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Acetylation of cell wall is required for structural integrity of the leaf surface and exerts a global impact on plant stress responses

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The epidermis on leaves protects plants from pathogen invasion and provides a waterproof barrier. It consists of a layer of cells that is surrounded by thick cell walls, which are partially impregnated by highly hydrophobic cuticular components. We show that the Arabidopsis T-DNA insertion mutants of REDUCED WALL ACETYLATION 2 (rwa2), previously identified as having reduced O-acetylation of both pectins and hemicelluloses, exhibit pleiotropic phenotype on the leaf surface. The cuticle layer appeared diffused and was significantly thicker and underneath cell wall layer was interspersed with electron-dense deposits. A large number of trichomes were collapsed and surface permeability of the leaves was enhanced in rwa2 as compared to the wild type. A massive reprogramming of the transcriptome was observed in rwa2 as compared to the wild type, including a coordinated up-regulation of genes involved in responses to abiotic stress, particularly detoxification of reactive oxygen species and defense against microbial pathogens (e.g., lipid transfer proteins, peroxidases). In accordance, peroxidase activities were found to be elevated in rwa2 as compared to the wild type. These results indicate that cell wall acetylation is essential for maintaining the structural integrity of leaf epidermis, and that reduction of cell wall acetylation leads to global stress responses in Arabidopsis.

Keywords: cell wall acetylation, trichomes, cuticles, epidermis, Botrytis cinerea, peroxidase, mRNA sequencing

Introduction

The epidermis of plants forms a protective layer against xenobiotics, ultraviolet light, and pathogens and provides a waterproof barrier (Liu, 2006; Kourounioti et al., 2013). The epidermis consists of a layer of cells surrounded by thick cell walls that are partially impregnated with the cuticle layer. The main cell wall components in the epidermis are complex polysaccharides: celluloses, hemicelluloses, and pectins. Cellulose forms paracrystalline microfibrils and provides the scaffold of
the cell wall; hemicelluloses, mainly xyloglucan in growing tissues such as epidermal cells, crosslink with cellulose to provide support to the cellulose network; while pectins not only crosslink these and other cell wall polymers but also serve as hydrated extracellular matrix components (Carpita and Gibeaut, 1993; Somerville et al., 2004). In contrast, the cuticle consists of highly hydrophobic long-chain hydrocarbons (e.g., cutins and waxes). These cuticle components are transported across the hydrophilic cell wall while partially impregnated within the cell wall (Yeats and Rose, 2013).

Pectins and hemicelluloses are subjected to modifications, of which O-acetylation has attracted growing attention in recent years because it significantly impacts a number of industrial applications including food, lumber, and biofuel industries (Klein-Marcuschamer et al., 2010; Gille and Pauly, 2012; Pawar et al., 2013). In pectic homogalacturonan and rhamnogalacturonan (RG) I, O-acetylation occurs at the O-2 and O-3 positions in the backbone galacturonic acid residues (Schols et al., 1990; Ishii, 1997), while in RG II sidechains aceric acid and fucose residues are O-acetylated (Whitcombe et al., 1995). In the hemicellulose xyloglucan, acetylation mainly occurs on the galactose residue in the sidechain with the exception that in Solanaceae and Poaceae glucose residues in the backbones are acetylated (Jia et al., 2005). In the hemicelluloses xylans and gluconomannans O-acetylation occurs in the backbone at O-2 and O-3 position in the xylosyl and mannosyl residues, respectively (Lundqvist et al., 2002; Perrin et al., 2003; Jia et al., 2005; Van Dongen et al., 2011; Gille and Pauly, 2012; Pawar et al., 2013; Xiong et al., 2013). In addition, acetylation of lignins has been reported in some angiosperms (Del Rio et al., 2007; Lu and Ralph, 2008).

Three classes of proteins are known to be involved in O-acetylation of cell wall polysaccharides in the Golgi apparatus. The REDUCED WALL ACETYLATION family proteins (RWA1 through 4 in Arabidopsis) are thought to be responsible for the translocation of acetyl-CoA across the Golgi membrane and appear to supply the acetyl-donor to both pectins and hemicelluloses, because knock-out of individual RWA s impacted the level of acetylation in both pectins and hemicelluloses (Lee et al., 2011; Manabe et al., 2011, 2013). For instance, homozygous rwa2 mutants of Arabidopsis exhibit approximately 20% reduction in the degree of acetylation in both pectins and hemicelluloses (Manabe et al., 2011). The TRICHOME BIREFRINGENCE-LIKE family proteins (TBR1 and TBL1 through 46 in Arabidopsis) are likely to confer polymer specificity, because different tbl mutants of Arabidopsis show polymer-specific reduction in the level of acetylation: altered xyloglucan 4 (axy4)/tbl27 and axy4L/tbl22 lack O-acetylation of xyloglucan (Gille et al., 2011), while tbl29/eskimo1(esk1) mutants have reduced O-acetylation of xylan and mannan (Gille et al., 2011; Xiong et al., 2013). Recently, in vivo acetyltransferase activity of TBL29 has been demonstrated, lending support to the above notion (Urbanowicz et al., 2014). A gene(s) responsible for pectin-specific acetylation has not yet been identified. Lastly, AXY9, a protein that shows a limited sequence similarity to TBL, was also found to be involved in acetylation of hemicelluloses (Schultink et al., 2015). The reported phenotypes of rwa2, axy4, and tbl29 are diverse; rwa2 shows enhanced resistance to the necrotrophic fungal pathogen Botrytis cinerea (Manabe et al., 2011), axy4 shows enhanced sensitivity to aluminum (Zhu et al., 2014), while tbl29 shows enhanced freezing tolerance and dwarfism (Xin and Browse, 1998; Yuan et al., 2013). These observations indicate that cell wall acetylation plays roles in broad aspects of plant stress responses.

