Daqiu, Zhao, Saijie, Gong, Zhaojun, Hao, Jiasong, Meng, Jun and Tao (2015) Quantitative Proteomics Analysis of Herbaceous Peony in Response to Paclobutrazol Inhibition of Lateral Branching. *International Journal of Molecular Sciences*, **16**, 24332-24352.
- Duan, H., & Schuler, M. A. (2005) Differential expression and evolution of the Arabidopsis CYP86A subfamily. *Plant physiology*, **137**, 1067-1081.
- Edstam, M.M., Blomqvist, K., Eklf, A., Wennergren, U. and Edqvist, J. (2013) Coexpression patterns indicate that GPI-anchored non-specific lipid transfer proteins are involved in accumulation of cuticular wax, suberin and sporopollenin. *Plant Molecular Biology*, **83**, 625-649.
- Escamilla-Treviño, L.L., Chen, W., Card, M.L., Shih, M.C., Cheng, C.L. and Poulton, J.E. (2006) Arabidopsis thaliana beta-Glucosidases BGLU45 and BGLU46 hydrolyse monolignol glucosides. *Phytochemistry*, **67**, 1651-1660.
- Franke, R. and Schreiber, L. (2007) Suberin—a biopolyester forming apoplastic plant interfaces. *Current Opinion in Plant Biology*, **10**, 252-259.
- Fu, Y., Zhang, H., Mandal, S.N., Wang, C. and Ji, W. (2015) Quantitative proteomics reveals the central changes of wheat in response to powdery mildew. *Journal of Proteomics*, **130**, 108-119.
- Gamble, J., Jaeger, S.R. and Harker, F.R. (2006) Preferences in pear appearance and response to novelty among Australian and New Zealand consumers. *Postharvest Biology & Technology*, **41**, 38-47.
- Graça, J. and Santos, S. (2010) Suberin: A Biopolyester of Plants' Skin. *Macromolecular Bioscience*, **7**, 128-135.
- Heng, W., Liu, L., Wang, M.D., Jia, B., Liu, P., Ye, Z.F. and Zhu, L.W. (2014) Differentially expressed genes related to the formation of russet fruit skin in a mutant of 'Dangshansuli' pear (*Pyrus bretschneideri* Rehd.) determined by suppression subtractive hybridization. *Euphytica*, **196**, 285-297.
- Hou, Z., Jia, B., Li, F., Liu, P., Liu, L., Ye, Z., Zhu, L., Wang, Q. and Heng, W. (2018) Characterization and expression of the ABC family (G group) in 'Dangshansuli' pear (*Pyrus bretschneideri* Rehd.) and its russet mutant. *Genetics and Molecular Biology*, **41**, 137-144.
- Inoue, E., Kasumi, M., Sakuma, F., Anzai, H., Amano, K. and Hara, H. (2006) Identification of RAPD marker linked to fruit skin color in Japanese pear (*Pyrus pyrifolia* Nakai). *Scientia Horticulturae*, **107**, 254-258.
- Jia, X.M., Zhu, Y.F., Hu, Y., Zhang, R. and Wang, Y.X. (2019) Integrated physiologic, proteomic, and metabolomic analyses of *Malus halliana* adaptation to saline–alkali stress. *Horticulture Research*, **6**, 91.
- Justin, L., Asaph, A. and Fabrizio, C. (2015) Genome investigation suggests MdSHN3, an APETALA2-domain transcription factor gene, to be a positive regulator of apple fruit cuticle formation and an inhibitor of russet development. *Journal of Experimental Botany*, **66**:6579-6589.
- Kaur, H., Shaker, K., Heinzl, N., Ralph, J., Galis, I. and Baldwin, I.T. (2012) Environmental stresses of field growth allow cinnamyl alcohol dehydrogenase-deficient *Nicotiana attenuata* plants to compensate for their structural deficiencies, **159**, 1545-1570

- Krishnamurthy, P., Ranathunge, K., Nayak, S., Schreiber, L. and Mathew, M.K. (2011) Root apoplastic barriers block Na<sup>+</sup> transport to shoots in rice (*Oryza sativa* L.). *Journal of Experimental Botany*, **62**, 4215-4228.
- Lara, I., Belge, B. and Goulao, L.F. (2015) A focus on the biosynthesis and composition of cuticle in fruits. *J Agric Food Chem*, **63**, 4005-4019.
- Legay, S., Guerriero, G., Deleruelle, A., Lateur, M., Evers, D., André, C.M. and Hausman, J.-F. (2015) Apple russetting as seen through the RNA-seq lens: strong alterations in the exocarp cell wall. *Plant Molecular Biology*, **88**, 21-40.
- Li, A.X., Han, Y.Y., Wang, X., Chen, Y.H., Zhao, M.R., Zhou, S.M. and Wang, W. (2015) Root-specific expression of wheat expansin gene TaEXPB23 enhances root growth and water stress tolerance in tobacco. *Environmental & Experimental Botany*, **110**, 73-84.
- Li, Y., Beisson, F., Koo, A.J.K., Molina, I., Pollard, M. and Ohlrogge, J. (2007) Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 18339-18344.
- Lotfy, S., Javelle, F. and Negrel, J. (1996) Purification and characterization of hydroxycinnamoyl- Coenzyme A:  $\omega$ - hydroxypalmitic acid O-hydroxycinnamoyltransferase from tobacco (*Nicotiana tabacum* L.) cell-suspension cultures. *Planta*, **199**, 475-480.
- MacLean, B., Tomazela, D.M., Shulman, N., Chambers, M., Finney, G.L., Frewen, B., Kern, R., Tabb, D.L., Liebler, D.C. and MacCoss, M.J. (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*, **26**, 966-968.
- Martinez, E., Lesur, A., Devis, L., Cabrera, S., Matias-Guiu, X., Hirschfeld, M., Asberger, J., Van Oostrum, J., Casares de Cal, M.d.l.Á. and Gómez-Tato, A. (2017) Targeted proteomics identifies proteomic signatures in liquid-biopsies of the endometrium to diagnose endometrial cancer and assist in the prediction of the optimal surgical treatment. *Clinical Cancer Research An Official Journal of the American Association for Cancer Research*, clincanres, **23**:6458-6467.
- Norris, R.F. and Bukovac, M.J. (2010) Influence of cuticular waxes on penetration of pear leaf cuticle by 1-naphthaleneacetic acid. *Pest Management Science*, **3**, 705-708.
- Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H. and Kanehisa, M. (1999) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res*, **27**, 29-34.
- Perez-Enciso, M., Toro, M.A., Tenenhaus, M. and Gianola, D. (2003) Combining gene expression and molecular marker information for mapping complex trait genes: a simulation study. *Genetics*, **164**, 1597-1606.
- Pollard, M., Beisson, F., Li, Y. and Ohlrogge, J.B. (2008) Building lipid barriers: biosynthesis of cutin and suberin. *Trends Plant Sci*, **13**, 236-246.
- Riederer, M. and Schreiber, L. (2001) Protecting against water loss: analysis of the barrier properties of plant cuticles. *J Exp Bot*, **52**, 2023-2032.
- Scandiani, M.M., Luque, A.G., Razori, M.V., Lucila, C.C., Takayuki, A., Kerry, O.D., Cervigni, G.D.L. and Spampinato, C.P. (2015) Metabolic profiles of soybean roots during early stages of *Fusarium tucumaniae* infection. *Journal of Experimental Botany*, **391**.
- Secchi, F., Pagliarani, C. and Zwieniecki, M.A. (2017) The functional role of xylem parenchyma cells and aquaporins during recovery from severe water stress. *Plant Cell & Environment*, **40**, 858-871.
- Serra, O., Chatterjee, S., Figueras, M., Molinas, M. and Stark, R.E. (2014) Deconstructing a plant macromolecular assembly: chemical architecture, molecular flexibility, and mechanical performance of natural and engineered potato suberins. *Biomacromolecules*, **15**, 799-811.
- Shao, H.B., Chu, L.Y., Shao, H.B., Chu, L.Y., Shao, M.A. and Zhao, C.X. (2008) Advances in functional regulation mechanisms of plant aquaporins: Their diversity, gene expression, localization, structure and roles in plant soil-water relations (Review). *Molecular Membrane Biology*, **25**, 179-191.
- Schomburg, D., and Schomburg I. (2013).  $\omega$ -hydroxypalmitate o-feruloyl transferase 2.3.1.188. Springer Berlin Heidelberg.
- Shi, C.H., Qi, B.X., Wang, X.Q., Sheng, L.Y., Luo, J. and Zhang Y.X. (2019) Proteomic analysis of the key mechanism of exocarp russet pigmentation of semi-russet pear under rainwater condition. *Scientia Horticulturae*, **254**, 178-186.

