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Key Words:	cuticle biosynthesis, evolution, regulatory network, MYB transcription factor, papillate cells, plant terrestrialisation

The land plant-specific MIXTA-MYB lineage is implicated in the early evolution of the plant cuticle and the colonization of land.

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Abstract

- The evolution of a lipid-based cuticle on aerial plant surfaces protects against dehydration is considered as a fundamental innovation in the colonization of the land by the green plants. However, key evolutionary steps in the early regulation of cuticle synthesis are still poorly understood due to limited studies in early diverging land plant lineages.
- Here, we characterise a land plant specific subgroup 9 R2R3 MYB transcription factor MpSBG9, in the early diverging land plant model *Marchantia polymorpha*, that is homologous to MIXTA genes in vascular plants.
- The MpSBG9 functions as a key regulator of cuticle biosynthesisby preferentially

regulating expression of orthologous genes for cutin formation, but not wax biosynthesis genes, implying conserved MYB transcriptional regulation in controlling the cutin biosynthesis pathway as a core genetic network in the common ancestor of all land plants.

- The MpSBG9 homolog also promotes the formation of papillate cells on the adaxial surface of *M. polymorpha*, which is consistent with its canonical role in vascular plants.
- The identification of this conserved role in regulating cuticle synthesis implicates the land-plant specific MIXTA MYB lineage in the early origin and evolution of the cuticle.

Key words: cuticle biosynthesis, evolution, regulatory network, MYB transcription factor, papillate cells, plant terrestrialisation

Introduction

The colonization of land by plants was a milestone in the evolution of life, which has had a profound impact on global ecosystems (Kenrick & Crane, 1997; Bateman *et al.*, 1998). Water availability is one of the major limitations of terrestrial habitats. In plants, the evolutionary transition from aquatic to terrestrial has been accompanied by a suite of adaptations to minimize water loss, facilitating survival and reproduction in a desiccating environment (Corner, 1964; Graham *et al.*, 2000). The plant cuticle is one such adaptation, synthesized and secreted by epidermal cells, and forming a continuous hydrophobic layer at the interface between plant and environment (Raven, 1977; Kolattukudy, 1980). The primary function of this waterproof layer is to limit non-stomatal transpiration, providing protection against water loss from plants to the atmosphere (Kolattukudy, 2001; Nawrath, 2006). In addition, cuticle can also serve as a physical barrier against UV radiation, pathogen attack and mechanical damage (Kolattukudy, 2001; Nawrath, 2006). The cuticle is considered to be one of the first of the key innovations necessary to colonize land (Corner, 1964), however, compared to

other land plant traits such as stomata (Chater *et al.*, 2016), vasculature (Xu *et al.*, 2014; Lu *et al.*, 2020), and roots (Menand *et al.*, 2007; Hetherington & Dolan, 2018), relatively little is known about the genetic pathways underpinning development of the cuticle in early diverging land plants..

The cuticle in vascular plants is mainly composed of cutins and waxes (Yeats & Rose, 2013). In most vascular plants, cutins contain a high amount of hydroxylated fatty acids of carbon chain length C16 and C18, with the addition of moieties such as glycerol and phenyl-propanoids (Nawrath, 2006; Li-Beisson *et al.* 2013). These components form a network of cross-linked polyesters, which are covalently linked to polysaccharides on the surface of epidermal cell walls (Fich *et al.*, 2016; Philippe *et al.*, 2020). Cutin is insoluble and resistant to degradation, serving as a scaffold for impregnation by waxes, which further contribute to the structure and properties of the cuticle. Cuticular waxes are soluble complex mixtures, consisting of very-long chain fatty acids (VLCFAs) and their derivatives, including ketones, alkanes, primary and secondary alcohols, aldehydes, and wax esters, with mostly carbon chain length ranging from C24 to C40 (Bernard & Joubès, 2013; Yeats & Rose, 2013). Cuticular waxes, depending on their location, are further defined as intra-cuticular waxes, which are impregnated in the cutin matrix, and epi-cuticular waxes, which are assembled as free wax crystals on the surface of the cutin matrix. Cutins and waxes start as long-chain fatty acids in the endoplasmic reticulum but are synthesized by different pathways, and their formation can be roughly divided into three steps: elongation and modification; transport of monomers to the epidermal surface; and subsequent assembly on the outer surface of epidermal cell walls (Bernard & Joubès, 2013; Li-Beisson *et al.* 2013).

Recent studies have made considerable progress in understanding the biochemical and genetic basis of cuticle formation in vascular plants (Yeats & Rose, 2013; Domínguez *et al.*, 2017; Fich *et al.*, 2016;). Formation of cutin monomers mainly involves esterification of free fatty acids C16 and C18 with Coenzyme A (CoA) by Long-chain acyl-CoA synthetase (LACS), hydroxylation of acyl-CoA by CYP86A and CYP77A proteins from the cytochrome P450 enzyme family, as well as acyl

transfer of activated fatty acids to glycerol by glycerol-3-phosphate acyltransferase (GPAT) to produce mono-acyl glycerol (MAGs) (Wellesen *et al.*, 2001; Schnurr *et al.*, 2004; Li *et al.*, 2007; Li-Beisson *et al.*, 2009; Lu *et al.*, 2009; Li-Beisson *et al.*, 2013). The cutin monomers are subsequently transported to the cell surface by plasma membrane localized ATP-binding cassette subfamily G (ABCG) transporters (such as ABCG11 and ABCG32) as well as lipid transfer proteins (LTPs), before undergoing polymerization, catalyzed by the GDSL-motif lipase/hydrolase family protein (GDSL) cutin synthase (CUS) (Debono *et al.*, 2009; Bessire *et al.*, 2011; Panikashvili *et al.*, 2011; Yeats *et al.*, 2012; Yeats *et al.*, 2014). The biosynthesis of cuticular waxes constitutes a largely distinct pathway. Sharing the first step with the cutin pathway, the carbon chain of acyl-CoAs is extended to generate a wide range of chain lengths by the Fatty Acid Elongase (FAE) complex consisting of β-ketoacyl-coA synthase (KCS), β-ketoacyl-coa reductase (KCR), 3-hydroxyacyl-coA dehydratase (HCD), and enoyl-coA reductase (ECR) (Millar *et al.*, 1999; Todd *et al.*, 1999; Zheng *et al.*, 2005; Bach *et al.*, 2008; Beaudoin *et al.*, 2009; Kim *et al.*, 2013). The very long chain acyl-CoA precursors then feed into two different pathways. The alcohol forming pathway for the production of primary alcohols and wax esters is catalyzed by ECERIFERUM4 (CER4) and wax synthase/diacylglycerol diacyltransferase1 (WSD1) (Rowland *et al.*, 2006; Li *et al.*, 2008). The alkane forming pathway for the production of aldehyde, alkanes, secondary alcohols, and ketones is catalyzed by a complex composed of CER1, CER3, and cytochrome B5 (CYTB5) and further transformed into secondary alcohols and ketones by midchain alkane hydroxylase 1 (MAH1), a cytochrome CYP95A enzyme (Aarts *et al.*, 1995; Chen *et al.*, 2003; Bernard *et al.*, 2012). All these wax components are exported to the apoplast by ABCG transporters and LTPs, where they are assembled into cuticular waxes (Debono *et al.*, 2009; Bessire *et al.*, 2011; Panikashvili *et al.*, 2011).

Although the majority of genetic studies of the cuticle have taken place in vascular plant systems, a limited number of studies have confirmed the role of genetic orthologs in the synthesis of cuticle in early diverging land plants. Genetic knock-out of an ATP-binding cassette protein from subfamily G (*Pp*ABCG7) from the model

moss *Physcomitrella patens* resulted in impaired cuticle deposition and reduced tolerance to dehydration stress (Buda *et al.*, 2013). Disruption of glycerol-3-phosphate acyltransferases (*Pp*GPAT2 and *Pp*GPAT4) in *P. patens* results in growth retardation, reduced cuticle permeability, and reduced tolerance to drought, osmotic and salt stress (Lee *et al.*, 2020). A recent study has identified a member of the cytochrome P450 gene family, CYP98, as important in the synthesis of the unusual phenolic-rich composition of the cuticle in *P. patens* (Renault *et al.*, 2017). Finally, orthologues of cutin synthase (*PpCUS1*) from *P. patens* have been shown to perform the requisite polyester synthase activity *in vitro*, although their *in planta* phenotype is untested (Yeats *et al.*, 2014). However, notably all of these studies have taken place in the context of the model moss *P. patens*, and to date, to our knowledge, there has been no genetic insight into cuticle formation in other early diverging model species such as *Marchantia polymorpha*.

