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Thi Ngoc Hanh Nguyen, Mélanie Fortier, Sophie Bernard, Jérôme Leprince, Azeddine Driouich, et al. Sweet specificities of the root extracellular trap of perennial ryegrass (*Lolium perenne*), a fructan accumulating plant. *Environmental and Experimental Botany*, In press, 10.1016/j.envexpbot.2024.105743 . hal-04516244

HAL Id: hal-04516244

<https://hal.science/hal-04516244v1>

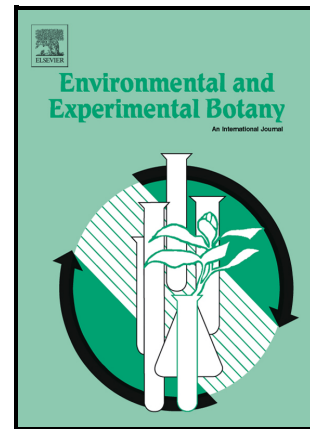
Submitted on 22 Mar 2024

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PII: S0098-8472(24)00101-1

DOI: <https://doi.org/10.1016/j.envexpbot.2024.105743>

Reference: EEB105743

To appear in: *Environmental and Experimental Botany*

Received date: 23 December 2023

Revised date: 28 February 2024

Accepted date: 19 March 2024

Please cite this article as: Thi Ngoc Hanh Nguyen, Mélanie Fortier, Sophie Bernard, Jérôme Leprince, Azeddine Driouich, Marie-Pascale Prud'homme, Marie-Laure Follet-Gueye, Annette Morvan-Bertrand and Maïté Vicré, Sweet specificities of the root extracellular trap of perennial ryegrass (*Lolium perenne*), a fructan accumulating plant, *Environmental and Experimental Botany*, (2024) doi:<https://doi.org/10.1016/j.envexpbot.2024.105743>

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Sweet specificities of the root extracellular trap of perennial ryegrass (*Lolium perenne*), a fructan accumulating plant.

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Keywords: arabinogalactan proteins (AGPs), fructans, *Lolium perenne*, osmotic stress, root defense, root extracellular trap (RET).

Abstract

Perennial ryegrass (*Lolium perenne*) is a fructan-accumulating plant constituting one of the most important grassland species with high herbage production, nutritive value and digestibility for grazing cattle. Although fructans were reported to be involved in plant defense acting as antioxidants or stress signals, their contribution in root protection is still to be explored. In roots, atypical defense is provided by the “Root Extracellular Trap” or “RET” at the root-soil interface. The molecular composition and structural organization of the RET are essential to provide root defense against pathogen attacks and abiotic stresses. The RET was reported to be mainly composed of polysaccharides (homogalacturonan, xylogalacturonan, xyloglucan) and proteoglycans such as arabinogalactan proteins (AGPs). Our aim is to characterize the RET composition of *L. perenne* using cell imaging techniques and a wide range of monoclonal antibodies directed against epitopes from cell wall glycomolecules and to investigate the potential presence of fructans. Interestingly, we found that both mucilage and cell wall surface

of border cells were enriched in AGP epitopes. An increased amount of the AGP-containing mucilage was produced by *L. perenne* root tip in response to both elicitor and osmotic stress. Fructan epitopes were also detected in root cap cells and appeared to be released in the RET under stress conditions. Taken together our findings suggest that AGPs together with fructans are involved in root response of *L. perenne* to environmental stresses.

1. Introduction

Lolium perenne or perennial ryegrass is a Poaceae of agronomic interest due to its role as a grassland forage plant and its high nutritional forage quality and yield. The refoliation ability of this plant, following mowing and animal grazing allows for several cut-regrowth cycles throughout the growing season (Meuriot et al., 2022). However, there is still a growing demand to increase shoot biomass production in this pasture crop while limiting the use of fertilizers such as urea (Zaman et al., 2016). The current context of global warming, with reduced water availability, might also have an impact on leaf growth and consequently forage productivity of perennial ryegrass (Jaškūnė et al., 2020). However, the consequences of environmental stresses on leaf and root growth and yield production of this temperate grassland species are poorly understood (Rigui et al., 2019). Perennial ryegrass is one of the species that accumulate carbon reserves mainly in the form of fructans, which are water-soluble polymers of fructosyl residues linked by β -(2,1) and/or β -(2,6) linkages with one external or internal glucosyl residue (Pavis et al., 2001). In addition to their role as storage carbohydrates, fructans play a protective role for the plant such as in drought tolerance, by providing membrane protection and acting as antioxidants (Hincha et al., 2007; Stoyanova et al., 2011; Nemati et al., 2018). It was previously reported that fructans could also play a particular role in the concept of “sweet immunity” or “sugar-enhanced defense” which supports the idea that sugar metabolism and signaling involved in plant immunity are tightly interconnected (Bolouri Moghaddam and Van den Ende, 2013; Trouvelot et al., 2014; Tarkowski et al., 2019; Svara et al., 2020). Although most studies in perennial ryegrass were performed on leaves, the root system is known to play a vital role in maintaining plants health and survival in their natural environment. External conditions such as temperature (Ahmed and Escobar-Gutiérrez, 2022) and cadmium or aluminum exposure were shown to impact root development in *L. perenne* (Lambrechts et al., 2014; Vera-Villalobos et al., 2020). Patchett and Newman (2021) also reported significant modifications in perennial ryegrass root exudation in response to fungal endophyte exposure. Such data highlight the need

for a better understanding of the relationship between root physiology and their surrounding environment for *L. perenne*.

A particularity of the root system defense is to rely on atypical cells termed root “border cells” (BCs) for its protection due to their localization at the interface between root and soil. The presence of BCs has been reported in more than 35 species belonging to 11 different families including fructan-accumulating Poaceae (oat, wheat, barley) (Hawes et al., 2002; Tamás et al., 2005). Different populations of BCs can be delivered by the root tips of a single plant species, e.g. pea (*Pisum sativum*) or soybean (*Glycine max*) released spherical cells, intermediate-size cells and elongated cells (Cannesan et al., 2011; Ropitiaux et al., 2020). Vicré et al. (2005) described for the first time the presence of cells that, unlike BCs, remain associated together into small groups of cells and organized in a sheath-like pattern after release from the root tips of *Arabidopsis thaliana*. Based on their unusual organization and adhesion to the root cap, they were named “border-like cells” or BLCs (Vicré et al., 2005; Driouich et al., 2007; Durand et al., 2009). The presence of BLCs was subsequently discovered in other species belonging to Brassicaceae family including rapeseed (*Brassica napus*), Brussels sprout (*Brassica oleraceae*), mustard (*Sinapis alba*), and radish (*Raphanus sativus*) (Driouich et al., 2007, 2010, 2012). This type of BLC organization was also reported in flax (*Linum usitatissimum*) where three morphotypes were described: spherical border-like cells (sBLC), elongated border-like cells (eBLC) and filamentous border-like cells (fBLC) (Plancot et al., 2013). For simplification, the term root associated-cap derived cells (AC-DCs) was proposed by Driouich et al. (2019) to include the different cell types previously described (Hawes et al., 2000, 2002; Driouich et al., 2007, 2010; Endo et al., 2011; Karve et al., 2016; Wang et al., 2017).

AC-DCs are embedded by a thick layer of mucilage enriched in cell wall polysaccharides including homogalacturonan, xylogalacturonan, xyloglucan and proteoglycans such as arabinogalactan proteins (AGPs) and extensins (Knee et al., 2001; Durand et al., 2009; Cannesan et al., 2012; Mravec et al., 2017; Ropitiaux et al., 2019). This mucilage together with AC-DCs form a protective structure defined as the Root Extracellular Trap (RET) by analogy with the Neutrophil Extracellular Trap (NET) involved in mammalian immunity (Bowdish et al., 2005; Urban et al., 2009; Driouich et al., 2013). The RET is characterized by the presence of diverse antimicrobial molecules such as reactive oxygen species (ROS) (Plancot et al., 2013), proteins (Ma et al., 2010; Weiller et al., 2017) and extracellular DNA (exDNA) (Wen et al., 2009; Tran et al., 2016). The role of the RET in immune defense mechanism is thought to occur through a wide range of mechanisms including either attraction, repulsion or neutralization of

microbial pathogens (Driouich et al., 2013, 2019). Ropitiaux et al. (2020) demonstrated that the RET from soybean acts as a physical (or even chemical) barrier preventing zoospores of *Phytophthora parasitica* from reaching and colonizing the root cap. The involvement of RET in the root protection towards abiotic environmental stresses, e.g. toxicity of some heavy metals such as aluminum, cadmium, and copper (Deiana et al., 2003; Cai et al., 2011) or drought although less studied was reported (Carreras et al., 2019). However, the precise function of molecules involved in such mechanisms, providing correct RET functioning, remained to be clearly established.

