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Changes in wax composition but not amount enhance cuticular transpiration

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Abstract

This study focuses on the role of the qualitative leaf wax composition in modulating the cuticular water loss using a *Populus* × *canescens cer6* mutant line, which accumulates C34–C46 wax ester dimers and is reduced in wax monomers >C24. The two literature-based hypotheses to be tested were the importance of the amount of wax esters and the weighted mean carbon chain length in restricting cuticular water loss. The main results were acquired by chemical analysis of cuticular wax and gravimetric cuticular transpiration measurements. Besides additional physiological measurements, the leaf surface properties were characterised by scanning electron microscopy and spectrophotometric light reflectance quantification. Mutation of the *CER6* gene resulted in striking changes in qualitative wax composition but not quantitative wax amount. Based on the strong accumulation of dimeric wax esters, the relative proportion of esters increased to >90%, and the weighted mean carbon chain length increased by >6 carbon atoms. These qualitative alterations were found to increase the cuticular transpiration of leaves by twofold. Our results do not support the hypotheses that enhanced amounts of wax esters or increased weighted mean carbon chain lengths of waxes lead to reduced cuticular transpiration.

KEYWORDS

3-ketoacyl-CoA synthase (KCS), cuticular wax, cutin, ECERIFIUM (CER), leaf development, light reflectance, poplar, *Populus* × *canescens*, residual and cuticular transpiration

1 | INTRODUCTION

The cuticle, located on the outer epidermal cell wall of all primary aerial organs of plants (Kunst, 2003), developed around 400 million years ago together with the exploration of the continents by the first terrestrial plants (Riederer, 2006). As a biological interface, it is in direct contact with the atmosphere, being characterised by lower humidity, lower mechanical stability and higher solar radiation than the hydrosphere. Thus, its primary function in forming the border between the plant and the surrounding abiotic environment is the protection against these harmful influences by restricting water loss, providing sturdiness and reflecting excessive radiation, respectively

(Bargel et al., 2004; Pfündel et al., 2006; Shepherd & Wynne Griffiths, 2006). The cuticle's hydrophobic chemical composition additionally aids in providing water repellence, which in turn may establish self-cleaning properties and prevent the leaching of solutes (Barthlott & Neinhuis, 1997; Tukey, 1966). Although uncertainties prevail in modelling the exact physical structure of the cuticle (Fernández et al., 2016), two major functional domains can be distinguished from one another: the stability-providing cutin matrix and the associated cuticular waxes conveying the increased resistance against the uncontrolled loss of water (Riederer & Schreiber, 2001).

When immobile plants encounter a situation where more water is evaporating from the shoot than can be supplied by the roots, their first line of defense to minimise the amount of water lost by the transpiration of the leaves is the actively regulated stomatal closure (Polle et al., 2019; Silim et al., 2009). Once stomata are closed, the remaining water loss of the leaves is defined as residual foliar transpiration or minimum leaf conductance (Kerstiens, 1996). Relying on the efficiency of stomatal closure, which appears to be species-specific and even cultivation condition-specific, residual transpiration can but must not represent cuticular transpiration (Burghardt, 2003; Duursma et al., 2019), which in turn appears to be mainly modulated by the cutin-associated cuticular waxes (Schreiber & Riederer, 1996). True cuticular transpiration can be measured with enzymatically isolated astomatous cuticular membranes (CMs) exceptionally well (Karbulková et al., 2008; Schönherr & Lendzian, 1981), which are experiments that due to amphistomaty or failure of enzymatic cuticle isolation can only be performed with a limited number of species. In the case of *Populus* × *canescens*, a previous study has illustrated that residual and cuticular transpiration are indeed highly similar in the case of ex vitro developed leaves of climate chamber, greenhouse and outdoor cultivated plants but not with in vitro developed leaves of tissue culture propagated plants (Grünhofer, Herzig, et al., 2022). Even during the process of acclimatisation from tissue culture to soil these in vitro leaves are not able to fully acquire the previously irrelevant ability of stomatal closure (Fanourakis et al., 2013; Santamaria et al., 1993).

The aliphatic cutin polyester is mainly composed of bi-functional even-chained omega-hydroxy fatty acids (ω-OH acids), alpha, omega-dicarboxylic fatty acids (α,ω-diacids), di-hydroxy fatty acids (di-OH acids), as well as mono-functional even-chained fatty acids, primary alcohols and 2-hydroxy fatty acids (2-OH acids) conjugated to glycerol to form a three-dimensional polymer network (Franke et al., 2005; Kolattukudy, 1981). In contrast, aliphatic cuticular waxes typically consist of monomeric even-chained fatty acids, aldehydes, primary and secondary alcohols, ketones and odd-chained alkanes, as well as dimeric even-chained esters, which are conjugates of fatty acids and alcohols (Samuels et al., 2008). Aside from these differences in functional groups, what sets cutin apart from wax is the commonly found chain length of constituents. While cutin monomers mainly comprise 16 or 18 carbon atoms, wax monomer and dimer chain lengths vary from C16 to C64 (Fich et al., 2016; Kunst, 2003).

C16 and C18 monomers originating from the plastidial fatty acid synthesis have to be elongated by a microsomal fatty acid elongation (FAE) machinery to yield these very long chain wax constituents. Here, C2 moieties are added to the substrates by a cyclic multistep enzymatic reaction (Kunst & Samuels, 2009), of which the first step is believed to provide chain length specificity (Samuels et al., 2008). The enzyme driving step one is a 3-ketoacyl-CoA synthase (KCS), which in *Arabidopsis thaliana* constitutes a gene family with 21 known members (Haslam & Kunst, 2013). Two of which (*KCS5* or *CER60* and *KCS6* or *CER6*) also belong to the *ECERIFIUM* (*CER*) genes (Joubès et al., 2008), a group that has long been known to cause significant perturbations in wax composition, wax amount and also male and female fertility when mutated (Koornneef et al., 1989). The fully elongated fatty acids can finally be converted into, among other monomers, primary alcohols via the acyl reduction or into aldehydes and alkanes via the decarbonylation pathway (Kunst, 2003; Shepherd & Wynne Griffiths, 2006). Previous studies have identified the epidermis-specific *KCS6* gene (or *CER6*; initially also termed *CUT1*, Millar et al., 1999) to affect lipid contents on *A. thaliana* pollen and stem (Fiebig et al., 2000) and to be stimulated by environmental factors such as light exposure and water deficit (Hooker et al., 2002). *A. thaliana cer6* mutants were decreased in >C24 stem wax monomers (Millar et al., 1999) and >C28 pollen coat lipids (Fiebig et al., 2000), and tomato leaf and fruit cuticles were reduced in wax compounds >C30 and >C28, respectively (Leide et al., 2007; Vogg et al., 2004). Thus, the species-specific and potentially also tissue-specific participation of *CER6* in the FAE process was substantiated.

It has been convincingly shown in the past that cuticular wax is the rate-limiting factor for water loss across the lipophilic cuticle (Schönherr & Lendzian, 1981) and that the amount of cuticular wax of a given species is not indicative of the barrier properties (Bueno, Sancho-Knapik, et al., 2019; Grünhofer, Herzig et al., 2022). The latter aspect was evaluated by cultivating *Populus* and *Quercus* plants in different environmental growth conditions, resulting in significantly altered cuticular wax amounts (up to 12.5-fold difference) but no apparent changes in residual (cuticular) transpiration. But what about relative compositional changes, even if the absolute cuticular wax amount is not significantly altered? The available literature suggests that the proportion of hydrophobic long-chained wax ester dimers (Buen, Alfarhan, et al., 2019; Patwari et al., 2019), as well as the weighted mean carbon chain length of the cuticular wax (Macková et al., 2013; Riederer & Schneider, 1990), might be of great importance in modulating the cuticular permeance. Here, the latter is a concept that considers which absolute amounts of all compounds differing in carbon chain length are present while estimating the overall mean cuticular wax chain length. In theory, a larger proportion of wax ester dimers and a longer weighted mean carbon chain length should, due to increased hydrophobicity, lead to decreased rates of cuticular permeance. In this study, we intended to test these two hypotheses with a single species, a single set of environmental conditions, but with a *cer6* mutant line that accumulates C34–C46 wax ester dimers and decreases >C24 wax

monomers, therefore, exhibiting a significantly increased wax ester fraction and weighted mean carbon chain length of the cuticular wax.