Although cuticle is present on the epidermis of aerial tissues in all land plant species, the composition, structure, and thickness of cuticle vary greatly among tissues and developmental stages (Lee *et al.*, 2015). Cuticle deposition is regulated in coordination with plant development (Lee *et al.*, 2015; Fich *et al.*, 2016). In flowering plants, the first transcription factor responsible for wax deposition was reported as WAX INDUCER1 (WIN1)/SHINE1 (SHN1), an AP2/ERF family protein in *Arabidopsis thaliana* (Aharoni *et al.*, 2004; Broun *et al.*, 2004). Further analysis of WIN1/SHN1 also showed that cutin production was increased by overexpression of WIN1/SHN1 (Kannangara *et al.*, 2007). MIXTA, an R2R3 MYB transcription factor from *Antirrhinum majus,* was previously identified as a regulator of the development and formation of the conical shape of petal epidermal cells (Noda *et al.*, 1994). However, in addition to their role in epidermal cell differentiation, recent studies report that proteins related to MIXTA, such as MYB106, control cuticle formation in *A. thaliana* by direct activation of cuticle biosynthesis genes or indirect upregulation via SHN genes (Oshima *et al.*, 2013). These findings are supported by studies in *Solanum lycopersicum* where SlSHN3 targets expression of SlMIXTA-like protein (Lashbrooke *et al.*, 2015). Additional MYB transcription factors are also implicated in

cuticle induction in response to various environmental signals and biotic stresses. For example, MYB94 and MYB96 upregulate cuticular wax formation to enhance tolerance to drought in *A. thaliana*, mediated by ABA signals (Seo *et al.*, 2009; Lee *et al.*, 2016). *MYB41* is expressed under osmotic and high salinity conditions, and overexpression of *MYB41* affected the permeability of cuticle in the leaf in *A. thaliana* (Cominelli *et al.*, 2008). Finally, MYB30 has been reported to play a positive role in pathogen response by targeting the VLCFA biosynthesis pathway to influence cuticular wax formation (Raffaele *et al.*, 2008).

Studies from vascular plant species have therefore revealed a genetic network regulating cuticle biosynthesis, in which MYB transcription factors play a central role. A handful of orthologous structural components of the downstream genetic pathway have been shown to operate in early-diverging land plants. To date, however, the extent to which a similar transcription factor network controlling cuticle biosynthesis is operating in the earliest diverging land plant lineages is unclear. In the present study, by identification and functional characterization of a MYB transcription factor MpSBG9 protein in the early diverging land plant *Marchantia polymorpha*, we show that a core component of the genetic network regulating cuticle biosynthesis and epidermal cell fate is highly conserved across land plants, with a phylogenetic origin that is specific to land plants.

Materials and Methods

Plant materials and growth conditions

The 'Cambridge' strain *M. polymorpha* (Pollak *et al.*, 2017) was grown on half strength Gamborg's B5 media with vitamins (pH5.8) containing 1% (w/v) agar at 22°C under 16 h-white light and 8 h-dark regime. To induce reproductive organs, *M. polymorpha* gemmae were propagated on agar plates for 10 days, and then transplanted into soil at 22°C under 16 h-white light and 8 h-dark conditions in a growth room. After 14 days, the plants were supplemented with far red light (Philips).

Nicotiana. tabacum was grown at 25°C under 16 h-white light and 8 h-dark conditions in a growth room.

Phylogenetic analysis

A previously published MYB gene dataset including all R2R3 MYB genes from *A. thaliana*, plus a selection of functionally characterized R2R3 MYB genes from additional plant models (Stracke *et al.*, 2014), was combined with a number of predicted annotated R2R3 MYBs from an early draft of the *M. polymorpha* genome. The dataset comprised 218 sequences, and was translated to amino-acid sequence, and then aligned by MAFFT. The alignment was subject to analysis by FASTtree, with associated calculation of Fasttree SH support values. The immediate Marchantia homolog of the Subgroup9A clade (containing MIXTA and MIXTA-like genes) was identified, with 99% support value.

DNA/RNA extraction and cDNA synthesis

About 100mg of fresh thalli from 10-day-old plants was ground frozen using a Tissue Lyer II homogenizer (Qiagen). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), and RNA contamination was removed by the DNase-Free RNase set (Qiagen). RNA extraction was performed using the RNeasy Plant Mini Kit (Qiagen), and DNA contamination was removed using the TURBO DNA-free Kit (Ambion). cDNA synthesis was carried out using Bioscript Reverse Transcriptase (Bioline Reagents).

Microscopy and image analysis

Thallus tips of 28-day-old transgenic Marchantia plants expressing yellow fluorescent protein Venus driven by the promoter of *MpSBG9* were observed using a stereo fluorescence microscope M205 FA (Leica). To detect the expression of Venus at tissue level, the thalli imaged in the previous observation were fixed in 100 mM sodium phosphate buffer (pH7.0) containing 4% paraformaldehyde at 4 °C for 1 hour. The fixed thalli were embedded in 5% (w/v) agar. Sections with 50 μm thickness

were made using a vibratome HM34OE (Thermo Scientific), and images were acquired using a confocal microscope SP5 (Leica) excited at 515 nm. Imaging of TB stained plants was performed with an optical microscope VHX-5000 (Keyence). Epidermis of thallus tips from WT, *MpSBG9*, and *OX-MpSBG9* (*MpSBG9* overexpressor) lines were observed by a Cryo-SEM EVO HD15 (Zeiss). Size of epidermal cells and number of conical cells were acquired using ImageJ software (http://rsb.info.nih.gov/ij/).

Toluidine Blue staining and quantification

Thalli of 28-day-old Marchantia plants from wild type (WT), *MpSBG9*, and *OX-MpSBG9* lines grown on plate were sampled and stained with Toluidine Blue (TB) solution as described (Tanaka *et al.*, 2004). The plant materials were incubated in staining solution with 0.05% (w/v) of TB (Sigma) at room temperature for 2 min, followed by gentle wash in water three times to remove excessive TB.

The quantification of TB staining was performed as previously described (Li *et al.*, 2016). The stained plants were ground in 400 µL extraction buffer containing 200 mM Tris-HCl (pH8.0), 250mM NaCl, and 25 mM EDTA. After adding 800 µL ethanol, the samples were vortexed and centrifuged, and the supernatant was examined by a scanning spectrophotometer SpectronicUV1 (Termo Electron Corp.) for absorbance at 630 nm (A_{630}) and 435 nm (A_{435}). Relative absorbance of TB by plants were obtained by calculating the ratio of A_{630}:A_{435}.

Water loss analysis

Thalli of 21-day-old Marchantia plants from WT, *mpsbg9*, and *OX-MpSBG9* lines grown on plates were detached carefully, and free water on the thalli surface was removed by filter paper. The samples then were transferred to a 9 cm petri-dish without a lid, and weight was monitored every 20 min for up to 4 h by a microbalance at room temperature. Water loss was presented as a percentage of initial fresh weight.

To test contribution of the *MpSBG9* gene to drought tolerance, 10-day-old thalli grown on plates from WT, *mpsbg9*, and *OX-MpSBG9* lines were transplanted into soil. 14 days later, the lid of the tray was removed and plants were left without watering. After 5 days, the plants were harvested and water content was calculated.

Cutin polyester analysis

The thalli of 21-day-old Marchantia plants from WT, *mpsbg9*, and *OX-MpSBG9* lines grown on plates were carefully harvested for cutin polyester analysis as previously described (Lu *et al.*, 2009).