The aim of the present work was to gain a better understanding of the role of cell wall acetylation in plant stress responses. To this end, we have carried out a series of phenotypic, microscopic, biochemical and transcriptomic analyses on the panel of Arabidopsis (ecotype Columbia-0) mutants defective in cell wall acetylation. We discovered that in the rwa2 mutants the architectures of the cell wall-cuticle layer was altered and the trichomes were fragile and collapsed, while the other (tbl29 and axy4-3) mutants appeared similar to the wild type. Furthermore, global transcriptome reprogramming including up-regulation of a large set of stress related genes and the concomitant accumulation of peroxidase activities was observed in the rwa2 mutant. These effects underpin the importance of cell wall acetylation in the leaf surface integrity and stress responses in plants.

### Materials and Methods

#### Plant Material and Growth

*Arabidopsis thaliana* L. Heyn. ecotype Colombia-0 and mutants were grown in soil in Percival climate chamber with a 12 h dark/light cycle at 22°C and 70% relative humidity. When grown on plates, seeds were surface-sterilized as previously described (Weigel and Glazebrook, 2002). Analysis of root growth inhibition was performed according to Weigel and Glazebrook (2002) on half-strength Murashige Skoog (MS) medium containing 0.5% (v/v) sucrose and 0.8% (v/v) agar in the vertical orientation for 1 week. The seedlings were transferred to new MS-agar plates containing hormones (indole-acetic acid and trans-zeatin in the range between 0.01 and 10 µM; ABA in the range between 0.3 and 1.5 µM; JA, 10 and 25 µM), grown vertically. For quantification, the plates were scanned and the root lengths were measured using the software ImageJ (http://rsweb.nih.gov/ij/).

#### Staining and Light Microscopy

To assess cuticle permeability, detached leaves were floated on the toluidine blue solution, 0.025% (w/v), for 15 min and rinsed with distilled water before imaging with the Stereoscope (Leica EZ4D, Leica, Denmark). Alternatively, soil grown plants were sprayed with the toluidine blue solution, incubated for 45 min and rinsed with excess water to remove the unbound dye.

#### Electron Microscopy

Leaf pieces of approximately 1 × 3 mm were taken from the tip part leaves having approximately 20 mm long leaf blades from three independent plants in each genotype. Samples were fixed for 4 h in Karsnovsky’s fixative [5% (v/v) glutaraldehyde, 4% (v/v) paraformaldehyde, 0.1 M sodium cacodylate buffer, pH 7.3],
washed in the buffer, and post-fixed in 1% (v/v) osmium tetroxide in the 0.1 M sodium cacodylate buffer for 8 h at 4°C. After washing in the buffer and water, the samples were dehydrated in a graded acetone series and embedded in Spurr resin. The resin was polymerised in an oven at 60°C for 8 h. Ultrathin sections (40 nm thick) were cut with a diamond knife using a Reichert-Jung/LKB Supernova ultramicrotome and sections were contrasted with 1% (v/v) uranyl acetate and lead citrate [2.7% (v/v) in 3.5% (v/v) sodium citrate] and examined in a Philips CM 100 TEM at 80 kV. Quantification of cell wall thickness and cuticle thickness was performed with ImageJ. Five regions from the outer cell wall of the epidermis cells, avoiding trichomes, were analyzed for each genotype, and within each region 20 pairs of measurements were made at the magnification of 64,000 folds. For scanning electron microscopy (SEM), leaf sections were fixed and washed as indicated above, and were dehydrated in ascending concentrations of acetone reaching 100% (v/v) acetone at the final step and dried in an EMS 850 CP drier. Specimens were mounted onto metal stubs, sputter-coated with gold:palladium (1:1) in a Polaron SC 7640 (Quorum Technologies, Newhaven, UK) automated sputter coater, and viewed in a Quanta 200 SEM (FEI CompanyTM) at 10 kV. Contrast adjustments were carried out using Adobe Photoshop CS5 and final mounting of images were done with Adobe Illustrator C52.

