Elucidating the molecular basis of exodermis development to create a *Zea mays* variety with a multiseriate exodermis

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 **Summary: 291 words**
Global atmospheric CO₂ concentrations are rising rapidly due to human activities and an increasing world population. This puts increasing pressure on crops we rely on for food and other necessities. Plants capture CO₂ in order to assimilate biomolecules like sugar for energy. Suberin is one of these biomolecules, and it is a hydrophobic polymer deposited in barrier cell types, like root endodermis and exodermis, where it forms a barrier. Some plants are known to possess multiple of these exodermis layers; a multiseriate exodermis (*MEX*). Molecularly, the exodermis is poorly characterized compared to the endodermis, because the model plant species *Arabidopsis thaliana* has none. However, the exodermis increases plant drought resistance by increasing water retainage of roots and even has the potential of alleviating the rising atmospheric CO₂ levels by storing suberin in soils. The more suberin a plant deposits in its root system, the more atmospheric CO₂ becomes sequestered underground. This means that plants with a *MEX* generally have a larger potential of storing CO₂. If crops like maize, *Zea mays,* which has a single exodermis layer, can obtain multiple exodermis layers this might increase carbon sequestration and drought resistance. Maize is suitable for this approach, since maize roots are typically left underground after harvest. In this proposed work, we aim to generate a new maize variety that possess a *MEX*. By investigating the molecular mechanisms behind (multiseriate) exodermis development we aim to elucidate what (transcription) factors influence development of the exodermis. Using paired-end deep RNA sequencing and genome walking of (dry- and hydroponically grown) maize roots and *Chrysopogon zizanioides*, a maize relative with a *MEX*, we will generate transcriptomic data of both species. Lastly, we aim to find genes we can use to introduce a *MEX* in transgenic maize.
 **Overall aim:**
Identifying molecular regulators of multiseriate exodermis and designing a new *Zea mays* crop variety that has a suberized multiseriate exodermis, which sequesters more atmospheric carbon and is more drought tolerant.

**Key objectives:**
**1**: Identify a plant species that has a suberized multiseriate exodermis and thrives in typical *Zea mays* environmental conditions.
**2**: Generate novel transcriptomic data from *Zea mays* and related species.
**3**: Identify genetic factors responsible for (multiseriate-) exodermal development.
**4**: Transform *Zea mays* to create a new variety with a multiseriate exodermis, increasing carbon sequestration and drought resistance.

**Keywords:** *exodermis, Zea mays, suberin, drought tolerance, carbon sequestration*
**Introduction**

***The exodermis and suberin***
In 2021, Earth’s northern hemisphere temperatures reached 1,5 degrees Celsius above pre-industrial temperatures which is largely caused by human CO₂ emissions (‘Climate Change 2021, the physical science basis’, IPCC, 2021). With the global human population almost reaching 8 billion, both greenhouse gas emissions and food demands are expected to increase in the future. This puts increasing pressure on plant crop performance, which is depending on plant energy use. Plants **sequester carbon** (C) to assimilate it into biomolecules like sucrose and cellulose for energy storage and/or structure. One of the most persistent and slow-degrading of these molecules is **suberin** (Figure 1A), a hydrophobic biopolymer consisting of fatty acid chains linked via phenolic structures (Graça, 2015). Suberin is mostly found in cell walls of adapted cells that serve as protective barriers, in leaves aboveground and the endodermis and exodermis in roots (Graça, 2015). Both the exodermis and endodermis are lignified and suberized, but the endodermis to a smaller extent than the exodermis in some plants (Kajala *et al.,* 2021). The exodermis is the outermost cortex cell layer of the roots, while the endodermis layer is located just inside the cortex layer(s). Exodermis is involved in retaining water during drought or inhibiting Radial Oxygen Loss (ROL) during floods (Ma and Peterson, 2003). In both exodermis and endodermis, lignin is deposited around cells to form a sealed barrier known as Casparian Bands (Hose *et al.,* 2001), whereas suberin is found as coating around individual cell walls (Meyer *et al*., 2009).