The presence of RET in plants presenting the particularity to accumulate fructans, has never been investigated. Here, we provide the first detailed characterization of the occurrence of cell-wall glycomolecules in root BCs and mucilage of perennial ryegrass using immunocytochemistry. The histochemical distribution of fructans within plant organs is poorly investigated due to the lack of specific probes. It is therefore not known whether fructan epitopes are part of the cell walls of BCs and/or the mucilage in perennial ryegrass. To this end we previously developed monoclonal antibodies (mAbs) recognizing fructans from plants (Nguyen et al., 2023). Root tips of *A. thaliana*, together with two other fructan-accumulating Poaceae timothy (*Phleum pratense*) and wheat (*Triticum aestivum*), were also used for comparison in order to determine whether the RET of perennial ryegrass presents some particularities. The response of the RET of perennial ryegrass to polyethylene glycol (PEG)-induced drought stress and to the presence of the bacterial elicitor flagellin22 (flg22; Millet et al., 2010) was also investigated in order to assess its potential properties in root protection. The most important findings are: (i) perennial ryegrass root tip released BCs of different morphologies and an abundant mucilage; (ii) mucilage secretions present an atypical composition and are enriched in AGPs (iii) fructans are released within the RET in response to PEG-induced osmotic stress and flg22 treatment.

2. Materials and Methods

2.1. Plant material and growth conditions

Seeds of perennial ryegrass (*Lolium perenne* var. Delika), timothy (*Phleum pratense* var. Aturo) and wheat (*Triticum aestivum* var. Cheignon) were surface sterilized with 70% (v/v) ethanol (5 min), then with 0.9% (v/v) sodium hypochlorite (2 min). They were washed 6 times in sterile water before being sown onto Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Duchefa Biochemie) containing 1% (w/v) agar (European Bacteriological Agar,

Condalab). *Arabidopsis thaliana* (Col-0) seeds were sterilized and sown on Arabidopsis medium (Duchefa Biochemie) containing 1% (w/v) agar supplemented with 2 mL $\text{Ca}(\text{NO}_3)_2$. All Petri dishes with seeds were then placed vertically in continuous light ($120 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 21°C in 16-h-day/8-h-night, to avoid the roots penetrating the agar and the subsequent loss of border and border-like cells, as described by Vicré et al. (2005). For treatment with the Yariv reagent, which specifically binds and precipitates arabinogalactan proteins (AGPs), the 6-d-old perennial ryegrass seedlings (with the same root length) were transferred into a MS medium containing 1% (w/v) agar and supplemented with 1 or $10 \mu\text{M}$ β -glucosyl-Yariv (βglcY) or 1 or $10 \mu\text{M}$ β -galactosyl-Yariv (βgalY) (Biosupplies Australia).

2.2. Water deficit treatment

The 6-d-old perennial ryegrass seedlings (with the same root length) were transferred to a plate containing 30 mL solidified MS $\frac{1}{2}$ medium soaked with 45 mL of MS $\frac{1}{2}$ liquid medium containing polyethylene glycol (PEG) (300 g of PEG in 1L of MS $\frac{1}{2}$ liquid medium) (molecular weight 8,000; Sigma) prepared the day before the experiment, following the protocol described by van der Weele et al. (2000) and Verslues et al. (2006). Another set of 6-d-old perennial ryegrass seedlings was sown on 30 mL solidified MS $\frac{1}{2}$ medium mixed with 45 mL of MS $\frac{1}{2}$ liquid medium without PEG for the “well-watered” control medium. Then, all the plates were sealed with surgical tape and placed vertically in a phytotron at 21°C under a photoperiod of 16h-day/8h-night. After 6 days of treatment, 12-d-old roots were collected lately for visualizing mucilage and for the immunolabeling experiment.

2.3. Elicitation treatment by flg22

The synthetic peptide flagellin 22 (flg22) was used at $1 \mu\text{M}$ and deposited on the roots for elicitation treatment (Felix et al., 1999; Millet et al., 2010). After 48h of treatment, the 12-d-old roots were collected for visualizing mucilage and for the immunolabeling experiment as previously described (Plancot et al., 2013).

2.4. Morphotypes and location of root border cells

For this technique, root tips from 12-d-old perennial ryegrass seedlings were selected with ultra-fine tweezers and mounted on Superfrost microscope slides (Thermo Scientific) in a drop of water for direct examination for morphological analyses using an inverted bright-field microscope (Leica DMI6000B, Wetzlar, Germany). In this experiment, 24–30 roots were observed to ensure representativity for each set of observations.

2.5. Visualization of mucilage by counterstaining with India ink

India ink (Black star Hi-Carb, 1.0 oz) produced from carbon black, serves as a negative stain to visualize mucilage (Miyasaka and Hawes, 2001; Curlango-Rivera et al., 2013). The root tips were collected and placed on Superfrost microscope slides (Thermo Scientific). A 0.05% (w/v) India ink solution was added by capillary action between the slide and the coverslip from the ends to the center of the sample. After 10 min, the samples were observed under an inverted bright field microscope (DMI6000B). In this experiment, 4 to 5 technical replicates and 6 biological replicates were performed.

2.6. Analysis of the mucilage and cell wall monosaccharide composition

Root tips (including root cap and RET) and elongation zone fragments (approximately 0.2 to 0.3 mm) of 12-d-old perennial ryegrass seedlings were selected with ultra-fine tweezers, ground in 70% ethanol and incubated at 70°C for 15 min for the Alcohol Insoluble Residues (AIR) extraction. The pellet obtained after centrifugation at 4,000 xg for 10 min was washed three times with 70% ethanol, dissolved in 5 mL of methanol:chloroform (1:1, v/v) and incubated for 1h at room temperature (RT) and overnight in acetone. For gas chromatography (GC) analysis, 1.0 mg of the dried AIR pellet was used.

The monosaccharide composition of the AIR samples, composed of non-cellulosic cell wall polysaccharides was determined according to the protocol developed by Cannesan et al. (2012) with GC analysis of trimethylsilyl methylglycoside derivatives after hydrolysis in 2M trifluoroacetic acid (York et al., 1986). Inositol served as an internal standard, and a mixture of external standard sugars, including arabinose (Ara), fucose (Fuc), galactose (Gal), galacturonic acid (GalA), glucose (Glc), glucuronic acid (GlcA), mannose (Man), rhamnose (Rha) and xylose (Xyl) was used. The injection was carried out in the GC-FID (gas chromatography-flame ionization detector) system, equipped with a CP-Sil5-CB capillary column (Agilent Technologies). The trimethylsilylated derivatives were eluted by a constant flow of helium according to the following temperature program: 40°C for 3 minutes, rise to 160°C at 15°C.min⁻¹, rise to 220°C at 1.5°C.min⁻¹ and finally up to 280°C at a rate of 20°C.min⁻¹.

2.7. Primary antibodies against cell wall polysaccharides and fructans

Primary monoclonal antibodies (mAbs) recognizing epitopes associated with different cell wall polysaccharides were mainly provided by Plant Probes (University of Leeds, UK) and Biosupplies Australia (<http://www.biosupplies.com.au>). A summary table of the antibodies

used in this project is presented in **Supplemental Table S1** providing details of the epitopes recognized by the antibodies and their associated references.

The mAbs termed BTM15A6, recognizing specifically β -(2,6) and β -(2,1) linked fructans (Nguyen et al., 2023), was also used.

2.8. Immunolabeling of root border cells and mucilage on the surface of root tips

To label the polysaccharides, glycoproteins, and proteoglycans present in the cell wall of root border cells and the mucilage, an indirect surface immunolabeling protocol has been developed, as recently described by Castilleux et al. (2018). Roots of 12-d-old seedlings were placed onto sterile 10-well diagnostic microscope slides (Thermo Scientific). The wells were then filled up with 30 μ L of phosphate-buffered saline (PBS) for 5 min to initiate detachment of root border cells. Next, the liquid was removed using an Eppendorf micropipette (P200), by taking from the severed end of the root. All of the root tips were fixed for 40 min in 4% (w/v) PFA (paraformaldehyde), in 50 mM PIPES pH 7 (piperazine- N, N'-bis [2-ethanesulfonic acid], Alfa Aesar) containing 1 mM CaCl₂. Roots were washed 4 times for 10 min each wash at RT in PBS 1x containing 1% (w/v) bovine serum albumin (BSA) (Aurion) to eliminate the maximum of PFA, and to allow the saturation of the non-specific sites of the sample. After having eliminated the last wash, 30 μ L of a solution of primary antibody were added and incubated overnight at 4°C in a humid chamber (dilution 1:5 in 1x PBS containing 1% w/v BSA for mAbs listed in **Supplemental Table 1**, excepted 1:20 for BTM15A6). The primary antibody solution was removed before performing four washes with PBS+1% BSA at RT for 10 min.

