Bundle sheath suberisation is required for C₄ photosynthesis in a Setaria viridis mutant

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C₄ photosynthesis provides an effective solution for overcoming the catalytic inefficiency of Rubisco. The pathway is characterised by a biochemical CO₂ concentrating mechanism that operates across mesophyll and bundle sheath (BS) cells and relies on a gas tight BS compartment. A screen of a mutant population of Setaria viridis, an NADP-malic enzyme type C₄ monocot, generated using N-nitroso-N-methylurea identified a mutant with an amino acid change in the gene coding region of the ABCG transporter, a step in the suberin synthesis pathway. Here, Nile red staining, TEM, and GC/MS confirmed the alteration in suberin deposition in the BS cell wall of the mutant. We show that this has disrupted the suberin lamellae of BS cell wall and increased BS conductance to CO₂ diffusion more than two-fold in the mutant. Consequently, BS CO₂ partial pressure is reduced and CO₂ assimilation was impaired in the mutant. Our findings provide experimental evidence that a functional suberin lamellae is an essential anatomical feature for efficient C₄ photosynthesis in NADP-ME plants like S. viridis and have implications for engineering strategies to ensure future food security.
C₄ photosynthesis has independently evolved more than 60 times, providing one of the most widespread and effective solutions for overcoming the catalytic inefficiency of Rubisco.²,³ The pathway is characterised by a biochemical CO₂ concentrating mechanism that involves coordinated functioning of mesophyll (M) and bundle sheath (BS) cells within a leaf.¹ CO₂ is initially assimilated into C₄ acids by phosphoenolpyruvate (PEP) carboxylase in the mesophyll cells. These acids then diffuse to and are decarboxylated in BS cells where CO₂ is concentrated. This serves to enhance Rubisco carboxylation while at the same time inhibiting Rubisco oxygenation. Passive leakage of CO₂ out of the BS limits the efficiency of the system and represents an energy cost to the leaf as ATP is required to regenerate PEP.⁴

It has been hypothesised that low conductance to CO₂ diffusion across the M and BS interface is an essential feature of the C₄ photosynthetic CO₂ concentrating mechanism.⁵,⁶ The evolution of C₄ biochemical CO₂ concentration mechanism(s) has been accompanied by a suite of anatomical modifications broadly termed Kranz anatomy, an increase in vein density, and development of photosynthetic BS.⁹ However, the anatomical characterisation essential for low BS conductance is poorly understood.¹⁰ It has often been speculated that the interspecific diversity in gₛ may in part be due to variation in the presence of secondary thickening and suberisation of BS cell wall but diffusion path length and positioning of organelles may also be important.¹⁰ In NADP-malic enzyme (ME) and PEP carboxykinase C₄ subtypes, there is a deposition of the lipophilic heteropolymer suberin in the parenchymatous BS cell wall.¹¹ Chloroplasts in BS cells are centrifugally oriented only in suberised C₄ species, thus BS suberisation is thought to function in the restriction of CO₂ leakage across the M–BS interface during C₄ photosynthesis.¹³,¹⁴ The efficiency of the C₄ photosynthetic concentrating mechanism is intimately linked to BS thickness, ϕ, defined as the fraction of CO₂ generated by C₄ acid decarboxylation in the BS that subsequently leaks out. An increase in BS thickness results in an increase in photosynthetic carbon isotope discrimination.¹⁶ However, comparison of BS thickness of species with and without suberised BS cell wall have shown no difference, likely because of the differences in diffusion path length and organelle positioning in the species used.¹⁰

Setaria viridis (L.) P. Beauv. (green foxtail millet) is an NADP-ME type C₄ monocot species and its BS cell wall is suberised.¹² The principle of this screen was to expose NMU-treated and organlle positioning in the species used.¹⁰