2 | MATERIALS AND METHODS

2.1 | Plant material, cultivation conditions and leaf sampling

All experiments of this study were performed with leaves of the wildtype (WT) or transgenic lines (*CER6* knock-out by CRISPR/Cas9) of INRA 717-1B4, a clone of the grey poplar *Populus* × *canescens* (Aiton) Sm. (*Populus tremula* × *Populus alba*) (Mader et al., 2016). Initial plant propagation and cultivation were carried out in a long-day climate chamber with tightly controlled environmental conditions. During the illumination phase (16 h per day), the light intensity was approx. 100 µmol m^{-2} s^{-1}, the relative humidity was 50% and the temperature was 21°C on average. During the dark period (8 h per day), the mean relative humidity increased to 67%, whereas the mean temperature decreased to 19°C. Axenic tissue culture propagation was achieved on solidified nutrient medium (Woody Plant Medium, Duchefa Biochemie; 2% saccharose, Carl Roth; 0.5% Phytagel, Sigma-Aldrich), which in the case of the mutants was supplemented with antibiotics (see paragraph 'Generation of transgenic poplar lines'). After axenic cultivation for 6–8 weeks, the tissue culture plants were transplanted into soil (Einheitserde Classic Type Topf 1.5, Einheitserde Werksverband e.V.), initially covered with a lid to ensure high ambient humidity, and subsequently acclimatised for 2 weeks to the lower humidity of the climate chamber. Soil cultivation in the climate chamber was continued for further 4–6 weeks before the plants were translocated into a greenhouse where all plants developed under identical environmental conditions. Shortly after the translocation to the greenhouse, the primary shoots of all plants were cut back to about 50% of plant height to facilitate the development of new shoots and ensure a synchronised development of all newly budding leaves. These freshly developing leaves were monitored and marked each week, which guaranteed the precise determination of leaf ages for the following experiments.

A kinetic series was performed to acquire better insights into the developmental surface area expansion and the concurrent adaxial cuticular wax deposition of young leaves. In this kinetic, leaves of 1, 2, 3, 4, 5 and 6 weeks of age were harvested, their lamina surface area was determined (see paragraph '2.3 Leaf morphology and physiology'), and their adaxial cuticular wax was analysed (see paragraph '2.4 Analytics'). All other experiments of this study were then performed exclusively with 6-week-old leaves since, based on the results of the kinetic series, they promised to be the best compromise of (i) a finished development (excluding 1-, 2- and 3-week-old leaves), (ii) yet still a young age without potential senescence symptoms (excluding leaves >6 weeks of age) and (iii) the highest relative amount of wax ester dimers and the highest weighted mean carbon chain length, especially in the mutant line (excluding 4- and 5-week-old leaves).

2.2 | Generation of transgenic poplar lines

The *Populus* × *canescens* clone INRA 717-1B4 was chosen for the leaf disk transformation because it has previously been characterised for its good in vitro cultivation ability and transformability (Leple et al., 1992), and the availability of the complete genome sequence (Mader et al., 2016). The target gene of the CRISPR/Cas9-based knock-out approach was *CER6*, AT1G68530, of *Arabidopsis*. As a result of the known whole-genome duplication in *Populus* about 65 million years ago, a set of two ortholog gene targets (*CER6_1*, Potri.010G125300; *CER6_2*, Potri.008G120300) were selected based on the genome of *Populus trichocarpa* (Tuskan et al., 2006). Consecutively considering both alleles of the *Populus* × *canescens* hybrid for both orthologs, four targets had to be addressed (Grünhofer, Stöcker, et al., 2022; Supporting Information: Figure S1).

The leaf disk transformation with CRISPR/Cas9 transformation vectors harbouring a *nptII* resistance cassette for subsequent Kanamycin selection as well as guide-RNA for two targets (T1, T2) specifically tailored for *CER6* (Supporting Information: Figure S1) was performed following the protocol described in Bruegmann et al. (2019). Methodological details and the results of the subsequent analytical screening for cuticular waxes and of the target gene editing analysis by sequencing are given in Supporting Information: Figure S2.

2.3 | Leaf morphology and physiology

To thoroughly monitor the morphology and physiology of developing leaves of defined ages, different assessments were carried out either with leaves still attached to the plant or after leaf abscission. The chlorophyll content was measured using the Force A device (Dualex Scientific). Stomatal conductances were measured using the LI-600 leaf porometer (LiCor). The lamina surface area was determined using a scanner (Canon) and ImageJ (Abramoff et al., 2004). Fresh and dry weight (FW and DW, respectively) of leaves were evaluated with an analytical balance (resolution of ±0.01 mg, Sartorius), and the relative water content (RWC) was calculated following RWC = (FW–DW) FW^{-1}.

The stomatal density of abaxial leaf sides (the adaxial side had previously been shown to be devoid of stomata in climate chamber and greenhouse cultivation, Grünhofer, Herzig, et al., 2022) was estimated with Collodion (4%–8% in ethanol/diethyl ether, Fluka) imprints. Since abaxial leaf surfaces of grey poplar are densely covered by trichomes, these had to be ripped off with a first drop of Collodion. The second drop was used to prepare imprints of the smoothened area, which were then evaluated for the presence and density of stomata under a light microscope.

To acquire high-resolution images of the adaxial and abaxial leaf sides, both surfaces were prepared for scanning electron microscopy (SEM; S200, Cambridge Instruments). Leaf segments of 1 cm^2 were fixed on aluminium sample holders, dried over activated silica gel and sputter-coated with palladium for 60 s using 35 mA current (Leica EM

ACE200, Leica), resulting in approx. 15 nm palladium layer thickness, and examined by the SEM with a working distance of 11 mm and an accelerating voltage of 15 kV.

Additional contact angle measurements were carried out to investigate further the surface properties of the adaxial leaf sides investigated by the SEM. Here, fresh leaf samples were fixed to glass slides with double-sided adhesive tape (abaxial side facing down towards the slide, adaxial side facing up), and water droplets of 10 µL volume were placed and analysed on each adaxial leaf surface with a Drop Shape Analyzer (Krüss).

Finally, the light reflectance properties of the adaxial leaf surface were analysed using a spectrophotometer (AvaSpec 2048, Avantes) connected to a light source with a precisely known (200–1100 nm; AvaLight-DHS Deuterium-Halogen Light Source, Avantes) light spectrum (Vitt et al., 2020). The 200 µm fibre-optic probe was firstly placed on a 98% (300–700 nm) Spectralon white standard (WS-2, Avantes) and subsequently on different spots (tip, middle, base) of several leaves per line at a 45° angle. This way, the light reflectance in both the ultraviolet (UV-A and UV-B) range (280–380 nm) and in the photosynthetically active radiation (PAR) spectrum (400–700 nm) could be investigated.

2.4 | Analytics

Sampling for all cuticular wax analyses always followed the same methodological pattern. For the cuticular wax extraction, chloroform-filled (spiked with 10 µg internal standard; Tetracosane, Fluka) broad-rim vials of 1.28 cm^2 opening were tightly placed onto the investigated leaf surface, supported by polytetrafluoroethylene (PTFE) plates from underneath the sample (Grünhofer et al., 2021). Repeated turning of the leaf upside down for 10 s ensured a sufficient wax extraction based on extraction kinetics for leaf waxes conducted previously (Baales et al., 2021). Four extracts per leaf were pooled to yield one biological replicate. To prepare the samples for gas chromatographic (GC) analysis, the sample volumes were completely evaporated with nitrogen at 60°C before derivatization with BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide, Macherey-Nagel) and pyridine (Sigma-Aldrich) to mask reactive acid and alcohol groups by trimethylsilyl esters and ethers, respectively (Hauke & Schreiber, 1998). GC analysis for qualification of wax compounds using a coupled mass spectrometer (GC-MS: 7890B) and GC analysis for quantification of wax compounds using a coupled flame ionisation detector (GC-FID: 6890 N) was achieved by on-column injection of 1 µL derivatized sample at 50°C, followed by a specifically designed temperature programme (Kurdyukov et al., 2006). Finally, fragmentation patterns of wax compounds (GC-MS) were qualified with an in-house created mass spectral library, and acquired signal intensities of wax compounds (GC-FID) were quantified by relation to that of the known internal standard.

A slight deviation of this protocol was the analysis-preceding transesterification of the below-specified samples with BF_3-methanol at 70°C overnight and stopping the reaction with $NaHCO_3$ (Zeier & Schreiber, 1998). To analyse the polymeric leaf cutin composition and amount, punched-out leaf disks (LD; 2.55 cm^2), comprising both the adaxial and abaxial leaf side, were fully extracted of soluble lipids in chloroform:methanol for several days before being transesterified with BF_3-methanol to deconstruct the three-dimensional polyester structure of cutin and release methylated monomers. For harvested adaxial cuticular wax samples, the same treatment resulted in the splitting of wax ester dimers and the immediate conjugation of released monomers to methanol. Thus, the shorter-chained acid and alcohol constituents of very long-chained wax ester dimers (being C34–C46 in the cuticular wax of grey poplar) could efficiently be analysed. After repeated chloroform extractions, these two protocol modifications were continued as described above.