**Water Loss Assay**
Rosette leaves from 4-weeks-old plants were cut at the petiole and immediately weighed in plastic weighing boats. The leaves were incubated at room temperature on the laboratory bench and weighed every 20 min. The amount of water loss was calculated as percentage of weight loss compared to original weight.

**Leaf Gas Exchange**
Photosynthesis and transpiration were measured 30 days after germination. A fully expanded leaf, still attached to an intact plant, was placed in the cuvette of a CIRAS-2 portable photosynthesis and transpiration monitor (CIRAS-2 Portable Photosynthesis System; PP Systems, Amesbury, USA). The concentration of CO2 in the cuvette was maintained at 400 ppm, humidity at 80% and light level was 150 µmol photons m\(^{-2}\) s\(^{-1}\). Four independent plants were measured for each line.

**Stomatal Aperture Measurement**
Detached rosette leaves from 4-week old plants were floated in opening buffer (5 mM KCl, 10 mM MES, pH 5.6) for 2 h in the light. Hundred microliters of 50 µM ABA in 2% (v/v) ethanol or 2% (v/v) ethanol (control) was added and the leaves were incubated for 2 h. To measure the response to drought, 4-week-old plants were exposed to 100% humidity for 12 h. Rosette leaves were detached from the plant and incubated on the lab bench for 1 h. Following treatment, the leaves were grinded in opening buffer with a polytron and the homogenate was filtered through nylon cloth (30 µm mesh size). The isolated epidermal fragments were transferred to microscope slides and viewed under the microscope (Leica DM750, Leica, Denmark), 400x magnification.

**Analysis of Cuticle Composition**
To extract wax and cutins, 5–10 leaves per plants were used. The extraction and derivatization of wax and cutin monomers were performed as previously described (Bonaventure et al., 2004; Molina et al., 2006). For the GC-FID analysis the following conditions were used. HP-5 capillary column (30 m, 0.32 mm ID, 0.25 µm film thickness) was used and helium carrier gas at the flow rate of 2 ml min\(^{-1}\) with the gradient oven temperature programmed from 140 to 310°C at increment of 3°C min\(^{-1}\) followed by 10 min at 310°C. Samples were injected in split mode (30:1 ratio, 310°C injector temperature) and peaks quantified on the basis of their FID ion current. For GC-MS, the same column was used with helium carrier gas at 2 ml min\(^{-1}\) and a gradient oven temperature programmed from 110 to 300°C at the increment of 10°C min\(^{-1}\). Split-less injection was used and the mass spectrometer operated in scan mode over 40–500 amu (electron impact ionization) with peaks quantified on the basis of their total ion current.

**Pathogen Infection Assay**
Detached leaves of 3–4 weeks old plants were inoculated with *B. cinerea* IK2018 spore solution (5 * 10^6 spores ml\(^{-1}\) in half strength PDB) as previously described (Denby et al., 2004). To quantify lesion sizes, high quality digital images were acquired and processed with ImageJ.

**mRNA Sequencing**
Wild type and rwa2-3 treated with mock (PDB alone) or infected with *B. cinerea* in PDB were planted in a randomized complete block design and individual leaves chosen from different plants. Half of the leaves were infected with *B. cinerea* while the other halves were inoculated with the half strength PDB as a control as previously described (Manabe et al., 2011). Three independent samples per genotype were harvested at 0 h as a control ("untreated"). Thereafter three samples were taken per genotype per treatment at 24 and 48 h post treatment ("mock" and "B. cinerea"). Each sample was independently extracted for total RNA using Spectrum RNA kit (Sigma-Aldrich, Denmark) with on-column DNAase treatment and the RNA integrity checked by gel and quantified with NanoDrop 2000 (ThermoScientific, USA). The RNA was then converted into sequencing libraries and sequenced at the Beijing Genome institute. This provides three independent RNASeq samples per genotype per treatment at per timepoint. All reads were mapped against a synthetic transcriptome that combined the *A. thaliana* Ensembl (TAIR10) transcriptome with the predicted *Botrytis cinerea* B05.10 transcriptome using TopHat v2.0.8 (Trapnell et al., 2009) and default settings. Mapped reads were counted using HTSeq (Anders et al., 2014) using the setting -m intersection-nonempty. Differential expression was analyzed with EdgeR (Robinson et al., 2010) using a model that accounted for the genotype at RWA2 and directly tested for an interaction of the genotypes with the treatment (mock vs. *B. cinerea*) and an interaction with time point. All P values were adjusted to a FDR of 0.05 within EdgeR using the factorial model and are presented along with the mean corrected cpm per transcript per genotype (Supplementary Table 1).
Localization of Hydrogen Peroxide and Peroxidase Activity

Hydrogen peroxide and peroxidase activity were visualized with the use of 3,3′-diaminobenzidine (DAB; Thordal-Christensen et al., 1997). The DAB precipitate was visualized by light microscopy and stereoscope. Extracellular peroxidase activity was measured using TMB (tetramethylbenzidine) (Barcelo, 1998). Detached rosette leaves from 4-week old plants were either used directly for the assay or incubated with B. cinerea or PDB as described above. The leaves were floated on the TMB solution for 30 min. The TMB solution was removed to cuvettes and the absorbance at 654 nm was recorded.