**Results**

**Compromised growth in S. viridis mutant is a result of the mutation in the ABCG transporter gene.** Mutant plants grew poorly in ambient CO₂ concentration but when grown at 2% CO₂ concentration, grew at similar height to wildtype plants (Fig. 1a and Table 1) consistent with the growth response to high CO₂ concentration observed in other photosynthetically impaired NMU candidate mutants.¹⁹ However even in a 2% CO₂ growth environment, production of tillers and, therefore, shoot biomass remained significantly reduced in mutant plants compared to wildtype plants (Table 1). Root biomass was also significantly reduced in mutant plants compared to wildtype plants, making up to 25% of the total biomass in wildtype plants but only 13% in mutant plants (Table 1). To characterise the inheritance of the mutant phenotype, a segregating backcrossed population was generated (BC₁F₂). From the BC₁F₂ population, 25% of the segregants showed impaired chlorophyll fluorescence yield following low CO₂ treatment in the light, which is characteristic of the mutant phenotype (Supplementary Fig. 1). This result suggests that a single gene or locus is responsible for the mutant phenotype. Mapping-by sequencing analysis identified two candidate genes from the trait-associated locus of chromosome 9 (Supplementary Fig. 2). The first one (Sevir.9G451500) had a SNP in the 3’UTR, whereas the second one (Sevir.9G451500) had a SNP that caused an amino acid change from a positively charged Arginine to a more polar Serine (Fig. 1b). The former gene has an unknown specific function but is assigned to a co-expression network of genes induced by ammonia treatment (Supplementary Fig. 3). The mutation in this gene, however, is judged unlikely to have an effect on gene function as the mutation is not in the coding sequence or in the promoter region. On the other hand, Sevir.9G451500 encodes for an ABCG transporter gene. Peptide sequence analysis of Sevir.9G451500 showed that it is a half transporter as it contained only one nucleotide binding domain (NBD) and transmembrane domain (TMD) arranged in reverse orientation (Supplementary Fig. 4), a unique domain arrangement characteristic of members of the ABCG subfamily.²⁸ Protein sequence and structure analysis of ABCG protein family members reveal that the mutated residue Arginine (R552) is highly conserved across members of different kingdoms (frequency of ~66%), and when substituted, it is mostly by another positively charged residue, Lysine (frequency of ~16%) (Supplementary Table 1), indicative of its important functional role. Peptide sequence analysis of Sevir.9G451500 showed that R552 is located a few amino acids upstream of TMD in an amphipathic α-helix (Supplementary Fig. 4), which is previously reported to be a key component of the transmission interface essential for ABCG protein folding, ATP hydrolysis (via NBD), and substrate binding and transport (via TMD).²⁹ Expression analysis performed using existing maize²¹, rice²¹ and S. viridis²² data showed that this particular ABCG transporter gene is highly expressed in the basal region of Zea mays and S. viridis leaves, but not in Oryza sativa leaf (Fig. 1c). This corroborates the genetic association of the ABCG transporter gene with C₄ photosynthesis and leaf anatomy, most likely in BS wall suberisation as Sevir.9G451500’s direct orthologues in monocot and dicot C₄ plants (Supplementary Fig. 5) have previously been reported to be involved in suberin transport.³⁰–³³.

**Mutation in ABCG transporter gene impedes proper BS suberisation in S. viridis leaf.** Suberised BS in leaves of NADP-ME C₄ grasses is characterised by the presence of a dark, osmiophilic band deposited in the outer tangential wall and radial wall of the BS–M interface that can be visualised under an electron microscope. This contrasts with periderm and suberised cork.
cells, which have distinct layers of alternating light and dark lamellae corresponding to aliphatic suberin compounds and aromatic lignin-like compounds, respectively. Transmission electron microscopy (TEM) performed on wildtype and mutant leaf tissues revealed disrupted and thinner suberin lamellae in the BS cell wall of mutant compared to wildtype (Fig. 2a). Consequently, there was a significant reduction in BS cell wall thickness of mutant leaf relative to the wildtype leaf (Table 1). Disrupted dark lamellae are typically associated with defects in the aliphatic component of suberin. Nile Red staining, which targets the aliphatic suberin compounds, showed a lower signal in the BS of mutant leaves compared to wildtype (Fig. 2b) consistent with the reduced Fluorol Yellow staining in the mutant compared to the wildtype (Supplementary Fig. 6). Since the polyaromatic moiety of suberin shares similarities with lignin, staining after clearing using lignin stain (Basic Fuchsin) was performed. Basic Fuchsin staining showed the presence of lignin around BS of both wildtype and mutant leaves (Fig. 2c), most probably from the lignocellulosic tertiary wall but also likely contributed too by the hydroxycinnamic acids, ferulate and coumarate, which are lignin-like aromatic compounds abundant in suberin. To further investigate specific changes between the aliphatic and aromatic components of suberin in mutant leaves, particularly in the BS, gas chromatography/mass spectrometry (GC/MS) was performed. GC/MS analysis of suberin monomers obtained after BF3-methanol depolymerisation of solvent-extracted residue from leaf and isolated BS strands revealed a significant reduction of up to 50% in the aliphatic suberin compounds (C16–C26) in mutant relative to wildtype plants (Fig. 3 and Supplementary Table 2).
plants is mainly attributed to the BS as evidenced by the proportional increase in monomer concentrations obtained from the isolated BS strands compared to the leaf (Table 1 and Supplementary Table 2). In addition, there was no significant difference between the cutin layer of wildtype and mutant leaves (Supplementary Fig. 7) suggesting that cutin deposition in leaves of mutant plants is not affected by the mutation. There was no significant difference between wildtype and mutant aromatic suberin monomers in leaf and isolated BS strands (Supplementary Fig. 7) suggesting that cutin deposition in leaves of mutant plants presumably due to direct CO2 diffusion through the BS.