- Vishwanath, S.J., Delude, C., Domergue, F. and Rowland, O.** (2015) Suberin: biosynthesis, regulation, and polymer assembly of a protective extracellular barrier. *Plant Cell Rep*, **34**, 573-586.
- Wang, G., Xu, J., Li, L., Guo, Z., Si, Q., Zhu, G., Wang, X., and Guo W.** (2019) GbCYP86a1-1 from gossypium barbadense positively regulates defence against *Verticillium dahliae* by cell wall modification and activation of immune pathways. *Plant Biotechnology Journal*, **18**.
- Wang, Y.Z., Dai, M.S., Zhang, S.J. and Shi, Z.B.** (2014a) Exploring Candidate Genes for Pericarp Russet Pigmentation of Sand Pear (*Pyrus pyrifolia*) via RNA-Seq Data in Two Genotypes Contrasting for Pericarp Color. *Plos One*, **9**, e83675.
- Wang, Y.Z., Dai, M.S., Cai, D.Y. and Shi, Z.B.** (2020) Proteome and transcriptome profile analysis reveals regulatory and stress-responsive networks in the russet fruit skin of sand pear. *Horticulture Research*, **7**, 16.
- Wang, Y.Z., Dai, M.S., Cai, D.Y., Zhang, S. and Shi, Z.B.** (2016) A review for the molecular research of russet/semi-russet of sand pear exocarp and their genetic characters. *Scientia Horticulturae*, **210**, 138-142.
- Wang, Y.Z., Zhang, S., Dai, M.S. and Shi, Z.B.** (2014b) Pigmentation in sand pear (*Pyrus pyrifolia*) fruit: biochemical characterization, gene discovery and expression analysis with exocarp pigmentation mutant. *Plant Molecular Biology*, **85**, 123-134.
- Wei, X., Lu, W., Mao, L., Han, X., and Xu, C.** (2019). ABF2 and MYB transcription factors dominate feruloyl transferase fht gene involved in aba-mediated wound suberization of kiwifruit. *Journal of Experimental Botany*, **71**, 305-317.
- Wu, M., Si, M., X.Li, Song, L.Y., Liu, J.L., Zhai, R., Cong, L., Yue, R.R., Yang C.Q., Ma, F.W., Xu, L.F. and Wang Z.G.** (2019). Pbcop1.1 contributes to the negative regulation of anthocyanin biosynthesis in pear. *Plants*, **8**, 39.
- Zhai, R., Wang, Z., Zhang, S., Meng, G., Song, L., Wang, Z., Li, P., Ma, F. and Xu, L.** (2016) Two MYB transcription factors regulate flavonoid biosynthesis in pear fruit (*Pyrus bretschneideri* Rehd.). *J Exp Bot*, **67**, 1275-1284.

**Table 1. The overlapping differentially expressed genes (DEGs) related to the biosynthesis of cutin, suberin and wax identified between the russet ‘Zaoshengxinshui’ (ZS) and green non-russet ‘Cuiyu’ (CY) (ZS vs CY); semi-russet ‘Cuiguan’ (CG) and green non-russet CY (CG vs CY); rainfall-treated (R) and non-treated (C) russet ZS (ZS (R vs C), and rainfall-treated (R) and no-rainfall-treated (C) of semi-russet CG (CG (R vs C) pear fruit. Plus values indicate upregulation, minus ones downregulation. ZS vs CY, between the russet ‘Zaoshengxinshui’ (ZS) and green non-russet ‘Cuiyu’ (CY); CG vs CY, between the semi-russet ‘Cuiguan’ (CG) and CY; ZS (R vs C), between rainfall-treated and non-rainfall-treated russet ZS; CG (R vs C), between rainfall-treated (R) and non-rainfall-treated (R) russet CG pear fruit.**