Expression Analysis of the RWA2 Promoter

The DNA fragment covering the 1668 base pairs upstream of the RWA2 start codon was selected to be the promoter region (Prwa2) and it was PCR amplified using primers with USER overhangs: forward 5′-GGCTTAAUaaattgccttaaatccagcg-3′ and reverse 5′-GGCTTAAUttccgatcagagaagca-3′. The resulting PCR fragment was inserted in the USER cassette of the pLIFE41 vector containing the Kanamycin resistance gene and the BASTA® resistance gene and the resulting vector was introduced in Agrobacterium tumefaciens. The wild type Arabidopsis was transformed by floral dipping and BASTA® was used to select for positive transformants and the presence of the Prwa2:GUS construct was verified by PCR with the primers: forward 5′-ggtctctattacgacacg-3′ and reverse 5′-ccggcatagtaagaatact-3′.

Histochemical staining was performed as previously described (Harholt et al., 2006).

Results

Resistance Against B. cinerea, Surface Permeability, and Trichomes of Acetylation Mutants

The responses of the wild type, rwa2-3, tbl29, and axy4-3 mutants to B. cinerea were analyzed side-by-side. rwa2-3 developed smaller lesions as compared to the wild type upon infection (Figure 1A) as previously shown (Manabe et al., 2011). In contrast, axy4-3 developed lesions comparable in size and appearance to the wild type, whereas tbl29 developed significantly

![FIGURE 1](https://example.com/figure1.png)

**FIGURE 1** | Resistance to B. cinerea and toluidine blue staining of wild type and cell wall acetylation mutants (rwa2-3, tbl29, and axy4-3). (A) B. cinerea infection defined by lesion area measured. Relative mean values to the wild type are shown and the error bars represent standard deviations (N > 8). An asterisk indicates statistically significant difference from the wild type by Student’s t-test (P < 0.05). (B) Toluidine blue staining pattern of 3-week old plants. The same results were obtained in three independent analyses. The arrowhead indicates the staining of a trichome.
larger lesions relative to the wild type, indicating that tbl29 is more susceptible to B. cinerea as compared to the wild type.

Enhanced resistance against B. cinerea is often observed among mutants defective in assembly or biosynthesis of the cuticle layer (Chassot et al., 2007; Tang et al., 2007; Voisin et al., 2009; Curvers et al., 2010; Bessire et al., 2011; Suo et al., 2013). In order to test the integrity of the cuticular layers in the acetylation mutants, toluidine blue was applied to the leaf surface. Toluidine blue is a cationic dye that does not stain leaves with an intact cuticular layer due to repulsion by the highly hydrophobic cuticle (Tanaka et al., 2004); however it stains cuticle mutants due to irregularity in the cuticle layer. When treated with toluidine blue, rwa2-3 retained the dye predominantly in trichomes (Figure 1B). In contrast, neither the wild type, axy4-3, nor tbl29 retained the dye (Figure 1B). In order to further assess leaf permeability of the rwa2 mutants, detached leaves of rwa2-1 and rwa2-3 were incubated at room temperature and the weight loss was measured over time. The rwa2 mutants lost weight faster than wild type (Figure 2A). Leaf gas exchange measurements were conducted in order to quantify transpiration rate and stomatal conductance across lamina. Transpiration rate is a measure of the actual net water loss, while stomatal conductance is a measure of conductivity for water transport across lamina, and both measures depend on the ratio of open/closed stomata but also on the integrity of the cuticle layer. Since the phenotypes of rwa2-1 and rwa2-3 were largely indistinguishable, only rwa2-3 was analyzed. The stomatal conductance and transpiration rate were increased up to 50% of the wild-type level in rwa2-3 (Figures 2B,C). It should be noted that the increased water loss and transpiration is not due to misregulation of guard cells as no difference in the stomatal aperture was observed between the wild type and rwa2 neither under drought conditions nor upon treatment with abscisic acid (ABA), which is a major regulator of stomata closure (Acharya and Assmann, 2009) (Figure 2C). There was no difference in the stomatal density between the wild type and rwa2-3 (data not shown). These results show that the leaf surface of rwa2, particularly in trichomes, is damaged and more permeable to transpiration.

Impairment in cuticle integrity is also known to cause collapsed trichomes. Many trichomes on rwa2 leaves were found to be thinner and more transparent as compared to the wild type and were often partially or fully collapsed (Figures 3B–D). The number of fully collapsed trichomes was about 30% of the total number of leaf trichomes in rwa2 while no collapsed trichomes were observed on wild-type leaves (Figure 3C). The remaining 70% of the rwa2 trichomes were not collapsed but often appeared fragile. Macroscopically the rwa2 mutant plants (rwa2-1 and rwa2-3) appear comparable to the wild-type plants under the standard growth conditions in the green house and growth chambers (Figure 3A). Collapsed trichomes has also been seen in the tbr-2 mutant (Suo et al., 2013), wherein the deposition of paracrystalline cellulose is impaired in trichomes, resulting in loss of trichome birefringence under UV light (Potikha and Delmer, 1995). Trichome birefringence of rwa2 appeared indistinguishable from that of wild type under the conditions tested (Figure 3D). Apart from these morphological differences, trichomes on rwa2 leaves develop normally and have the typical three-branched structure with a similar height to those in the wild type.