Absence of functional suberin lamellae resulted in leaky BS in *S. viridis* mutant. CO2 assimilation rates were reduced in the mutant plants compared to wildtype plants (Fig. 4a). Although the initial slope of the CO2 response of CO2 assimilation is similar for mutant and wildtype plants, CO2 assimilation rates of the mutant fail to increase to the same degree as wildtype above an intercellular CO2 of 100 µbar. The reduced CO2 assimilation rate of the mutant is also apparent in the light response of CO2 assimilation rate (Fig. 4b). Stomatal conductance is also reduced in the mutant plants (Fig. 4c), but the intercellular CO2 partial pressure is similar between mutants and wildtype leaves so that reduced CO2 access is not the cause of the reduction in CO2 assimilation rates. The reduction in CO2 assimilation rates cannot be explained by reduced photosynthetic biochemistry as the in vitro PEPC, Rubisco and carbonic anhydrase activities were similar between wildtype and mutants (Table 1). The reduction of stomatal conductance in the mutant was observed despite increased stomatal density (Table 1) pointing to a possible mechanical effect of reduced suberin on stomatal opening.

**Table 1 Comparative analysis of different plant and leaf properties between *Setaria viridis* wildtype and mutant grown at 2% CO2 environment.**

| Properties                          | Units          | n     | Wildtype | Mutant | Effect size | p-Value |
|-------------------------------------|----------------|-------|----------|--------|-------------|---------|
| **Plant growth and biomass measurements** |                |       |          |        |             |         |
| Plant height                        | cm             | 5-6   | 61.7 ± 2 | 58.7 ± 2 | 0.9452     | 0.36924 |
| Tiller number                       | no.            | 5-6   | 12.4 ± 0.7 | 7.3 ± 0.8** | 4.69928 | 0.00112 |
| Shoot dry weight                    | g              | 5-6   | 8.8 ± 0.7 | 6.5 ± 0.5* | 2.71083   | 0.02396 |
| Root dry weight                     | g              | 5-6   | 3.0 ± 0.7 | 1.0 ± 0.2* | 3.14176   | 0.01189 |
| **Leaf anatomical measurements**    |                |       |          |        |             |         |
| BS cell wall thickness              | µm             | 34-40 | 0.21 ± 0.01 | 0.17 ± 0.01*** | 4.7862 | 8.80445E−6 |
| M cell wall thickness               | µm             | 32-38 | 0.21 ± 0.01 | 0.22 ± 0.01 | −0.80949 | 0.42106 |
| Sb                                  | m² m⁻²         | 14-17 | 1.4 ± 0.03 | 1.4 ± 0.03 | 0.10757   | 0.91508 |
| Sw                                  | m² m⁻²         | 14    | 10.3 ± 0.3 | 9.5 ± 0.4  | 1.38164   | 0.17884 |
| Pit field area per M-BS cell interface area | %       | 9     | 15.9 ± 0.5 | 16.1 ± 0.2 | −0.40047 | 0.69411 |
| Stomatal density                    | no. mm⁻²       | 20    | 122.5 ± 3 | 135.5 ± 3** | −3.29462 | 0.00214 |
| Stomatal index                      | %              | 20    | 20.2 ± 0.3 | 20.6 ± 0.2 | −1.20009  | 0.23753 |
| **Gas exchange measurements**       |                |       |          |        |             |         |
| CO2 assimilation rate               | µmol m⁻² s⁻¹   | 4     | 30.2 ± 2.0 | 18.4 ± 1.2** | 5.16319   | 0.00209 |
| Stomatal conductance                | mol H₂O m⁻² s⁻¹ | 4     | 0.17 ± 0.02 | 0.12 ± 0.01* | 3.25543   | 0.01735 |
| Quantum yield                       | mol CO₂ (mol incident quanta)⁻¹  | 4     | 0.05 ± 0.001 | 0.04 ± 0.001*** | 7.75341   | 2.14969E−4 |
| Ratio of intercellular to ambient CO₂|                | 8     | 0.41 ± 0.02 | 0.52 ± 0.04*  | −2.33862  | 0.03471 |
| C/CC                               | %              | 8     | 3.55 ± 0.1 | 3.93 ± 0.2 | −1.65824  | 0.1195 |
| Δ¹³C measured with gas exchange     | %e             | 8     | 0.29 ± 0.01 | 0.34 ± 0.01* | −2.54362  | 0.02341 |
| Bundle sheath leasiness              | %              | 8     | 20.5 ± 1.2 | 21.8 ± 2.0  | −0.53415  | 0.61244 |
| **Biochemical measurements**        |                |       |          |        |             |         |
| Rubisco maximal activity            | µmol m⁻² s⁻¹   | 4     | 20.5 ± 1.2 | 21.8 ± 2.0  | −0.53415  | 0.61244 |
| Rubisco sites                       | µmol m⁻²       | 4     | 5.4 ± 0.1  | 6.0 ± 0.3  | −1.69453  | 0.1411 |
| PEPC maximal activity               | µmol m⁻² s⁻¹   | 4     | 167.0 ± 7.5 | 184.3 ± 15.5 | −1.00683  | 0.35288 |
| Carbonic anhydrase (rate constant)  | mol m⁻² s⁻¹ bar⁻¹ | 4   | 12.6 ± 1.1 | 11.2 ± 1.1  | 0.86485   | 0.42035 |