2.5 | Transpiration

Since the enzymatic isolation of CMs (used to measure true cuticular transpiration) previously shown for a grey poplar tree growing outdoors for several years (Schreiber et al., 2006) was not possible with greenhouse-grown plants of several months of age in this study, the complexity of residual (cuticular) leaf transpiration was approached from different angles.

At first, stomatal behaviour after leaf abscission was investigated by measuring the decline of stomatal conductance with the L-I600 leaf porometer (LiCor). This would theoretically result in residual leaf transpiration after almost full stomatal closure, which cannot be measured with the LI-600 due to insufficient resolution. The measurement intervals were every 5 min in the first 30 min and every 10 min between 30 min and 3 h after leaf abscission.

The residual (cuticular) leaf transpiration was then determined using whole leaves (WLs), following protocols in Schuster et al. (2016). Here, leaves were abscised in the greenhouse and transported to the lab in sealed plastic bags, leaf petioles were sealed with PTFE paste and the leaf FW and lamina surface area were determined as soon as possible. During the subsequent weight measurements every 20 min in the first 3 h and then every 60 min up to 7 h, the detached leaves were stored in plastic boxes over activated silica gel (resulting in 2% relative humidity) at 25°C. At the end of the experiment, the leaves were dried at 60°C overnight, and the DW was determined the next day. Permeances P ($m\,s^{-1}$) can generally be calculated by dividing the slopes ($g\,m^{-2}\,s^{-1}$) of surface area-normalised water loss ($g\,m^{-2}$) versus time (s) by the driving force (density of water; $10^6\,g\,m^{-3}$ at 25°C) of water (Schreiber & Schönherr, 2009). These slopes can be estimated incrementally between each sequential gravimetric measurement, resulting in many incremental permeances per leaf. The acquired data allowed visualisation in two different ways: plotting the incremental permeance versus the relative water deficit (RWD) of leaves (Figure 6b) or the weight loss kinetic as surface area-normalised weight loss versus time (Figure 6c). The RWD was calculated from the measured combined weight loss at a given increment related to the leaf FW. Especially the former

visualisation allows for a better estimation of persisting stomatal contribution in residual (cuticular) transpiration after leaf detachment. Following an initially high stomatal contribution shortly after leaf abscission (approx. 0–0.1 RWD), plateauing lower permeance values (approx. 0.1–0.4 RWD) indicate that the majority of stomata must be closed to a high degree in a steady-state condition. To test the limitations of this whole leaf method, the influence of the theoretical decrease in the driving force of water during leaf desiccation (theoretically altering the calculation of P; Grünhofer & Schreiber, 2023) was investigated with isolated CMs of *Prunus laurocerasus* (Supporting Information: Figure S3a). It has been observed that the decreasing driving force of water did not result in significantly altered permeances, and thus the calculations of the main experiments were carried out using a constant driving force for water of 10^6 g m^{-3}.

For some but not all of five investigated species (Burghardt, 2003) and also for *Populus* × *canescens* (Grünhofer, Herzig, et al., 2022), it has been shown previously that the residual (cuticular) transpiration indeed was highly similar to the cuticular transpiration. However, when working with WLs, especially of newly created mutant lines, the possible contribution of not fully closed stomata can never be excluded entirely. This is why in addition to WLs also punched-out LDs of intact leaves were mounted into nonrefillable transpiration chambers with an opening of 1.13 cm^2 as an approximation for measurements with CMs. Since grey poplar leaves are hypostomatous, in this setup, water can only transpire through the astomatous adaxial leaf side facing the atmosphere (the stomatous abaxial leaf side is facing the water-filled inside of the chamber). Since, in contrast to isolated CMs, punched-out LDs also represent a living tissue, the gravimetric measurements were carried out in shorter intervals of 20 min in the first 3 h and every 60 min up to a final experimental duration of 7 h, just like with WLs. In the meantime, the chambers were also stored at 25°C and 2% relative humidity. In contrast to the data acquired with WLs, the calculation of RWDs (Figure 6b) with transpiration chamber-mounted LDs is not possible, and thus only the water loss kinetic as surface area-normalised water loss versus time can be compared (Figure 6c,d). Also for this LD method, different additional experiments were carried out with LDs of the WT (Supporting Information: Figure S3b). But since neither the vacuum infiltration nor the mechanical abaxial roughening of LDs resulted in methodological optimisations, the main experiments were carried out with untreated LDs.

Finally, all individual incremental permeances of the plateauing region of the graphs (0.1–0.4 RWD) of the WLs (Figure 6b) and all individual incremental permeances of the linear region of the graphs (0.5–7 h) of the LDs (Figure 6d) were used to calculate the mean permeance. In addition, the available literature was used to determine previously published mean residual (cuticular) permeances for *Populus* × *canescens* (Grünhofer et al., 2021; Grünhofer, Herzig, et al., 2022; Schreiber et al., 2006) and the lowest (residual; 0.9×10^{-9} m s^{-1}) and highest (cuticular; 2.7×10^{-9} m s^{-1}) mean permeances were used as references (Figure 7).

2.6 | Statistical analysis

The preparations of figures as well as the statistical analyses were performed in OriginPro 21b (OriginLab Corporation). After assessing the normality of data with the Shapiro–Wilk test, normally distributed data were visualised using means with standard deviations, whereas not normally distributed data were presented with boxplots. Analysis of significant differences between sample means (the sample sizes are given in the respective figure legends) was carried out with a one-way analysis of variance (ANOVA) with Fisher's least significant difference post hoc test (normal distribution of data) or with a Kruskal–Wallis ANOVA (no normal distribution of data). All statistical tests were carried out at $p < 0.01$, and different letters indicate significant differences.

3 | RESULTS

3.1 | Transgenic poplar lines

After 15 transformed and putatively edited plants had been regenerated, a set of six lines that appeared promising after a first genotyping (the presence of *cas9* and/or *nptII* were checked by polymerase chain reaction) were included in a chemical analytical screening to check for differences in the cuticular wax amount and composition as well as in a more elaborate genetic screening performed by DNA sequencing. Since only one single mutant line (termed *cer6_1,2*) was found to be remarkable in both analyses, a transformed but not edited control (termed *CER6_1,2*) was also chosen to be included in further experiments in addition to the WT (Supporting Information: Figure S2).

3.2 | Leaf morphology and physiology

Only a few phenotypical differences were evident between the WT, the transformation control (*CER6_1,2*), and the mutant (*cer6_1,2*) line (Figure 1). The leaves of the main experiment, which budded after cutting back the plants, developed normally in size and reached their final lamina surface area at 4 weeks of age (Figure 1a). Six-week-old leaves (the leaf age all experiments of this study except the developmental leaf surface area and wax accumulation kinetic were focused on) hardly differed in FW and DW per area (Figure 1b,c), the resulting RWC (Figure 1d) or the chlorophyll content (Figure 1e). The abaxial stomatal densities were slightly higher in *CER6_1,2* and even higher in *cer6_1,2* when compared to the WT (Figure 1f), which was also reflected in the trends of the not significantly increased stomatal conductances of *CER6_1,2* and *cer6_1,2* (Figure 1g).