**Cuticle and Cell Wall Architectures and Trichome Morphology are Altered in rwa2**

To test if cuticle content and/or composition have been affected in rwa2, the chemical composition of the cuticle was determined by gas chromatography (Bessire et al., 2007). No significant
difference in the composition or content of the cuticular wax and cutin monomers was observed between the wild type and the rwa2 mutants (Figures 4A,B). Therefore, biosynthesis and delivery of the wax and cutin components appear to occur normally in rwa2.

In order to gain insights into the ultrastructure of the cuticle and cell wall, transmission electron microscopy (TEM) was performed on the leaf epidermal cells. In the wild type, the cuticle appeared compact and highly electron dense while the cell wall layer underneath was relatively electron opaque (Figure 5A). The cuticle layers in rwa2-1 and rwa2-3 appeared notably more diffused than that in the wild type (Figures 5B,C) with the average thickness increased by approximately 50% of that of the wild type (Table 1). The similar thickness values and the difference between the genotypes were observed in two independent experiments using independently grown plants. Apart from the change in the cuticle layer, the cell wall layer in rwa2 showed marked difference from the wild type. The cell wall layer in rwa2 was interspersed with electron-dense deposits, which were rarely seen in the wild type (Table 1). In addition, the
average cell wall thickness in rwa2 was approximately 30–50% larger than that in the wild type (Table 1). Hence, we conclude that both the cuticle and cell wall ultrastructures were altered in rwa2.

Given the enhanced permeability and structural impairments of rwa2 trichomes (Figures 6A, 3B), the traverse sections of trichomes were analyzed by TEM. It was noticed that the base of the trichomes in rwa2-3 was notably larger than that in the wild type (Figures 6C, 5B). The average width of three traverse sections, each cutting through the middle of a trichome increased in rwa2-3 by 50% of the wild type level (Table 1). The increase in size of the rwa2 trichome base was confirmed by SEM of trichomes (Figures 6C, 5D). SEM analysis revealed additional anatomical differences between the wild-type and rwa2-3; the papillae were either entirely missing or reduced in size in rwa2-3 as compared to the wild type (Figures 6C–F). Furthermore, in some cases the trichome base in rwa2-3 was sunken (Figure 6F).

Global Transcriptomic Reprogramming Occurred in rwa2

We hypothesized that the surface damage caused by the rwa2 mutation could have a significant impact on plant stress responses and that transcriptome profiling would shed light on which stress response(s) is affected. Wild-type and rwa2-3 leaves were either untreated or treated with mock [potato dextrose broth (PDB) only] or B. cinerea spore solution in PDB for 24 and 48 h and mRNA sequencing was performed (Supplementary Table 1). The largest difference in the transcriptome profile was observed between the untreated wild type and rwa2-3, indicating that the untreated rwa2-3 perceives the environment differently from the wild type (Table 2). Out of 21,178 transcripts that were sequenced in both the wild-type and rwa2-3, 1650 transcripts showed statistically significant differential abundance: 857 genes up-regulated and 793 genes down-regulated ([log2 fold ≥ 2, false discovery rate (FDR) ≤ 0.05]). The mock and B. cinerea treatments led to smaller differences between rwa2-3 and the wild type in terms of the number of genes with altered expression levels at both 24 h and 48 h after infection (Table 2). Principal component analysis showed that genotype and treatments describe 86% of the total transcriptomic variance detected (Figure 7). The first vector (PCA vector 1) largely describes the differences between the genotypes at the untreated and early mock-treated samples (untreated wild type vs. rwa2-3), while the second vector (PCA vector 2) largely describes the response of the genotypes to infection with B. cinerea (untreated, mock, and B. cinerea treatments). In the wild type, the mock treatment (detachment of leaves followed by incubation in a water-agar medium and application of PDB) caused a notable change in the transcriptome profiles. Interestingly, both the untreated and mock-treated rwa2-3 for 24 h showed a significant overlap with the mock-treated wild type. Upon treatment with B. cinerea,
the transcriptomes of the wild type and rwa2-3 showed an even higher degree of overlap (Figure 7). This indicates that the rwa2-3 responds to B. cinerea similarly to the wild type even though their initial transcriptomes are highly divergent.