| Acc. no.  | Gene name               | Description                                                   | Log <sub>2</sub> (FC) |          |             |             |
|-----------|-------------------------|---------------------------------------------------------------|-----------------------|----------|-------------|-------------|
|           |                         |                                                               | ZS vs CY              | CG vs CY | ZS (R vs C) | CG (R vs C) |
| 103966555 | <i>PbHHT1-like</i>      | PREDICTED: omega-hydroxypalmitate O-feruloyl transferase-like | +2.65                 | +3.68    | +0.21       | +3.21       |
| 103948102 | <i>PbCYP749A22-like</i> | PREDICTED: cytochrome P450 CYP749A22-like                     | +5.10                 | +3.45    | +1.10       | --          |
| 103949661 | <i>PbKCS10-like</i>     | PREDICTED: 3-ketoacyl-CoA synthase 10-like                    | -8.15                 | -3.43    | -1.36       | -2.94       |
| 103943280 | <i>PbCCR SNL6-like</i>  | PREDICTED: cinnamoyl-CoA reductase-like SNL6 isoform X1       | -5.57                 | -3.35    | -1.07       | -2.76       |
| 103928845 | <i>PbWSD1-like</i>      | PREDICTED: O-acyltransferase WSD1-like                        | -7.18                 | -3.67    | -1.79       | -2.89       |
| 103929753 | <i>PbCER1-like</i>      | PREDICTED: protein ECERIFERUM 1-like isoform X1               | -5.86                 | -3.07    | -1.83       | -4.65       |
| 103934278 | <i>PbABCG10</i>         | PREDICTED: ABC transporter G family member 10-like            | +2.85                 | +3.31    | +1.12       | +4.62       |
| 103963796 | <i>PbABCG15</i>         | PREDICTED: ABC transporter G family member 15-like            | -8.25                 | -3.16    | -1.82       | -3.45       |
| 103942099 | <i>PbABCG15</i>         | PREDICTED: ABC transporter G family member 15-like            | -5.26                 | -3.44    | -2.21       | -3.09       |
| 103955899 | <i>PbABCG15</i>         | PREDICTED: ABC transporter G family member 15-like            | -7.15                 | -2.69    | -3.28       | -2.98       |
| 103961207 | <i>PbnsLTP-like</i>     | PREDICTED: non-specific lipid-transfer protein-like           | -5.86                 | -3.53    | -1.30       | -3.26       |
| 103938613 | <i>PbnsLTP</i>          | PREDICTED: non-specific lipid-transfer protein                | -7.16                 | -4.09    | -2.17       | -3.23       |
| 103961206 | <i>PbnsLTP</i>          | PREDICTED: non-specific lipid-transfer protein                | -6.99                 | -3.65    | -1.78       | -3.20       |

**Table 2. Top hits of common DEGs and DEPs identified from RNA-seq and iTRAQ analysis of different samples.** Plus values indicate upregulation, minus values downregulation. ZS vs CY, between the russet ‘Zaoshengxinshui’ (ZS) and green non-russet ‘Cuiyu’ (CY); CG vs CY, between the semi-russet ‘Cuiguan’ (CG) and green non-russet ‘Cuiyu’ (CY); ZS (R vs C), between rainfall-treated (R) and non-rainfall-treated (C) russet ZS; CG (R vs C), between rainfall-treated (R) and non-rainfall-treated (C) semi-russet CG pear fruit.

| Acc. no.<br>(gene) | Acc. no.<br>(protein) | Description                                                                                  | Log <sub>1.2</sub> (FC) (protein) |             |                |                | Log <sub>2.0</sub> (FC) (gene) |             |                |                |
|--------------------|-----------------------|----------------------------------------------------------------------------------------------|-----------------------------------|-------------|----------------|----------------|--------------------------------|-------------|----------------|----------------|
|                    |                       |                                                                                              | ZS<br>vs CY                       | CG<br>vs CY | ZS<br>(R vs C) | CG<br>(R vs C) | ZS<br>vs CY                    | CG<br>vs CY | ZS<br>(R vs C) | CG<br>(R vs C) |
| 103966555          | 694406379             | PREDICTED:<br>omega-hydroxypalmitate O-feruloyl<br>transferase-like [Pyrus x bretschneideri] | +1.69                             | +4.48       | +4.35          | +4.10          | +2.65                          | +3.68       | +0.21          | +3.21          |
| 103952998          | 694312604             | PREDICTED:<br>3-ketoacyl-CoA synthase 10-like [Pyrus<br>x bretschneideri]                    | +0.41                             | -1.16       | -2.30          | -1.77          | -5.23                          | -7.86       | -4.34          | -0.79          |
| 103938613          | 694440153             | PREDICTED:<br>non-specific lipid-transfer protein [Pyrus<br>x bretschneideri]                | -0.41                             | -4.06       | -8.54          | -7.57          | -7.16                          | -4.09       | -2.17          | -3.23          |
| 103961207          | 694393124             | PREDICTED:<br>non-specific lipid-transfer protein-like<br>[Pyrus x bretschneideri]           | -0.21                             | -4.66       | -7.78          | -6.66          | -5.86                          | -3.53       | -1.30          | -3.26          |

## FIGURE LEGENDS

**Figure 1. Phenotypic difference between the russet ‘Zaoshengxinshui’ (ZS), semi-russet ‘Cuiguan’ (CG) and green non-russet ‘Cuiyu’ (CY), and between the rainfall treated and non-treated russetting of ZS and CG fruit.**

A. Phenotypic difference between ZS, CG and CY pear fruit. Left panel, images of the whole fruit; middle panel, scanning electron microscopy observation of the fruit surface; right panel, transmission electron microscopy observation of the fruit peel. SL, Suberin lamella; SC, stratum corneum; CW, cell wall; Tc, tannin cells; LAMB, liposomes; Cu, cutin layer; Chl, chloroplast; V, vacuole.

B. Effect of rainfall on russetting of russet ZS (top) and semi-russet CG (bottom) pear fruit. Left panel, non-rainfall-treated control fruit; right panel, rainfall-treated fruit.

C. Scanning electron microscopic images of the surface of pear fruit. Left panel, control, non-rainfall treated fruit; right panel, rainfall-treated fruit. SL, suberin lamella; SC, stratum corneum; CW, cell wall; Tc, tannin cells; LAMB, liposomes; Cu, cutin layer.

D. The different russet index between the three different pear genotypes. ZS, ‘Zaoshengxinshui’ (russet); CG ‘Cuiguan’ (semi-russet); CY, ‘Cuiyu’ (non-russet). Data are expressed as the means  $\pm$  SD of three biological replicates.  $**p < 0.01$ .

