

**University of South Bohemia in České Budějovice**  
**Faculty of science**

**Screening of Reactive Oxygen Species Production  
Across the Plant Kingdom in Response to Treatment  
with Microbe-Associated Molecular Patterns**

Bachelor thesis

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## **Annotation**

This thesis uses a luminol-based detection method to examine ROS production triggered by flg22 in 194 plant species. A bioinformatic analysis was also performed to find potential FLS2, flg22 receptor, orthologs in the plant species.

## **Declaration**

I declare that I am the author of this bachelor thesis and that in writing it I have used only the sources and literature displayed in the list of used sources. I additionally declare that I have used the DeepSeek generative artificial intelligence tool to assist with rephrasing or correcting the English language in this work. After using this tool, I have reviewed and edited the content as necessary and assume responsibility for the content.

České Budějovice, 08.05.2025

Julie Hlaváčová

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# Abstract

Plants rely on induced immunity to protect themselves against microbial threats. The surface of plant cells contains Pattern Recognition Receptors (PRRs) that, upon recognition of Microbe-Associated Molecular Patterns (MAMPs), activate the plant's Pattern-Recognition Immunity (PTI). PTI includes a variety of responses which can occur within minutes after the induction with the MAMP or it can take days to fully develop. Among the earliest PTI responses is the production of extracellular Reactive Oxygen Species (ROS), particularly superoxide converted to hydrogen peroxide. One of the most well-studied MAMPs is flg22, a 22-amino acid peptide derived from a conserved region of bacterial flagellin. Flg22 is recognized by the PRR Flagellin-Sensing 2 (FLS2). Upon binding of flg22, FLS2 triggers the PTI. Although the flg22-FLS2 interaction has been extensively studied in model plants such as *Arabidopsis thaliana* and *Solanum lycopersicum* L., based on our knowledge, large-scale screening for ROS production across the plant kingdom has not yet been conducted. In this study, a luminol-based assay was used to detect hydrogen peroxide bursts in 194 plant species upon flg22 treatment as an indicator of PTI activation. Additionally, bioinformatic analysis was conducted to identify the putative presence of FLS2 in the tested plants. The results revealed ROS bursts in 29 plant species that had not been previously shown, confirming the presence of a functional FLS2 ortholog. These findings provide a broader variety of plants for studying the effects of flg22.

# Table of contents

|       |                                                                               |    |
|-------|-------------------------------------------------------------------------------|----|
| 1.    | Current state of knowledge .....                                              | 1  |
| 1.1   | Plant immunity.....                                                           | 1  |
| 1.1.1 | Pattern-triggered immunity .....                                              | 2  |
| 1.1.2 | Effector- triggered immunity .....                                            | 3  |
| 1.2   | PTI in detail .....                                                           | 3  |
| 1.2.1 | MAMPs.....                                                                    | 3  |
| 1.2.2 | PRRs .....                                                                    | 5  |
| 1.3   | Case study: flg22- FLS2 .....                                                 | 6  |
| 1.3.1 | Flagellin 22 .....                                                            | 6  |
| 1.3.2 | FLS2.....                                                                     | 8  |
| 1.3.3 | Early apoplastic ROS production in plant immunity .....                       | 9  |
| 1.4   | Methods for detection of ROS produced by plants .....                         | 10 |
| 1.4.1 | Chemical probes measurements.....                                             | 10 |
| 1.4.2 | Fluorescent biosensor.....                                                    | 12 |
| 1.4.3 | Electron Paramagnetic Resonance Spectroscopy .....                            | 12 |
| 2.    | Aims.....                                                                     | 14 |
| 3.    | Experimental part.....                                                        | 15 |
| 3.1   | Materials and methods .....                                                   | 15 |
| 3.1.1 | Plant material .....                                                          | 15 |
| 3.1.2 | Measurement of apoplastic H <sub>2</sub> O <sub>2</sub> .....                 | 15 |
| 3.1.3 | Bioinformatics.....                                                           | 15 |
| 3.1.4 | Literature survey .....                                                       | 16 |
| 3.1.5 | Construction of phylogenetic tree .....                                       | 16 |
| 3.2   | Results.....                                                                  | 17 |
| 3.2.1 | Typical kinetic course of ROS production after flg22.....                     | 17 |
| 3.2.2 | Response to flg22 across species .....                                        | 18 |
| 3.2.3 | Different responses to flg22 between “young” and “well developed” leaves..... | 24 |
| 3.3   | Discussion.....                                                               | 24 |
| 3.3.1 | Typical ROS response to flg22 .....                                           | 24 |
| 3.3.2 | Response to flg22 across species .....                                        | 25 |
| 3.3.3 | Effects of the age of the leaves .....                                        | 28 |
| 3.3.4 | Effects of temperature .....                                                  | 28 |
| 4.    | Conclusion .....                                                              | 29 |
| 5.    | Bibliography .....                                                            | 30 |

# 1. Current state of knowledge

## 1.1 Plant immunity

Plants, as sessile organisms, have multiple strategies to protect themselves from possible predators. Mechanical protection, such as bark, waxy cuticle or thorns, is effective against herbivores, however, they do not help against threats on a cellular level. Unlike animals, plants lack mobile immune cells, and an adaptive immune system dedicated to combating pathogen attacks. Therefore, they rely on the innate immunity of individual cells and the systemic signals emitted from infectious sites [1,2].