Values are the mean ± SE (see Supplementary Data). The number of measurements made is indicated by n. Asterisks denote statistically significant difference (*p < 0.05, **p < 0.01, ***p < 0.001) of mutant relative to wildtype according to two-sample t-test (OriginPro 9.1, OriginLab Corporation).

*Measured at a leaf temperature of 25 °C, relative humidity of 55%, 21% O₂, ambient CO₂ concentration of 380 µmol mol⁻¹ and irradiance of 2000 µmol quanta m⁻² s⁻¹.

**Calculated as the initial slope of the CO2 assimilation rate at 0, 25, 50, 75, 100, 150, and 200 µmol quanta m⁻² s⁻¹ irradiances.

*Measured at 2% CO2, 380 µmol mol⁻¹ CO2 leaf temperature of 25 °C, irradiance of 1500 µmol quanta m⁻² s⁻¹ and relative humidity of 55%.
mutant compared to the wildtype commensurate with a loss of photosynthetic efficiency (Table 1).

**Discussion**

We have investigated a C₄ photosynthesis mutant (NM03966), identified using chlorophyll fluorescence-based high throughput phenotyping platform, for its underlying genetic alteration and mapped an ABCG transporter gene, Sevir.9G451500, as the causal gene. Our phylogenetic analysis revealed that the direct orthologues of this gene in monocot and dicot plants/crops have previously been reported in suberin transport. Our gene expression analysis also revealed that this gene is highly expressed in NADP-type C₄ leaves but not in C₃ leaves. We have used TEM, two recognised suberin stains, and GC/MS to confirm the reduction in leaf suberin, particularly in the BS cells, of Sevir.9G451500 or ABCG transporter gene mutant. The reduction in suberin is leaf-specific and does not affect suberin in the roots, suggesting that most of the differences in growth observed in mutant plants compared to wildtype plants are due to reduced CO₂ assimilation rate. This increased BS leakiness, consequently increasing the oxygen sensitivity of the CO₂ assimilation. While the physiological phenotypes of this mutant are largely predicted by models of C₄ photosynthesis (Supplementary Fig. 10), the decreased CO₂ assimilation rate in the mutant, particularly at high CO₂ and irradiance, is greater than can be predicted from an increase in gs alone, suggesting that other metabolic impairments are occurring (comparison of Fig. 4g and Supplementary Fig. 10). The moderate increase in carbon isotope discrimination observed here would indicate that BS leakiness increased by only 17%. As leakiness, \( \phi \), is defined as