E. The effect of artificial rainfall treatment on the russet index of the semi-russet CG pear fruit. R, rainfall-treated, C, non-rainfall treated controls. Data are expressed as the means  $\pm$  SD of three biological replicates.  $**p < 0.01$ .

F. The effect of rainfall treatment on the thickness of suberin lamellae of ZS pear fruit. R, rainfall-treated, C, non-rainfall-treated controls. Data are expressed as the means  $\pm$  SD of three biological replicates.  $**p < 0.01$

**Figure 2. KEGG enrichment of the differentially expressed metabolites (DEMs) obtained from the LC-MS analysis of the russet ‘Zaoshengxinshui’ (ZS), semi-russet ‘Cuiguan’ (CG) and non-russet ‘Cuiyu’ (CY), and from the artificial rainfall-treated ZS and CG sand pear fruit.**

A. Bubble diagram of the KEGG enrichment of DEMs from ZS, CG and CY fruit.

B. Bubble diagram of the KEGG enrichment of DEMs related to russetting of the rainfall-treated (R-) and non-treated control (C-) ZS and CG fruit.

**Figure 3. Differentially expressed metabolites (DEMs) by LC-MS between the russet ‘Zaoshengxinshui’ (ZS), semi-russet ‘Cuiguan’ (CG) and green non-russet ‘Cuiyu’ (CY), and from the rainfall-treated ZS and CG sand pear fruit.**

A. Heatmaps of DEMs related to russetting between the three different pear genotypes of ZS, CG and CY fruit (n=8) (top), and between the rainfall-treated (R) and non-treated control (C) ZS and CG fruit (bottom) (n=8).

B. Venn diagrams to identify overlapping DEMs between ZS, CG and CY, and between the rainfall-treated (R) and non-rainfall-treated control (C) ZS and CG fruit.

C. Core russetting related DEMs between the ZS, CG and CY, and between the rainfall-treated (R) and non-rainfall-treated ZS and CG control (C) fruit. Enhanced DEMs are indicated in red and inhibited DEMs in green.

**Figure 4. Differentially expressed genes (DEGs) by RNA-seq analysis between russet ‘Zaoshengxinshui’ (ZS), semi-russet ‘Cuiguan’ (CG) and green non-russet ‘Cuiyu’ (CY), and between the rainfall treated and non-treated ZS and CG fruit.**

A. Heatmap of russetting related DEGs between ZS, CG and CY, and between the rainfall-treated (R) and non-rainfall-treated control (C-) of ZS and CG fruit.

B. Transcript abundance of DEGs related to russetting in the peels of ZS, CG and CY fruit.

C. Transcript abundance of DEGs related to russetting in rainfall-treated (R) and non-rainfall treated control (C) of ZS and CG sand pear fruit (n = 3). The data are represented as the means  $\pm$  standard deviation. \*\*  $p < 0.01$ .

**Figure 5. Parallel Reaction Monitoring (RPM) identification of russetting related core proteins of absolute abundance in the peels of the rainfall-treated (R) and non-treated control (C) of ZS and CG sand pear fruit.** The data are represented as the mean  $\pm$  standard deviation. \*  $p < 0.05$ . \*\*  $p < 0.01$ .

**Figure 6. Transient expression of *PbHHT1* triggered russetting in the green young non russet of CG (semi-russet) and the green CY (non-russet) pear fruit.**

A. Transient expression of *PbHHT1* in CG and CY fruit. Observation was made 13 days after injection of transgenic Agrobacteria GV3101 harbouring the 35S:PbHHT1 construct. The russet spot around the injection site was clearly seen. Top panel, images of the whole fruit. The entire part of the russetting in the *PbHHT1* expressing CG fruit is marked with a yellow dotted line box while the injection sites with red circles. Bottom panel, fluorescence microscopy observation of the stained peel by aniline blue. Arrows indicate aniline blue stained suberin. PbHHT1, PbHHT1 containing agrobacteria; EV, empty vector containing agrobacteria; MMA, the infiltration buffer (10 mM MgCl<sub>2</sub>, 10 mM MES at pH 5.5 and 20 mM acetosyringone).

B. LC-MS identification and quantification of  $\omega$ -feruloyloxypalmitic acid.

C. Expression level of *PbHHT1* by qRT-PCR.

D. The heatmap of DEGs identified by RNAseq from the peel injected with 35S:PbHHT1 (H1) and empty vector (EV) of CG and CY fruit. Statistical significance was determined by the Tukey HSD test (n=5). \* $p < 0.05$ , \*\* $p < 0.01$ .

**Figure 7. Integrated network of metabolism of russetting in the rainfall treated russet ‘Zaoshengxinshui’ (ZS) and semi-russet ‘Cuiguan’ (CG) sand pear fruit.**

A. Russetting metabolism in the rainfall treated russet ZS sand pear fruit.

B. Russetting metabolism in the rainfall-treated semi-russet CG sand pear fruit.

The nodes represent omics measurements and edges correlation-based interactions between omics measurements. The five major clusters I–V are represented by different colored block area, I. phenylpropanoid biosynthesis, in blue; II. cutin, suberin and wax biosynthesis, in green; III. fatty acid metabolism, in pink; IV, flavonoid biosynthesis, in purple and V. fatty acid elongation, in yellow. Circles represent genes/proteins, squares signal pathways, triangle arrows metabolic metabolites. Map node size represents the degree that is the most direct measurement to describe the node centrality in the network analysis. The larger and darker the node size the higher the degree centrality of the node.

Figure 8. Proposed model of russetting in sand pear fruit.

Russetting was attributed by both the PAL (Phenylalanine ammonia lyase) phenylpropanoid lignin biosynthesis pathway and the LCFAs (Long-chain fatty acids)-monoacylglyceride (16-feruloyloxypalmitic acid) for suberin biosynthesis pathway. The involvement of the phenylpropanoid biosynthesis pathway was evidenced by the increased content of lignin, p-cinnamic acid and the catalytic products, coumarine and 3-O-p-trans-Coumaroylaliphitollic acid of BGLU ( $\beta$ -Glucosidases). On the other hand, the biosynthesis and transportation of very-long chain alkanes (triacontan, C30) of stratum corneum related waxes were suppressed. Subsequently, the suberin monomer /oligomer, lignin monomer and small phenolic molecules were transported out of the membrane by the active ABC transporters where they were polymerized by the polyester synthases (PS) and finally assembled into a macromolecular suberin polymer on the cell wall. Its accumulation leads to russetting on the peel of sand pear fruit. Note that PbHHT1 plays important roles by acting as a ‘bridge’ between the phenylpropanoid lignin biosynthesis and the LCFAs-monoacylglyceride for suberin biosynthesis pathways.