The goat anti-rat IgG secondary antibody conjugated to Alexa Fluor 594 (Invitrogen) was diluted to 1:50 in the solution of PBS+BSA 1 % and incubated with the samples for 2 h at 25°C in the dark. For the BTM15A6 mAb, the same procedure was followed with the donkey anti-mouse IgG secondary antibody, conjugated to Alexa Fluor 594 (Invitrogen). Four washes with PBS+BSA 1% were then carried out at RT for 10 min to eliminate the secondary antibody, followed by a final wash with PBS at RT for 10 min. To avoid photobleaching, citifluor (Agar scientific) was delicately deposited on the sample using a pipette tip of 200 μ L. The samples were observed under an epifluorescence microscope (Leica DMI6000B, Wetzlar, Germany; λ Excitation: 591 nm; λ Emission: 614 nm). Controls were routinely performed by incubation of the roots with the secondary antibody only (**Supplemental Fig. S1**). For this experiment, 3 to 4 technical replicates and 4 to 6 biological replicates were carried out. A semi-quantification of the fluorescence intensity obtained with the monoclonal antibodies was performed using ImageJ software on epifluorescence microscopy images. This approach is based on grey value

measurements (Jung et al., 2020; Pelikh et al., 2021). The grey values represent the light intensity for each pixel, ranging from 0, corresponding to low light intensity, to 255, indicating high light intensity. The maximum grey value was determined on 8-bit images within a delimited region of interest, corresponding to the root tip and RET. An average was performed on measurements from 10 to 20 images for each mAb. Four ranges of maximum grey values were chosen to compare the fluorescence labeling according to the growth conditions and species (**Supplemental Fig. S2**).

2.9. High-Pressure Freezing/Freeze Substitution (HPF) sample preparation

Dissected 12-d-old root tips of *Arabidopsis* and perennial ryegrass grown *in vitro* were transferred into the cavity of copper cupules (100 μm in depth; 0.6 mm in diameter and 200 μm in depth; 0.6 mm in diameter, respectively). All of the cupules were coated with soybean (*Glycine Max*) lecithin (100 $\text{mg}\cdot\text{mL}^{-1}$ in chloroform). The excess medium was removed using filter paper. The sample carriers were tightened securely to the pod of the sample holder by using a horizontal loading station. Then, samples were frozen using a high-pressure freezing HPF-EM HPM 100 (Leica Microsystems) according to a maximum cooling rate of $20,000^{\circ}\text{C}\cdot\text{s}^{-1}$, an incoming pressure of 7.5 bar, and a working pressure of 4.8 bar. Cupules containing frozen samples were stored in liquid nitrogen until the freeze-substitution procedure was initiated.

After high-pressure freezing, samples were transferred to a freeze-substitution automate (AFS2, Leica Microsystems) precooled to -110°C . Samples were substituted in anhydrous acetone with 0.5% uranyl acetate at -90°C for 96h (Ovide et al., 2018). Using a gradient of $+2^{\circ}\text{C}\cdot\text{h}^{-1}$, the temperature was gradually raised from -90 to -15°C with two intermediate steps at -60 and -30°C . Samples were washed twice at RT with fresh anhydrous ethanol. Resin infiltration was processed at -15°C in a solution of ethanol/London Resin White (LRW) with successive baths as followed: 8h with LRW 25%; 16h with 50%; 8h with 75%; 16h with 100%. Finally, samples were infiltrated 2 x 24 hours in LRW with 0.5% (w/v) of benzoin methyl ether (UV catalyst), and polymerized. at -15°C under ultraviolet light for 48h. Using a Leica ultramicrotome EM-UC7 (Leica Microsystems), semithin sections (0.5 μm) were cut and adhered onto 10-well diagnostic microscope slides (Thermo Scientific) pre-coated with Poly-L-Lysine (dilution 1:10 in filtered water). The HPF sections were stained with toluidine blue to highlight components.

2.10. Immunolabeling of root border cells and mucilage on the HPF sections

Semithin sections (0.5 μm) of 12-d-old roots and leaf bases on 10-well Teflon microscope slides coated with Poly-L-Lysine were blocked in PBS 1x with 0.1% (v/v) Tween 20 (PBST)

containing 3% (w/v) BSA and normal goat (or donkey for anti-fructans immunolabeling) serum (NGS-dilution 1:20) for 30 min at RT. Then, sections were carefully washed 5 times for 5 min with 0.1 % PBST containing 1% BSA. After washing, sections were incubated overnight at 4°C with primary antibody (dilution 1:2 in 0.1% PBST containing 1% BSA and NGS (diluted 1:20)). On the next day, sections were washed 5 times for 5 min with 0.1 % PBST containing 1% BSA before being incubated with goat anti-rat IgG (or donkey anti-mouse IgG for anti-fructans immunolabeling) secondary antibody conjugated to Alexa Fluor 594 (Invitrogen) at 1:200 dilution in 0.1% PBST containing 1% BSA and NGS (diluted 1:20) for 2 h at 25°C. At RT, sections were rinsed 5 times for 5 min with 0.1 % PBST containing 1% BSA and two final washes for 5 min at RT with ultrapure water. Then, a droplet of ultrapure water was added to the section of each well. Epifluorescence of the immunostained tissue sections was observed on an epifluorescence microscope (Leica DMI6000B, Wetzlar, Germany; λ Excitation: 591 nm; λ Emission: 614 nm). Control experiments were performed by omission of primary antibodies (**Supplemental Fig. S3**). For this experiment, 3 technical replicates and 4 biological replicates were performed.

2.11. Ultrastructural and immunogold analyses using transmission electron microscopy

Ultrathin sections (90 nm; EM UC6 Leica microsystems) of perennial ryegrass root tips from HPF samples prepared previously are collected on nickel formvar-coated grids. For immunogold analysis, sections are blocked in PBS 1x containing 3% BSA for 30 min at RT. Sections were then incubated with the primary antibody (JIM13, PlantProbes; dilution 1:2 in PBS 1x containing 0.3% BSA overnight at 4°C in a humid chamber). After washing in PBS 1x containing 0.3% BSA, grids were incubated for 1h at 37°C with the goat anti-rat secondary antibody conjugated to 10 nm gold particles (dilution 1/20 in PBS 1x containing 0.3% BSA; British Biocell International). Before transmission electron microscopy observation, all sections were stained with classical staining using uranyl acetate (0.5% in methanol) and Reynolds lead citrate (Delta microscopies). Observations were made with a FEI Tecnai 12 Biotwin transmission electron microscope operating at 80 kV, with ES500W Erlangshen CCD camera (Gatan).

2.12. Statistical and image analysis

The results of monosaccharide composition and root lengths of the seedlings were analyzed with the GraphPadPrism software (version 7.05), with two-way ANOVA and Tukey's multiple comparisons tests. The RET surfaces were measured on the images acquired under the inverted

microscope with India ink counterstaining. Results were determined using ImageJ 1.53p software on 60 root tips by measuring the total area of the RET containing the root cap and then subtracting the root cap surface. The data were analyzed with R software (version 4.0.0). Statistical significance was calculated by using the Kruskal–Wallis test. The statistical effects were considered significant with p -value <0.05 .

3. Results

3.1. An abundant mucilage is released at the root tip of perennial ryegrass

The negative staining with India ink revealed the presence of an abundant mucilage along the root tip (**Fig. 1A**). Most of the mucilage is concentrated at the root cap and meristematic zone (**Fig. 1D**) and to a lesser extent in other root zones (**Fig. 1B, 1C**). Observation of the root tip with a bright-field microscope revealed that perennial ryegrass released large numbers of individual root BCs from the periphery of the root cap in the presence of water (**Fig. 2**). Two cell morphotypes based on their size and shape were observed depending on their localization at the root tip. Small spherical border cells (sBC) were abundantly present among the BC population and mostly associated to the root cap zone (**Fig. 2C, 2D**). The presence of few elongated border cells (eBC) with an elongated shape and slightly curved was also found along the meristematic and the elongation zones (**Fig. 2A, 2B**). The sBC represent around 65% and the eBC 35% of the whole BCs population.

3.2. Fructan epitopes are detected in root cap cells but not in the RET.

Indirect immunofluorescence of fructan epitopes with BTM15A6 mAb indicated labeling at the surface of epidermal cells from meristematic and elongation zones while no labeling was observed at the RET surface including BC and mucilage (**Fig. 3B**). Labeling was also performed on longitudinal section of root tips prepared by high pressure freezing, freeze substitution and subsequent resin embedding (**Fig. 3C**). While the RET surface was deprived of fluorescence, root cap cells present a uniform pattern of distribution of the epitopes recognized by BTM15A6 mAb (**Fig. 3B, 3C**). Punctate labeling together with a faint diffuse fluorescence was present in the cytoplasm whereas no staining of the cell walls was detected in root cap cells (**Fig. 3C**).