A B

C

**Figure 1: *Suberin structure and distribution in root cross-sections of single- and multi-layered exodermis layers.***

A: Schematic representation of chemical structure of suberin. (Yao *et al.,* 2012)
B: Cross-section of *Zea mays* roots grown in perlite and sand mixture (PER) with Sudan Red 7B staining of suberin, visible in endodermis-, epidermis- and exodermis cell layers. (Tylova *et al.,* 2017)
C: Cross-section close-up of the *MEX* in *Iris pumila* with Sudan Red 7B staining of suberin. Red arrowheads indicate suberin lamellae. Epi=epidermis, ex=exodermis (Meyer *et al.,* 2009).

***The potential of a multiseriate exodermis***
93% of all flowering plants have an exodermis layer in roots (Peterson and Perumalla*.,* 1990) but the model plant species *Arabidopsis thaliana* has not, which is why the exodermis has not been studied as thoroughly. The exodermal rate of maturation is highly responsible to environmental cues (Meyer *et al.,* 2009), meaning that when plant roots experience drought, the exodermis matures faster. Some monocot plant species are known to have multiple exodermal cell layers in their roots (a **multiseriate exodermis, *MEX***). Plants with *MEX* have greater suberin content than plants with a uniseriate exodermis, because deposition of suberin lamellae is not restricted to the outermost exodermis layer (Meyer *et al.,* 2009). Given that suberin is stored in roots and decomposes slowly, roots with a *MEX* sequester more atmospheric carbon in soils than roots with a single or no exodermis. After a plant has died, the suberin molecules in the roots can remain in the soil for a long time, since it degrades slower than most other biopolymers (Harman-Ware *et al*., 2021). In this way, atmospheric CO₂ can be stored in the soil via deposition of suberin, which could help decreasing the rising atmospheric CO₂ levels that cause climate change. Using this potential of the (multiseriate) exodermis could be advantageous when applied widely, such as on agricultural crops. Therefore, expanding our knowledge of molecular mechanisms that underlie exodermis differentiation and -development is key in developing crops that help tackling climate change. Next to that, more frequent drought events are expected to take place in the future (Sheffield and Wood, 2007), which increases pressure on crop performance. By introducing a *MEX* to widely used crops in dry areas, these extra root barriers can increase plant drought tolerance by reducing the transmembrane water transport of roots (Ma and Peterson, 2003), which helps increase water retainage during drought. Another possibility is to use this principle to fertilize soils. When suberin eventually breaks down, it can be used for agriculture by fertilizing soils with organic matter, although this reduces the effect of atmospheric carbon sequestration.

***Applying a MEX to a crop***
Some important considerations of applying a *MEX* to a crop is that the plant roots must remain in the soil after harvest and the crop is preferably annual for better results. Maize (*Zea* mays) is an annual food- and biofuel crop which is cultivated mostly in dry areas. During harvest, shoot material is removed, with roots staying behind in the soil. Like the majority of flowering plants, maize has an exodermis layer in roots which contains suberin lamellae (Peterson and Perumalla*,* 1990, Meyer *et al.,* 2009). By introducing *MEX* to maize, this can help increase carbon capture from the atmosphere, while increasing crop performance during drought stress.
Like in the (single-layered) uniseriate exodermis, **molecular foundations of *MEX*-development** are unknown. *Chrysopogon zizanioides,* or vetiver, is a grass species closely related to maize which possesses a *MEX,* which makes it a useful candidate for studying *MEX*-anatomy. Another uniseriate exodermis monocot crop that could benefit from multiple exodermal layers in roots is rice (*Oryza sativa*). Maize seems more favorable however, because maize can be cultivated alternately with other crops like beans and other legumes. These could benefit from the soil fertilizing potential of extra carbon sequestration. However, the molecular factors responsible for development of single- or multilayered exodermal cells is largely unknown to date (Kajala *et al.,* 2021). In this work, we propose to **investigate the molecular mechanisms of exodermis development**. Factors involved in these mechanisms could act in different ways on exodermis development. Genes that increase suberin deposition in outer cortex cells, increase cell division in the root meristem or protein gradients are all examples of how gene expression could establish different exodermal anatomies across plant species. Also, we aim to apply these newly found factors to *Zea mays* by *Agrobacterium tumefaciens* mediated transformation in order to obtain a maize variety with a *MEX*.