Results

Phylogenetic analysis of MYB transcription factors regulating cuticle biosynthesis across land plants

MYB domain proteins form a super family, and function as master regulators in a variety of processes in plant development as well as responses to biotic and abiotic stresses (Dubos *et al.*, 2010). Recent studies have revealed a group of R2R3 MYB transcription factors as a key switch controlling cuticle biosynthesis to maintain plant development or mediate responses to drought tolerance and pathogen attack (Aharoni *et al.*, 2004; Raffaele *et al.*, 2008; Seo *et al.*, 2009; Lee *et al.*, 2016). To understand the evolution of the core genetic network underlying land plant cuticle biosynthesis, cuticle-regulating MYB transcription factors in Arabidopsis were used as bait to search against a database of genomes from Phytozome (https://phytozome.jgi.doe.gov) and transcriptomes from the 1KP sequencing project (Matasci *et al.*, 2014). Phylogenetic analysis clearly identified a MYB protein Mp0096s0058 homologous to MIXTA (Fig. S1; Bowman *et al.*, 2017), which has previously been implicated in cuticle regulation in vascular plants, in the early diverging land plant model *M. polymorpha*, thereafter referred to *M. polymorpha* subgroup 9 protein (MpSBG9, following the nomenclature of Brockington *et al.*, 2013).

Expression pattern of the *M. polymorpha* cuticle-related *MpSBG9* Ortholog

To better understand the putative function of the MpSBG9 protein in *M. polymorpha*, a cassette expressing the yellow fluorescent protein Venus fused with a nuclear localization signal (NSL) driven by the *MpSBG9* promoter (*proMpSBG9:Venus-NLS*), containing an ~ 4.0 kb-long genomic fragment upstream of the ATG start codon of the *MpSBG9* gene, was assembled (Fig. S2a; Methods S1). Transgenic *M. polymorpha* plants were generated and the expression pattern of the *MpSBG9* gene was investigated by visualization of Venus protein. Expression of Venus was detected in the dorsal surface of thallus, with a higher intensity in the apical notch and surrounding tissues as well as air pore cells (Fig. 1a, b, d). Close observation showed that Venus signals were predominantly restricted to the dorsal and ventral epidermis, filamentous photosynthetic cells within the air chamber, and scales at the ventral surface (Fig. 1c). It was noted that *proMpSBG9* activity was also detected in papillate cells, that resemble conical petal epidermal cells in shape and are widely distributed across the dorsal surface of the growth tip (Fig. 1d). In addition, the Venus signals were also visualized in epidermal cells of both male and female reproductive organs, antheridiophores and archaegoniophores (Fig. 1e, f). In conclusion, the *MpSBG9* gene was expressed in epidermal cells and in epidermal structures such as air pores and projections directly interacting with external environments, as would be expected for a gene potentially involved in cuticle biosynthesis in *M. polymorpha*.

Morphologies of *mpsbg9* mutants and *MpSBG9* overexpression lines

In order to elucidate MpSBG9 function in *M. polymorpha*, we produced *MpSBG9* loss of function mutants and transgenic plants overexpressing the *MpSBG9* gene. Two independent *mpsbg9* mutant lines were established using the CRISPR/Cas9-mediated genome editing system (Fig. S2c, d; Methods S1). Frame shift mutations induced by the CRISPR/Cas9 system at the N' end of the protein introduced a premature stop

codon in both lines, and led to complete loss of the R2R3-MYB DNA binding domain of the MpSBG9 protein (Fig. S3). There were no distinguishable differences in growth between either *mpsbg9* mutant line and wild-type plants at a macroscopic level (Fig. S4).

In addition, transgenic *M. polymorpha* plants overexpressing the *MpSBG9* gene under control of the *M. polymorpha Elongation Factor 1α* promoter (*proEF1α*) were generated (Fig. S5; Methods S1). Five independent lines were analysed. 10-day-old overexpressor plants with higher abundance of *MpSBG9* transcripts exhibited growth retardation and abnormal thallus development (Fig. S5). In wild-type *M. polymorpha*, air chambers were specified by several rows of cells, and the thallus margin in a smooth shape comprises a few rows of cells. However, in *MpSBG9* overexpression lines, the partitions between each air chamber were increased, and the cells at the thallus margin underwent excessive proliferation, leading to an irregular shape.

Essential role for MpSBG9 in *M. polymorpha* cuticle development

Cuticle is deposited on the plant surface as a barrier to prevent water loss, and as a result plant surface permeability can be monitored as a proxy for cuticle defects (Tanaka *et al.*, 2004). To test whether cuticle formation was affected in established transgenic plants, a toluidine-blue (TB) staining assay was carried out with 28-day-old *M. polymorpha* thalli from *mpsbg9* mutants and *MpSBG9* overexpressors. The thalli from *MpSBG9* mutants were heavily stained by TB (Fig. 2a). In contrast, only a few dots with slight staining were observed at the base of the thallus lobes in wild-type plants and overexpressors (Fig. 2a). These observations were further supported by measurements of ratio A_{630}:A_{435}, which represented a relative quantification of TB dye binding to plant samples (Fig. 2b). It also showed that *MpSBG9* overexpressors were much repellent for staining than wide-type plants. To confirm the cuticle defect phenotype, the 28-day-old thalli were gently detached and subjected to a water loss assay as previously described (Aharoni *et al.*, 2004). It was observed that *mpsbg9*

mutant thalli lost water much more rapidly through cuticular transpiration than the wild-type plants and *MpSBG9* overexpressors, but there was no difference in water loss rate between *MpSBG9* overexpression lines and wild-type plants (Fig. 2c), even though *MpSBG9* overexpressors were relatively less heavily stained by TB dye (Fig. 2b). These results indicated that MpSBG9 affects permeability of the epidermis in *M. polymorpha*, most likely by altering cuticle permeability.

Cutin biosynthesis is controlled by MpSBG9 in *M. polymorpha*

To further determine whether altered cuticle permeability in *MpSBG9* mutants is due to changes of cuticle composition or total amount, cutin and wax composition of 28-day-old thalli from both *MpSBG9* overexpressors and *MpSBG9* mutants was analysed and measured by gas chromatography mass spectrometry (GC-MS) (Fig. 3). The cutin mainly consists of fatty acids with chain length of even carbon number C16 to C24, and dicarboxylic acids (DCA) and hydroxy fatty acids (HFA) with chain length of C16 and C18. Similar to the cutin constituents in higher land plants, the main constituents of cutin were detected and dominated the cutin matrix of *M. polymorpha* thallus, but the content of these constituents was much lower than that in higher land plants. Overexpression of the *MpSBG9* gene significantly elevated accumulation of all cutin constituents detected in this study, except for C16:0 DCA, which showed a moderate decrease in *MpSBG9* overexpressor plants. By contrast, loss of *MpSBG9* function induced a dramatic decrease of cutin constituents, in particular the contents of C18:1 FA, C18:2 FA, and C18:0 HFA, which declined to nearly an undetectable level. Unlike other constituents, the amount of C16:0 DCA exhibited a strong increase in *mpsbg9* mutants. However, wax was not detected in *M. polymorpha* plants grown under experimental condition in this study. These observations demonstrated that MpSBG9 plays a key role in regulating cutin biosynthesis in *M. polymorpha*, and that altered cuticle composition may underpin previously discussed changes in permeability

The contribution of the cuticle layer to drought tolerance in *M. polymorpha*.

A key role of cuticle is to maintain water balance within plants, by preventing excessive non-stomatal water loss. Therefore, plants deprived of cuticle are prone to growth defects, for instance in stature, and leaf shape and size (Aharoni *et al.*, 2004; Oshima *et al.*, 2013). However, no discernible differences between wild-type plants and *mpsbg9* mutants in growth were evident under experimental condition in this study (Fig. S4). Therefore, we tested whether cuticle contributes to drought tolerance in *M. polymorpha*. To do so, 24-day-old plants of wild type, *MpSBG9* over-expressors, and *mpsbg9* mutants grown in soil were exposed to a dehydration condition by removal of the lid from the tray and depletion of watering for 5 days. The drought treatment reduced the whole-plant growth in all treated plants (Fig. 4a). Both *mpsbg9* mutants and *MpSBG9* over-expressors were more sensitive to drought than wild type plants, and exhibited a much more severe wilting phenotype, such as frequent occurrence of brownish tissues at the flank and tip of the thallus, likely due to excessive water loss (Fig. 4a). Compared to the control, the water content in *mpsbg9* mutants and *MpSBG9* over-expressors significantly declined to approximate 70%, but water content of wild-type plants was still maintained at approximate 90%, only a 5% decrease (Fig. 4b). These results suggest the importance of MpSBG9 in regulating water balance and through regulation of the cuticle as a physical barrier against water loss in *M. polymorpha*.