High morphological similarity between all three lines was further confirmed by macroscopically (Figure 2a) as well as scanning electron microscopically (Figure 2b,c) observing the leaves, as it did not reveal any visible alterations in the leaf shape or the ultrastructural surface properties, which translated well also into the contact angles of water

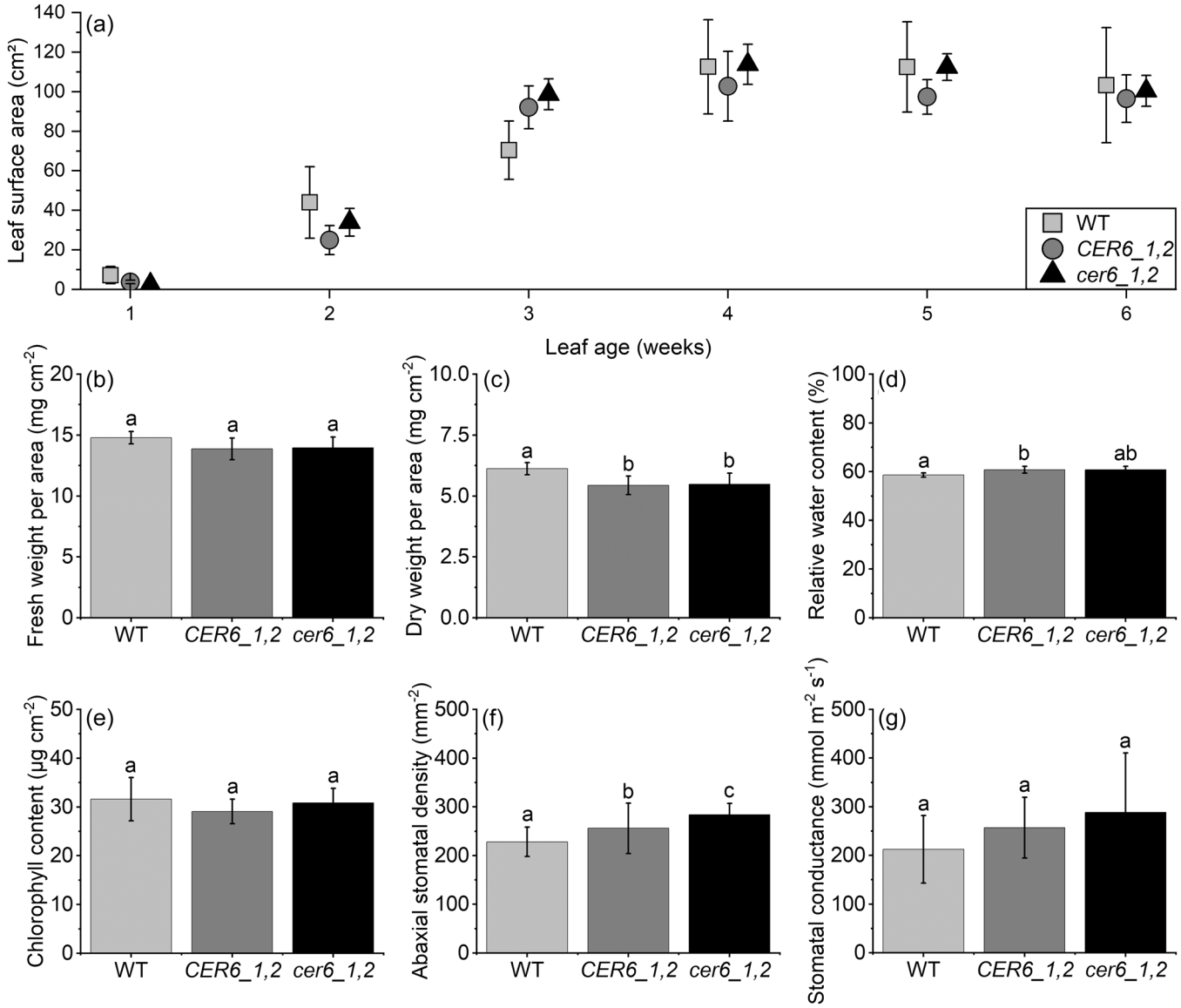

FIGURE 1 Leaf development and morphology. The age-dependent development of the leaf surface area (a) and fresh weight per surface area (b), dry weight per surface area (c), relative water content (d), chlorophyll content (e), abaxial stomatal density (f) and stomatal conductance (g) of 6-week-old leaves were investigated. Means with standard deviations [$n = 4–6$ (a–e), 30 (f), 10 (g)] are shown and different letters indicate significant differences at $p < 0.01$. *CER6_1,2*, transformation control; *cer6_1,2*, mutant line; WT, wildtype.

droplets placed on the adaxial cuticular surface not being significantly different (Figure 2d). However, the relative reflectance of different wavelengths in the UV (280–380 nm) and PAR (400–700 nm) range indicated a first peculiar difference in the surface properties of the *cer6_1,2* mutant, which appeared to reflect less light across the whole investigated spectrum with most remarkable differences between 500 and 700 nm (Figure 2e).

Two noteworthy exceptions of the above-mentioned normal leaf development of *cer6_1,2* were (i) sporadically observed necrotic leaf tips of some leaves of some *cer6_1,2* mutant plants during their primary (before the cut-back) shoot growth (Supporting Information: Figure S4) and (ii) a notably slower overall height growth of some *cer6_1,2* mutants. Although not precisely monitored, the weekly growth rate of some but not all of the *cer6_1,2* mutants appeared to be about 20% reduced. However, as this was not observed consistently in all mutant plants and was not the main focus of this study, these two phenomena were not quantified in greater detail.

3.3 | Analytics

In contrast to the leaf morphology, the chemical cuticular wax and cutin analyses yielded more significant differences (Figures 3 and 4).

When the adaxial cuticular wax composition of 6-week-old leaves was sorted for chain lengths and then functional groups, it was evident that the *cer6_1,2* mutant was almost entirely lacking any

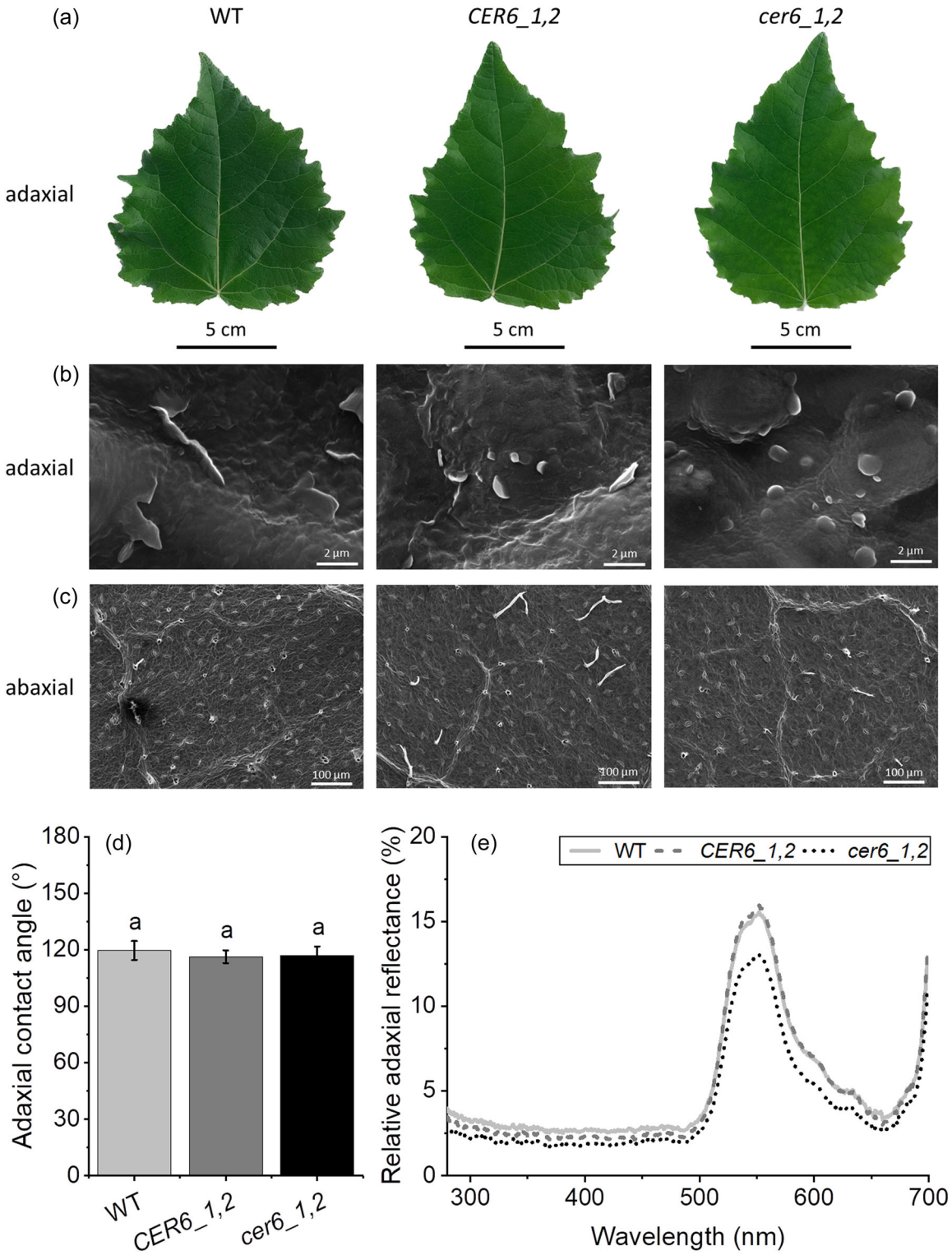

FIGURE 2 Macroscopical and microscopical leaf properties. Six-week-old leaves were analysed in regard to shape and appearance (a), adaxial (b) and abaxial (c) surface quality, contact angles of water on adaxial leaf sides (d) and adaxial light reflectance characteristics (e). Means with (d) or without (e) standard deviations [$n = 9$ (d), 15 (e)] are shown and different letters indicate significant differences at $p < 0.01$. *CER6_1,2*, transformation control; *cer6_1,2*, mutant line; WT, wildtype.