**Hypotheses:**
**1**: performing assays and studying suberin content of *Zea mays* related plant roots will bring forward a candidate species (for RNA-seq) with a multiseriate exodermis. **2**: We can identify molecular regulators of multiseriate exodermal development, like SHORTROOT (SHR) and SCARECROW (SCR) regulate endodermal development.
**3**: When *Zea mays* obtains multiple exodermal cell layers with suberin, it will capture more carbon and becomes more drought resistant.

**Originality**
In this work, development of the multiseriate exodermis will for the first time be molecularly characterized by performing RNA sequencing. The focus lies on genes that express in the exodermis- and cortex cells during exodermal development. If new molecular (multiseriate) exodermis markers or transcription factors are found, they will be applied to maize by transformation in order to create a newly designed maize variety that has *MEX* (multiseriate exodermis) or more suberin in the native uniseriate exodermis. If this succeeds, the new maize crop variety will capture more atmospheric carbon and will be more resistant to drought at the same time. Also, new transcriptome- and cell type-specific gene expression data of the species *Chrysopogon zizanioides* will be generated, next to identifying new promoters in both *C. zizanioides* and *Zea mays*. *C. zizanioides* is of interest for many industries. It be used to extract oil, livestock feed or erosion control in soils prone to degradation. Generating a novel reference transcriptome for *C. zizanioides* is of value for research with these applications in mind.

Methods and techniques
**Work package 1:** ***Finding Zea mays relatives with MEX***
The first work package (WP) aims to find the best candidate plant species for RNA-seq experiments. The maize variety we will be using is A188, because this inbred line shows the highest transformation efficiency when using *Agrobacterium tumefaciens* (around 50%, Ishida *et al.,* 2007). Since maize is a member of the *Poaceae* order of plants, the focus of WP 1 will lie on plants around this group. We will take the following criteria into account when selecting species:
- does the plant species have a multiseriate exodermis?
- is the plant species (closely) related to *Zea mays*?
- does the plant live in similar conditions as *Zea mays*?
- data and resource availability of the species

Aims and methods
1 Grow species of interest in typical *Zea mays* growth conditions
Maize is a member of the *Poaceae* family of grasses, which contains species living in different environments where abiotic factors like temperature and water availability can differ greatly. A *MEX* is found in several species closely related to maize, as is shown in figure 2.Because exodermal development is responsive to the environment (Meyer *et al.,* 2009), all species of interest in figure 2 will be grown in conditions similar to those in typical maize fields. This will take place in the greenhouse cabinets in the Botanical Gardens at Utrecht University. Temperatures are kept close to 25 degrees Celsius, with 16- and 35 degrees Celsius as lower and upper limits. Plants will grow in long day conditions (sowing in March, with growing lights supplying extra light when needed). Water will be supplied twice, since plants will grow for only 4 weeks. At least 20 individuals per species will be grown to collect sufficient amount of material for further experiments, preferably more. We will grow plants on patches or trays of 1m2 to resemble agricultural light competition. All plants will be grown on vermiculite soil.