Activation of putative cuticle biosynthesis genes by MpSBG9 in *M. polymorpha*

To obtain a comprehensive understanding of how the MpSBG9 protein promotes cuticle biosynthesis in *M. polymorpha*, genome-wide expression analysis was carried out using 10-day-old thallus from plants grown in tissue culture. Ten plants of each line (WT, *MpSBG9*-1, and *OX-MpSBG9*-1) were pooled for each of 3 biological replicates (Methods S2). The expression of 1060 and 965 genes were statistically up- and down-regulated in *OX-MpSBG9* overexpressors and *mpsbg9* mutants respectively

(p-value <0.01) (Tables S1, S2). Transcripts of many genes encoding enzymes putatively involved in lipid metabolism were overrepresented among top 50 transcripts with dramatically decreased expression in *mpsbg9* mutants, e.g. genes encoding GDSL proteins (Mapoly0003s0312 and Mapoly0012s0165), ABCG transporter (Mapoly0109s0011), and CYP704B subfamily protein (Mapoly0005s0090) (Table S3).

In this study, we focused on the genes putatively associated with cuticle deposition. The putative genes orthologous to well-known cuticle biosynthesis genes in vascular plants have recently been identified in *M. polymorpha* (Table S4) (Bowman *et al.*, 2017). Data analysis showed that the expression profiles in *M. polymorpha* were dramatically changed, with selective up-regulation and down-regulation of cuticle biosynthesis genes in *MpSBG9* overexpressor and *mpsbg9* mutants respectively (Fig. 5; Table S5). Further analysis demonstrated that MpSBG9 regulates a wide range of genes involved in cutin biosynthesis pathway, including orthologs of biosynthesis gene *CYP77A* and *GPATs*, extracellular transporter genes *ABCG11s* and *LTPs*, as well as assembly genes *Cutin Synthase* (*CUS*) and *Bodyguard* (*BDG*) (Fig. 5, Table S5). KCS and ECR are two enzymes serving in FAE complex for elongation of acyl-CoAs in wax biosynthesis pathway. The expression of these two genes exhibited a MpSBG9-dependent manner, but MpSBG9 did not appear to regulate the expression of other orthologues of genes known to function in the wax biosynthesis pathway (Fig. 5, Table S5). These results may indicate that MpSBG9 serves as a key regulator of cuticle formation in *M. polymorpha*, primarily through cutin formation as opposed to wax biosynthesis more generally. This implies the presence of a transcription factor-controlled cutin biosynthesis pathway as a core genetic network for cuticle formation in common ancestors shared by land plants.

Functional equivalency between MpSBG9 and vascular plant MIXTA proteins.

Cuticle deposition has been reported to influence development of epidermal cells and

their derivatives in vascular plants, and MIXTA family genes were originally identified as regulators of epidermal cell differentiation (Brockington *et al.*, 2013; Javelle *et al.*, 2011; Noda *et al.*, 1994). To investigate whether MpSBG9 is able to affect epidermal cell differentiation in bryophytes, epidermal morphology of 24-day-old *M. polymorpha* thallus was observed using cryo-scanning electron microscopy (Cryo-SEM) (Fig. 6a, b, c; Fig. S6). In *M. polymorpha*, papillae that resemble conical petal cells in shape are dotted across the dorsal surface of the thallus (Fig. 6d; Fig. S6a). These cells are unicellular, and more prolific in the vicinity of the apical notch and growing apex with a frequency of ~6.9 papillae per 100 epidermal cells (Fig. 6d, j). No papillae are found when the thallus is mature (Fig. 6g). Overexpression of *MpSBG9* induced ectopic formation of papillae on the dorsal surface of mature thallus to a frequency of ~9.6 papillae per 100 epidermal cells (Fig. 6e, h, j; Fig. S6b). These papillae were highly similar to those usually found at the growing apex (Fig. 6e). Papillae formation on the dorsal surface of the growth tip showed an approximate 1.8-fold increase in frequency (Fig. 6j). To confirm that MpSBG9 is able to induce papillae formation in *M. polymorpha*, the *mpsbg9* mutants were examined. The development of papillae was highly disrupted, and papillae were rarely found at either growth tip or on mature thallus (Fig. 6f, i; Fig. S6c). The papillae frequency sharply dropped to ~0.1 papillae per 100 epidermal cells (Fig. 6j). In addition, quantitative analysis of epidermal cell size on the dorsal thallus surface revealed that overexpression of the *MpSBG9* gene led to a 1.9-fold and 2.9-fold increase in epidermal cell size at the growth tip and on mature thallus, respectively (Fig. 6k).

To test the functional conservation between MpSBG9 and MIXTA family proteins in higher land plants, transgenic *Nicotiana tabacum* plants constitutively expressing the *MpSBG9* gene were generated (Fig. S7). No discernible differences were seen in overall morphology between wild-type *N. tabacum* plants and *MpSBG9* expressors. In wild-type *N. tabacum*, the ovary epidermal cells range from flat to gently rounded in shape (Fig. 7c). However, in transgenic *N. tabacum* plants expressing *MpSBG9*, these cells relatively uniformly underwent outgrowth and transformed into conical shapes

(Fig. 7d). These results demonstrate that MpSBG9 protein is sufficiently similar to the vascular plant MIXTA orthologues to act as a trans-acting factor for papillate cell gene networks in vascular plants.

DISCUSSION

Significant research has been undertaken to understand how early plants protected themselves from terrestrial environments, ultimately leading to the colonization of land (Rensing *et al.*, 2008; Banks *et al.*, 2011; Bowman *et al.*, 2017; Nishiyama *et al.*, 2018; Cheng *et al.*, 2019; Zhang *et al.*, 2020; Li *et al.*, 2020; Jiao *et al.*, 2020). The evolution of a lipid barrier that controls water loss was essential for the early plants surviving in water-limited environments. Despite recent studies revealing a genetic network underlying cuticle formation in vascular plants, it is still unclear to what extent regulation of this genetic network is conserved in early land plants. In this study, we report that an R2M3-MYB protein MpSBG9, which plays an established role in cuticle regulation in higher plants, also regulates cuticle formation in the early diverging land plant species *M. polymorpha*. Over-expression and mutant lines of MpSBG9 showed altered permeability to aqueous dye, altered rates of water loss in simulated drought experiments, and display altered levels of typical cuticle monomers. Together, these results suggest a conserved role for the MYB MIXTA-like lineage, in the regulation of the cuticle over 450 million years of land plant evolution.

Furthermore, through analysis of the gene expression profiles in over-expression versus mutant lines we have revealed a core genetic network regulated by MpSBG9 in controlling cuticle formation in early land plants (Fig. 5). To some extent, this genetic regulatory gene network contains likely orthologues of known genes involved in the cuticle biosynthesis pathway in vascular plants, however, many of the genes and their orthologues have not been functionally analyzed in either vascular plants or early diverging land plants (Table S1). From this putative MIXTA-regulated cuticle gene network, so far, only GPAT (Lee *et al.*, 2020) and ABCG (Buda *et al.*, 2013) have been studied in *P. patens*, with the function of *P. patens* CUS only tested *in vitro* (Yeats *et*

480 *al.*, 2014). However, we identify numerous additional putative homologs of cuticle
481 synthesis genes, which lie within the MpSBG9 transcriptional network, but which are
482 functionally uncharacterised. These data therefore provide a rich source of hypotheses
483 with which to further dissect the conservation of cuticle genetic pathways across land
484 plants, including an understanding of lineage specific diversification and evolution of
485 the cuticle pathway in the early diverging land plant, *M. polymorpha.*

486 Our analyses of the cuticle biochemistry in *M. polymorpha* represent the first
487 comprehensive analysis of chemical composition in this early diverging land plant
488 model. The cutin monomers in *M. polymorpha* consist of DCA (C16-C18), HFA
489 (C16-C18), and fatty acids (C16-C24), and their constituent categories are very
490 similar to those in higher land plants, for example in *A. thaliana* (Fig. 3). Fatty acids
491 are the most abundant constituent, suggesting their critical role in maintaining the
492 cutin matrix of land plants. These data are also consistent with recent observations on
493 *Klebsomidium nitens*, which is a green alga species that is able to grow in both aquatic
494 and terrestrial environments (Hori *et al.*, 2014). It was reported recently that *K. nitens*
495 produces a considerable amount of fatty acids with simple linear carbon chain length
496 from C16 to C18 that attach to cell wall components, when grown on solid medium
497 (Kondo *et al.*, 2016). Intriguingly, the moss *P. patens* at the protonema stage produces
498 a smooth hydrophobic layer of only fatty acids, with C16 as a major constituent, that
499 resembles the secretions of *K. nitens*, while gametophores develop a cutin layer (Lee
500 *et al.*, 2019). Our observations support the idea that fatty acids were already a well-
501 established component in the common ancestors of land plants, with the potential to
502 form a more complex polyester matrix by interaction with other well-known cutin
503 constituents in land plants, including DCA and HFA.