Because the major difference was found in the untreated rwa2-3 and wild type, further analysis focused on these samples. Analysis of gene ontology (GO) categories by the AmiGo software (Ashburner et al., 2000) showed that a large fraction of the up-regulated genes in rwa2-3 belong to categories relating to both abiotic and biotic stress responses, with notable examples of responses to and transport of organic and inorganic substances, as well as detoxification processes and oxidative stress responses (Supplementary Table 2). Several of the genes that are up-regulated in untreated rwa2-3 have been shown to be important for resistance against B. cinerea (Table 3 and references therein). This includes lipid transfer proteins (AT4g12470, AT4G12480, AT4g12490) and peroxidases (AT2g37130, AT5g39580, and AT5g64129) that were up-regulated in a cutinase-overexpressing Arabidopsis transgenic line as compared to the wild type (Chassot et al., 2007). Moreover, overexpression of each of these transcripts in the wild-type background was sufficient to cause enhanced resistance against B. cinerea (Chassot et al., 2007). Hence the enhanced resistance of rwa2-3 against B. cinerea can be explained by the constitutive and coordinated up-regulation of these defense related genes.

In contrast to the up-regulated GO categories, the most overrepresented biological process category among the down-regulated genes was “response to hormones” (Supplementary Table 3). Manual inspection of genes that specifically respond to treatment with auxin, ABA, brassinosteroid (BR), cytokinin (CK), ethylene, gibberellic acid, and jasmonic acid (JA) (Nemhauser et al., 2006) showed that several transcripts that are up-regulated upon treatment with CK, auxin, and JA were coordinately down-regulated in rwa2-3 untreated leaves (Supplementary Figure 1). However, the rwa2-3 mutant was found to retain the wild-type level of response to these hormones; when seedlings were grown on nutrient agar plates supplemented with the hormones JA, CK, auxin or ABA no difference in general growth or root inhibition was observed between wild type and rwa2-3 (Supplementary Figure 2).

The transcript profile of the untreated rwa2-3 relative to the untreated wild type was compared to publically available microarray data by using Signature Tool in the Genevestigator software (Zimmermann et al., 2004). The 300 genes that showed the highest differential expression as compared to the wild type and with the lowest FDR values, hence highest confidence, were subjected to the analysis. Among the top 30 transcript datasets that showed similarities to the rwa2-3 transcript profile, experiments inducing oxidative stress (growth under high light, cold, drought; CAT2HP1 overexpressor; catalase2-1) were highly represented (Supplementary Table 4). In addition, consistent with the GO analysis, transcript profiles upon changes in lipid metabolism or signaling (suppressor of SA insensitivity-1, application of phytoprostane A1), exogenous application of xenobiotics (phenanthrene, fenclorin, sulfometuron methyl), alteration of

TABLE 2 | Number of genes with altered expression in rwa2 as compared to the wild type as identified by mRNA sequencing (log2 fold ≥ 2, FDR ≤ 0.05).

| Treatment         | Up-regulated | Down-regulated |
|-------------------|--------------|----------------|
| Un-treated        | 857          | 793            |
| Mock treatment    | 24 h         | 320            | 40             |
|                   | 48 h         | 134            | 10             |
| Botrytis treatment| 24 h         | 415            | 319            |
|                   | 48 h         | 32             | 5              |

FIGURE 7 | Global transcript analysis. (A) Shown is a principal component analysis of the entire dataset with the individual samples plotted. Wild type samples are shown as diamonds and rwa2 samples are shown as squares with the blue to orange transition showing the time course of the experiment. The first two PCA vectors are utilized that explain 86% of the total variance. (B) VENN diagrams showing the overlap of genes differentially expressed (left side: upregulated genes, right side repressed genes) in rwa2 control vs. wild type control, and wild type infected with B. cinerea vs. wild type mock across the time points. The genes that are differentially expressed in rwa2 control vs. the corresponding wild-type samples have a high overlap with the expression profile of genes in wild type infected with B. cinerea. The numbers in brackets represent the overlap expected by chance. The overlaps are highly significant both for induced (P < 0.001) and for repressed genes (P < 0.001) as determined by χ²-test.
phytohormones (ARR22 overexpression, application of salicylic acid), and microbial infection (Golovinomyces cichoracearum, Alternaria brassicicola) were found.

Extracellular Peroxidases Accumulate in the rwa2 Mutant

To test if rwa2 experiences increased oxidative stress, we conducted 3,3’-diaminobenzidine (DAB) staining to visualize H2O2 production (Thordal-Christensen et al., 1997). DAB staining did not show notable accumulation of H2O2 on the leaf surface of the wild type and the rwa2 mutants (Figure 8A). On the other hand, the mock treatment (PDB) resulted in an elevated H2O2 production in rwa2-3 leaves as compared to the wild type (Figure 8A). To test peroxidase activities, the leaves from the same developmental stage were incubated with DAB in the leaf surface. Intense DAB staining around the trichome base and secondary xylem in stem (Lee and Rose, 2011; Manabe et al., 2011) with specific expression in the xylem in roots and secondary xylem in stem (Lee and Rose, 2011; Manabe et al., 2011) shows that the rwa2-3 mutant accumulates a higher level of peroxidase activity around the trichome base and secondary xylem in stem (Lee and Rose, 2011; Manabe et al., 2011).