HHT1,  $\omega$ -hydroxypalmitate-O-feruloyl transferase; COMT, caffeic acid 3-O-methyltransferase; 4CL, 4-coumarate-CoA ligase; PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaric acid:CoA A ligase; HCT, the lignin synthase; C3H, p-coumaryl coenzyme A3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydroge-nase; LACS, long-chain acyl-CoA synthetase; BGLU44,  $\beta$ -Glucosidase.

Solid dots indicate metabolites, genes and proteins identified in this study where blue dots indicate down-regulation while red up-regulation of DEGs, DEPs and DEMs. Arrows indicate the direction of the pathways where the solid lined arrows indicate the characterized and published whilst the dotted lined arrows the reported yet to be published metabolic pathways on KEEG.

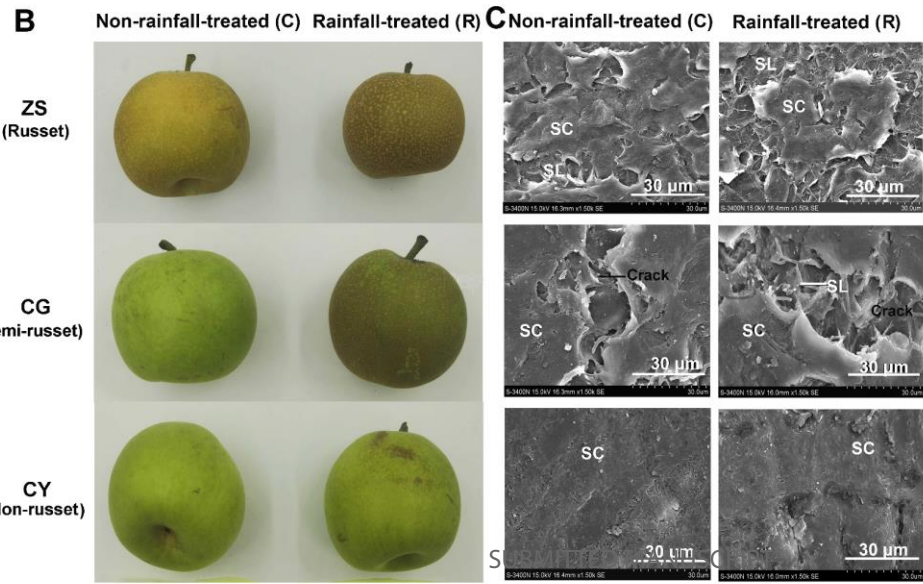
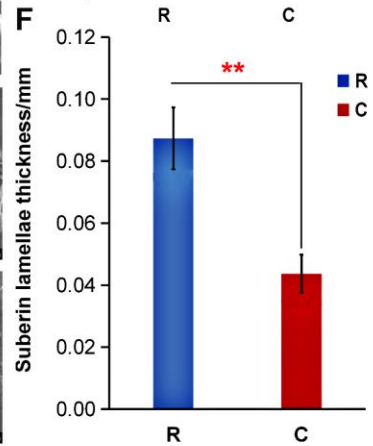
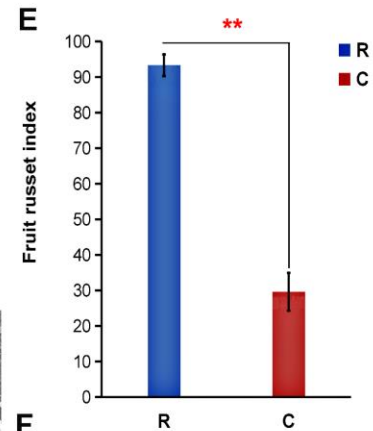
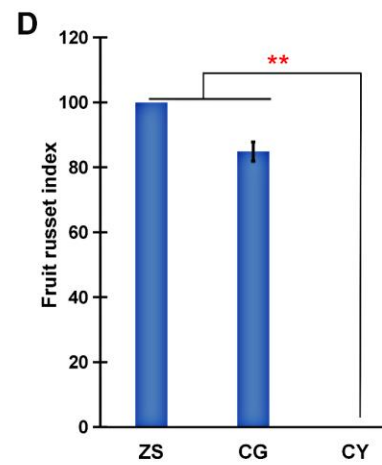
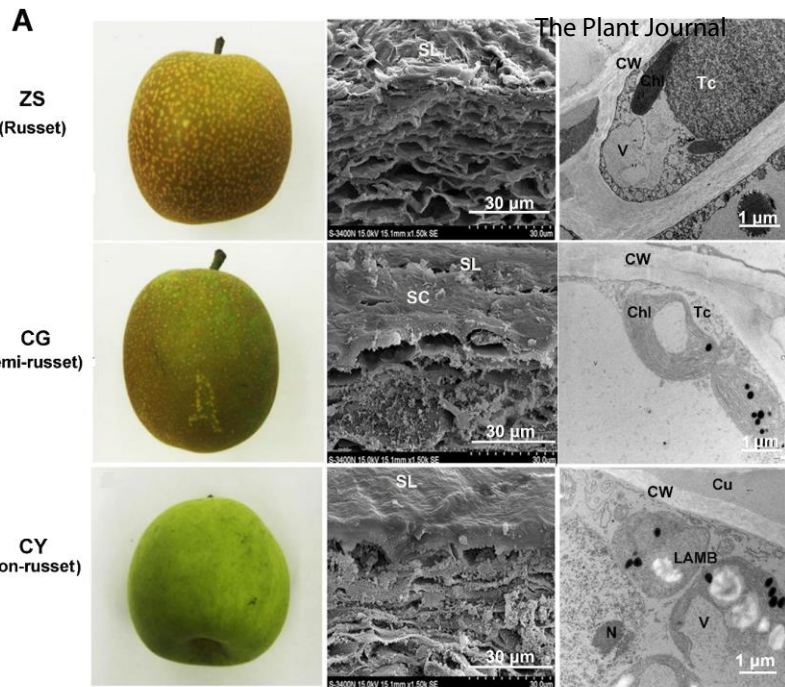