504 Recent studies of cuticle in bryophytes have shown the presence of free waxes on
505 some species of mosses and liverworts (Schönherr & Ziegler, 1975; Cook & Graham,
506 1998; Budke *et al.*, 2011). Using microscopic and histochemical analysis, at least in
507 some mosses, a free wax layer was observed on the surface of gametophores and
508 sporophores. This layer is well developed and resembles the cuticular wax in higher
509 land plants (Busta *et al.*, 2016). For three liverwort species, *M. polymorpha*, *M.*

510 *paleacea*, and *Plagiochasma elongatum*, the waxes were preferentially deposited on
511 air pore cells (Schönherr & Ziegler, 1975). In this study, we were unable to detect free
512 waxes in lab-grown samples of *M. polymorpha* using GC-MS, although we have
513 observed them in wild-grown populations. It has been well documented that the
514 amount and composition of cuticular waxes vary greatly among plant tissues as well
515 as plant species (Jenks & Ashworth, 1999; Bernard & Joubès, 2013). It could be that
516 the amount of wax on the surface of *M. polymorpha* thalli used in lab growth
517 conditions was too low to be detected, as biosynthesis of cuticular wax is greatly
518 affected by growth conditions (Bernard & Joubès, 2013; Yeats & Rose, 2013). Plants
519 generally promote production of cuticular waxes in response to dry environments. The
520 *M. polymorpha* thalli used for chemical analysis of waxes in the present study were
521 grown at a favourable condition with high humidity. Thus, the wax biosynthesis
522 pathway may not be fully activated to accumulate free waxes to a degree that was
523 detectable. Alternatively, these results may imply that protection against water loss in
524 *M. polymorpha* is mainly due to the cutin layer, and also suggest that the hydrophobic
525 lipid layer in *M. polymorpha* is a proto-type cuticle of land plants, in which free
526 waxes play a less substantial role. Further involvement and stronger integration of
527 free waxes via recruitment of the wax biosynthetic pathway may have arisen later in
528 the evolution of the typical land plant cuticle, allowing adaptation of land plants to
529 dehydrating environments. As discussed below, this interpretation may be supported
530 by the analyses of the MpSBG9 expression network, in which orthologues of the
531 known components of the wax biosynthesis pathway are less apparent (Fig. 5).
532 Previous studies in Arabidopsis and tomato have shown that the interplay
533 between MIXTA and SHN serves an important role in orchestrating cuticle deposition
534 (Oshima *et al.*, 2013; Lashbrooke *et al.*, 2015). Significantly, in Arabidopsis, MIXTA
535 family proteins activate both the cutin and the wax biosynthesis pathways directly, or
536 indirectly through SHN protein to control cuticle formation (Oshima *et al.*, 2013);
537 while in tomato, MIXTA family proteins function downstream of SHN protein to
538 specifically switch on the cutin biosynthesis pathway (Lashbrooke *et al.*, 2015). The
539 regulatory relationship between MIXTA and SHN proteins suggests a complex

evolution of the genetic network underlying cuticle formation in vascular plants. In *M. polymorpha*, our data show a central role for MpSBG9 in regulating cuticle formation (particularly cutin) but MpSBG9 does not seem to activate the wax biosynthesis pathway or at least not detectably in lab grown systems (Figs 3, 5). Interestingly, genome-wide analysis did not identify SHN orthologues in *M. polymorpha*, although the SHN orthologue appears to have first evolved prior to the divergence of mosses (Bowman *et al.*, 2017). Thus, further functional exploration of SHN orthologues in mosses may provide insights into the genetic changes underlying cuticle evolution and identify the emerging complexity of the interactions between SHN and MIXTA in the control of cuticle formation.

In addition to their role in cuticle formation, MIXTA orthologs have been shown to be both positive and negative regulators of trichome and petal conical cell development in a wide range of vascular plant species (Noda *et al.*, 1994; Glover *et al.*, 1998; Brockington *et al.*, 2013; Oshima *et al.*, 2013; Shi *et al.*, 2018; Galdon-Armero *et al.*, 2020). The epidermis is a layer of highly differentiated cells on the surface of land plants, playing important roles in interaction with external environments. Epidermal cells have evolved into different shapes with diverse functions contributing to their multiple roles, such as trichomes, which serve a protective function against dehydration and herbivore attacks, and petal conical cells, which generate visual and tactile cues that attract animal pollinators (Serna & Martin, 2006; Fattorini & Glover, 2020). We observed that mutation and over-expression of *MpSBG9* led to respective decreases and increases in short papillate protrusions in *M. polymorpha* (Figs 6, S6). Furthermore, heterologous expression of *MpSBG9* in tobacco (*N. tabacum*) led to induction of conical cells mainly on the ovary, which is typically the strongest location for conical cell phenotypes is these types of heterologous assay (Fig. 7). The ability of MpSBG9 protein to induce the ectopic formation of papillae in both *M. polymorpha* and tobacco indicates conservation of the pleiotropic properties of MIXTA-like proteins to activate both epidermal outgrowths and cuticle formation. To what extent this pleiotropic activity is developmentally and genetically based, or whether the occurrence of epidermal

outgrowths is a consequence of secondary effects of cuticle modification, is an interesting and active research question.

In summary, we have identified the first transcriptional regulator of cuticle formation in early diverging bryophytes, and shed light on the gene network it controls, opening multiple research avenues to further dissect cuticle synthesis in early diverging land plants. Our study on *MpSBG9* from the liverwort *M. polymorpha* implies a conserved genetic mechanism underpinning this process in the common ancestor of all land plants, both in control of the cuticle and in epidermal outgrowths. The MYB transcription factor clade containing MpSBG9 is land-plant specific, therefore its recruitment to specify control of the cuticle may have been an early event in land plant evolution.