Based on the “zig-zag” model plant immunity can be separated into two layers. The first layer is initiated by the recognition of Microbe-Associated Molecular Patterns (MAMPs) through cell surface-localized receptors, referred to as Pattern Recognition Receptors (PRRs) [2,3]. This recognition triggers Pattern Triggered Immunity (PTI) responses that vary from immediate signaling events to transcriptional reprogramming leading to production of antimicrobial compounds, which can take up to hours to fully unfold [1,4]. However, pathogens secrete effectors into the cell cytoplasm, suppressing the PTI and promoting microbial fitness and virulence [2]. This suppression activates the second layer of plant immunity, in which mainly cytoplasmic Nucleotide binding Leucine-rich repeat Receptors (NLRs) recognize effectors, triggering the Effector-Triggered Immunity (ETI) [2]. Although ETI shares similar types of responses as PTI, which it is more specific and often, in coordination with PTI, leads to Hypersensitive Response (HR), a specific type of programmed cell death [2,4]. ETI and PTI were traditionally thought to be two separate branches of immune signaling. However, in recent years, it has become evident that they are dependent on each other [5].

### 1.1.1 Pattern-triggered immunity

As mentioned above, PTI is considered as the first layer in plant immunity. One of its first responses, which occurs within 30 seconds to five minutes upon MAMP treatment is  $\text{Ca}^{2+}$  burst. It involves an influx of extracellular  $\text{Ca}^{2+}$  into the cytosol, which consequently leads to the opening of other membrane transporters, leading to an influx of  $\text{H}^+$  and efflux of  $\text{K}^+$ ,  $\text{Cl}^-$  and  $\text{NO}_3^-$ . These concentration changes lead to extracellular alkalization and depolarization of the plasma membrane [6]. Additionally,  $\text{Ca}^{2+}$  concentration increase acts as a positive regulator for Reactive Oxygen Species (ROS) production, which is the following PTI response [7,8].

ROS burst first occurs within two three to three minutes after the MAMP application with a peak around 10-15 minutes in *Arabidopsis thaliana* (*A. thaliana*). ROS are produced due to their toxicity towards pathogens and to act as signaling molecules however, in high concentrations they are toxic to the plant itself and may trigger cell death. Thus, their concentration is tightly regulated, and ROS production is temporary [7,9,10].

Phytohormones play another crucial part of PTI since they act as signaling molecules within and between cells, coordinating their responses. The traditional phytohormones in plant defense are jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). SA is considered to be most effective against biotrophs and hemibiotrophs [11]. Its production begins within three to six hours after MAMP treatment, peaking at around nine hours [12]. On the other hand, JA and ET are effective against necrotrophs. Ethylene production begins earlier, around one hour after MAMP treatment, with its peak around four hours [7,13].

The final part of PTI is transcriptional reprogramming, mediating the signaling pathway into defense responses. MAMP recognition triggers genome-wide changes in gene expression. In *A. thaliana* flg22, a well-studied MAMP, affects up to four thousand genes, with initial changes occurring within 15 minutes after treatment [7,14,15].

### 1.1.2 Effector- triggered immunity

Effectors, historically also called avirulent proteins, get recognized directly or indirectly by NLRs, therefore activating ETI [5]. ETI offers effective resistance to pathogens upon the effector recognition. Most NLRs comprise N-terminal variable domain, which determines their type, a middle nucleotide binding domain and a C-terminal Leucine rich repeat (LRR) domain. The major types of NLRs are Coiled-coil- type NLRs (CNL), Toll/interleukin-1 receptor/ Resistance protein-type NLR (TNL) and Resistance to powdery mildew 8-like domain-type NLR (RNLs) [3].

As stated before, ETI and PTI have overlapping immune responses, however there are some distinctions. Although the  $\text{Ca}^{2+}$  burst in the cytosol represents the fastest immune response during PTI, it is slower with a more prolonged influx during ETI. Similarly, the ROS burst occurs in two phases, with the second peak occurring around 90 minutes after activation, typically being stronger and more sustained [3,5]. Additionally, studies have shown that exposure to MAMP is necessary for the second ROS burst, demonstrating the complexity of the plant immune system [3,16,17]. Lastly, transcriptional reprogramming triggered by ETI is generally the same as in PTI but with a more robust and increased expression pattern. Despite recent discoveries highlighting the interconnection of ETI and PTI, the specific mechanisms of their crosstalk remain unclear. Even though ETI plays a crucial role in plant immunity, this thesis will focus solely on PTI and its early responses.

## 1.2 PTI in detail

### 1.2.1 MAMPs

Pathogens and microbes have components that are crucial for their life. These components could have structural regions called MAMPs, which are ideal targets for the recognition and subsequent triggering of immune responses [18]. The recognition of MAMPs is highly specific for each plant due to evolutionary divergence in receptor specificity. Even plants that belong to the same family might be unable to recognize the same MAMP. Although MAMPs are conserved regions, mutations or sequence divergences can occur. This can then cause the plant to fail to recognize the MAMP altogether and therefore, lack the immune response [19]. MAMP chemical structure could be any of the major biomolecules, such as proteins, carbohydrates, or lipids, as seen in Table 1 [20,21].