\[
\phi = \frac{g_{bs}(C_{bs} - C_m)}{V_p}
\]

where \( C_m \) and \( C_{bs} \) are M and BS CO₂ partial pressures and \( V_p \) is the rate of PEP carboxylation, one must assume that some impairment in the rate of PEP carboxylation has occurred, restricting the capacity of the M reactions to respond and elevate CO₂ supply to the BS. As the extractable activity of PEPC and Rubisco from the mutant plants did not differ from that found for the wildtype plants (Table 1), either the provision of PEP to PEP carboxylase must become limiting at higher fluxes (potentially via a limitation in ATP supply) or posttranslational downregulation of enzyme activity in the M is occurring. Notably, in transgenic *Flaveria bidentis* with reductions in Rubisco activity due to *RbcS* gene suppression, greater BS CO₂ leakiness occurred due to elevated BS CO₂. While \( V_p \) was reduced in these plants, this was not commensurate with the reduction in net CO₂ assimilation. In the case of *Flaveria* plants, where NADP-ME activity was reduced by gene suppression, a reduction in BS leakiness to CO₂ was observed as predicted. However, the amount of both Rubisco and PEPC actually increased despite a substantial decrease in \( V_p \) being observed. While we have not previously had access to C₄ plants where BS cell wall properties were genetically altered rather than

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**Fig. 2** Mutation in ABCG transporter gene Sevir.9G451500 impedes proper bundle sheath suberisation in *Setaria viridis* leaf. a Electron micrographs of transverse leaf sections of *Setaria viridis* wildtype and mutant showing disrupted and thinner suberin lamellae (black arrows) in mutant bundle sheath (BS) cell wall. M, mesophyll; P, plastids; M, mesophyll; *, plasmodesmata. Bars = 0.5 µm. b Orthogonal view of z-stacks of cleared leaf tissue of *S. viridis* wildtype and mutant stained with Nile Red (magenta, aliphatic component of suberin) and Calcofluor White (cyan, cell wall) showing decreased level of aliphatic suberin staining in mutant bundle sheaths (see Supplementary Videos 1 and 2). Line graphs show the profile of Nile Red fluorescence along the leaf tissue, with wildtype showing peaks where bundle sheaths (white arrows) are present. Bars = 50 µm. c Transverse hand section of leaf of *S. viridis* wildtype and mutant stained with Basic Fuchsin (yellow, lignin) and Calcofluor White (cyan, cell wall) showing unaltered lignin deposition in mutant leaf. Bars = 50 µm.
cell-specific enzyme activities, taken together these experiments suggest that a variety of regulatory processes may be occurring to match BS and M biochemical fluxes and minimise CO2 leakage.

To conclude, the *S. viridis* ABCG transporter gene *Sevir.9G451500* mutant provided the opportunity to analyse the effect BS cell wall properties have on the C4 photosynthetic CO2 concentrating mechanism. We have shown that the disruption of the suberin lamellae has increased BS conductance to CO2, which resulted in reduced CO2 assimilation rates due to reduced BS CO2 partial pressure. Anatomical and biochemical assays showed few other differences between mutant and wildtype plants other than stomatal function, which is accounted for in gas exchange measurements. This work experimentally demonstrates the importance of a functional suberin lamella around the BS for efficient C4 photosynthesis in the leaves of *S. viridis*, which have centrifugally oriented chloroplasts, and represents a central discovery for understanding the C4 photosynthetic mechanism, the evolution of C4 photosynthesis and the engineering of this process into C3 crops.

**Methods**

**Plant material and growth conditions.** *M*$_5$ seeds were grown in Australia between March and August 2019. The seeds were germinated in garden soil mix fertilised with Osmocote (Scotts, Australia) in small containers before being transferred to individual 2 L pots. Plants were grown in controlled environmental chambers, irradiance 500 µmol photons m$^{-2}$ s$^{-1}$, 16-h photoperiod, 28 °C day, 24 °C night, 2% CO2. Pots were watered daily.

**Plant growth and biomass measurements.** The whole plant was removed from the pot and the roots were washed with water to remove any adhering soil. Plant height was measured from the base of the shoot using the main tiller. After counting the number of tillers per plant, the roots and shoots were dried separately at 80 °C oven for 5 days and weighed.