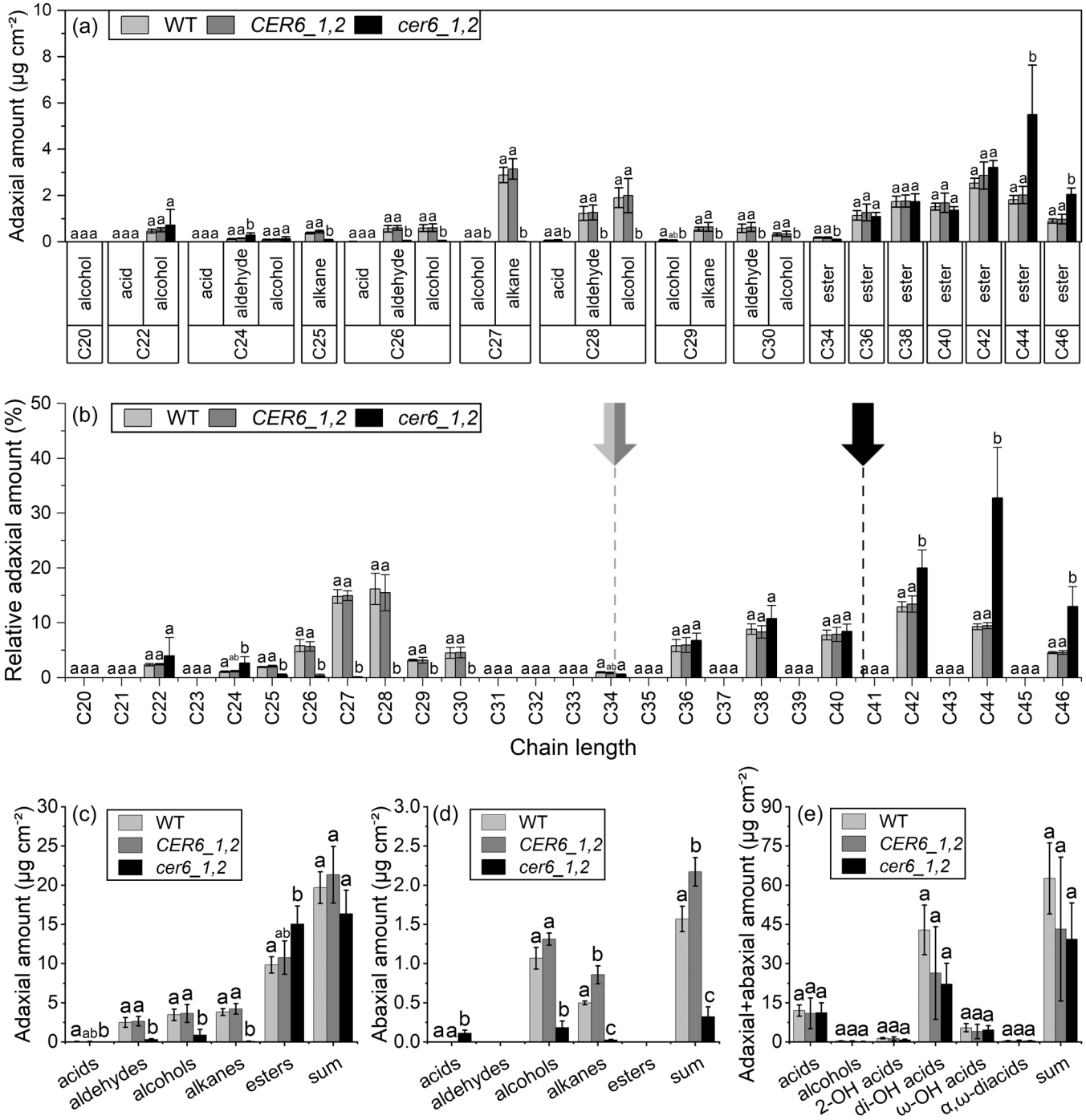

FIGURE 3 Aliphatic cuticular wax and cutin quality and quantity. Six-week-old leaves were analysed for the adaxial cuticular wax monomer composition (a), relative adaxial distribution of different chain lengths (b) and functional groups of the cuticular wax of the adaxial (c) and abaxial (d) leaf side, as well as functional groups of the cuticular cutin of both leaf sides combined (e). Differently coloured arrows in (b) indicate the weighted mean carbon chain length. Means with standard deviations [$n = 4$ (a–c,e), 3 (d)] are shown and different letters indicate significant differences at $p < 0.01$. *CER6_1,2*, transformation control; *cer6_1,2*, mutant line; WT, wildtype.

monomers (acids, alcohols, alkanes and aldehydes) with a chain length >C24 (Figure 3a). Instead, the C24 aldehyde as well as the C44 and C46 ester dimers accumulated significantly (Figure 3a). When all compounds of the same chain length were combined and related to the total wax amount, it became evident that primarily the increase in the C44 and C46 wax ester dimers and the decrease in most >C24 monomers resulted in a significant increase of the weighted mean carbon chain length from 34.1 in the WT and *CER6_1,2* to 40.7 in *cer6_1,2* (Figure 3b). In addition, the absolute reductions (basically almost absence) of the >C24 monomers resulted in significant reductions in the functional groups of acids, alcohols, alkanes and aldehydes, but the significant absolute accumulation of the C44 and