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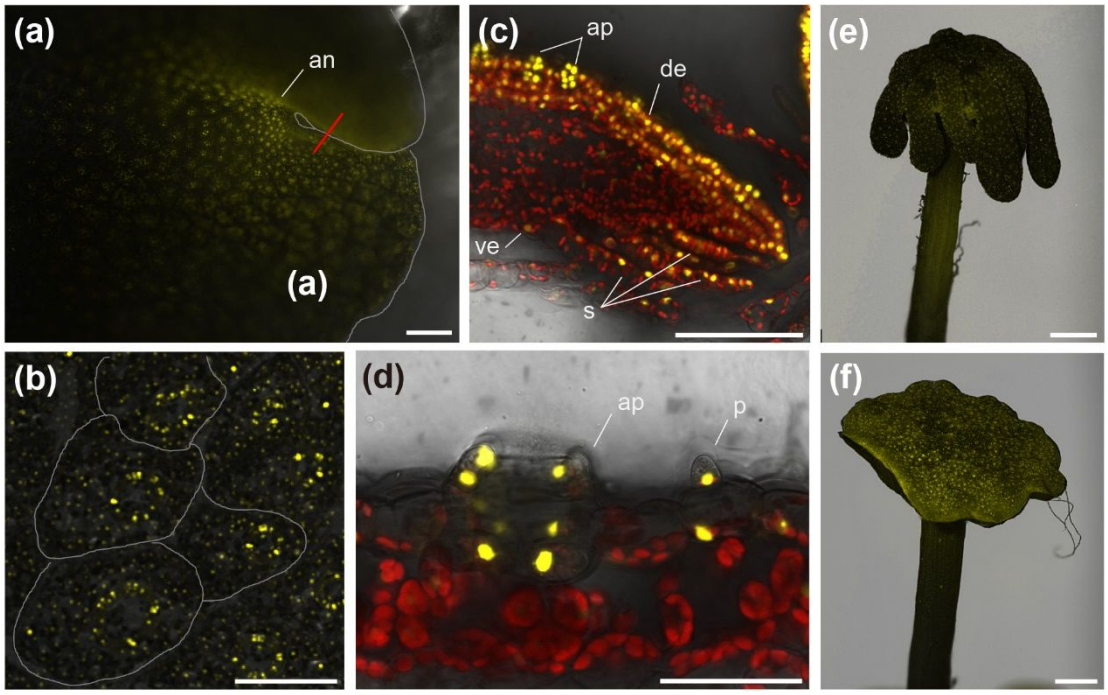
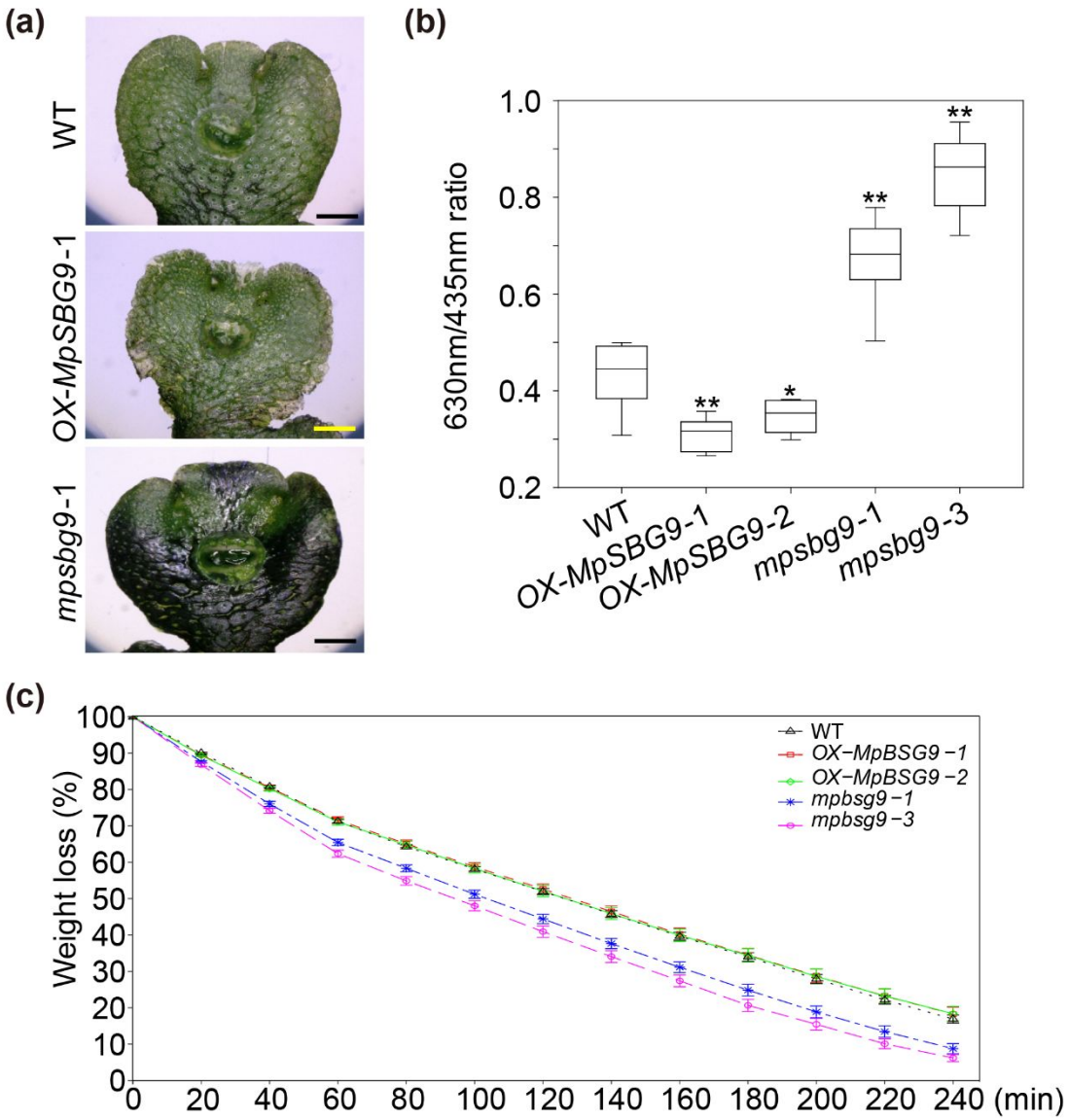


Fig. 1 Expression of *MpSBG9* in *M. polymorpha*. Visualisation of Venus expression in vegetative tissues (a-d) and reproductive organs (e-f) from representative pro*MpSBG9*:*Venus-NLS* lines. (a) Dorsal view of a 28-day-old thallus tip grown on half strength Gamborg's B5. Thallus tip is outlined in gray. (b) Close-up view of thallus surface in (a). Air chambers are indicated by gray lines. (c-d) Transverse sections of the developing region indicated by the red line in (a). an, apical notch; ap, air pore; de, dorsal epidermis; ve, ventral epidermis; s, scale; p, papilla. (e) Antheridiophore. (f) Archaegoniophore. Scale bars, 1 mm in (a), (e), and (f); 200 μm in (b); 100 μm in (c); and 50 μm in (d).



888 **Fig. 2** Surface permeability of wild type (WT), *OX-MpSBG9*, and *mpsbg9 M.*
889 *polymorpha* plants. (a) 28-day-old thalli grown on half strength Gamborg's B5 plate
890 stained by Toluidine blue solution (0.05%, w/v). Top, WT; middle, *OX-MpSBG9* line
891 1; bottom, *mpsbg9* mutant line 1. Scale bars, 2 mm. (b) Quantification of Toluidine
892 blue staining. Each measurement represents mean ± standard error (SE) of 8
893 biological replicates. The thalli from 3 plants were harvested for each replicate. *P <
894 0.05 and **P < 0.01 (Welch's *t* test). All comparisions were performed against WT.
895 (c) Water loss assay. 28-day-old thalli grown on half strength Gamborg's B5 were
896 carefully detached and placed in a weighting boat in air at room temperature. The
897 weight was monitored every 20 min up to 4 h. Water loss was presented as a ratio to
898 fresh weight at the start. Each measurement represents mean ± standard error (SE) of

four biological replicates. The thalli from at least 3 plants were harvested for each replicate.