**Generation of segregating population and DNA sequencing of bulked segregants.** Homozygous mutant *M*$_5$ plants were crossed with wildtype to generate F$_1$ heterozygous lines. The F$_1$ lines were selfed to produce segregating F$_2$ populations (BC$_1F_2$). From a BC$_1F_2$ population of 300 plants (Supplementary Fig. 1), equal quantities of DNA were pooled from 45 individual plants exhibiting the suberin phenotype (homozygous mutant pool) and 54 individuals without the suberin phenotype (pool of azygous or hemizygous lines). DNA was also pooled from 50 individual WT plants (WT pool). The genomic DNA was extracted from young

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**Fig. 3 Mutation in ABCG transporter gene *Sevir.9G451500* affects the synthesis of aliphatic suberin monomers in *Setaria viridis* mutant.** Relative suberin monomer composition of leaf and bundle sheath strands of *Setaria viridis* wildtype and mutant showing reduced aliphatic monomers in mutant, n = 5. Note the difference in y-axis scale between leaves and bundle sheath strands plots. Statistically different values according to one-way analysis of variance are denoted by * at p < 0.05, ** at p < 0.01 and *** at p < 0.001. Numerical values of the relative average concentration expressed as µg monomer per g tissue, standard error and p-value according to one-way analysis of variance of each suberin monomer of *S. viridis* wildtype and mutant leaves and bundle sheath strands after BF$_3$-MeOH depolymerisation are provided in Supplementary Table 2. Individual measured values are provided in Supplementary Data.
leaves mid-tillering using a cetyltrimethylammonium bromide (CTAB) protocol. The DNA quantity and quality were evaluated using a Nanodrop ND-8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and agarose electrophoresis. The three samples were sequenced using Illumina HiSeq2000 platforms at The Beijing Genome Institute (BGI Tech Solutions (Hongkong) CO. Limited, Shenzhen, China). The reads were paired-end (PE) of size 125 base pairs. Number of paired-end reads generated were ~108.1 million for WT pool, ~91 million for azygous pool and ~107.3 million for mutant pool yielding 23–33 Giga-bases of genomic sequence with a genome coverage of 45–65X.

Variation discovery. The raw sequence data were processed so that reads/bases with poor quality are either trimmed or filtered using Trimmomatic v0.32 (ref. 48). The minimum Phred quality score was set to 20, and reads containing partial/complete Illumina adaptor sequences were also trimmed. The processed reads were aligned against the S. viridis reference genome (version 1.1 from Phytozome database) using BWA aligner version 0.7.12 (ref. 49). The alignment was improved by discarding the duplicate read pairs with the PICARD tool (https://broadinstitute.github.io/picard/), and re-calibration of base quality using very high-quality variants as a gold standard using the GenomeAnalysisTK (GATK-3.7-0) tool90. Variant calling was also done by the same tool such that alleles were first called at all genomic positions, followed by variant calling51.

Filtering of variants to identify candidates. Variants with an allele frequency of 0.3 or less were considered to result from improper alignment of minority reads, thus, removed from the analysis. Only variants occurring in the pooled mutant samples with at least 10 reads and a genotype quality (GQ) of called variants of 30 or above were considered for downstream analysis. In order to discover allele(s) which were present in all individuals (that were pooled), the frequency of induced mutations (mutant alleles) in the paired samples were plotted along chromosome position. Theoretically, a mutation with allele frequency of 1 in the mutant pool should display a frequency of 0.5 or less in the azygous pool. Genomic regions/loci harbouring mutations qualifying above filters were extracted, and were called candidate mutations. Variants with an allele frequency of 1 were checked to see if neighbouring ones showed a typical linkage pattern.

Causal gene discovery. Candidate mutations with an allele frequency of 1 in the mutant pool were annotated with the S. viridis annotation from Phytozome 12 (https://phytozome.jgi.doe.gov/pz/portal.html). Mutations within a gene leading to alterations in protein structure were classified as candidate genes.