Table 1: Various types of known MAMPs and their receptors

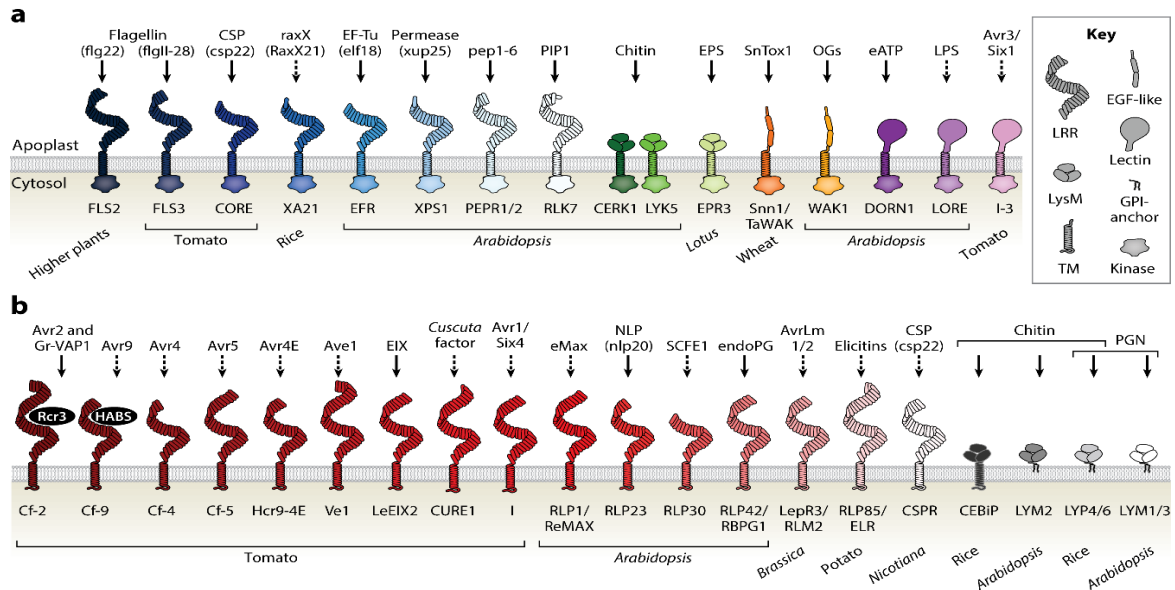
| Chemical type | MAMP                       | Original organism                            | Plant receptor | Plant*                                        | Reference   |
|---------------|----------------------------|----------------------------------------------|----------------|-----------------------------------------------|-------------|
| Carbohydrate  | EPSs                       | <i>X. campestris</i>                         | EPR3           | <i>Capsicum annuum</i>                        | [86,87]     |
| Fatty Acid    | me-3-OH-FAs                | <i>P. syringae</i>                           | LORE           | <i>A. thaliana</i>                            | [88]        |
| Glycopeptides | Invertase                  | <i>S. cerevisiae</i>                         | ?              | <i>S. lycopersicum</i>                        | [89]        |
| Glycoprotein  | CBEL                       | <i>P. parasitica</i> var. <i>nicotianae</i>  | ?              | <i>A. thaliana</i>                            | [90]        |
| Glycolipids   | LPSs                       | <i>P. fluorescens</i>                        | LORE           | <i>A. thaliana</i> , <i>D. curvophyllus</i>   | [91–93]     |
| Lipid         | Ergosterol                 | <i>C. fulvum</i>                             | ?              | <i>S. lycopersicum</i>                        | [94,95]     |
| Peptide       | Xyn25                      | <i>B. cinerea</i>                            | ?              | <i>N. tabacum</i> , <i>S. lycopersicum</i>    | [95]        |
| Peptide       | PeBL1                      | <i>B. laterosporus</i>                       | ?              | <i>N. benthamiana</i>                         | [96]        |
| Peptide       | nlp20                      | <i>P. parasitica</i>                         | RLP23          | <i>A. thaliana</i>                            | [97,98]     |
| Peptide       | Pep-13                     | <i>P. transglutaminases</i>                  | ?              | <i>P. crispum</i>                             | [99,100]    |
| Peptide       | flg22                      | <i>P. syringae</i>                           | FLS2           | <i>A. thaliana</i>                            | [25,28,75]  |
| Peptide       | flgII-28                   | <i>P. syringae</i>                           | FLS3           | <i>S. lycopersicum</i>                        | [73]        |
| Peptide       | flg15                      | <i>P. syringae</i>                           | FLS2           | <i>S. lycopersicum</i>                        | [75,28,101] |
| Peptide       | elf18/26                   | <i>P. syringae</i>                           | EFR            | <i>A. thaliana</i>                            | [102,103]   |
| Peptide       | csp22                      | <i>N. sylvestris</i>                         | CORE           | <i>N. tabacum</i>                             | [104,105]   |
| Peptide       | Xup25                      | <i>P. syringae</i>                           | XPS1           | <i>A. thaliana</i>                            | [106]       |
| Peptide       | Rax21                      | <i>X. oryzae</i> pv. <i>oryzae</i>           | XA21           | <i>O. sativa</i>                              | [107,108]   |
| Peptidoglycan | PGN                        | <i>A. tumefaciens</i> , <i>X. campestris</i> | LYM1/LYM3      | <i>A. thaliana</i>                            | [109,110]   |
| Protein       | Cellulase                  | <i>T. viride</i>                             | ?              | <i>Capsicum annuum</i>                        | [111]       |
| Protein       | Pectinase                  | <i>A. niger</i>                              | ?              | <i>Capsicum annuum</i>                        | [111]       |
| Protein       | harpin                     | <i>E. amylovora</i>                          | ?              | <i>N. tabacum</i>                             | [38]        |
| Protein       | PcF                        | <i>P. cactorum</i>                           | ?              | <i>S. lycopersicum</i> , <i>F. × ananassa</i> | [112]       |
| Protein       | Elicitin                   | <i>P. cryptogea</i> , <i>P. capsici</i>      | ELR            | <i>N. tabacum</i>                             | [113,114]   |
| Protein       | PoEli8                     | <i>P. oligandrum</i>                         | REli           | <i>N. benthamiana</i>                         | [115]       |
| Protein       | Ethylene inducing xylanase | <i>T. viride</i>                             | LeEIX1, LeEIX2 | <i>N. tabacum</i> cv <i>Xanthi</i>            | [116,117]   |
| Saccharide    | 1,3-β-d-(Glc)6             | <i>P. cucumerina</i>                         | CERK1          | <i>A. thaliana</i>                            | [118]       |
| Saccharide    | OGs                        | <i>P. syringae</i>                           | WAK1           | <i>G. max</i>                                 | [119,120]   |
| Saccharide    | Chitin                     | <i>Saccharomyces</i>                         | CERK1          | <i>S. lycopersicum</i>                        | [121,122]   |