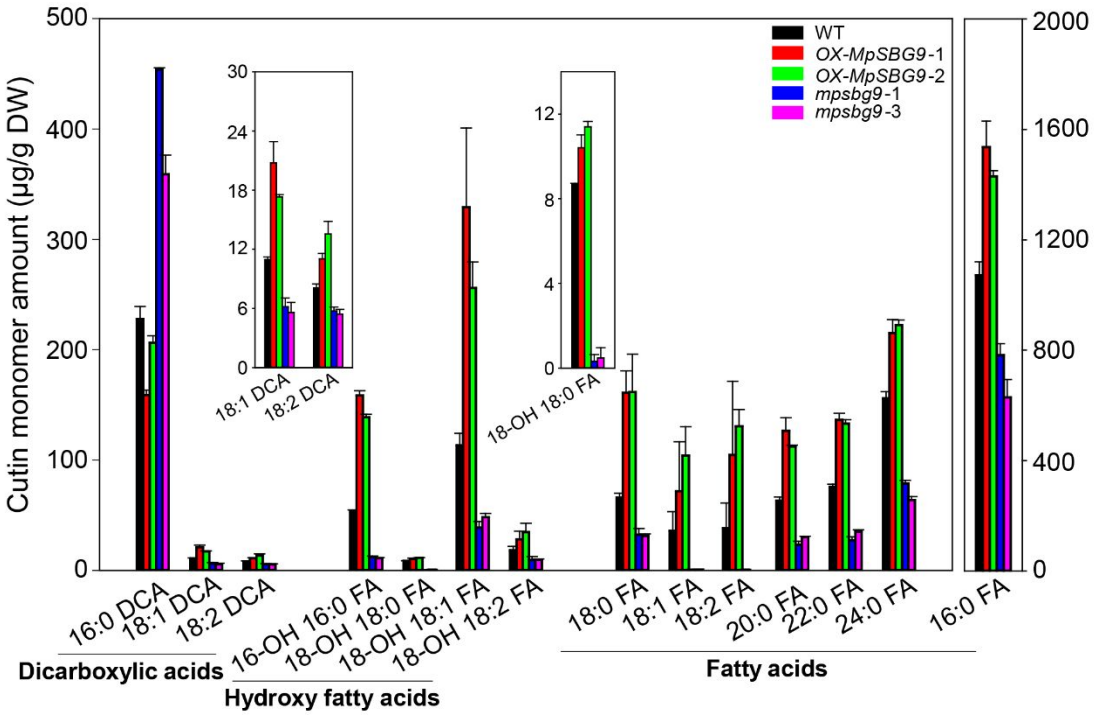
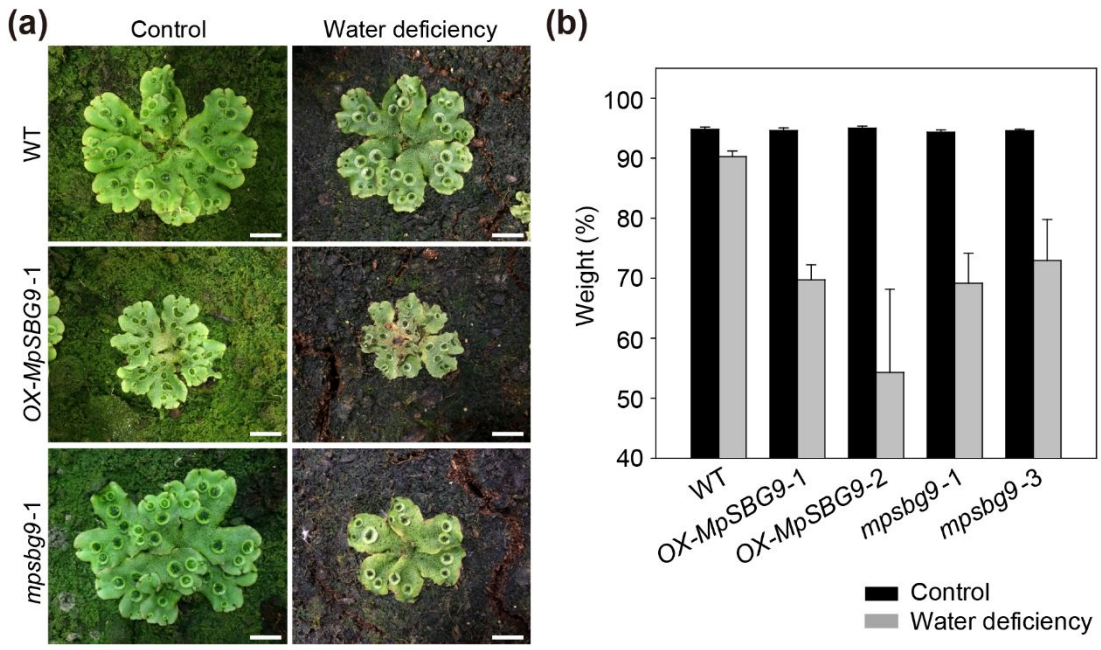
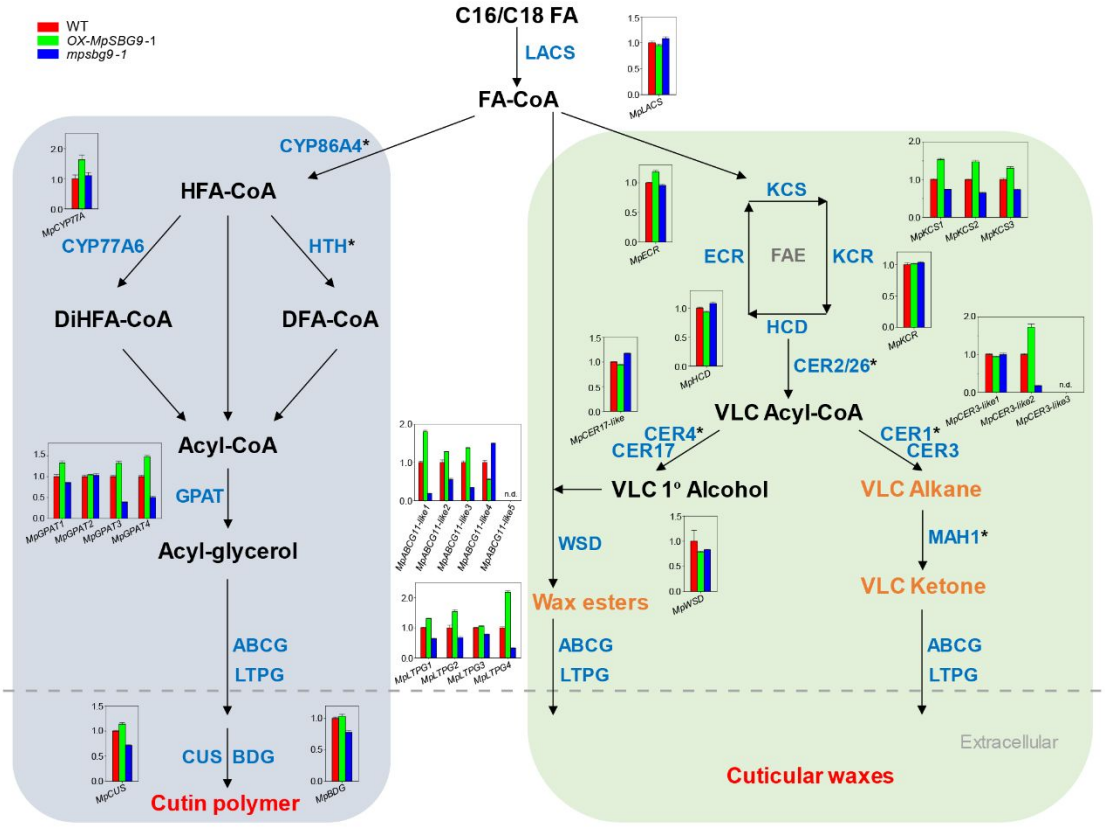


Fig. 3 Cutin polyester compositions and amounts in thalli of WT, *OX-MpSBG9*, and *mpsbg9 M. polymorpha* plants. 28-day-old thalli grown on half strength Gamborg's B5 plate were used for examination. Each measurement represents mean ± SE of three biological replicates. The thalli from at least 5 plants were harvested for each replicate.



906 **Fig. 4** Drought tolerance of WT, *OX-MpSBG9*, and *mpsbg9 M. polymorpha* plants. (a)
907 24-day-old plants (10-day propagation of gemmae on half strength Gamborg's B5
908 plate) were left without watering for 5 days. Scale bars, 1 mm. (b) Water content
909 measurement of plants in (a). Each measurement represents mean ± SE of three
910 biological replicates. The thalli from at least 3 plants were harvested for each
911 replicate.



912 **Fig. 5** Expression of putative *M. polymorpha* genes associated with cuticle deposition
913 in WT, *OX-MpSBG9*, and *mpsbg9 M. polymorpha* plants. A schematic demonstration
914 of the cuticle biosynthesis pathway. Relative expression of putative genes orthologous
915 to cuticle biosynthesis genes in *M. polymorpha* are displayed in the graphs. Each
916 measurement represents mean ± SE of three biological replicates. The thalli from at
917 least 10 plants were harvested for each replicate. FAE, fatty acid elongase complex. *
918 indicates no ortholog identified in *M. polymorpha.* Dashed line in gray represents
919 plasma membrane of epidermis, with the area below the dashed line representing the
920 extracellular space. n.d., not detected.