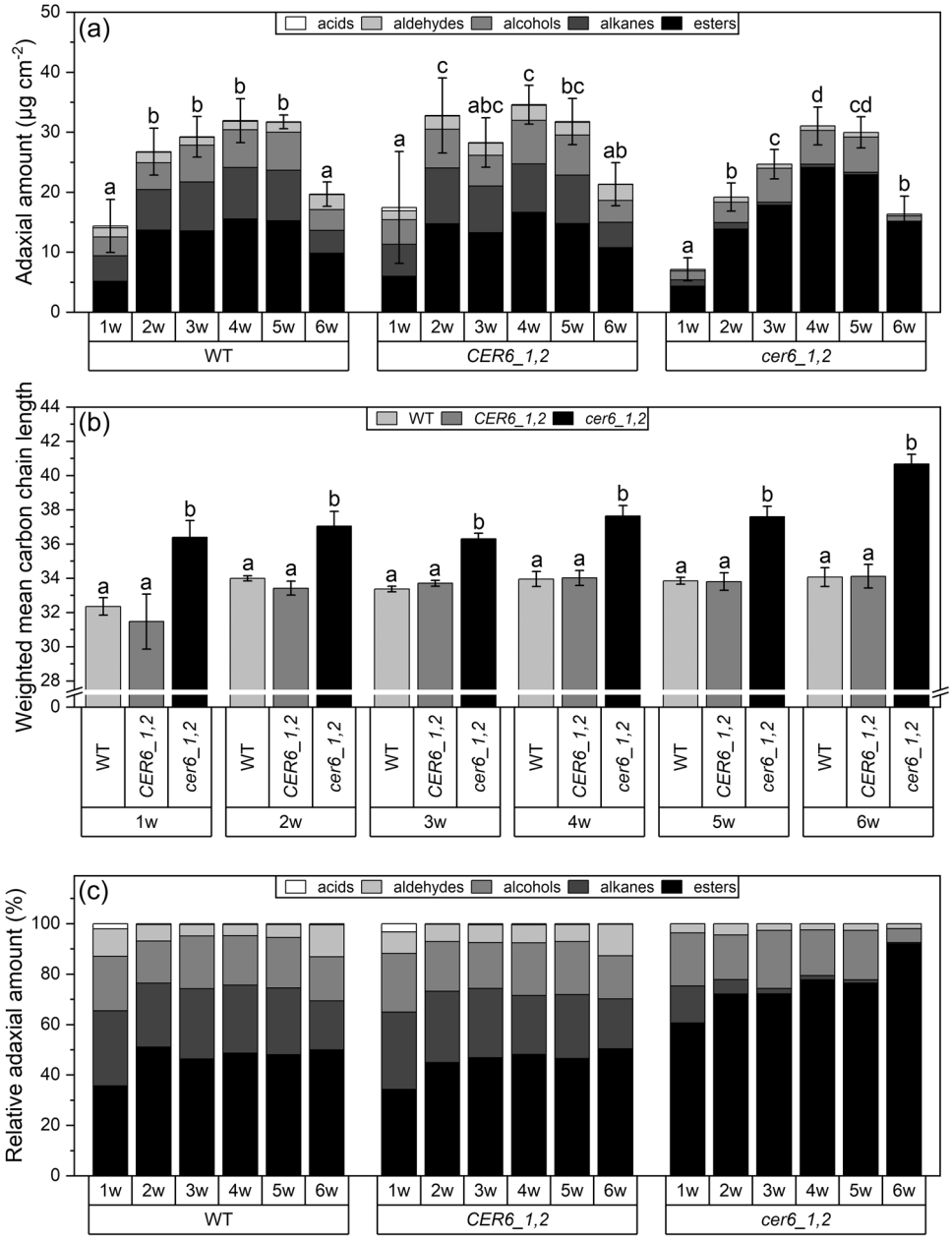

FIGURE 4 Age-dependent adaxial aliphatic cuticular wax accumulation. Leaves of different ages (1–6 weeks) were analysed in regard to their absolute cuticular wax amount (a), weighted mean carbon chain length (b) and relative cuticular wax composition (c). Means with standard deviations ($n = 4$) are shown and different letters indicate significant differences at $p < 0.01$. *CER6_1,2*, transformation control; *cer6_1,2*, mutant line; w, week; WT, wildtype.

C46 wax ester dimers led to a significant increase of all esters combined and thus the total adaxial cuticular wax amount with approx. 16.4–21.4 µg cm^{-2} was not significantly different between all three lines (Figure 3c). The same absence of >C24 wax monomers was also observed in the abaxial cuticular wax (Supporting Information: Figure S5a). However, in contrast to the adaxial side, considerably lower absolute amounts (about 10-fold less) and no aldehydes or wax ester dimers were detected (Figure 3d). These two major differences of adaxial and abaxial wax resulted in the total abaxial cuticular wax being approx. 1.6–2.2 µg cm^{-2} in the WT and *CER6_1,2* and only approx. 0.3 µg cm^{-2} in *cer6_1,2* because increased amounts of esters did not recover the total wax load this time (Figure 3d).

The amounts of all cutin monomers and also the total cutin amount of investigated LDs (adaxial and abaxial side combined) was not significantly affected (Figure 3e). This was most likely because, with the exception of the C26 2-OH acid, the leaf cutin was made up only of <C24 monomeric compounds (Supporting Information: Figure S5b), which seemed not to be affected by the mutation.

Despite these remarkable compositional differences, the developmental adaxial cuticular wax accumulation was not severely affected by the mutation (Figure 4). All leaves continuously accumulated cuticular wax in their first 4 weeks of age, which correlated very well with the observed leaf lamina surface area development (Figure 1a), and plateaued with total wax amounts of approx. 30–34.6 µg cm^{-2} (Figure 4a). In addition, all 6-week-old leaves exhibited significantly less adaxial cuticular wax (16.4–21.4 µg cm^{-2}) than their 5-week-old counterparts (Figure 4a). However, exactly these 6-week-old leaves were chosen to be the most interesting for further analyses, even though the weighted mean carbon chain length was significantly increased in *cer6_1,2* in all investigated leaf ages (Figure 4b). This decision was made because the highest absolute amount was not of interest for this study, and the 6-week-old leaves of *cer6_1,2* additionally exhibited the greatest relative proportion of wax ester dimers (>90%) and the smallest relative amounts of acids, aldehydes, alcohols and alkanes (Figure 4c).

To better understand why exactly these dimeric wax ester compounds accumulated in *cer6_1,2*, a transesterification analysis was performed for all three lines (Figure 5). Without affecting the total wax amount, this transesterification led to an entire absence of dimeric wax esters but a significant increase of the monomeric wax ester constituents acids and alcohols (Figure 5a). While even-numbered C14–C24 acids and even-numbered C16–C24 alcohols accumulated in the transesterifications of all three lines, it was evident that especially the C22 acid and the C22 and C24 alcohol accumulated the most in *cer6_1,2* when compared to the WT and *CER6_1,2* (Figure 5b).

3.4 | Transpiration

Despite the not significantly altered stomatal conductances while the 6-week-old leaves were still attached to the plant (Figure 1g), the decline of stomatal conductance after leaf abscission and thus the efficiency of stomatal closure was slightly worse in *cer6_1,2* (Figure 6a). After a steep decline in all lines in the first 45 min, the stomatal conductances of *cer6_1,2* plateaued on higher values than those of the WT and *CER6_1,2*. This was also supported by plotting the incrementally calculated permeances of WLs versus their RWDs, where leaves of *cer6_1,2* reached higher mean water deficits by constantly exhibiting higher mean permeances and thus indicating potential incomplete stomatal closure (Figure 6b). The RWD of approx. 0.6 reflects the end-point of the transpiration experiment after drying the leaves for 24 h and represents the counterpart of the previously reported RWC of approx. 60% (Figure 1d). Due to these observations, not only residual (cuticular) transpiration of WLs (Figure 6b,c), always being a composite quantity of stomatal and cuticular contribution, but also true cuticular transpiration of LDs was measured (Figure 6d).

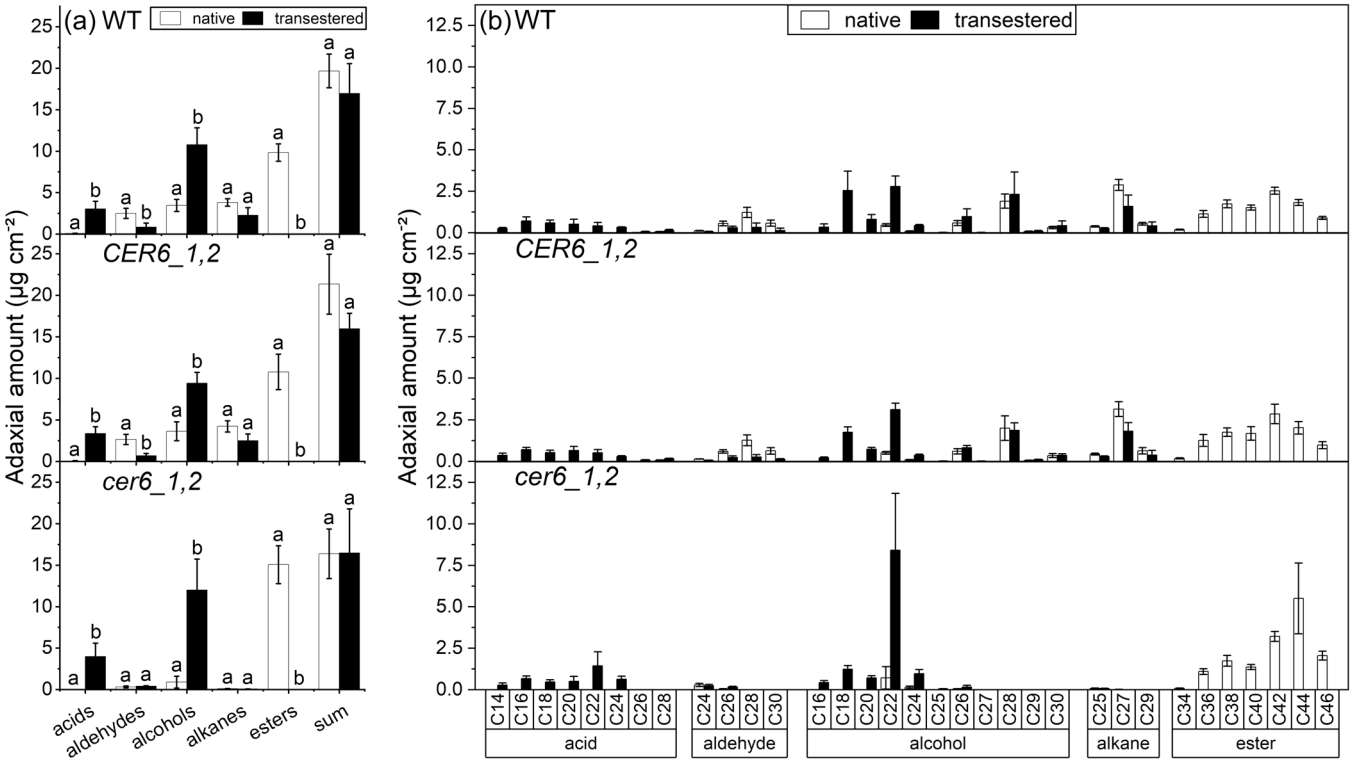

FIGURE 5 Transesterification analysis of adaxial cuticular wax. Harvested samples of 6-week-old leaves were transesterified to check the composition of the cuticular wax ester dimers. Functional groups (a) and individual monomers (b) are shown. Means with standard deviations ($n = 4$) are shown and different letters indicate significant differences at $p < 0.01$. *CER6_1,2*, transformation control; *cer6_1,2*, mutant line; WT, wildtype.