\*Plants where the first immune response has been detected

Additionally to MAMPs, plants can recognize their own molecules, which can be released into the extracellular space upon cell damage [20]. These so-called Damage-Associated Molecular Patterns, such as cell wall fragments or extracellular ATP, are secreted from dying cells and provide higher protection for the vulnerable, injured site [1,20,22]. They can induce PTI as well as MAMPs however, in this thesis, the focus will be mainly on bacterial MAMPs.

### 1.2.2 PRRs

PRRs can be divided into two groups based on their domain organization. The first class are receptor proteins, which contain an extracellular domain, a single pass transmembrane helix, and a short cytosolic tail. The second class is Receptor-like Kinases (RLKs), containing an extracellular domain, a single pass transmembrane helix, and a cytosolic kinase domain [23]. The type of extracellular domain, as seen in Figure 1, determines the specific molecule the PRR can recognize. For example, LRRs bind to proteins or peptides, while Lysine motifs (LysMs) recognize carbohydrate-containing molecules such as chitin or bacterial peptidoglycans, among others [1,24]. PRRs recruit co-receptors upon ligand recognition, creating a complex which triggers phosphorylation and the subsequent activation of the downstream signaling pathways [23]. This thesis will be focused on the Flagellin-sensing 2 (FLS2) receptor. It was one of the first ones to be discovered and therefore, is among the most extensively studied receptors [25].



Boutrot F, Zipfel C. 2017. *Annu. Rev. Phytopathol.* 55:257–86

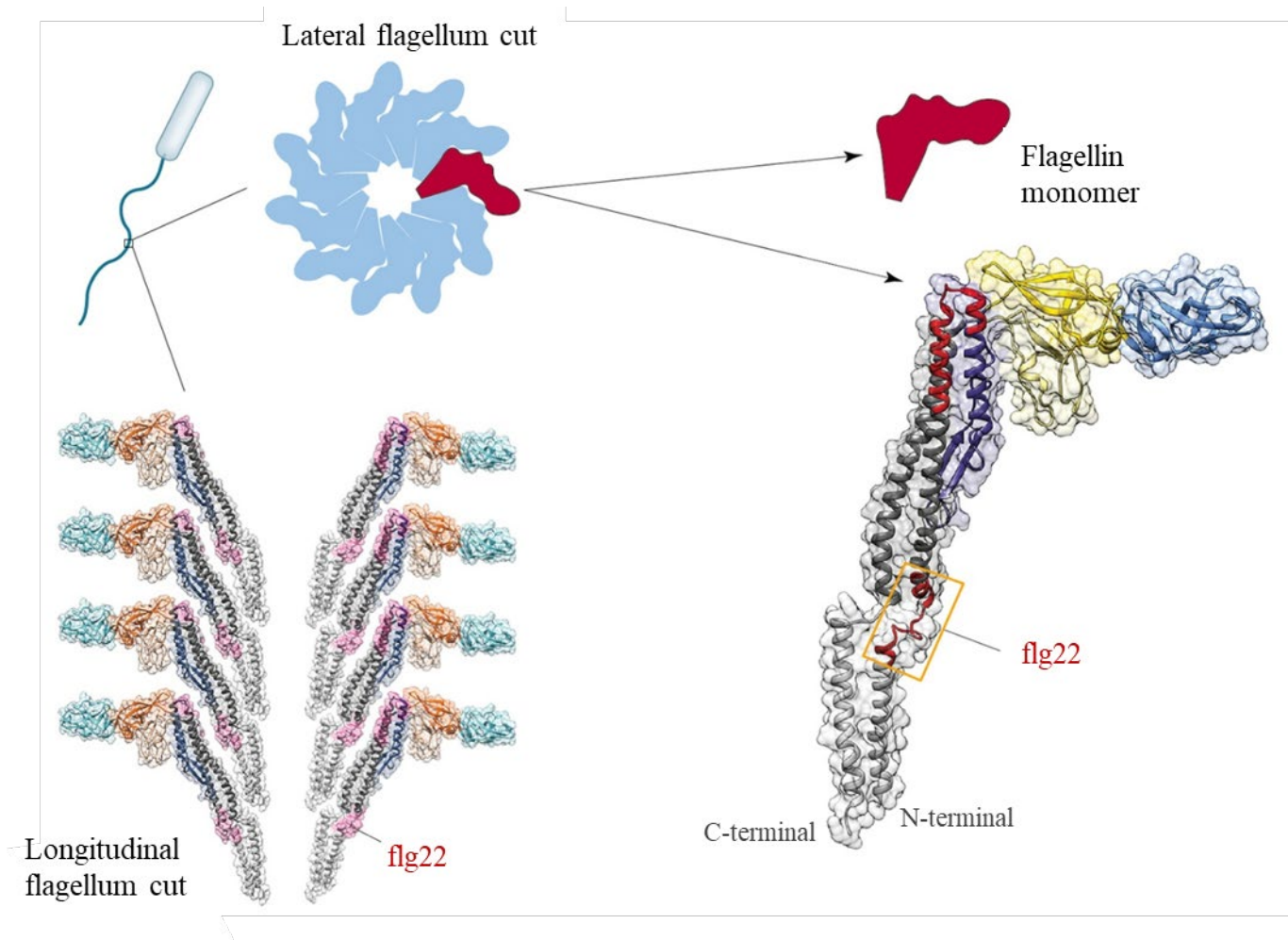
**Figure 1:** “Proven and potential plant pattern recognition receptors (PRRs) with known ligands/agonists. (a) Receptor kinases (RKs). (b) Receptor-like proteins (RLPs). Ligands; Solid arrows indicate demonstrated direct binding; dashed arrows indicate a current lack of evidence for direct binding. Abbreviations: EGF, epidermal growth factor; EIX, ethylene-inducing xylanase; EPS, extracellular polysaccharides; GPI, glycosylphosphatidylinositol; LPS, lipopolysaccharide; LRR, leucine-rich repeat; OGs, oligogalacturonides; PGN, peptidoglycan; TM, transmembrane.” Figure and caption taken from [21]