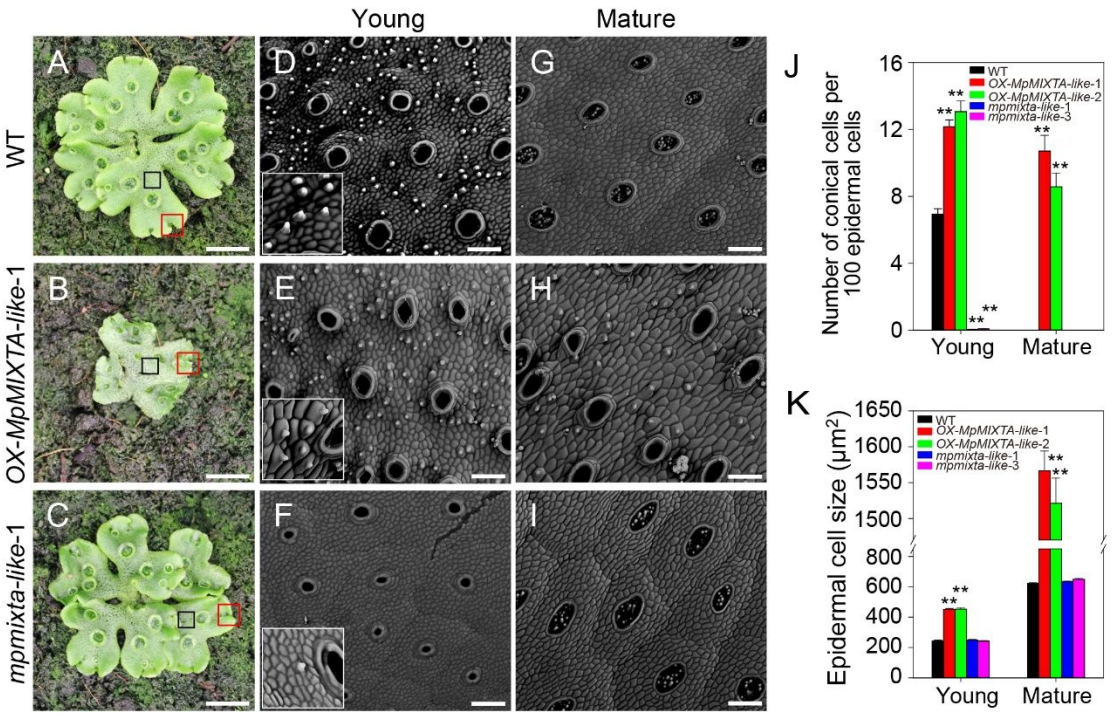


Fig. 6 Morphology of epidermis from WT, *OX-MpSBG9*, and *mpsbg9* plants. (a-c) Dorsal view of 24-day-old plants in soil (10-day propagation of gemmae on half strength Gamborg's B5 plate), showing WT (a), *OX-MpSBG9*-1 (b), and *mpsbg9*-1(c). (d-f) Cryo-SEM images of young tissues located close to the apical notch, indicated respectively by red box in (a-c). Insert in (d-f) is close-up view of papillate. (g-i) Cryo-SEM images of mature tissue at the base of thallus, indicated by black box respectively in (a-c). (j) The density of papillate in WT, *OX-MpSBG9*, and *mpsbg9* plants. Each calculation represents mean ±SE of three biological replicates. The thalli from 3 plants were randomly collected for each replicate. (K) Size of epidermal cells in WT, *OX-MpSBG9*, and *mpsbg9* plants. Each measurement represents mean ± SE of three biological replicates. At least fifty epidermal cells were randomly selected for each replicate. Scale bars, 1 cm in (a-c), 100 µm in (d-i); and 50 µm in insert of (d-f). **P < 0.01 (Welch's *t* test). All comparisions were performed against WT.

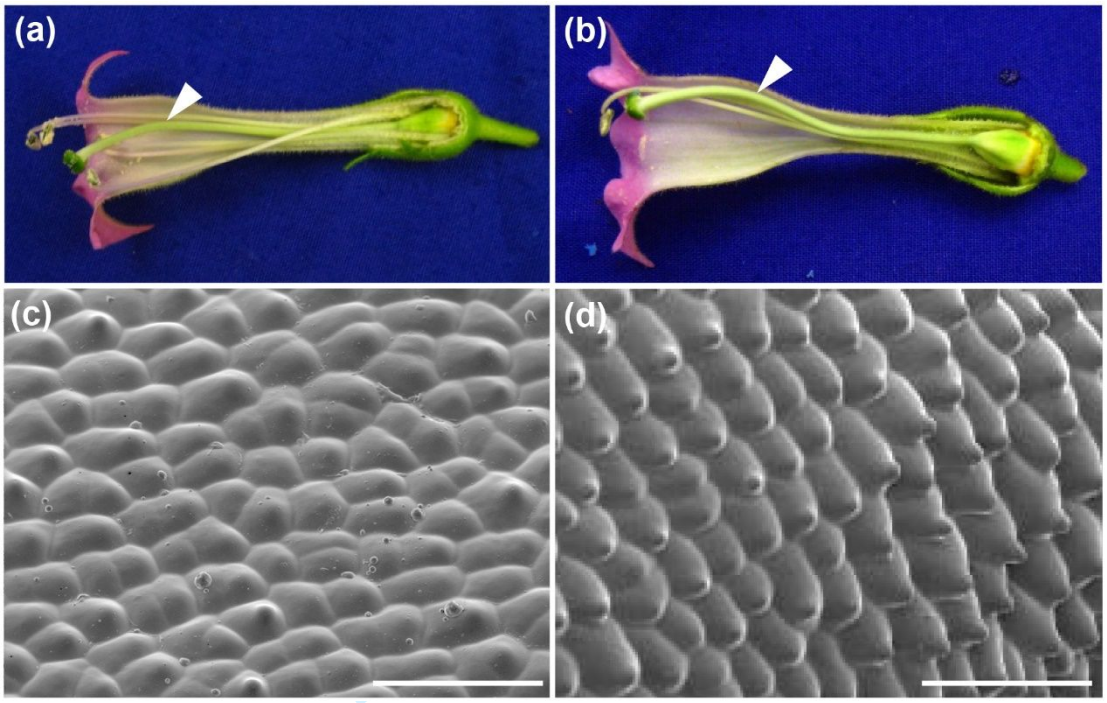


Fig. 7 Ectopic expression of the *MpSBG9* gene in tobacco. (a-b) Longitudinal tobacco flower dissections of WT (a) and *MpSBG9* expressing plants (b). A slight curve in the style (arrowhead) in (b) versus a fairly straight style of WT (arrowhead) in (a). (c-d) Cryo-SEM images of ovary abaxial epidermis from WT (c) and *MpSBG9* expressing plants (d). Epidermal cells range from flat to gently rounded in shape (c). Epidermal cells are relatively uniformly conical shapes in the transgenic lines (d). Scale bars, 100 μm.

Supporting Information

Fig. S1 Phylogeny of R2R3 MYB proteins.

Fig. S2 Schematic diagram of plasmids used for *M. polymorpha* transformation.

Fig. S3 Generation and identification of *mpsbg9* mutant *M. polymorpha* plants.

Fig. S4 Phenotype of WT and *mpsbg9* mutant *M. polymorpha* plants.

Fig. S5 Identification of transgenic *M. polymorpha* plants ectopically overexpressing the *MpMIXTA* gene.

Fig. S6 Epidermal morphology of WT, *OX-MpSBG9*, and *mpsbg9 M. polymorpha* plants.

Fig. S7 Characterisation of tobacco plants constitutively overexpressing the *MpSBG9* gene.

Methods S1 Plasmid construction and plant transformation.

Methods S2 RNA-seq analysis.

Table S1 Transcripts with increased expression in *M. polymorha* overexpressing MpSBG9.

Table S2 Transcripts with decreased expression in *M. polymorha mpsbg9* mutant.

Table S3 Top 40 transcripts with decreased expression in *M. polymorha mpsbg9* mutant.

971

972 **Table S4** Putative genes in *M. polymorpha* orthologous to well-know cuticle

973 biosynthesis genes in *A. thaliana*.

974

975 **Table S5** Digital gene expression.

976

977 **Table S6** Oligo primers used in this studies.

978

979 **Table S7** Raw counts. WT, wild type. OX, *OX-MpSBG9*-1. KO, *mpsbg9*-1.