Suberin and lignin staining. Tissue from the widest portion of the third fully expanded leaf of 10-day-old plant was fixed with 4% paraformaldehyde for 1 h at room temperature, sectioned, and stained along with transverse sections of the root. Aliphatic suberin staining was performed using 0.05% Nile Red in ClearSee solution (10% (w/v) xylitol, 15% (w/v) sodium deoxycholate, 25% (w/v) urea in water) while lignin staining used 0.2% Basic Fuchsin in ClearSee solution; 0.1% Calcofluor White was used in combination with Nile Red and Basic Fuchsin to visualise cell walls. Nile Red-stained and Basic Fuchsin-stained tissues and sections were mounted onto glass slides with ClearSee solution. Fluorol Yellow staining was performed according to Yadav et al.31. Slides were examined with a Leica SP8 multiphoton confocal microscope (Leica Microsystems). Nile Red was imaged at 561 nm excitation wavelength and detected at 600–620 nm. Basic Fuchsin was imaged at 561 nm excitation wavelength and detected at 600–650 nm. Fluorescence from Calcofluor White-stained cell walls was detected at 434–445 nm following excitation at 405 nm. Fluorol Yellow was imaged at 514 nm excitation wavelength and detected at 516–593 nm.

Anatomical measurements. Sample preparation for light microscopy and electron microscopy was performed using the middle portion of the youngest fully

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**Fig. 4 Absence of functional suberin lamellae resulted in leaky bundle sheath in Setaria viridis Sevir.9G451500 mutant.**

- **a** CO₂ assimilation rate over a range of intercellular partial pressures of CO₂ and **b** CO₂ assimilation rate and **c** stomatal conductance over a range of irradiances of Setaria viridis wildtype and mutant, n = 4. Time course of CO₂ assimilation rate of detached S. viridis wildtype and mutants leaves after feeding with 2–4 mM DCDP measured at 400 μbar CO₂, and subsequent measurements of CO₂ response curves of DCDP-fed leaves over a range of pCO₂, n = 4. The initial slope of the CO₂ response curve was used to calculate bundle sheath conductance (gbs)42. For wildtype gbs = 2.8 ± 0.3 mmol m⁻² s⁻¹ bar⁻¹ and for the suberin mutant gbs = 7.4 ± 0.7 mmol m⁻² s⁻¹ bar⁻¹. 

- **d** Carbon isotope discrimination, δ, as a function of the ratio of intercellular to ambient CO₂, C/Cₐ in S. viridis wildtype and mutant. Measurements were made at 2% O₂, 380 μmol mol⁻¹ CO₂, leaf temperature of 25 °C, irradiance of 1500 μmol quanta m⁻² s⁻¹ and relative humidity of 55% using combined tuneable diode laser spectroscopy and gas exchange measurements, n = 8. The lines depict the predicted theoretical relationship between δ and C/Cₐ during C₄ photosynthesis at infinite gₐₚ at a leakage, ϕ = 0.34 and 0.29 (Δ = 4.3 ± (−5.7 – 4.3 + 27.2 × δ)C/Cₐ)16,57. 

- **e** CO₂ assimilation rate over a range of intercellular partial pressures of CO₂ made consecutively at 21%, 2% and 10% O₂, n = 4. Suberin mutant gₐₚ = 7.4 mmol ± 0.7 m² s⁻¹ bar⁻¹

- **f** Ratio of intercellular to ambient CO₂, C/Cₐ for wildtype and suberin mutant leaves after feeding with 2 μmol nitrate. The lines depict the predicted theoretical relationship between C/Cₐ and C/Cₐ during C₄ photosynthesis at infinite gₐₚ at a leakage, ϕ = 0.34 and 0.29 (Δ = 4.3 ± (−5.7 – 4.3 + 27.2 × δ)C/Cₐ).
expanded leaf of 14-day-old plants. Light micrographs used to calculate BS surface area per unit leaf area (SBS) and M surface area exposed to intercellular airspace (SMB) were obtained using Leica DMD5000 compound microscope (Leica Microsystems). BS and M cell wall thickness were measured using electron micrographs of the transverse leaf sections imaged under Hitachi 7100 transmission electron microscope (Hitachi High Technologies America). Pit field area per cell interface area was used as a measure of plasmodesmata density. Stomatal measurements were taken from the abaxial side using regions close to the midrib within the widest portion of the youngest fully expanded leaves. Stomatal imprints were obtained as described and images were captured under 10× objective using Leica DMD5000 compound microscope. Stomatal density (number of stomata/unit leaf area) and stomatal index (proportion of epidermal cells that are stomata) were quantified. All anatomical measurements were performed using ImageJ.