3.3. Heteroxylan epitopes are part of the perennial ryegrass RET

We analyzed cell wall monosaccharide composition of the root tip (including the root cap and RET) in comparison with the root elongation zone of perennial ryegrass (**Fig. 4**). The major monosaccharides identified in the non-cellulosic cell wall polymers are xylose (Xyl), arabinose (Ara) and galactose (Gal) suggesting the presence of xylans, arabinoxylans or AGPs. The relative percentage of Ara and Xyl slightly differs between the root tip and root elongation zone probably reflecting differences in cell wall polymers between these two root compartments. Using a set of well-characterized mAbs in conjunction with fluorescence imaging, we investigated the occurrence of plant cell wall polysaccharides (hemicelluloses and pectins) in the RET of perennial ryegrass (**Table 1**).

We investigated whether the hemicellulosic polymers commonly found in grasses are present within the RET (**Fig. 5, Supplemental Fig. S4**). Mixed linked glucans epitopes recognized by mAb MLG were not detected in border cell walls nor in the mucilage of perennial ryegrass (**Table 1**). Xylan epitopes recognized by mAb LM10 and arabinoxylan and low-substituted xylan epitopes recognized by mAb LM11 were only slightly detected in the mucilage (**Table 1**). The LM12 feruloylated epitope also slightly bound to the mucilage of perennial ryegrass root (**Fig. 5B**). Among the antibodies tested, only the heteroxylan LM27 antibody bound to the cell walls of the different BC morphotypes of perennial ryegrass (**Fig. 5C, Table 1**). The same observations were made in the RET of the two other fructan-accumulating Poaceae studied, wheat and timothy (**Table 1**).

Among pectins, although homogalacturonans (HG) were previously demonstrated to be essential in border cells attachment in *A. thaliana* and pea (Vicré et al., 2005; Durand et al., 2009), low (or non) methylesterified HG epitope and high methylesterified HG epitope using LM19 and LM20 mAbs were not detected in the RET of perennial ryegrass nor in wheat and timothy (**Table 1, Fig. 6A, Supplemental Fig. S5C**), unlike *Arabidopsis* for which the labeling was intense (**Fig. 6B, Supplemental Fig. S5D**). Our data revealed that only LM5 epitopes corresponding to galactan side chains were detected but showed weak fluorescence with the cell wall of perennial ryegrass BCs (**Table 1, Fig. 6C**) and no labeling occurred with LM6 mAb specific to epitopes containing arabinan side chains (**Table 1, Supplemental Fig. S5E**). On the contrary, intense labeling of the cell wall of *Arabidopsis* BLCs was observed with LM5 and LM6 mAbs (**Fig. 6D, Supplemental Fig. S5F**).

3.4. The RET of perennial ryegrass is enriched in arabinogalactan proteins epitopes

In addition to polysaccharides, we investigated the occurrence of hydroxyproline-rich glycoproteins (HRGPs) epitopes including extensins and AGPs in the RET of perennial ryegrass.

No labeling or only a faint fluorescence was observed at the RET surface regarding extensin epitopes as previously described in the literature LM1, JIM11, JIM12, JIM19, and JIM20 (**Table 1, Supplemental Fig. S6**). Interestingly, such observations were also found to occur in the RET of the two other fructan-accumulating Poaceae studied, wheat and timothy (**Table 1**). Only the mAb JIM20 labeled the mucilage and border cells present in perennial ryegrass (**Supplemental Fig. S6E**).

Whereas AGP epitopes recognized by JIM16 present only a weak signal (**Fig. 7A**), the RET of perennial ryegrass was strongly labeled with the mAbs JIM8, LM2 and JIM13 (**Fig. 7C, 7E, 7G, Fig. 8A-C**). Similar observations were made with wheat and timothy labeling with intense staining observed at the RET surface (**Table 1**). In contrast, JIM13 only presents a restrictive label to the cell surface of BLCs of Arabidopsis (**Fig. 7H**) while no labeling was detected with JIM8 on the root surface (**Fig. 7D**), and an intense fluorescence was observed for the root with JIM16 and JIM13 (**Fig. 7B, 7F**). In perennial ryegrass root, fluorescence pattern with JIM8 and JIM13 mAbs presented the particularity to appear as a dense structured network surrounding the root cap and the meristematic zone (**Fig. 7C, 7G, Fig. 8A, 8C**).

Considering the strong detection of mAb JIM13 by the mucilage of perennial ryegrass root (**Fig. 7G**), we were interested in assessing the immunogold labeling of AGPs at the subcellular level using JIM13 on 12-d-old perennial ryegrass root. It is thus confirmed that the AGP epitopes recognized by JIM13 were extensively present in the mucilage associated with the cell wall of BCs (**Fig. 8D**). As shown in **Fig. 8E and 8F**, we observed the presence of a large number of Golgi stacks in BCs of perennial ryegrass root, suggesting that BCs could be actively secreting molecules such as complex polysaccharides and glycoproteins into their walls and the surrounding medium. Higher magnification observations revealed the localization of epitopes detected by the JIM13 mAb along the plasma membrane (**Fig. 8F**). Interestingly these epitopes were found to be associated with secretory vesicles emerging from *trans* Golgi apparatus cisternae indicating their active biosynthesis and secretion within the RET.

3.5. Precipitation of AGPs by β -glucosyl Yariv reagent impacts BC production and mucilage release in *L. perenne*

AGPs appear to be an essential component of the RET of *L. perenne*. In order to assess the importance of AGPs within the RET, we investigated the impact of disrupting AGPs using the

β -glucosyl Yariv reagent (β GlcY), a synthetic phenyl glycoside that interacts with AGPs. 4 days-old seedlings were transferred in medium containing β GlcY reagent and grown for 6 days. The uptake and binding of β GlcY were monitored by the red coloration of the roots and a reduction in root growth as compared to controls including untreated seedlings (**Fig. 9A**) and seedlings grown in β GalY, a Yariv reagent that does not bind to AGPs (**Fig. 9D, 9F**). This effect on root growth was dose-dependent (**Fig. 9B**). We selected the condition of 10 μ M β GlcY to investigate the impact of RET organization. Seedlings grown in 10 μ M β GlcY did not show any significant difference as compared to those exposed to a higher concentration of 30 μ M β GlcY. The epidermal cells of the roots of β GlcY-treated plants presented intense bulging and root hairs underwent extensive growth (**Fig. 9C, 9E**). Although root caps from *A. thaliana* seedlings did not show any morphological changes in β GlcY-treated plants (Willats and Knox, 1996), root cap cells from perennial ryegrass presented an important reddish color and the RET surface appeared to be reduced as compared to control seedlings. Together these findings corroborate the hypothesis that AGPs are essential in maintaining root growth and RET organization in perennial ryegrass.

3.6. Impact of PEG treatments and flg22 on AGPs and fructans and on the RET organization in perennial ryegrass

In order to investigate whereas the perennial ryegrass RET is impacted by environmental stresses, we investigated the effects of PEG-induced osmotic stress and the impact of flg22 on the presence of AGPs and fructans at the root tip of perennial ryegrass. We performed immunolabeling on 12-day-old plants using the LM2, JIM13, and JIM8 mAbs to explore potential change in AGP distribution (**Fig. 10, Supplemental Fig. S7**) and BTM15A6 mAb to follow fructan distribution (**Fig. 11**).

In control plants (PEG-free and non-elicited plants), all three AGP-recognizing mAbs strongly labeled the mucilage which forms a densely structured network covering the root cap (**Fig. 10C, 10E, Supplemental Fig. S7A, S7C, S7E, S7G**). It should be noted that an increase in the RET surface occurred in response to PEG-treatment (**Fig. 10B, Supplemental Fig. S8**). The labeling with AGP-recognizing mAbs (LM2, JIM13, and JIM8) in PEG-treated roots covered a wider area of mucilage area until the elongation zone (**Fig. 10D, Supplemental Fig. S7B, S7D**). We obtained such fluorescence pattern in flg22-elicited roots (**Fig. 10F, Table 2, Supplemental Fig. S7F, S7H**).

To compare the impact of flg22 on AGPs in perennial ryegrass root tip compared with that of Arabidopsis, we also used LM2, JIM13 and JIM8 mAbs to examine the distribution of AGPs

(Supplemental Fig. S9). In non-elicited Arabidopsis, the fluorescence labeling was observed with the mAb LM2 all over the roots including BLCs (**Supplemental Fig. S9C**). Interestingly, the JIM13 labeled only the BLCs (**Supplemental Fig. S9E**) and no labeling was observed with JIM8 (**Supplemental Fig. S9G**). When Arabidopsis roots were elicited with flg22, stronger staining was observed in the mucilage with LM2 and JIM13 (**Supplemental Fig. S9D, S9F**). Treatment of Arabidopsis roots with flg22 showed no labeling with JIM8 as the non-elicited roots (**Supplemental Fig. S9H**).