Fig 2

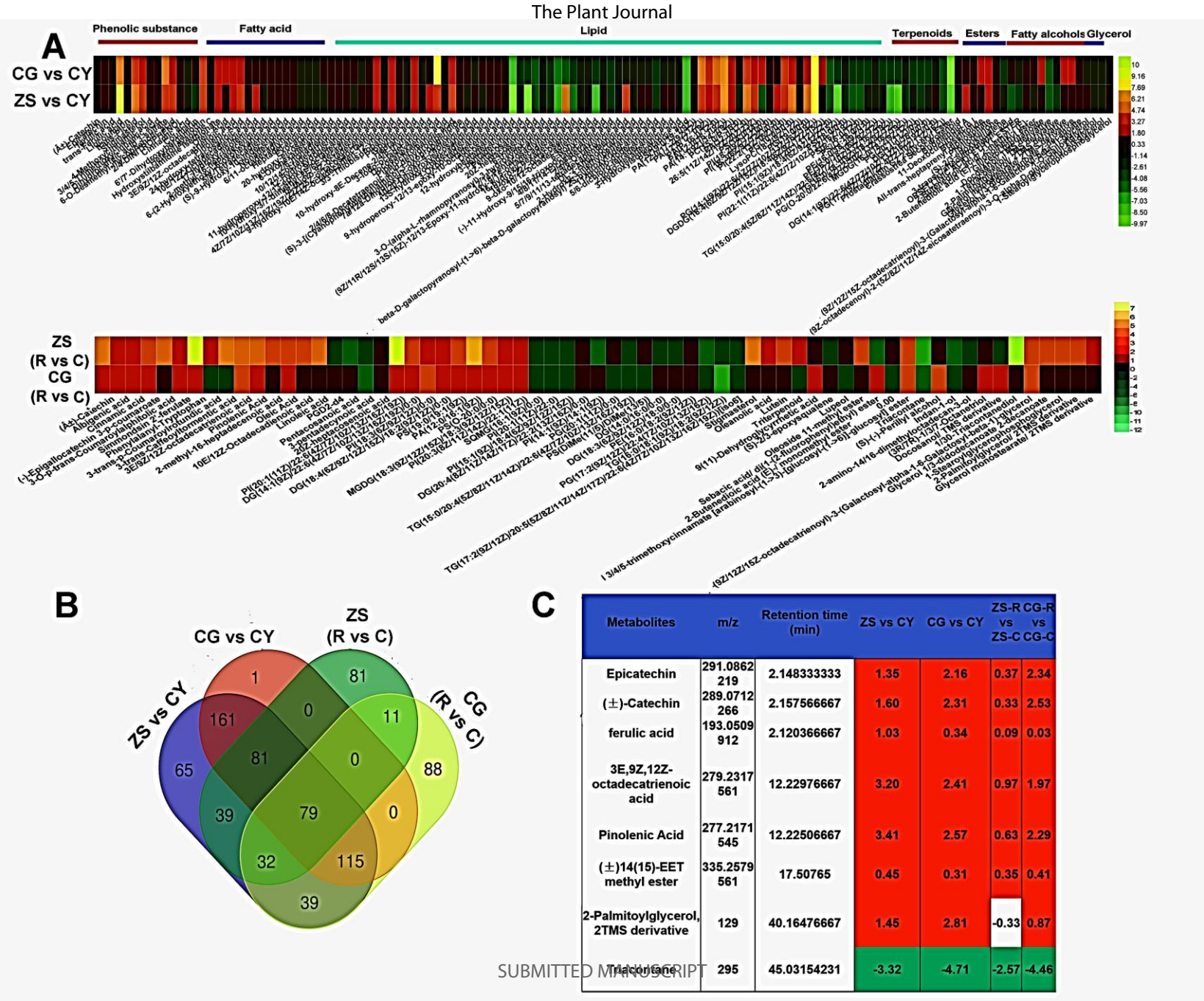


Fig 3

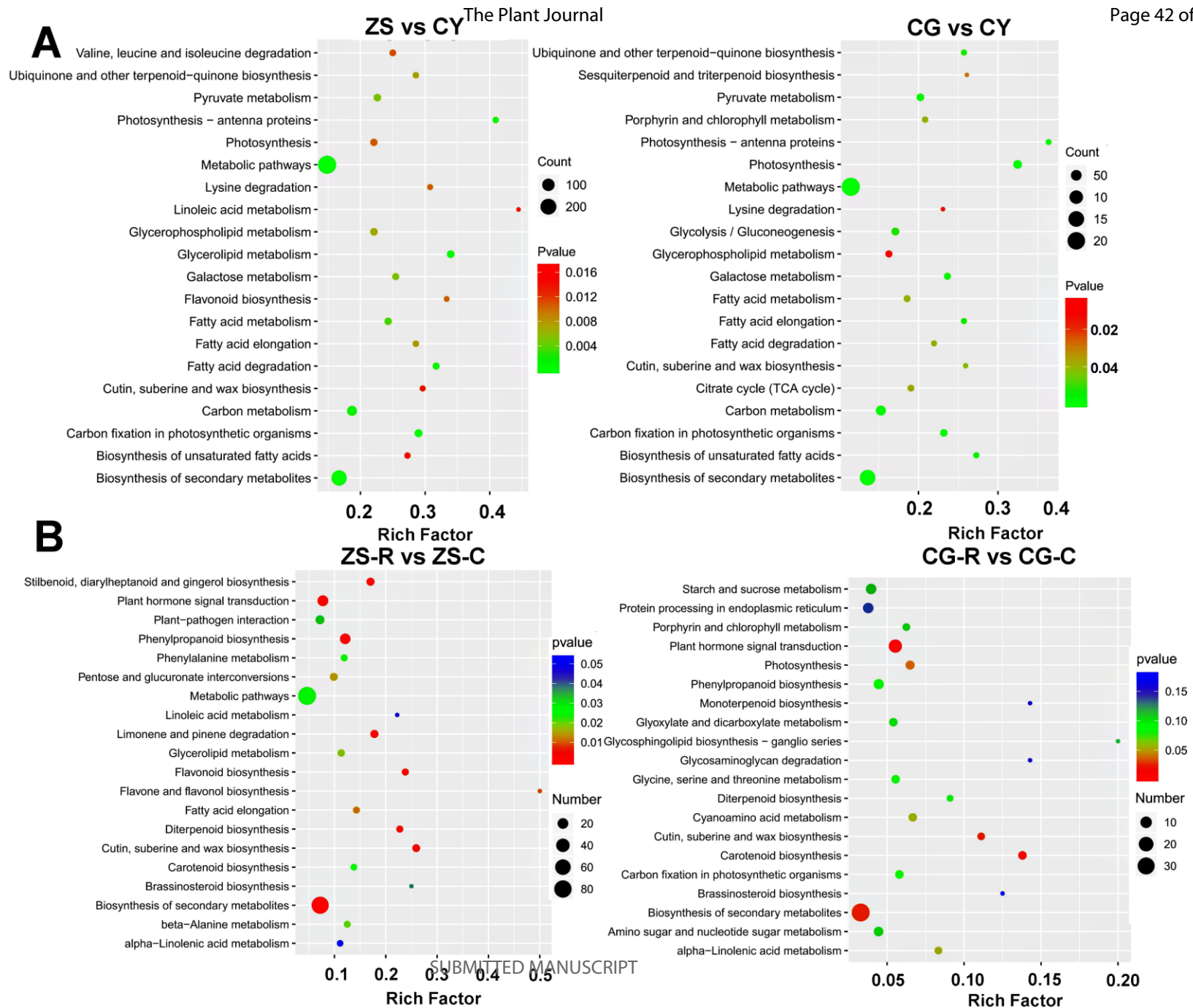