**GC/MS sample preparation.** Isolated BS strands were used as a measure of plasmodesmata density. Four 5 ms capillary column (CP9013); helium carrier gas; constant column gas flow 20 mL min⁻¹ at 25 °C, irradiance 1500 µmol quanta m⁻² s⁻¹. Gas exchange measurements combined with tunable diode laser measurement were made for 1°C isotope discrimination. Measurements were made at 2% O₂, 380 µmol mol⁻¹ CO₂, leaf temperature of 25 °C, irradiance of 1500 µmol quanta m⁻² s⁻¹ and relative humidity of 55%. Leakiness, defined as the ratio of CO₂ leak rate out of the BS over the rate of CO₂ delivery to the BS, was calculated from the simplest form of the equation relating leakiness to carbon isotope discrimination, \[ \Delta = 4.3 +(-5.7 - 4.3 + 27.2 x)C_4/C_3. \]

**PPEC inhibitor DCDP feeding and estimation of BS conductance, \( g_{bs} \).** DCDP feeding in detached leaves and estimation of BS conductance were performed using the first method described in ref. 42.

**Biochemical measurements.** Measurements of Rubisco, PEPc and carbonic anhydrase activity were made as described. Rubisco catalytic site content were measured for both non-DCDP-fed plants and DCDP-fed plants.

**Statistics and reproducibility.** Significance of all experimental data between wildtype and mutant were determined according to two-sample t-test or one-way analysis of variance using OriginPro 9.1 (OriginLab Corporation). No outliers were excluded in any statistical analysis. Figures were generated using OriginPro 9.1 (OriginLab Corporation) and Microsoft PowerPoint. Details of sample size or replication number were listed in Table 1 and described in the figure legends.

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**References**

1. Christin, P.-A. & Osborne, C. P. The recurrent assembly of C4 photosynthesis, an evolutionary tale. *Photosynth. Res.* 117, 163–175 (2013).
2. Sage, R. F., Sage, T. L. & Kocacinar, F. Photorespiration and the evolution of C₄ photosynthesis. *Annu. Rev. Plant Biol.* 63, 19–47 (2012).
3. Hatch, M. D. C₄ photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. *Biochim. Biophys. Acta Rev. Bioenerg.* 895, 81–106 (1987).
4. Furbank, R., Jenkins, C. & Hatch, M. C₄ photosynthesis: quantum requirement, C₄ acid overcycling and Qₐ. *Funct. Plant Biol.* 17, 1–7 (1990).
5. Berry, J. A. & Farquhar, G. D. In *Proc. Fourth International Congress on Photosynthesis* (eds Hall, D. et al.) 119–131 (Biochemical Society of London, 1978).
6. Jenkins, C. L. D., Furbank, R. T. & Hatch, M. D. Mechanism of C₄ photosynthesis, a model describing the inorganic pool in bundle sheath cells. *Plant Physiol.* 91, 1372–1381 (1989).
7. Sage, R. F. The evolution of C₄ photosynthesis. *New Phytol.* 161, 341–370 (2004).
8. Danila, F. R. et al. Multiple mechanisms for enhanced plastomesdmsata density in disparate subtypes of C₄ grasses. *J. Exp. Bot.* 69, 1135–1145 (2018).
9. Nelson, T. in *C4 Photosynthesis and Related CO₂ Concentrating Mechanisms* (eds Raghavendra, A. A. & Sage, R. F.) 147–159 (Springer, 2011).
10. von Caemmerer, S. & Furbank, R. T. The C₄ pathway: an efficient CO₂ pump. *Photosynth. Res.* 77, 191–207 (2003).
11. Hattersley, P. W. & Browning, A. J. Occurrence of the suberized lamella in leaves of grasses of different photosynthetic types. I. In parenchymatous bundle sheaths and PCR (“Kranz”) sheaths. *Protoplasma* 109, 371–401 (1981).
12. Dengler, N. G., Dengler, R. E., Donnelly, P. M. & Hattersley, P. W. Quantitative leaf anatomy of C₄ and grasses (Poaceae): bundle sheath and mesophyll surface area relationships. *Ann. Bot.* 73, 241–255 (1994).
13. Laetsch, W. M. The C₄ syndrome: a structural analysis. *Annu. Rev. Plant Physiol.* 25, 27–52 (1974).
Competing interests
The authors declare no competing interests.

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