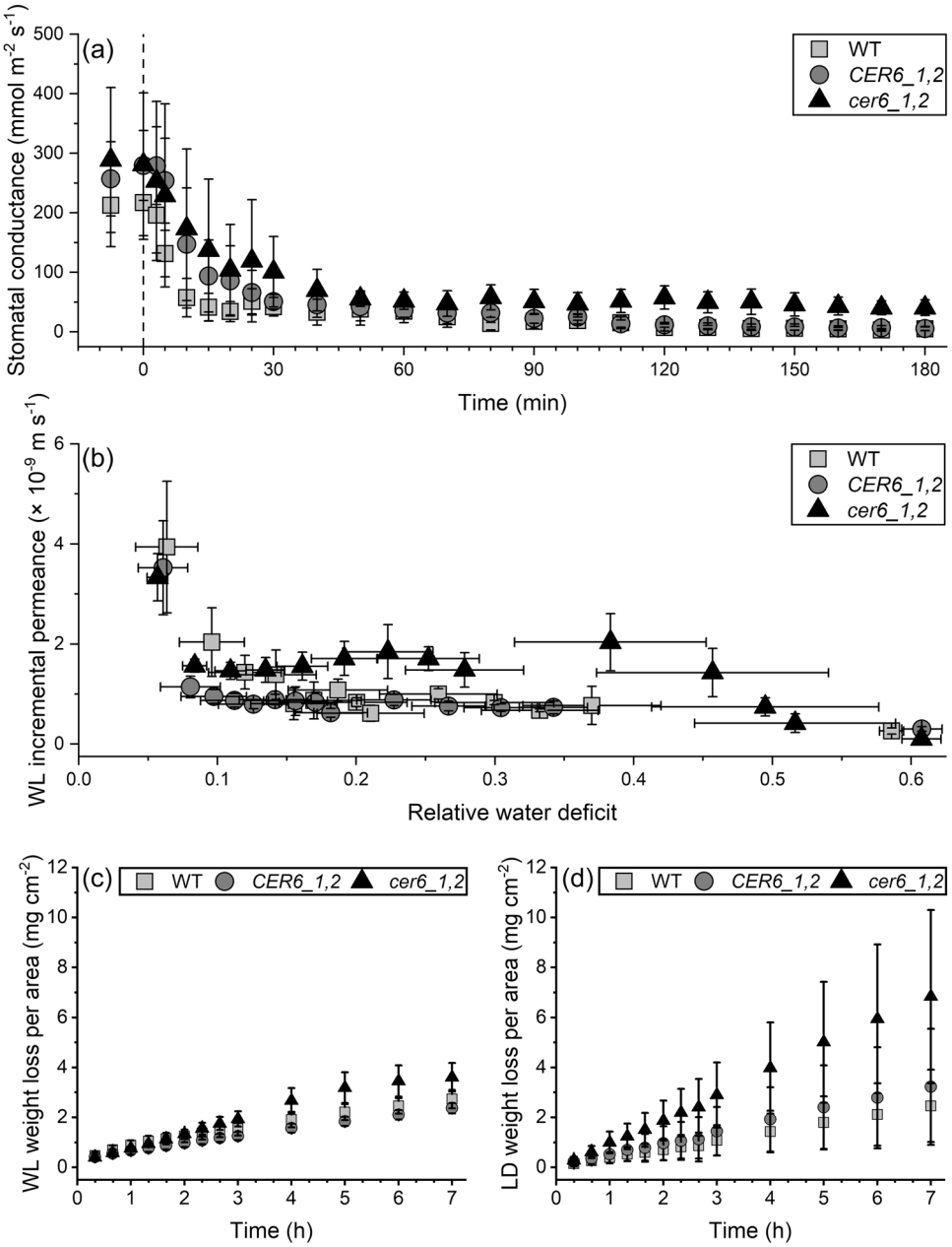

FIGURE 6 Transpiration measurements of whole leaves and punched-out leaf disks. The stomatal conductance (a), incremental permeance (b) and weight loss per area (c) was monitored for 6-week-old wholes leaves. Since a persisting stomatal contribution to the residual (cuticular) transpiration of whole leaves could not be excluded based on (b) and (c), also the weight loss per area of leaf disks mounted to transpiration chambers (d), where water could exclusively evaporate across the adaxial leaf side, was investigated. Plotting the data of the leaf disks (d) as shown in (b) was not possible, because this method did not allow the calculation of a relative water deficit. Means with standard deviations [n = 6 (a–c), 10 (d)] are shown. Dashed line in (a) = timepoint of leaf abscission. *CER6_1,2*, transformation control; *cer6_1,2*, mutant line; WT, wildtype.

When the incrementally calculated permeances of all lines and both experiments were combined, it became evident that the residual (cuticular) transpiration of *cer6_1,2* was about two fold higher than that of *CER6_1,2* and the WT, irrespective if WLs (1.58 vs. 0.82 and 1.09×10^{-9} m s^{-1}, respectively) or LDs (2.61 vs. 1.44 and 1.12×10^{-9} m s^{-1}, respectively) were measured (Figure 7). Nonetheless, the significantly increased mean permeance of *cer6_1,2* was still in the range of previously published mean residual (cuticular) transpirations ($0.9–2.7 \times 10^{-9}$ m s^{-1}) for other WTs of *Populus* × *canescens* (Figure 7).

4 | DISCUSSION

Since most of the evaluated morphological parameters were very similar between the WT, the transformation control (*CER6_1,2*) and the mutant (*cer6_1,2*) line (Figures 1 and 2), *cer6_1,2* appeared to be ideally suited to study the effects of a significantly shifted (significantly increased wax ester fraction and weighted mean carbon chain length) wax composition without alteration of the total wax amount (Figures 3 and 4) on the residual (cuticular) transpiration of leaves (Figures 6 and 7).

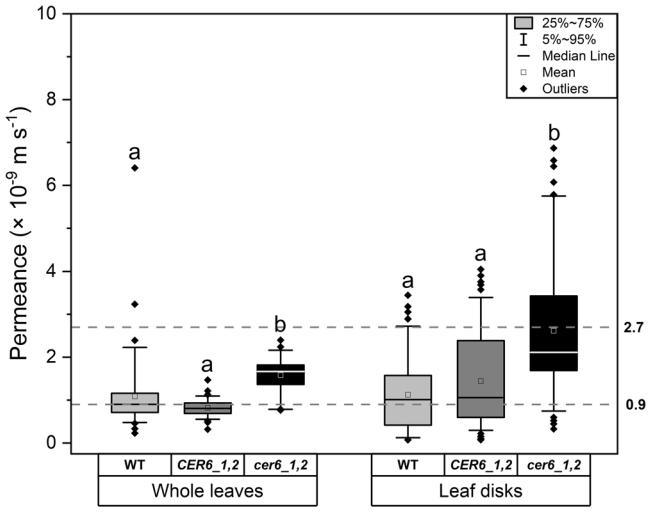

FIGURE 7 Permeances of whole leaves and punched-out leaf disks. Permeances of the whole leaves originate from the incremental permeances of the plateauing region (0.1–0.4 relative water deficit) of Figure 6b. Permeances of the leaf disks originate from the incremental permeances of the linear region (0.5–7 h) of Figure 6d. For whole leaves, a potential stomatal contribution to the residual (cuticular) transpiration cannot be excluded. But for the leaf disks mounted to transpiration chambers water can only be lost across the adaxial leaf side, resulting in the measurement of true cuticular transpiration. The dotted reference lines indicate the lowest (residual; 0.9×10^{-9} m s^{-1}) and highest (cuticular; 2.7×10^{-9} m s^{-1}) previously published mean residual (cuticular) permeances for *Populus* × *canescens* (Grünhofer et al., 2021; Grünhofer, Herzig, et al., 2022; Schreiber et al., 2006). Boxplots ($n = 47–115$) are shown and different letters indicate significant differences at $p < 0.01$. *CER6_1,2*, transformation control; *cer6_1,2*, mutant line; WT, wildtype.