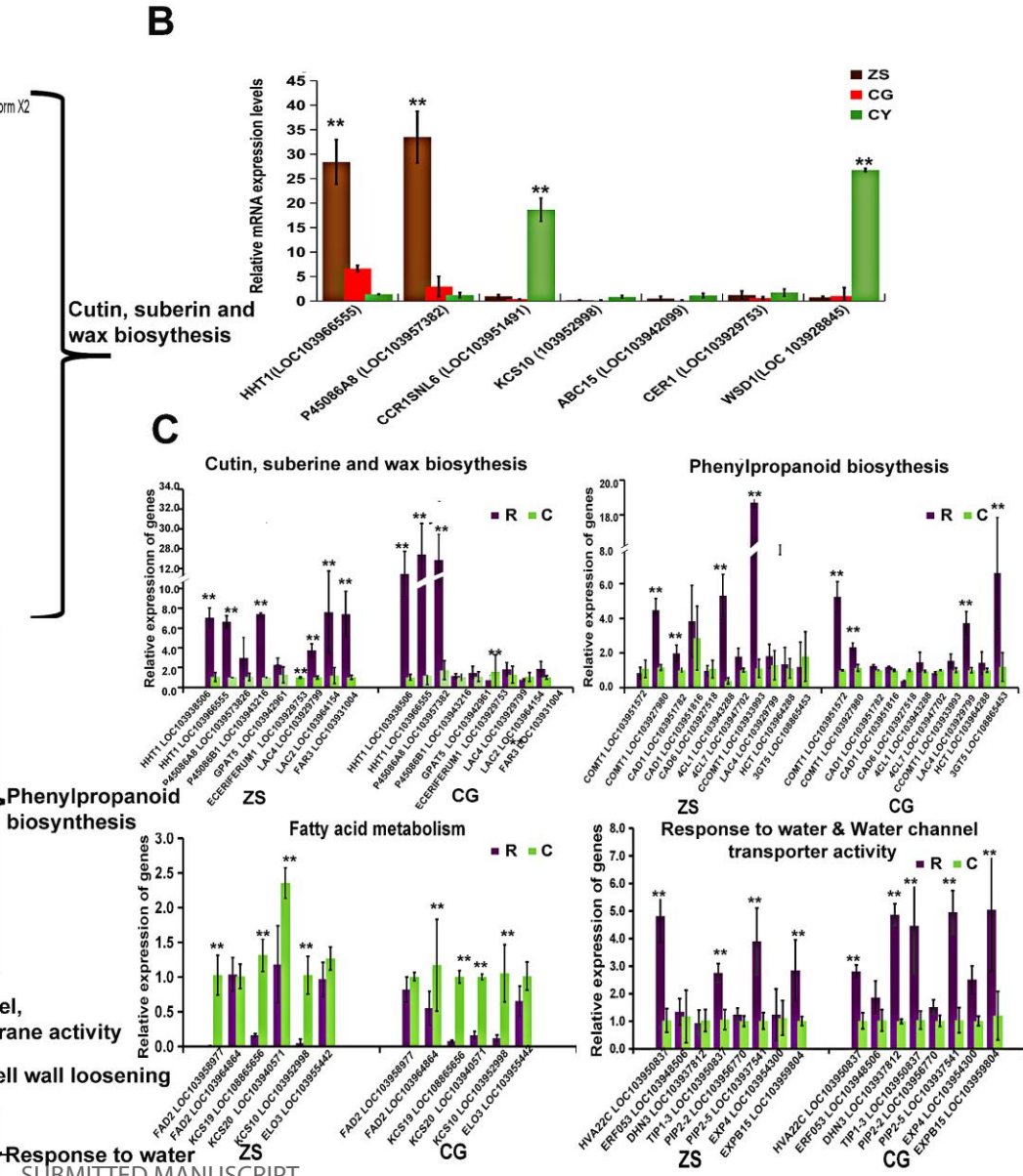
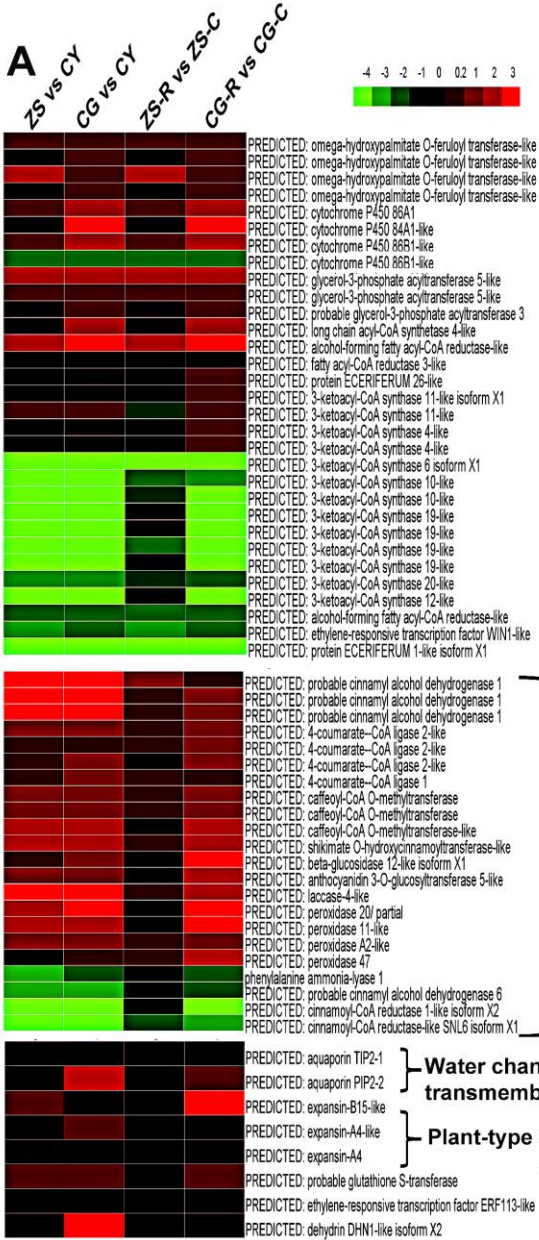