## 1.3 Case study: flg22-FLS2

### 1.3.1 Flagellin 22

Flagellin recognition and subsequent immunity cascade are extensively studied among all MAMPs. As seen in Figure 2, flagellin is a structural component of the bacterial flagellum, providing bacterial mobility [1,26,27]. The Flagellum consists of 11 protofilaments, each containing several thousand flagellin units [26]. While flagellin’s the N- and C-terminal regions are highly conserved, the central part can vary across different bacterial species. Plants typically recognize specific peptides derived from the flagellin unit, such as flg22, a 22-amino acid peptide from the conserved N-terminal region. It is observed that, when treating plants with the whole pathogen or the flagellin unit, the immune response is much slower [28]. In *A. thaliana* and *S. lycopersicum*, flg22 induced faster and greater immunity responses than the entire pathogen or Flagellin unit [18,27]. This is due to the enabled release of immunogenic compounds from the pathogen via secreting molecules such as Tobacco  $\beta$ -galactosidase 1 into the apoplast [1,29]. This leads to a slower immune reaction compared to treatment with flg22.

Furthermore, the specific Flagellin domain recognition can vary even among plants within the same family. For example, the *Escherichia coli* derived shortened version of flg22, called flg15, is highly active in *S. lycopersicum* but not in the related *N. tabacum* [27,30]. This just further shows the diversity in the plant immune responses.



**Figure 2:** Longitudinal and lateral flagellum cut illustrating the placement of flg22 in a flagellin unit; Figure taken from [123]

### 1.3.2 FLS2

Discovered in 2000, FLS2 was one of the first plant PRRs to be identified and remains among the most extensively studied PRRs to this day [25]. FLS2 recognizes bacterial flagellin, more specifically, flg22. FLS2 is expressed in roots, rosette leaves, stems, and flower petals, which was shown by labeling the FLS2 with a Green fluorescent protein (GFP) [31]. It localizes in the cell membrane, more frequently around possible bacterial entry points such as stomata lining guard cells, epidermal cells, and mesophyll [31]. Its ectodomain consists of 28 LRRs [32], which directly interact with regulatory LRR-RLK BRI1-associated receptor kinase 1 (BAK1)/ Somatic Embryogenesis RLK 3 (SERK3) and forms a complex. The flg22 acts as “molecular glue” stabilizing the complex. Although FLS2 is capable of binding flg22 independently, BAK1 is essential for the signaling required to activate the immune response. This was shown in *bak1* mutants of *A. thaliana*, while flg22 binding to FLS2 remained unaffected, the resulting early immune responses resulted in quantitative reduction and abnormalities [24,33].

PRR complexes often have Receptor-like cytoplasmic kinases (RLCKs). FLS2, and some other PRRs as well, interact with Botrytis-induced kinase 1 (BIK1). BIK1 is associated with the FLS2-BAK1 complex in the absence of flg22. Upon the flg22 perception, BIK1 is phosphorylated by BAK1. BIK1, in turn, phosphorylates the FLS2- BAK1 complex and then dissociates to start the immune signaling process. BIK1 belongs to the RLCK-VII family along with PBL1, PBL2, and PBL5, which are capable of regulating flg22-induced responses as well [1,24].