Whereas AGP epitopes were heavily detected at the RET surface including BC and mucilage of perennial ryegrass in control conditions, no labeling was detected with BTM15A6, recognizing fructan epitopes (**Fig. 11A**). Interestingly, a slight signal was observed in the RET in response to PEG treatment (**Fig. 11B**). In response to flg22 treatment, immunofluorescence of fructan epitopes was found to occur in the whole RET covering the elongation zone area (**Fig. 11C, Table 2**).

4. Discussion

The formation and release of RET from the root cap to the rhizosphere are essential for protecting the root against biotic and abiotic stresses (Haichar et al., 2014; Driouich et al., 2019). In this study, we provide the first in-depth characterization of the RET in perennial ryegrass (*Lolium perenne*), an important grassland forage plant that accumulates fructans. The RET from *L. perenne* consists of individual root border cells embedded in a thick mucilage. Different populations of BCs were previously found to be released at the root tip of pea (*Pisum sativum*) and soybean (*Glycine max*) including spherical cells, intermediate-size cells, and elongated cells (Cannesan et al., 2011; Ropitiaux et al., 2020). It was reported that small BC were probably the more efficient cells in protecting the root tip against pathogenic oomycetes *A. euteiches* (Cannesan et al., 2012). Here, we found two different morphotypes of border cells in perennial ryegrass with spherical cells (sBC) and elongated cells (eBC). In other plants belonging to the Poaceae family, including barley, spherical and elongated border cells were also reported with a high level of viability (Tamás et al., 2005). In maize, only border cells presenting a bell-shape morphology were previously described and their number increased upon exposure to humic acid in a dose-dependent manner (Canellas and Olivares, 2017).

It is worth noting that border cells produced by perennial ryegrass were surrounded by an abundant mucilage, mostly found covering the root cap and the meristematic zone. In rice, another Poaceae, the production of mucilage was shown to increase in response to aluminum

and silicon exposure (Xiao and Liang, 2022). Interestingly, we found that PEG-induced osmotic stress and flg22 elicitation increased the quantity of mucilage in perennial ryegrass which forms a larger halo at the root tip extending to the elongation zone. These results suggest that root mucilage from Poaceae might be involved in protecting the root tip against abiotic stress.

The degree of methylesterification of pectins present within the RET of rice, which does not accumulate fructans, was thought to be a key event in such protection against abiotic stress (Xiao and Liang, 2022). In perennial ryegrass, the presence of pectins was not or only very faintly detected in both mucilage and cell wall surface of border cells. Although pectins (especially homogalacturonans) play a major role in BC detachment and are abundantly reported in the mucilage of many eudicot species (Durand et al., 2009; Cannesan et al., 2011), they appear to be minor components in the RET of perennial ryegrass and also in wheat and timothy. This is further supported by the monosaccharide composition data showing low contents of galacturonic acid and rhamnose residues. It is then tempting to hypothesize that due to their hydroscopic and adhesive properties (Rozen et al., 2001; Wong et al., 2022), perennial ryegrass fructans, would partially fulfill the role of pectins during the process of BC detachment, although we cannot rule out the possibility that they may be involved in response to biotic or abiotic stresses.

Interestingly, we also failed to detect the presence of extensin-associated epitopes at the RET surface of perennial ryegrass, timothy and wheat. A distinct distribution of extensin epitopes was previously reported to occur at the cell surface of border cells from *A. thaliana* roots (Plancot et al., 2013; Castilleux et al., 2018). Furthermore, the signal intensity and/or extensin epitope distribution were found to be impacted in response to various stresses. We found that root responses to elicitors resulted in a loss of labeling with LM1 mAbs (Castilleux et al., 2020). We thus proposed to use extensin epitopes specifically recognized by LM1 mAb as a novel marker of the root immune response (Plancot et al., 2013; Castilleux et al., 2020). However, to the best of our knowledge, the presence of LM1 epitope has never been observed at the root level in Poaceae. This lack of knowledge in literature regarding extensin distribution in Poaceae requires further investigations and raises questions about the involvement of extensins in root Poaceae defense. However, we could speculate that AGPs, also belonging to HRGPs family, are key elements involved in RET architecture and adhesion in perennial ryegrass. In line with this hypothesis, it was previously reported that heteroxylan epitopes from Poaceae (oat) cell walls recognized by the mAb LM27 are likely to bind AGPs (Cornuault et al., 2015). Such linkages between heteroxylans and AGPs could also occur in the RET of perennial ryegrass. As a consequence, we cannot rule out that the high abundance of AGPs present in both the

mucilage and cell walls could result in masking of hemicellulosic epitopes, thus explaining the weak surface labeling of epitopes from xylans, arabinoxylans and mixed-linkage glucan (MLG). AGPs are central elements in root-microorganisms interactions (Nguema-Ona et al., 2013). The alteration of AGPs was found to significantly inhibit the attachment of the rhizobium bacteria to the surface of BLC and Arabidopsis root tip (Vicré et al., 2005). AGPs were also reported to be involved in the susceptibility of root to pathogenic cyst nematode in Arabidopsis (Baum et al., 2000; Bozbuga et al., 2018). Following flg22 treatment, the labeling of AGP epitopes was more spread out, creating a wider structure throughout the entire surface of the mucilage produced. It is thus likely that AGPs from perennial ryegrass contribute to interactions between the root and soil-borne microbes. The root tip from perennial ryegrass also produced an enhanced quantity and/or spreading of mucilage in response to PEG-induced osmotic stress. Previously, AGPs have been shown to be involved in abiotic stress responses such as salt adaptation processes (Olmos et al., 2017) and low-temperature tolerance (Yan et al., 2015). AGPs are known as extremely hydroscopic glycoproteins with a high water-holding capacity (Fincher et al., 1983; Showalter, 2001). We cannot exclude that the widespread distribution of AGP epitopes in the mucilage of perennial ryegrass might have a role in protecting the root tip in water deficit condition.

Other promising candidates in root protection of *Lolium perenne* are fructans. Although the presence of fructan epitopes were not detected within the mucilage of perennial ryegrass under control conditions, these epitopes were found in the root cap cells and also at the cell surface of elongation and meristematic zones. Moreover, fructan epitopes were detected in the RET following osmotic stress and elicitation treatments, suggesting that they are released from the root cap cells upon abiotic and biotic stresses. Knowing the roles of fructans in plant tolerance towards abiotic (Livingston et al., 2009) and biotic stresses (Bolouri Moghaddam and Van den Ende, 2013; Trouvelot et al., 2014; Tarkowski et al., 2019; Svara et al., 2020), our findings confirm that fructans, together with AGPs, contribute to root response to environmental stress in perennial ryegrass. As AGPs are known to be key elements serving as crosslinkers, covalently interconnecting diverse cell wall polymers (Tan et al., 2024), we cannot rule out the possibility that other glycomolecules such as pectins and/or xylans could also be part of perennial ryegrass responses to biotic or abiotic stresses.

Acknowledgments

We are grateful to Carole Plasson and Bruno Gügi (GlycoMEV laboratory, University of Rouen Normandy) for excellent technical assistance with plant cultures and GC analysis. Gaëlle Durambur (GlycoMEV laboratory, University of Rouen Normandy), Nicolas Elie (CMABio3, US EMerode, University of Caen Normandy) and Didier Goux (CMABio3, US EMerode University of Caen Normandy) are thanked for their technical assistance with microscopical analyses.

Author contributions

TNHN, MF, MV, MLFG, MPP, and AMB conceived and designed the experiments; TNH performed flg22 elicitation and immunocytochemistry experiments; MF performed GC analysis, water deficit treatment and immunocytochemistry for PEG treatment; SB performed the high-pressure freezing/freeze substitution sample preparation and MLFG performed their sections; JL synthesized the peptide flagellin flg22. TNH, MF, MV and MLFG analyzed the data; TNH wrote the first draft, AMB, MV, MF, AD, MLFG, SB and MPP edited and improved the manuscript.

Funding

This work was supported by the Universities of Caen and Rouen Normandie and the SFR Normandie Végétal FED 4277. The Normandie Council supported the work through the research projects EPURE (Enhancing Plant nutrition and Health, 2017-2019) and Normandy Plant Technologies (2018-2021). The Normandie Council and the European Union (in the framework of the ERDF-ESF operational program 2014-2020) supported the work through the research project BEER (Bactéries, Exsudats, Et Rhizodépôts, 2019-2022). Thi Ngoc Hanh Nguyen received a PhD grant (2018-2021) from the Normandie Council. Mélanie Fortier received a PhD grant (2020-2023) from the Doctoral School EDnBISE (École Doctorale normande de Biologie Intégrative Santé Environnement).