4.1 | Leaf morphology and physiology

Although the abaxial stomatal density of *cer6_1,2* was found to be significantly higher (Figure 1f) and potentially even influenced the nonsignificantly increased stomatal conductance while leaves were still attached to the plant (Figure 1g), the measured values of approx. 200–300 mm^{-2} stomatal density and approx. 200–300 mmol m^{-2} s^{-1} stomatal conductance were still very well in the range of previously reported reference values. Different poplars (both, between-species but also within-species) are known to vary widely in these traits, with abaxial stomatal densities of often more than 250 mm^{-2} (Al Afas et al., 2006; Dunlap & Stettler, 2001) and also high stomatal conductances of even up to about 1000 mmol m^{-2} s^{-1} (Pearce et al., 2006).

The observed differences in the adaxial light reflectance of *cer6_1,2* might be of greater biological importance. Since cuticular waxes are renowned for modulating the absorbance and reflectance of different light spectra (Pfündel et al., 2006; Shepherd & Wynne Griffiths, 2006), especially the harmful UV radiation (280–380 nm) and, to a lesser extent, the PAR range (400–700 nm), it was fascinating to observe that the significantly altered adaxial cuticular wax composition (Figure 3a–c) indeed consequentially seemed to decrease the light reflectance properties of the *cer6_1,2* leaves (Figure 2e). To what degree this observation is caused by either the high relative amount of wax esters or the almost complete absence of acids, aldehydes, alcohols and alkanes, and how biologically relevant this observation might be, can only be hypothesised now but warrants interesting future experiments.

4.2 | Analytics

The analytic results (Figures 3–5) generally support what has been reported in the literature previously (Fiebig et al., 2000; Leide et al., 2007; Millar et al., 1999; Vogg et al., 2004) and thus reconfirm *CER6* being a KCS elongase with substrate chain length specificity of ≤C24 in the case of poplar leaves. Interestingly, the very same difference in chain length distribution was also evident in the abaxial cuticular wax (Supporting Information: Figure S3a), but since there were no wax ester dimers on the lower leaf side, the total wax amount was not recovered (Figure 3d) as it was the case on the adaxial side (Figure 3c). Aside from a genetically predefined causation, the identified about 10-fold higher total wax amounts on the adaxial side (Figure 3c,d) might also indicate the already suggested higher importance of light radiation, in comparison to relative humidity and temperature (Grünhofer, Herzig, et al., 2022), in modulating the absolute amounts of accumulated leaf wax as this leaf side is constantly directly exposed to the sunlight.

The developmental leaf wax accumulation has previously been shown to be synchronised with the increasing leaf surface area and stop once leaves are fully expanded (Figures 1a and 4; Jetter & Schäffer, 2001; Kahmen et al., 2011; van Maarseveen et al., 2009). Also, the degradation of compounds which were identified by the comparison of 5-week- with 6-week-old leaves (Figure 4a) is not unknown and might be linked to wax-degrading environmental stimuli (abrasion or erosion) being stronger in a greenhouse or outdoors than in a climate chamber (Cameron et al., 2006; Neinhuis & Barthlott, 1998).

It can be assumed that, because wax ester dimers are not abundant in leaf and stem wax of *A. thaliana* (Jenks et al., 2002; Patwari et al., 2019), the significant adaxial increase of ester dimers upon *CER6* knock-out observed with *Populus* × *canescens* in this study has not been observed before. The explanation of this phenomenon was investigated using a transesterification approach to analyse the monomeric composition of the dimeric wax esters (Figure 5). Here, it was evident that transesterification indeed resulted in the complete disintegration of ester dimers and simultaneous accumulation of its acid and alcohol monomers (Figure 5a). Specifically acids and alcohols of C14–C24 accumulated in the extracts of all investigated lines (Figure 5b) and thus it was clear that in *cer6_1,2* the monomeric ≤C24 substrates of the knocked-out CER6 enzyme (e.g., especially the C22 acid and C22 alcohol) would tend to accumulate but are further metabolised to form the significantly increased fraction of dimeric wax esters (Figure 3a,c; e.g., C22 acid + C22 alcohol = C44 wax ester). Instead, in *A. thaliana*, the stem wax of *cer6* mutants was found to

accumulate exactly these ≤C24 monomers, and the total wax load was significantly decreased (Millar et al., 1999), probably due to the absence of a pronounced dimeric ester fraction compensating for these effects.

4.3 | Transpiration

Cutin is typically made of monomers with chain lengths ≤C24 (Pollard et al., 2008), as also identified in this study (Supporting Information: Figure S5b), which were not affected by the *CER6* mutation. Moreover, cutin was not altered in chain lengths and total amounts (Figure 3e, Supporting Information: Figure S5b). Thus, it appears reasonable to assume for the transpiration experiments that the polymeric cutin structures providing a three-dimensional matrix for the associated cuticular waxes were not different between all investigated lines and differences in the residual (cuticular) transpiration should be mainly due to the peculiar differences in the cuticular waxes. Based on the available literature, also focusing on cuticular wax composition and not only on the absolute amount, it was expected that a higher proportion of long-chained wax esters and a higher weighted mean carbon chain length, both theoretically increasing the cuticular hydrophobicity and crystallinity, thus strengthening the transpiration barrier, might lead to significantly reduced rates of cuticular water loss. Since a potential stomatal contribution to the measured residual (cuticular) transpiration could initially not be excluded when working with WLs, especially of *cer6_1,2* (Figure 6a–c), this hypothesis was additionally tested with punched-out LDs mounted to transpiration chambers (Figure 6d). These offer the great advantage that (i) abaxial stomata and (ii) the about 10-fold lower abaxial wax loads are entirely excluded as possible confounders and thus, due to hypostomaty, true adaxial cuticular transpiration is measured. The only downside was the weight loss measurements being closer to the resolution limit of the balance, as is reflected in the higher standard deviations around the sample means (Figure 6c,d). The observation that both (WLs and LDs) weight loss kinetics (Figure 6c,d) and resulting incremental permeances (Figure 7) looked very much alike proves no stomatal contribution to the residual (cuticular) transpiration measured with WLs. In fact, despite the abaxial leaf side exhibiting (i) stomata and (ii) a 10-fold lower wax amount of about 0.3–2.2 µg cm^{-2} (Figure 3d), the adaxial and abaxial cuticular permeance should be very similar, because the whole leaf residual (cuticular) permeance (adaxial + abaxial combined) and the LD cuticular permeance (only adaxial) were almost identical in both experiments (Figure 7). These data again seem to support the idea of a minimum effective amount for cuticular wax acting as a transport barrier (Grünhofer & Schreiber, 2023). The abaxial wax amount of this study is very much comparable to that of 1–1.5 µg cm^{-2} previously identified to be sufficient to establish an already fully efficient barrier for water loss even in very young leaves of *Populus* × *canescens* developing immediately after acclimatisation from tissue culture to growth in soil (Grünhofer, Herzig, et al., 2022). Additionally accumulating amounts of cuticular wax, in *Populus* × *canescens* especially on the adaxial leaf side (Figures 3c,d and 4a), might be beneficial in providing, for example, mechanical stability or UV reflectance properties (Shepherd & Wynne Griffiths, 2006).

Since absolute cuticular wax amounts of 6-week-old leaves were the same between all lines (Figure 3c), the observed compositional alterations (>90% wax esters, weighted mean carbon chain length of 40.7) in *cer6_1,2* (Figures 3a,b and 4b,c) must have been responsible for the twofold higher cuticular permeance of *cer6_1,2* leaves (Figures 6c,d and 7). Most interestingly, our observations were exactly opposite to the initial literature-based hypothesis. According to the model summarised in Bueno, Alfarhan, et al. (2019), increased amounts of esters could bridge together crystalline (established by alkyl chains of different wax monomers) and amorphous (established by functional groups of different wax monomers) domains of cuticular wax, thus reinforcing the transport-limiting barrier. However, our data do not support this model since here, with poplar, increased cuticular permeance was observed in parallel with an increased wax ester fraction. Instead, it might be speculated that due to mutation-induced structural perturbations of the wax composition of yet unknown nature, for example, the lateral heterogeneity of the cuticle could have been altered, resulting in higher amounts of aqueous pores (Remus-Emsermann, 2011; Schönherr, 2006) leading to the twofold higher cuticular transpiration. This idea might be tested in the future with the aqueous pore-sealing AgCl precipitation technique (Schreiber et al., 2006) in the case that more matured greenhouse-cultivated plants of *cer6_1,2* would allow enzymatic isolation of adaxial cuticles. Taken together, these results acquired with *Populus* × *canescens* generally support the idea of qualitative wax composition affecting cuticular permeance, but yet again show that the relationships between wax and permeance are not exactly straightforward.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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