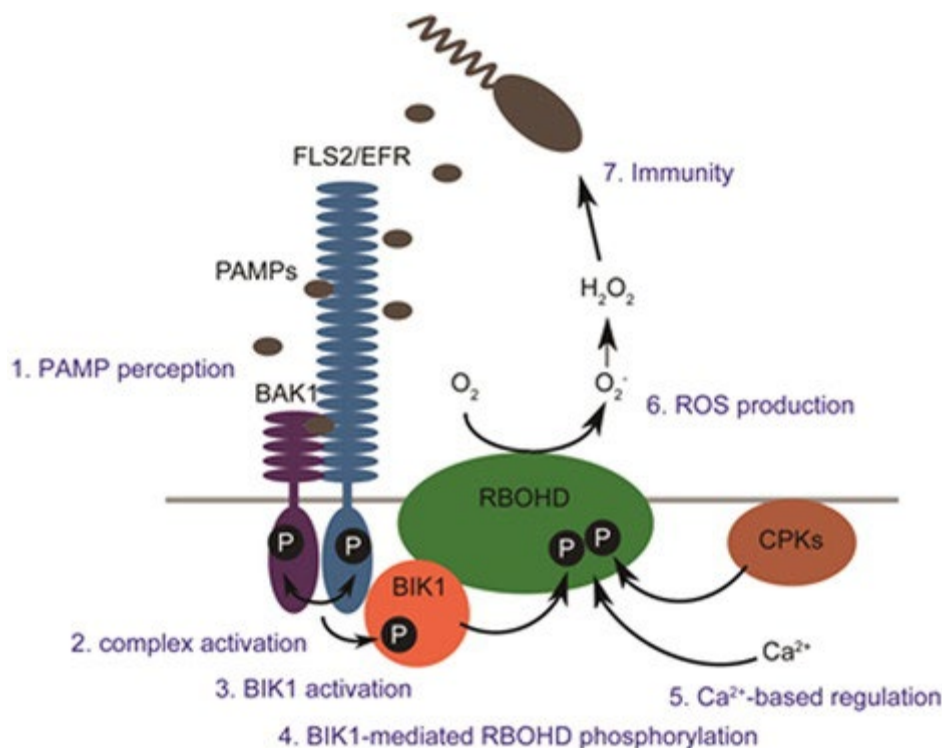
Besides *A. thaliana*, a FLS2 ortholog has been described or suggested by ROS burst upon flg22 treatment in *Oryza sativa* [34], *Solanum lycopersicum* [30,35], *Nicotiana benthamiana* [36], *Brassica rapa* ssp. *Rapa* [36], *Phaseolus vulgaris* [37], *Vitis rupestris* [38], *Vitis vinifera* cv 'Pinot Noir' [38], *Hordeum vulgare* [39], *Triticum aestivum* [40], *Glycine max* [41], *Solanum tuberosum* [42], *Phyllostachys nigra* [43], *Asparagus persicus* [43], *Lotus japonicus* [43].

### 1.3.3 Early apoplastic ROS production in plant immunity

As mentioned above, ROS burst is one of the earliest immune responses upon MAMP perception. One of the first documented ROS bursts in plants was in 1983 in potato tubers upon infection with *Phytophthora infestans* [8,44]. Since then, ROS burst has been intensively studied, as it is an important component of the immune response, contributing to defense against pathogen through its direct toxicity, limiting pathogen entry by stomatal closure, and strengthening the plant cell wall by cross-linking glycoproteins as well as acting as a signaling molecule [8,27,45]. ROS are produced by NADPH oxidases, which belong to the Respiratory Burst Oxidase Homolog (RBOH) family [1,8,23,45]. There are multiple types of RBOHs, each having a different function. Since RBOHDs are the main producers of ROS, they are the most expressed. Other examples of RBOHs include RBOHH, regulating pollen tube growth, or RBOHB, which is responsible for seed ripening, among others [8]. In Arabidopsis roots, ROS bursts are dependent on RBOHC and RBOHF .

RBOHD produces ROS by transferring electrons from cytosolic NADPH or NADH to apoplastic oxygen forming superoxide radical anion, which can then be converted to hydrogen peroxide ( $H_2O_2$ ) by superoxide dismutase [8,45]. Activated BIK1, which was previously dissociated from the FLS2-BAK1 complex, directly phosphorylates residues within the N-terminal part of the RBOHD [1,8,45]. Those residues are highly conserved among functional RBOHD orthologs in various plants. However, BIK1 phosphorylation alone is not sufficient to activate ROS production as it only primes the RBOHD for activation. The full activation requires  $Ca^{2+}$  binding to the EF-hand motifs of RBOHD. This induces conformational changes that are necessary for optimal NADPH oxidase activity [8].

Interestingly, in *S. tuberosum* and *A. thaliana*, RBOHD was shown to be phosphorylated by  $Ca^{2+}$ -dependent protein kinases (CPKs). It was demonstrated that *St*CDPK4 and *St*CDPK5 directly phosphorylate *St*RBOHB, RBOHD ortholog in *S. tuberosum*, in a  $Ca^{2+}$ -dependent manner [45,46]. In Arabidopsis, CPK5 phosphorylates RBOHD and regulates its activity, while other kinases such as CPK4, CPK6, and CPK11, which are similar to *St*CDPK4/5, also act as positive regulators of the ROS burst [8,45,47].



**Figure 3:** Scheme of ROS burst cascade; 1. Recognition of Pathogen-Associated Molecular Patterns (PAMPs)/MAMPs and their binding to a PRR 2. The activation of the PRR complex by phosphorylation upon PAMPs/MAMPs recognition 3. Dissociation of BIK1 from the FLS2-BAK1 complex 4. Phosphorylation of RBOHD by BIK1 5. RBOHD regulation by Ca<sup>2+</sup> and CPKs 6. Subsequent formation of ROS from apoplastic oxygen 7. The formation ROS production providing immunity against Microbes/Pathogens; Figure taken from [45]

## 1.4 Methods for detection of ROS produced by plants

### 1.4.1 Chemical probes measurements

The below mentioned methods are all based on the addition of a specific compound that interacts with ROS. These methods can be specific to one of the produced ROS types, since each ROS has its own lifetime, stability, and reactivity, or they can be non-selective.

Superoxide radical anion ( $O_2^{\cdot-}$ ) is the first ROS created during the immune response. It is highly reactive with a short lifespan. Due to these characteristics, there are a few chemical probes able to detect it. Tetrazolium dyes can be used to detect superoxide in plant tissues. Tetrazolium ring undergoes 4 electron reduction, which opens the ring up [48], causing a change of color that can be detected using standard microscope or reflection microscopy [48–50]. Another possible probe is dihydroethidium fluorescent dye. In its reduced state it is blue, however upon four electron oxidation it forms 2-hydroxyethidium. The 2-hydroxyethidium product inserts into DNA which leads to a shift in its excitation and emission spectra resulting

in fluorescence [51]. Derivates of this dye may be used to detect superoxide in specific cellular regions, such as MitoSOX dye, used to detect superoxide in mitochondria [52–54].