**Figure 2:** ***Phylogenetic tree of species of interest of the Poaceae family (grasses).*** All stated species have an exodermis, with barred species having multiple exodermis layers (*MEX*). Black colored species inhabit well-drained soils, blue colored species inhabit wet soils. \*=*Zea mays*. Derived from Meyer *et al.,* 2009; Soukup et al. (2007); Kroemer, 1903; Shiskoff, 1986

2 Drought assays
When maize plants are 4 weeks old, they are fit for experiments. We will perform dry treatments in combination with drought assays. Plants will be only watered once to simulate dry periods in which maize should be able to survive. As well as monitoring survival rate, we will measure relative water content of plants by using leaf samples we harvest after the dry period (Hao *et al.,* 2011). In this way, we will select the plants that match best with *Zea mays* in terms of water requirements for further experiments.
3 Study exodermal anatomy
Candidate species of which the majority of plants survived the drought assays will be used for further research. We will study exodermal pattern (uniform or dimorphic, Meyer *et al.,* 2009) and number of layers. We will harvest samples for microscopy from roots where the exodermis has fully matured, around 30 mm distal from the root tip (Hose *et al.,* 2001). We will fix, section and stain the samples for suberin using Sudan Red 7B. To study the different exodermal anatomies of the species, we will generate microscopic fluorescence images of the exodermis using Confocal Fluorescence Microscopy with the Carl Zeiss Airyscan microscope in the Kruyt building.
4 Quantification of microscopic data
We will perform quantification of fluorescent signal intensities using Image-J software to determine suberin content in the different species, as the intensities of the suberin stains are linearly correlated with suberin quantity. The species that show high suberin content will be investigated in WP2.

Figure 4 (side): two *Chrysopogon zizanioides* varieties. A: South Indian variant, B: Northern Indian variant.

Hypothesis: *Chrysopogon zizanioides* (Vetiver) is the best candidate species for RNAseq, since it is the closest C4 *Zea mays* relative with a suberized multiseriate exodermis (Meyer *et al*., 2009). It is also a hardy plant that can tolerate many abiotic stresses. Although perennial, *C. zizanioides* grows fast, with the South Indian type (A) reaching up to 2,5m. (Chakrabarty *et al*., 2015)

**Work package 2: *transcriptome analysis of exodermis***
In WP 2 we will generate transcriptome data of *Chrysopogon zizanioides* and *Zea mays*. We dividemaize plants into two groups: one that is grown in vermiculite soil like in WP1, and another group that is hydroponically grown (no exodermis suberization). When the three groups have grown for 4 weeks, we again divide them; one half of each group will be used for RNA-seq, the other for gene walking. By comparing transcript profiles of these three groups with different exodermal anatomies, chances are greatest of finding genetic factors that regulate exodermal development. For hydroponically grown maize we will use the outermost cortex layer for transcript profiling. Eventual transcription factors and promoters found here can be used for transformation in WP 3.

Aims and methods
1 Creating reference transcriptomes
*Chrysopogon zizanioides* and other *MEX* candidates in WP1 have little genomic- and transcriptomic data available, while maize has abundant transcriptome data available (Chakrabarty *et al.,* 2015, Martin *et al*., 2014, Hirsch *et al.,* 2014, Wang *et al.,* 2015), although parts of the maize transcriptome remain unclear. Therefore we will perform De novo transcriptome assembly using root tissues of *Chrysopogon zizanioides*.
2 LCM
To separate cell types, we will use laser capture microdissection with the Pix-Cell II LCM system (Kerk *et al.,* 2003) to separate cell types. After we isolate RNA and construct RNAseq libraries (Townsley et al 2015 / Kajala 2021), samples will be sent to USeq for high-throughput deep RNA sequencing with HiSeq.
 
**Figure 5: *Transcript categories.*** Diagram showing expected categories of relevant root transcripts from the three groups.