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	CW polymers		mAbs	Epitopes	<i>L. perenn e</i>	<i>P. pratens e</i>	<i>T. aestivu m</i>	<i>A. thalian a</i>
Hemicelluloses	Mixed linkage glucan Xylan		MLG LM10	(1,3; 1,4)- β -D-glucan (1,4)- β -xylosyl residues	- -	- -	- -	- -
	Arabinoxylan and low-substituted xylan		LM11	(1,4)- β -xylosyl residues	+	+	+	+
	Feruloylated polymers Glucuronoxylan Grass Heteroxylan		LM12 LM28 LM27	Feruloylated xylan Glucuronosyl residues of xylan Unknown	+ - ++	+ - ++	+ - ++	- - ++
	Homogalacturonan s (HG)		LM19 LM20	HG with low degree of esterification HG with high degree of esterification	- -	- -	- -	+++ +++
Pectins	Galactan chains		LM5	(1,4)- β -D-galactan, Rhamnogalacturonan -I (RG-I)	+	+	++	++
	Arabinan chains		LM6	(1,5)- α -L-highly branched arabinan, RG-I	-	-	+	++
	Extensin from rice		LM1	Unknown	- ⁽¹⁾	+	- ⁽¹⁾	++
Extensins	Extensin from carrot		JIM1 1	Unknown	- ⁽¹⁾	- ⁽¹⁾	- ⁽¹⁾	++
	Extensin from carrot		JIM1 2	Unknown	- ⁽¹⁾	-	- ⁽¹⁾	++
	Extensin from pea		JIM1 9	Unknown	-	-	-	-

Arabinogalactan proteins (AGPs)	Extensin from pea	JIM2 0	Unknown	– ⁽¹⁾	++	– ⁽¹⁾	+++
	AGP from rice	LM2	β-D-GlcpA	+++	+	++	++
	AGP from carrot	JIM1 3	β-D-GlcA-(1,3)-α-D-GalA-(1,2)-α-L-Rha	+++	+++	++	+
	AGP from carrot	JIM1 5	Unknown	–	–	–	–
	AGP from carrot	JIM1 6	Unknown	+	–	+	++
	AGP from sugar beet	JIM8	Unknown	+++	+++	++	–

The fluorescence intensity was measured with ImageJ software on epifluorescence microscopy images. Four ranges of maximum grey values (MGV) were chosen: $MGV < 90$, fluorescence labeling not detected (–); $90 \leq MGV \leq 120$, a weakly detected fluorescence labeling (+); $120 < MGV \leq 150$, a clearly detected fluorescence labeling (++); $MGV > 150$, a strongly detected fluorescence labeling (+++) (See Supplemental Figure S2).⁽¹⁾ Fluorescence labeling detected in the meristematic zone and the beginning of the elongation zone but absent from the root cap and the RET.

		Control	PEG	Flg22
Fluorescence intensity	AGPs	+++	+++	+++
	Fructans	–	+	++
	RET surface			nd

The fluorescence intensity was measured with ImageJ software on epifluorescence microscopy images. Four ranges of maximum grey values (MGV) were chosen: $MGV < 90$, fluorescence labeling not detected (–); $90 \leq MGV \leq 120$, a weakly detected fluorescence labeling (+); $120 < MGV \leq 150$, a clearly detected fluorescence labeling (++); $MGV > 150$, a strongly detected fluorescence labeling (+++) (See Supplemental Figure S2). The arrow pointing upwards indicates an increase of the RET surface following the PEG treatment. nd, not determined.

Figure captions

Fig. 1. Visualization of perennial ryegrass secreted mucilage (m) using India ink staining. (A) Localization of different root zones along the 12-day-old root tips with a stereomicroscope: (B) DZ, differentiation zone; (C) EZ, elongation zone; (D) MZ, meristematic zone and RC, root cap. An abundant slimy mucilage embedding border cells (BCs) is observed at the root tip. Scale bars: (A) 500 μm ; (B, C, D) 100 μm .

Fig. 2. Border cells of different morphotypes are observed in perennial ryegrass using bright-field microscopy. (A) Localization of different cell types along the root tip: EZ, elongation zone; MZ, meristematic zone; RC, root cap. (B) Elongated or curved border cells, eBC; (C) spherical border cells, sBC; (D) sBC mostly present at the root tip. Scale bars: (A) 100 μm ; (B–D) 20 μm .

Fig. 3. Immunolocalization of fructan epitopes using BTM15A6 mAb. (A) Control root tip without primary antibody; (B) immunolabeling at the surface of root tips and (C) on longitudinal section of cryofixed root of perennial ryegrass. BC, border cell; EZ, elongation zone; MZ, meristematic zone; RC, root cap. Scale bars: (A, B) 100 μm ; (C) 50 μm .

Fig. 4. Monosaccharide composition of root tip and elongation zone of perennial ryegrass roots. Error bars indicate the standard errors of the means. Asterisks on error bars indicate significant differences in relative concentrations of monosaccharides with two-way ANOVA and Tukey's multiple comparisons tests; p-value 95% confidence interval, **: $p < 0.01$ $n = 5$. Ara, arabinose; EZ, elongation zone; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose; Xyl, xylose.

Fig. 5. Immunolocalization of hemicellulose epitopes on the surface of root tips. (A) Control root tip without primary antibodies. (B, C) Immunofluorescence labeling of the cell wall and extracellular material in 12-d-old perennial ryegrass with the mAbs LM12 (B) and LM27 (C). Fluorescence labeling appears around the cells and faintly stained the mucilage of perennial ryegrass root. BCs, border cells; m: mucilage; RC, root cap. Scale bars: 100 μm .

Fig. 6. Immunolocalization of pectin epitopes on the surface of root tips of 12-d-old perennial ryegrass (A, C) and 10-d-old *Arabidopsis* root tips (B, D). The mAbs used are LM19 (A, B) and LM5 (C, D). Note the low level of detection of pectin epitope associated to the RET of *L. perenne* as compared to *A. thaliana*. BCs, border cells; BLCs, border-like cells; EZ, elongation zone; m: mucilage; MZ, meristematic zone; RC, root cap. Scale bars: 100 μm .

Fig. 7. Immunolocalization of arabinogalactan protein (AGP) epitopes at the surface of root tips of (A, C, E, G) perennial ryegrass and (B, D, F, H) *Arabidopsis* root tips. The mAbs used are JIM16 (A, B); JIM8 (C, D); LM2 (E, F); and JIM13 (G, H). Fluorescence labeling appears as a dense network surrounding the perennial ryegrass root tips. BCs, border cells; BLCs, border-like cells; EZ, elongation zone; m: mucilage; MZ: meristematic zone; RC, root cap. Scale bars: 100 μm .

Fig. 8. (A, B, C) Immunolocalization of arabinogalactan protein (AGP) epitopes on longitudinal section of cryofixed root of perennial ryegrass with the mAbs JIM8 (A); LM2 (B); and JIM13 (C). Fluorescence labeling of JIM13 and JIM8 appears as a dense network surrounding the BCs and the mucilage in perennial ryegrass root. (D, E, F) Immunogold labeling of AGP epitopes recognized by JIM13 at the transmission electron microscopy level. Arrowheads indicate the AGP epitopes recognized by JIM13. CW, cell wall; EZ, elongation zone; G, Golgi stack; M, mitochondria; m, mucilage; PM, plasma membrane; RC, root cap; sv, secretory vesicles; V, vacuole. Scale bars: (A, B, C) 100 μm ; (D, E) 1 μm ; (F) 0.5 μm .

Fig. 9. Impact of Yariv reagent on root growth of perennial ryegrass. Root tips are stained with India ink (A) control; (C, E) 1 and 10 μM β -glucosyl-Yariv (βglcY); (D, F) 1 and 10 μM β -galactosyl-Yariv (βgalY). The images chosen are representative of the 20 root tips observed for each condition. The RET surface is delimited by the blue dotted lines. Note that the presence of β -glucosyl-Yariv in the culture medium colors the BCs red, reduces mucilage production and modifies the morphology of the root tip, with root hairs closer to the root cap (red arrowheads) (C, E). BCs, border cells; EZ, elongation zone; m, mucilage; RC, root cap. Scale bars: 100 μm . (B) Ryegrass root length in response to Yariv treatment. Error bars indicate the standard errors

of the means. Different letters on error bars indicate significant differences between growth conditions with two-way ANOVA and Tukey's multiple comparisons tests; p-values 95% confidence interval $p < 0.05$ $n = 20$.