Hydrogen Peroxide ( $\text{H}_2\text{O}_2$ ) is the most stable ROS with the longest lifetime and the ability to cross plasma membrane [55]. Due to its stability, there is a high number of dyes and probes that can detect it. A luminol-based assay is used for rapid detection of extracellular hydrogen peroxide. It is based on the principle of chemiluminescence. In this case, luminol and  $\text{H}_2\text{O}_2$  react in the presence of horseradish peroxidase (HRP) to form excited 3-aminophthalate (3-APA) [56]. As the excited 3-APA decays to a lower energy state, it results in luminescence, which can then be detected with a luminometer. Although this method has a limited ability to quantify hydrogen peroxide directly, it still remains a convenient and precise method to detect it [52,55,57]. Another option to detect and localize the production of  $\text{H}_2\text{O}_2$  is 3,3'-diaminobenzidine (DAB) staining. The leaves uptake and equally distribute DAB through cuts. Subsequently, upon hydrogen peroxide production, DAB gets oxidized by peroxidases to form a polymer that precipitates. The precipitate has a dark reddish /brown color, which can be seen with a naked eye [58]. Additionally, fluorescent probes, such as Amplex Red, may be used for  $\text{H}_2\text{O}_2$  detection as well [59]. In the presence of  $\text{H}_2\text{O}_2$ , Amplex Red gets oxidized to form resorufin, which emits fluorescence [60]. Since Amplex Red is not membrane permeable therefore it can be used only to detect extracellular hydrogen peroxide. However, this method is not entirely reliable since light-mediated photochemical oxidation of resorufin might be caused by the presence of reducing metabolites such as glutathione or NADH [52,61].

Singlet oxygen ( $^1\text{O}_2$ ) is another type of ROS generated through the light-mediated activation of molecular oxygen ( $\text{O}_2$ ). It has a very short lifespan and can diffuse through membranes. Fluorescent probes are commonly used to detect singlet oxygen. For example, DanePy is a fluorescent probe whose emission is quenched upon interaction with singlet oxygen, hydrogen peroxide, and superoxide [62]. This reduces its specificity towards singlet oxygen however, it remains an accurate method for measuring ROS [52].

One of the options for the detection of total ROS within the cell is 2',7'-dichlorofluorescein ( $\text{H}_2\text{DCF}$ ).  $\text{H}_2\text{DCF}$  can freely pass into a cell where it is deacetylated and therefore trapped. Upon oxidization by ROS, it forms highly fluorescent 2',7'-dichlorofluorescein (DCF) which can then be detected with a fluorometer or fluorescence microscopy[63,64] .

## 1.4.2 Fluorescent biosensors

Fluorescent biosensors are genetically encoded in plants, which are then expressed as proteins or other biological molecules. They have the ability to bind distinct ROS, making them selective. Upon the ROS binding, they change their conformation, which leads to a measurable change. The first genetically encoded H<sub>2</sub>O<sub>2</sub>-specific probe was HyPer. HyPer was created by fusing circularly permuted Yellow Fluorescent Protein (cpYFP) with the hydrogen peroxide-sensing regulatory domain OxyR from *E. coli*. Hydrogen peroxide oxidizes Cys199 in the OxyR domain to sulfenic acid, inducing a conformational change that alters the fluorescence spectrum of cpYFP [52,65].

Another possible biosensor that has been used to detect H<sub>2</sub>O<sub>2</sub> in *A. thaliana* upon drought stress is cytoplasmically targeted redox-sensing GFP (c-roGFP1). By using a modified redox-sensing GFP it allows to monitor the real time redox status of plants' cytoplasm subjected to different stress conditions. The GFP has incorporated a pair of Cys residues which get oxidized by the H<sub>2</sub>O<sub>2</sub> and form a disulfide bond, changing the protein's conformation. Unlike dyes used to detect ROS under stress, c-roGFP1 allows for back-titration, thereby mitigating the issue of irreversible oxidation [52,66,67].

## 1.4.3 Electron Paramagnetic Resonance Spectroscopy

One of the uses of Electron Paramagnetic Resonance (EPR) spectroscopy is to detect unpaired electrons, including ROS. The first application was in 1974 to quantify superoxide chloroplast production in spinach; [68] however, it may be used in various eukaryotic systems. The working principle of EPR is based on the absorption of microwave radiation by paramagnetic centres as their magnetic moments interact with an external magnetic field [69]. The benefit of EPR is its ability to measure highly reactive ROS with a very short lifespan using Spin traps or Spin probes[52].

The spin trap method is applied along with EPR spectroscopy to detect or quantify unstable ROS. It works by the addition of a diamagnetic molecule (the spin trap) that reacts with the free radicals and forms a more stable paramagnetic spin product called spin adduct, which can then be detected via EPR [69,70]. There are various substances that can be used as spin traps. Since plants can produce multiple different radicals simultaneously, it is important to use spin

traps, which are able to detect various radicals. For example, DMPO or DEPMPO spin traps are used to detect  $O_2^{\cdot -}$  and  $OH^{\cdot}$  produced in plants. Firstly, DMPO and DEPMPO react with  $O_2^{\cdot -}$  and form a spin adduct, which can further react with  $OH^{\cdot}$  [69].

Spin probes work in opposite manner to spin traps. They are relatively stable paramagnetic species, which upon reaction with the free radical form diamagnetic species. This then decreases the EPR signal [71]. Even though the use of EPR spectroscopy to measure ROS in plants has been limited, it remains a powerful and versatile tool for studying oxidative processes in biological systems [52].