Figure 5 shows categories of transcripts we expect to find in the samples. The transcripts where chances of finding a *MEX* development regulator are greatest in *Chrysopogon zizanioides*. Between the two maize groups, we expect to find differentially expressed ABA-related transcripts (abscisic acid), since ABA is involved in drought stress responses in roots (Vallabhaneni *et al.*, 2010). Cortical identity-transcripts are expected to be found in every plant group.
3 Promoter identification
To identify promoters of the genes of interest, we will use genome walking with PCR on *C. zizanioides* and both maize groups. By using the exonic RNA sequences of transcripts found in WP2, we aim to find the corresponding coding sequences in the plant genomes to find flanking promoter sequences with PCR and sequence specific primers.

**Table 1: *example list of genes and -promoters***

|  |  |
| --- | --- |
| Promoters | Predicted target genes |
| ‘novel *MEX*’ TF promoter | ‘novel *MEX*’ TF |
| ‘novel uniseriate exodermis’ TF promoter | ‘novel uniseriate exodermis’ TF |
| monocot PEP homolog promoter | Maize *Os*MYB93 homolog |
| Maize *Os*LSI1 homolog promoter |  |

Table 1 states a number of genes and promoters our RNA-sequencing and genome walking experiments might identify. The ‘novel (multiseriate) exodermis’ genes we hope to find are regulators of exodermal development, like SHORTROOT and SCARECROW control endodermis development (Koizumi *et al.,* 2012).
4 Transcript data analysis
We will use Trans-ABySS to assemble paired-end sequencing data. When RNA-sequence data of the three groups and different tissues is collected, we will compare transcript abundances in a cell type specific manner, as well as between the different plant groups.
– *cell type-specific transcript comparison*
First, we will gather data of total root RNA as a reference. After that we will look for transcripts that are more abundant in exodermis- or cortex cells compared to other root cell types. Comparing transcriptome data of exodermal cells and outer cortex cells will provide new insights in the genetic basis of exodermis differentiation.
*–transcript comparison across species*
Next, we will compare cortex- and exodermis transcript abundances in a species-species way. Transcript profiles of soil grown maize exodermis will be compared to *C. zizanioides-*exodermis transcript abundances.

Hypothesis: (multiseriate) exodermis development is regulated by transcription factors, like the endodermis is regulated by SCR and SHR.

**Work package 3: *creating a maize variety with a MEX***The final WP aims to transform maize plants to create *MEX*-gain-of-function mutants. We will use *Agrobacterium tumefaciens* to transform maize plants with constructs expressing the *MEX* factors. Successfully transformed plants will be grown and tested for favorable characteristics like drought tolerance and suberin content like in WP 1 to determine effects of the newly obtained *MEX* on carbon sequestration and drought resistance.

Aims and methods
1 Generating transformed maize plants
We will use RNA obtained in WP2 to create strands of complementary DNA. The coding sequences of putative *MEX* genes will be cloned into maize expression vectors under selected promoters, and then into the T-DNA sequence of binary vector pSB131. We will transform maize plants using the *Agrobacterium tumefaciens* transformation protocol described by Ishida *et al.* (2007), who showed transformation frequency of around 50 % in maize*.* The bacterial strain we use is LBA4404. The selection agent will be Basta herbicide (Bayer Crop Science), which contains phosphinothricin (PPT). Transformed tissues will be redifferentiated to generate new plants. simlutaneously, a new set of non-transformed A-188 maize plants will be grown to use as control group in part (2) and (3) of this WP.
2 Investigating exodermal anatomy of new maize varieties
We will grow transformed (T2) plants in typical maize field conditions in the Botanical Gardens at Utrecht, similar to conditions and protocols in WP1. We will survey transformed plant roots for the presence of multiple exodermis layers. We will take samples from older root parts, where the exodermis is expected to have fully matured (Graça, 2015). We will use Sudan Red7B to stain suberin and generate microscopic images using Confocal fluorescence microscopy as described in WP1. We will quantify microscopic data using Image-J and/or ICY. Suberin amounts and exodermal layer numbers of transformed maize roots will be compared to non-transgenic roots. Also, we will determine total root biomass by weighing dried roots, same as we measure dry leaf mass in WP 1 (Hao *et al.,* 2011).
3 Evaluation of characteristics of new maize variety
We will also test and compare crop yield and drought resistance of the transformed plants to the original A188 variety. Plants will be grown fully mature and produce maize kernels. Crop yield will be tested by weighing the total amount of kernels per plant after removing them from the ears. Drought tolerance will be tested like in WP1 (2).