| Gene description | Locus tag | Fold change rel. to WT |
|------------------|-----------|------------------------|
| Lipid transfer proteins | At4g12480<sup>a,b</sup> | 301 |
| | At4g12490<sup>a</sup> | 492 |
| | At2g38530<sup>a</sup> | 10 |
| | At4g12470/AZ1<sup>a</sup> | 37 |
| | At2g37870<sup>c</sup> | 111 |
| Peroxidases | At2g37130<sup>a</sup> | 5.5 |
| | At5g64120<sup>a</sup> | 6.5 |
| Defensins | At5g44430/PDF1.2<sup>d</sup> | 4.5 |
| | At1g75830/PDF1.1<sup>1,3</sup> | 315 |
| Protease inhibitors | At2g38870<sup>a</sup> | 5 |
| | At5g43580<sup>a</sup> | 256 |
| Trypsin inhibitors | At2g43510<sup>a</sup> | 10 |
| | At1g73260<sup>d</sup> | 12 |
| ELI-3 defensive protein | At4g37990<sup>d</sup> | 10.5 |
| PR4 | At3g04720<sup>h</sup> | 3 |
| GDSL lipase 1 (GLIP1) | At5g40990<sup>j</sup> | 96 |
| PROPEP3 | At5g64905<sup>j</sup> | 12 |
| Glycolate oxidase 3 (GOX3) | At4g18360<sup>k</sup> | 41 |

<sup>a</sup>Chassot et al., 2007; <sup>b</sup>Li et al., 2012; <sup>c</sup>Hernandez-Blanco et al., 2007; <sup>d</sup>De Coninck et al., 2010; <sup>e</sup>Rutak and Mengiste, 2011; <sup>f</sup>Li et al., 2006; <sup>g</sup>Kiedrowiak et al., 1992; <sup>h</sup>Thomma et al., 1998; <sup>i</sup>Oh et al., 2005; <sup>j</sup>Huffaker et al., 2006; <sup>k</sup>Rojas et al., 2012.

**FIGURE 8** Increased peroxidase activity and hydrogen peroxide accumulation in rwa2. (A) DAB staining for hydrogen peroxide in untreated (left) and in response to treatment with potato dextrose broth (PDB) (right). Five micro liters of 2% (v/v) PDB was placed on each side of the mid vein for 48 h followed by DAB staining. Chlorophylls were extracted in 96% (v/v) ethanol. (B) Peroxidase activities accumulate at trichome bases in uninfected rwa2 leaves. Leaves were stained with DAB in the presence of 0.1% (v/v) H2O2 for 1 h. Chlorophylls were extracted in 96% (v/v) ethanol. (C) Extracellular peroxidase activity in the wild type and rwa2-3 leaves. Detached leaves were treated with mock (PDB) or B. cinerea for 24 and 48 h. To measure peroxidase activity, the leaves were floated with the adaxial side downwards in TMB and H2O2. Peroxidase activity was measured as absorbance at 654 nm. The data is average of three samples ± SD. The asterisks indicate significant difference between wild type and rwa2-3 as determined by Two-Way ANOVA test (*P < 0.05; **P < 0.01).
However, tissue-type specific expression pattern of RWA2 in leaves has not yet been reported. Ten independent lines of transgenic lines expressing a transcriptional fusion of the RWA2 promoter region (from −1600 to +50 bp relative to A in the start codon) and the reporter gene β-glucuronidase (GUS) were analyzed. As shown in Figure 9, histochemical staining with 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide revealed promoter RWA2:GUS expression in leaves and notably in trichomes. In light of the rwa2 trichome phenotypes, strong GUS expression in this tissue supports the notion that RWA2 expression is required for structural integrity of leaf surface and particularly trichome bases.

Discussion

We discovered that the structural integrity of Arabidopsis leaf surface was severely impaired in rwa2 mutants. The cell wall layer in the epidermis was morphologically distinct from that of the wild type with accumulation of numerous electron-dense deposits (Figure 5), indicative of abnormal cell wall architecture. Notably, rwa2 showed enhanced permeability to the cationic dye toluidine blue exclusively in trichomes (Figure 1), and trichomes appeared fragile; the base of the trichome was enlarged, often either ruptured or sunken (Figure 6) and a large fraction of the trichomes on rwa2 was collapsed (Figure 3). In addition, the papillae on the trichome surface were dramatically reduced in size (Figure 6). These phenotypes are often associated with mutants that are defective in cuticle integrity and biosynthesis (i.e., transgenic CUTE plants that overexpresses a cutinase and lacs2, lcr, fdh, pec1, and gl1 mutants) (Serrano et al., 2014). Although the cutin monomer and wax compositions were not altered in rwa2 as compared to the wild type, the cuticle layer in epidermis was notably diffused and thicker (Table 1, Figure 5). Moreover, mRNA sequencing revealed a massive reprogramming of the transcriptome in the rwa2-3 mutant characterized by coordinated induction of genes related to abiotic and biotic stress responses (Table 3, Figure 7). These results demonstrate that cell wall acetylation plays an important role in maintaining the surface structural integrity in Arabidopsis leaves and its impairment results in global stress responses in plants. Because it has previously been proposed that RWA2 functions as an acetyl-CoA transporter localizing to the Golgi apparatus (Manabe et al., 2011), it was possible that the cytosolic acetyl-CoA pool had been altered in rwa2 and this might have caused the observed phenotypes. In the cytosol, acetyl-CoA is converted to malonyl-CoA that feeds into the synthesis of a range of phytochemicals including wax and cutins (Oliver et al., 2009). It has been shown that overexpression of an ATP citrate lyase that synthesizes acetyl-CoA in the cytosol of Arabidopsis resulted in 30% increase in wax loading and the cutin monomer octadecadie-1,18-dioic acid content (Xing et al., 2014). In contrast, rwa2 contained the wild-type level of cutin and wax components (Figure 4). Therefore, it was concluded that the cytosolic acetyl-CoA pool is unlikely to be altered at a significant extent and to cause the observed phenotypes.