Fig. 10. Impact of PEG and flg22 treatments on AGP epitopes distribution in the RET of perennial ryegrass. (A, B) Perennial ryegrass root tips stained with India ink. The RET surface is delimited by the blue dotted lines. (C, D, E, F) Immunostaining of AGP epitopes recognized by the mAb JIM13 at the root surface (C) control; (D) PEG treatment (300 g.L^{-1}); (E) elicited with sterilized water only and (F) elicited with $1 \mu\text{M}$ flg22. Note the presence of a dense mucilage observed in the perennial ryegrass root tip. Arrows point to BCs and mucilage. BCs, border cells; EZ, elongation zone; m, mucilage. Scale bars: $100 \mu\text{m}$.

Fig. 11. Immunolocalization of fructan epitopes using BTM15A6 mAb on the surface of ryegrass root tips: (A) control; (B) PEG treatment (300 g.L^{-1}) and (C) elicited with $1 \mu\text{M}$ flg22. Note the presence of a dense mucilage observed in the perennial ryegrass root tip delimited by the white dotted lines. Arrows point to BCs and mucilage. BCs, border cells; EZ, elongation zone; m, mucilage; MZ: meristematic zone; RC, root cap. Scale bars: $100 \mu\text{m}$.

Table 1. Immunolabelling of major glyco-polymers in the RET of *Lolium perenne*, *Phleum pretense*, *Triticum aestivum* and *Arabidopsis thaliana* using immunofluorescence microscopy.

Table 2. Changes in ryegrass RET composition based on semi-quantification of fluorescence intensity and RET surface in response to polyethylene glycol (PEG) treatment and flagellin 22 (flg22) elicitation.

Figure 1.

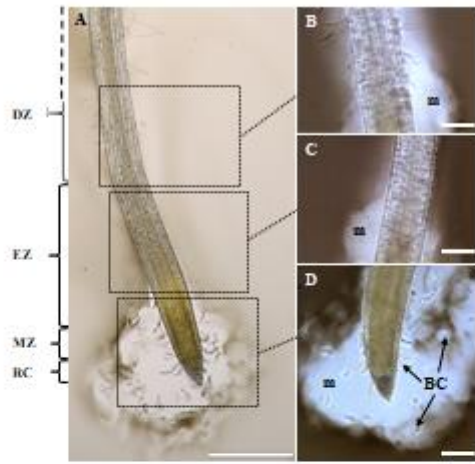


Figure 2.

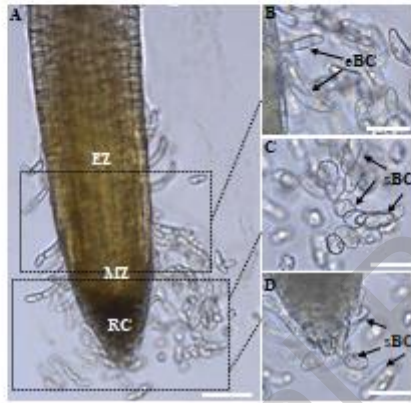


Figure 3.

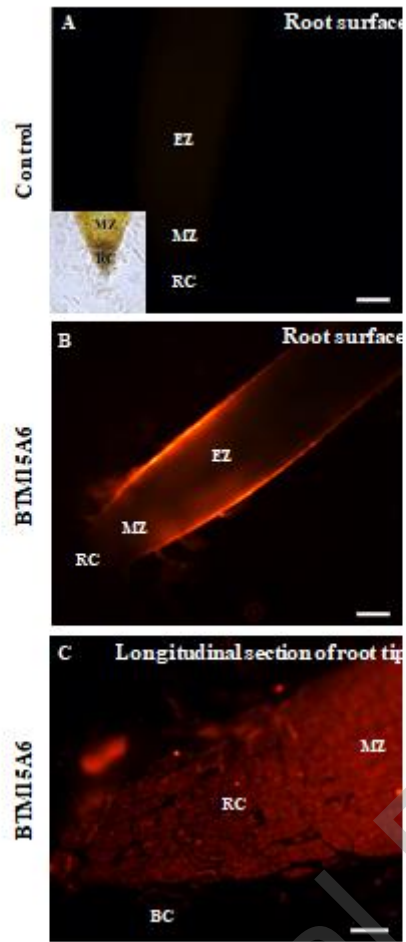
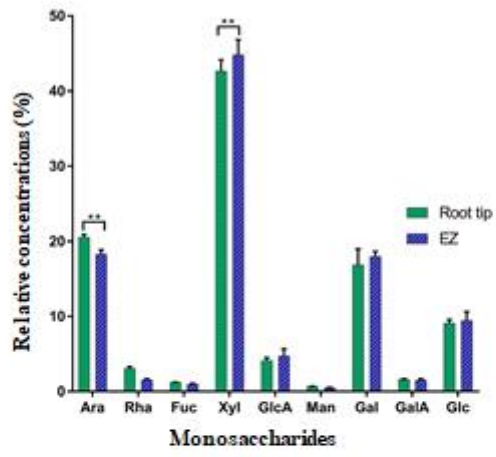


Figure 4.



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Figure 5.

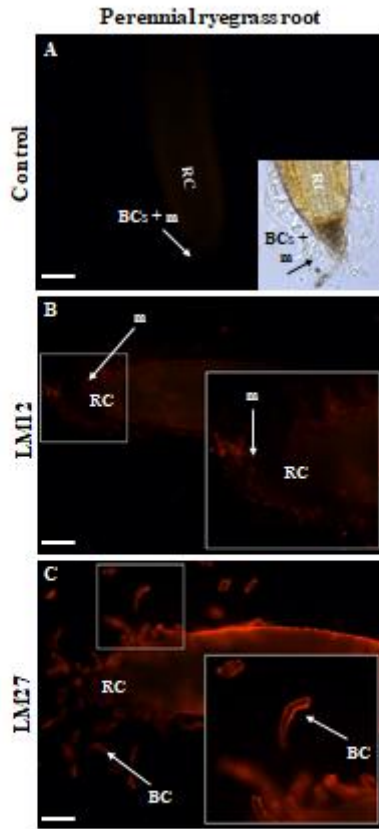
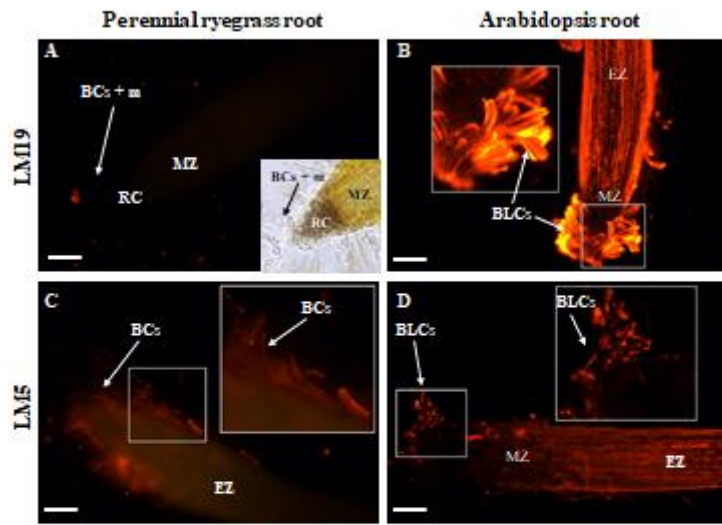


Figure 6.



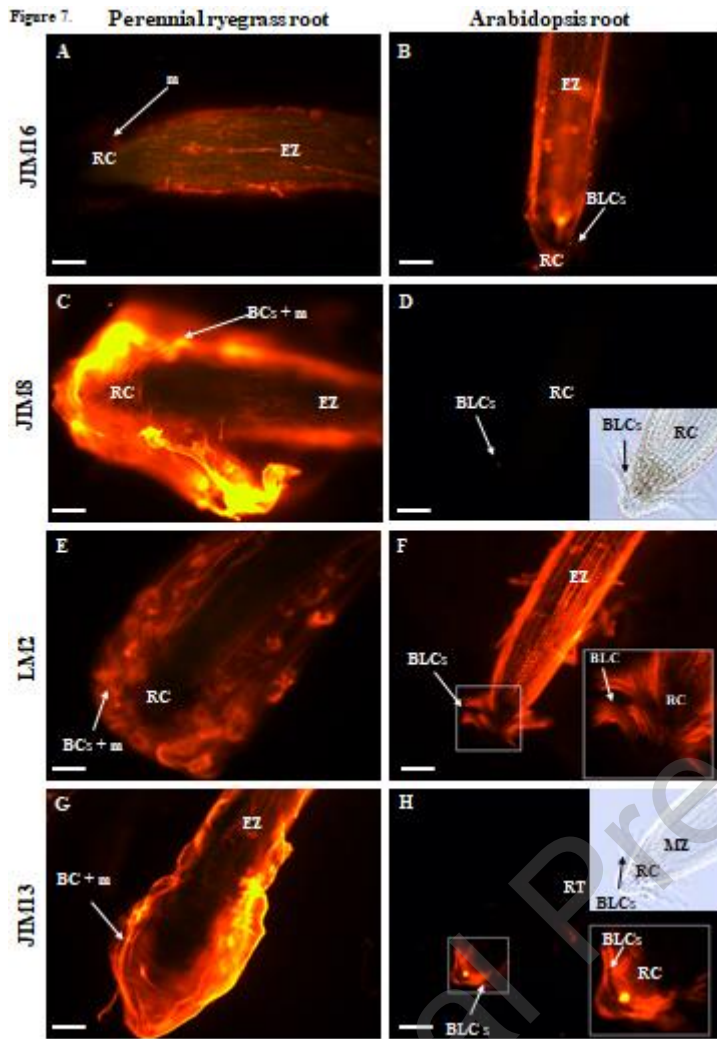


Figure 8.