A B

***Figure 6: Transformation strategy*** (Ishida *et al.,* 1996):

6A: T-DNA of the pSB131 vector. BR=right border, BL=left border, BAR=PPT resistance gene (phosphinothricin, selection agent), 35S=35SCaMV promoter, TNOS=nopaline synthase terminator. Restriction sites: B=BamHI, E=EcoRI, H=HindIII. ‘Gene of interest’ is where DNA coding sequence will be cloned and ‘promoter’ is where the selected promoter will be cloned.
6B: selection of maize leaves on medium containing Basta herbicide (phosphinothricin). R=an example of a successful transformation (Resistant), S=non-transgenic leaf (Susceptible).

Hypothesis: if transformation succeeds, the new maize variety captures more atmospheric carbon and is more drought tolerant, thanks to obtaining a multiseriate exodermis.



Risk assessment
If a regulator of multiseriate exodermal development cannot be found using the described experiments, there is another possibility to increase suberin content in maizeroots without the multiseriate approach. In 2016, an exodermis- and endodermis specific promoter was found in rice (*Oryza sativa*); the LSI1 promoter (Mitani-Ueno *et al.,* 2016). By transforming maize with a construct containing the LSI1 promoter in combination with a gene that can increase suberin deposition, suberin content of *Zea mays* roots can be increased. An example of a gene likely involved in regulating suberin synthesis in rice is *Os*MYB93. *Os*MYB93 is chosen because rice is monocot like maize. This option is saved as risk assessment experiment because adding extra exodermis layers to roots probably yields more gain of suberin than a uniseriate exodermis with increased suberin content. Next to that, *Os*MYB93 function is not yet explored specifically (Lashbrooke *et al*., 2016). Nevertheless, this option could also yield a maize variety with increased suberin content in roots, which sequesters more atmospheric carbon in soils and increases drought resistance.

Scientific and societal impact
The exodermis serves as a protective cell layer in plant roots. While the exodermis makes up the outermost cortex layer in 93% of all flowering plant roots, *Arabidopsis thaliana* roots possess none. This has caused molecular knowledge of the exodermis and its establishment to lag behind. By creating a new reference transcriptome for roots of monocot grasses like *Chrysopogon zizanioides,* we collect new data about an important crop relative. By comparing transcriptome data of the different species, this proposed work will be useful for evolutionary studies of grasses. Next to that, transcriptome data from WP2 could offer insights in the evolutionary paths of exodermis- and cortex development. Examples of new findings include transcripts of transcription factors, regulators and/or genes that influence suberin synthesis or cell divisions creating the exodermis. Also, gene walking in both species can yield unidentified promoters.
On top of that, this proposed work applies the newly gathered knowledge on a crop (maize) to help fighting climate change while improving crop resilience and soil-fertilizing potential. In numerous plants, the exodermis contains substances like suberin and lignin, of which suberin has the most potential in terms of increasing drought tolerance, since it is hydrophobic (Graça, 2015). This potential is a welcome addition when climate change causes more drought. Suberin of harvested crop roots can remain in the soil for longer periods of time, storing carbon. Another option is to use this carbon to fertilize soils. If suberin is in fact decomposed, it can help enrich carbon content in soils. Soils that fall short in terms of organic matter requirements can be improved to sustain more nutrient-demanding crops by first growing crops which enrich soils. Maize is often cultivated alternately with other crops such as legumes which may benefit from the enriched soil organic matter left behind by the new maize variety. This is why creating a maize variety with a *MEX* can be extra useful compared to other crop species that could benefit from extra exodermis layers like rice (*Oryza sativa*).

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