![Figure 9](image9.png)

**FIGURE 9 | RWA2 is expressed abundantly in leaves, particularly in trichomes in Arabidopsis.** RWA2 expression was analyzed by GUS staining in 3-weeks old Arabidopsis plants, 10 independent transformed lines were tested. (A) The negative control (promoter-less GUS line). (B) A representative RWA2 promoter:GUS fusion line. (C,D) Close-up images of leaves and trichomes in a representative RWA2 promoter:GUS fusion line.
Because the increased permeability and enhanced resistance to B. cinerea were only observed in rwa2 and not in tbl29 and axy4-3 (Figure 1), we speculate that the reduced acetyl groups in pectins contribute to the observed phenotypes by interfering with the normal cell wall and cuticle assembly. Earlier studies have shown that during the development of the cuticle, cutin polymers become progressively impregnated with the cell wall polysaccharides, particularly pectins (Schieferstein and Loomis, 1959). Pectinase treatment has been reported effective for releasing the cuticles (Orgell, 1955; Baker and Procopiou, 1975) and isolated cuticle from pear leaf stems with ruthenium red, a stain widely used to detect pectins (Norris and Bukovac, 1968). Treatment with EDTA and oxalic acid or ammonium oxalate, which are often used for isolation of pectins, have also been shown to be effective in isolating cuticles (Huelin and Gallop, 1951). In rwa2, the excess hydroxyl groups, as a result of reduced acetylation, may form atypical crosslinking with the cutins leading to abnormal cuticle assembly. Alternatively, though mutually nonexclusive, cell wall acetylation may impact the transport of cutin and wax across the cell wall. Both cutins and waxes are long chain fatty acid derivatives and must cross the highly hydrophilic cell wall layer and the transport process is thought to involve apoplastic carriers, possibly lipid transport proteins (LTPs) (Yeats and Rose, 2013; Hurlock et al., 2014). Notably, the expression levels of 10 genes coding for LTPs and LTP like proteins were shown to be up-regulated in rwa2 as compared to the wild type, which may represent a compensatory response (Table 3).

Transcriptomic profile of the rwa2-3 mutant was dramatically different from that of the wild type (Table 2, Figure 7) with a coordinate up-regulation genes involved in responses to detoxification and oxidative stress (Supplementary Table 2). Trichomes are involved in a range of protective mechanisms from adverse environmental conditions including protection from UV and excessive light (Karabourniotis and Bornman, 1999; Franke et al., 2005), and heavy metals detoxification (Freeman et al., 2006; Sarret et al., 2009; Marmiroli et al., 2010). Moreover, disturbance of cuticle biosynthesis or overexpression of a cutinase, as well as mechanical stress of leaf epidermis (i.e., wounding by forceps, soft rubbing by fingers), have been shown to induce reactive oxygen species and resistance to B. cinerea (L’Haridon et al., 2011; Benikhlef et al., 2013). It is likely that altered cell wall and cuticle assembly, as discussed above, causes structural and functional impairments in the trichomes and cuticle layer causing the induction of detoxification and oxidative stress responses, and this may ultimately lead to enhanced resistance against B. cinerea in rwa2. This notion is further supported by the previous results that ectopic overexpression of individual peroxidase genes induced in rwa2-3 was sufficient to cause enhanced resistance against B. cinerea in Arabidopsis (Chassot et al., 2007). Peroxidases are thought to cause cell wall stiffening in the presence of H2O2 through oxidative formation of covalent bonds between aromatic cell wall components (Francoz et al., 2015 and reference therein). It is possible that the induced levels of H2O2 and peroxidases (both transcripts and activities) (Figure 8, Table 3) might cause an enhanced cell wall fortification in and around the trichomes in the rwa2 mutants, causing restricted infection by the fungus.

Author Contributions

All authors contributed to either the conception, design of the work or the acquisition, analysis and interpretation of data, drafted and approved the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00550

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