Fig 4

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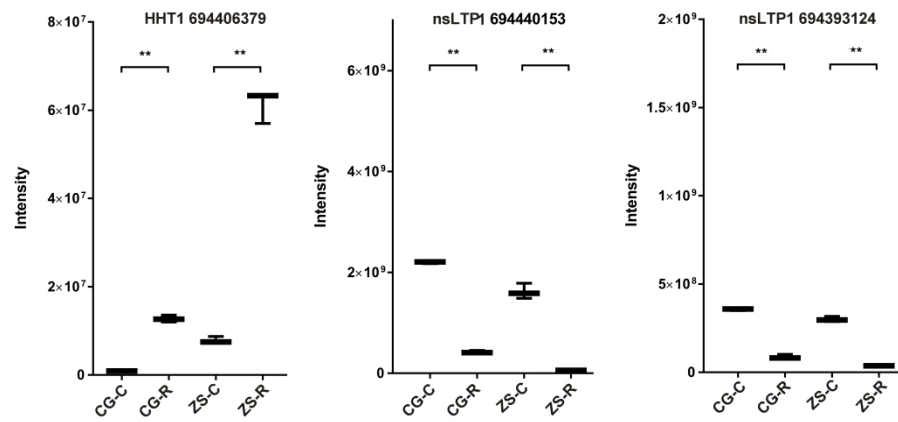


Figure 5. Parallel Reaction Monitoring (RPM) identification of russetting related core proteins of absolute abundance in the peels of the rainfall-treated (R) and non-treated control (C) of ZS and CG sand pear fruit. The data are represented as the mean  $\pm$  standard deviation. \*  $p < 0.05$ . \*\*  $p < 0.01$ .

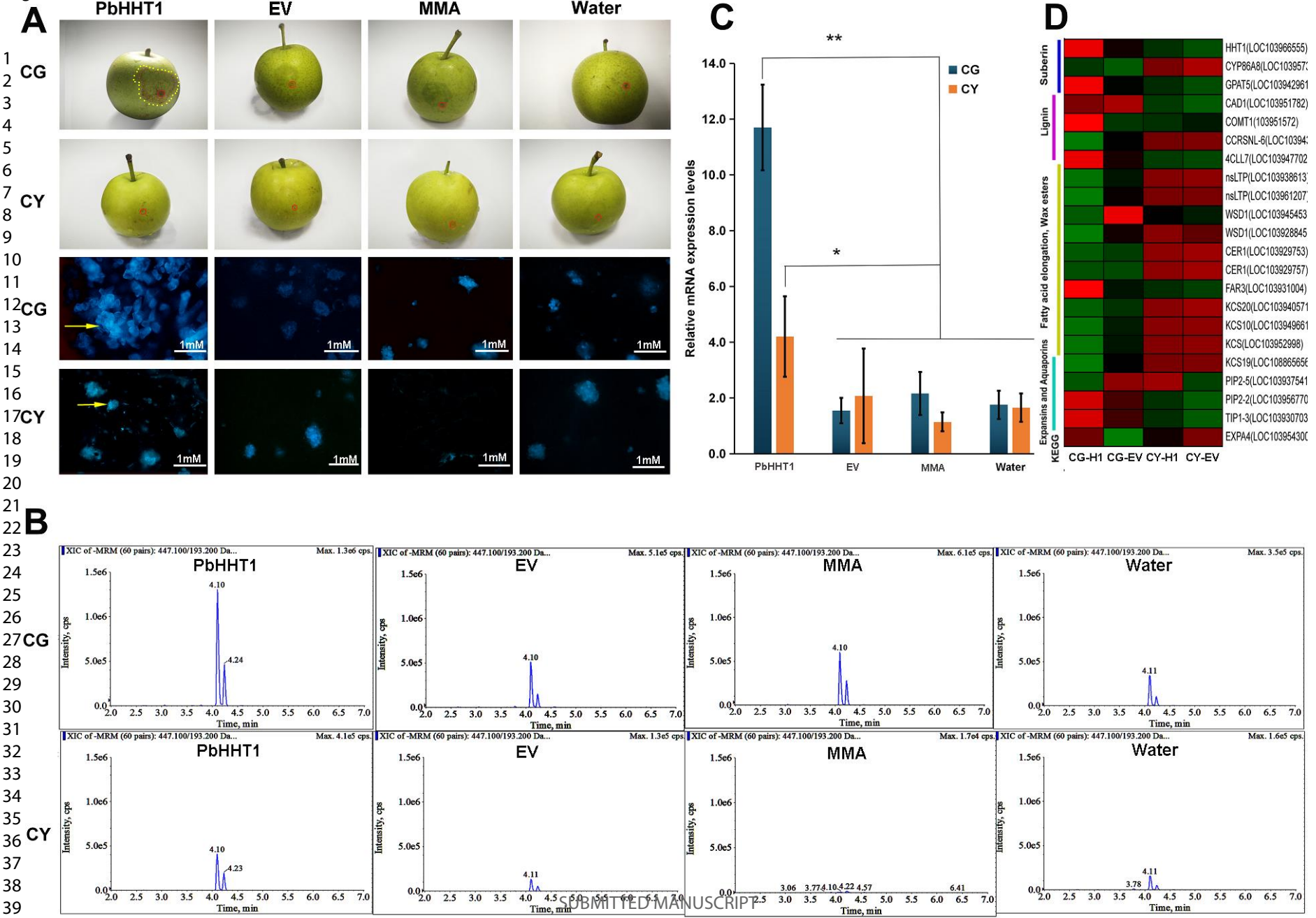


Fig 7