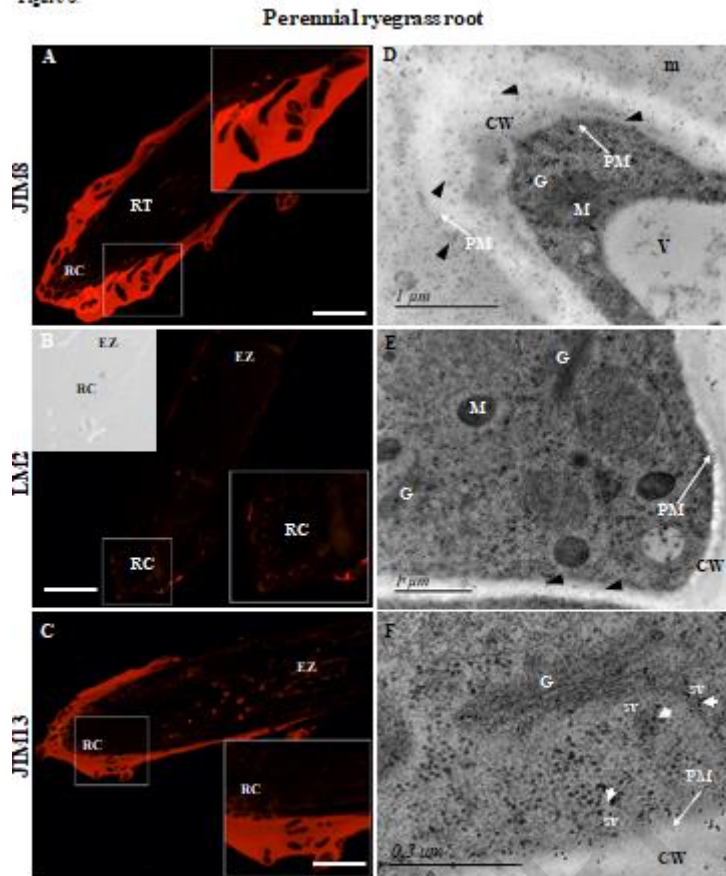


Figure 9.

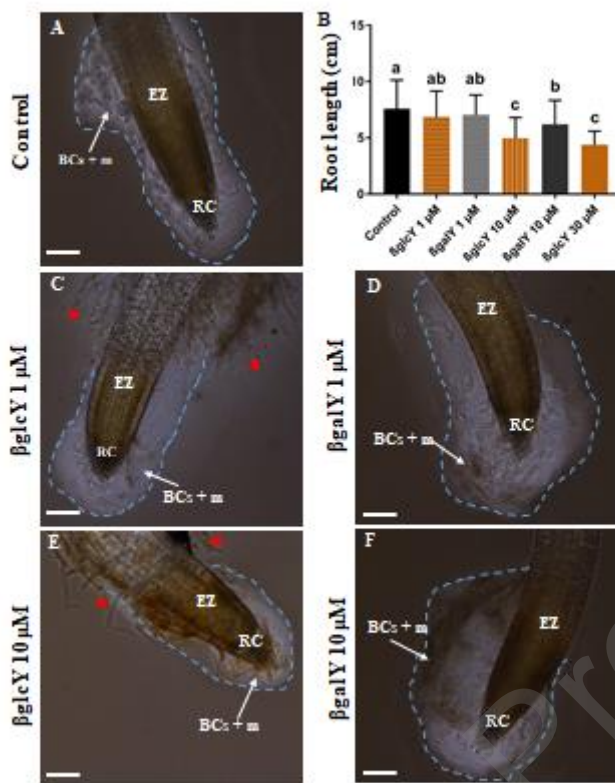


Figure 10.

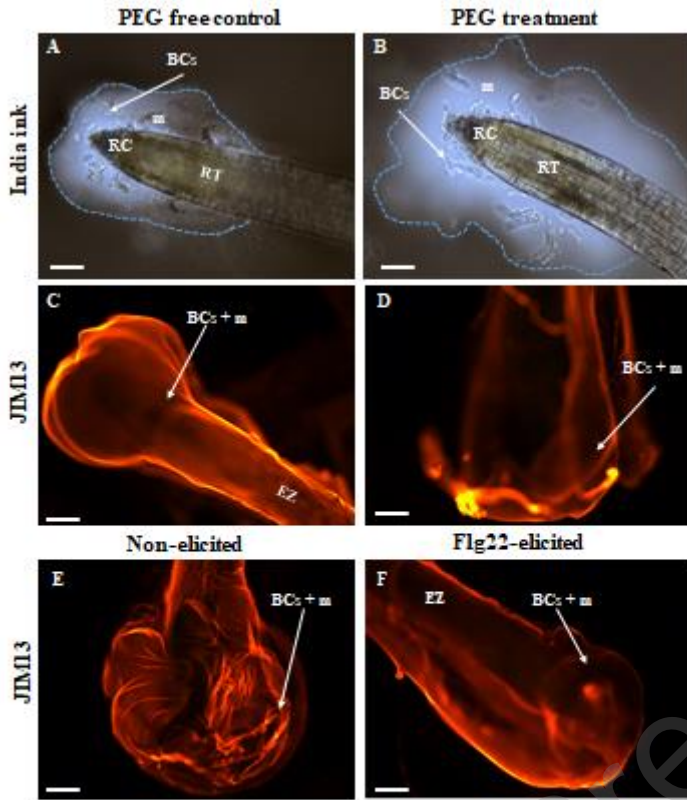
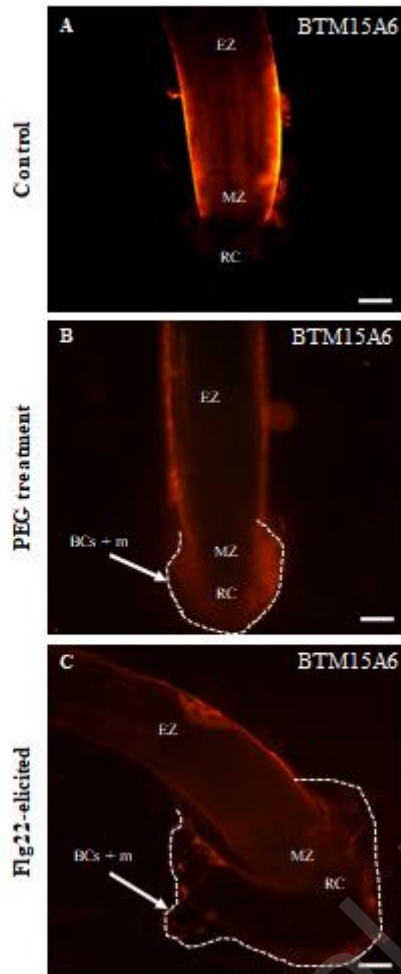


Figure 11.



Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

TNHN: Conceptualization, methodology, validation, formal analysis, investigation, writing-original draft, visualization

MF: Conceptualization, methodology, validation, formal analysis, investigation, writing-review and editing, visualization

SB: Methodology, validation, investigation, writing-review and editing

JL: Resources

AD: Resources, writing-review and editing, funding acquisition

MPP: Conceptualization, validation, resources, writing-review and editing, supervision, funding acquisition

MLFG: Conceptualization, methodology, validation, resources, writing-review and editing, supervision, funding acquisition

AMB: Conceptualization, validation, resources, writing-review and editing, supervision, funding acquisition

MV: Conceptualization, validation, resources, writing-review and editing, supervision, funding acquisition

Highlights :

- The root extracellular trap (RET) of perennial ryegrass contained border cells of different morphologies and an abundant mucilage enriched in arabinogalactan protein (AGP) epitopes.
- Fructan epitopes are released within the RET in response to PEG-induced osmotic stress and flagelin 22 elicitation.

Fructans, together with AGPs are promising candidates in root response to environmental stress in perennial ryegrass.