## 2. Aims of the thesis

- Conduct a literature review on the production of reactive oxygen species (ROS) in the context of plant immune responses, with a special emphasis on the early production of ROS in the extracellular space following the recognition of Microbe-Associated Molecular Patterns (MAMPs). The review should include an exhaustive list of known MAMPs and their descriptions.
- Perform a screening of ROS production after treatment with flg22 across a broad spectrum of plant species. Use the luminol-based method to measure ROS.
- Conduct a bioinformatical analysis to investigate the presence of orthologs of the FLS2 gene (receptor of flg22) across the plant kingdom.
- Clearly present and discuss the obtained results.

## 3. Experimental part

### 3.1 Materials and methods

#### 3.1.1 Plant material

Tested plants were all collected either at the Botanical Garden in Třeboň or in the area of the University of South Bohemia (USB) viz Table S1. From the collected plants in total 6 discs (4 mm diameter) were cut from mature leaves and placed in a 96-well plate with each well containing 150  $\mu$ L of distilled water. The last row of wells was left empty for a positive control sample which represents *A. thaliana* leaf discs. The plate was covered with a lid and placed in the dark overnight at room temperature.

#### 3.1.2 Measurement of apoplastic H<sub>2</sub>O<sub>2</sub>

To measure the concentration of apoplastic H<sub>2</sub>O<sub>2</sub> we used luminol-based assay inspired by Smith et al. 2014 [57]. In our protocol immediately before the measurement the distilled water in 96-well plate containing plant discs was replaced with the solution containing 200  $\mu$ M luminol (Serva, 28085.02), 20  $\mu$ g/mL horseradish peroxidase (HRP, Apollo Scientific, Aposbitp1327) and 1  $\mu$ M flg22 (EZBiolab; QRLSTGSRINSKDDAAGLQIA; [32]). Produced H<sub>2</sub>O<sub>2</sub> is monitored as luminescence intensity correlating with the concentration of H<sub>2</sub>O<sub>2</sub> and described as relative luminescence unit (RLU). For luminescence measurement, a luminometer Tecan SparkCyto 300 (Tecan, Switzerland) was used.

#### 3.1.3 Bioinformatics

The bioinformatics analysis was done using Basic Local Alignment Search Tool for Proteins (BLASTP). The amino acid sequence from Arabidopsis FLS2 (AtFLS2; Fig. S1) was obtained from TAIR database (Arabidopsis.org) and then BLASTP was used to find putative orthologs in every tested plant. The resulting hits were then sorted into 3 categories based on different values as seen in Table 2. Additionally, plants that resulted in no hits were put in the category “No hit” and plants without a genome in the BLASTP database were in the category “No genome”.

Table 2: The parameters used to determine the quality of a bioinformatics hit

| E-value             | Query coverage | Category     |
|---------------------|----------------|--------------|
| $< 10^{-6}$         | $>70\%$        | Good hit     |
| $10^{-3} - 10^{-6}$ | 40-70 %        | Moderate hit |
| $>10^{-3}$          | $<40\%$        | Weak hit     |

\* Plants that showed low E-value ( $<<10^{-6}$ ) as well as low query coverage ( $<40\%$ ) were sorted into the “Moderate hit” category.

### 3.1.4 Literature survey

To identify plant species exhibiting ROS bursts upon flg22 treatment, a search was conducted using Google Scholar. Only studies reporting direct experimental evidence (e.g., luminescence) were considered.

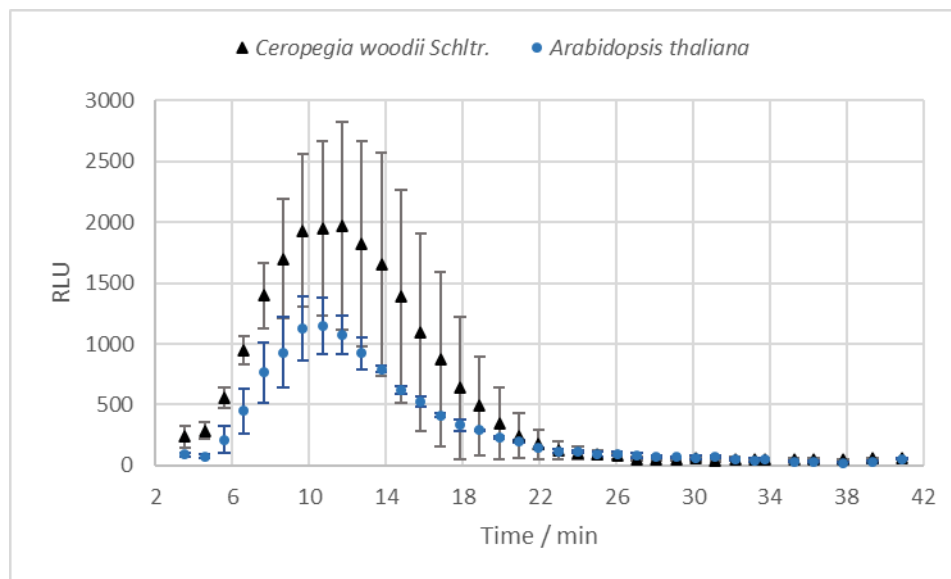
### 3.1.5 Construction of phylogenetic tree

The phylogenetic tree was firstly constructed using taxonomic data from National Center for Biotechnology Information (NCBI) from the list of all tested species. The tree was then imported into the Interactive Tree of Life (iTOL) for further customization.

## 3.2 Results

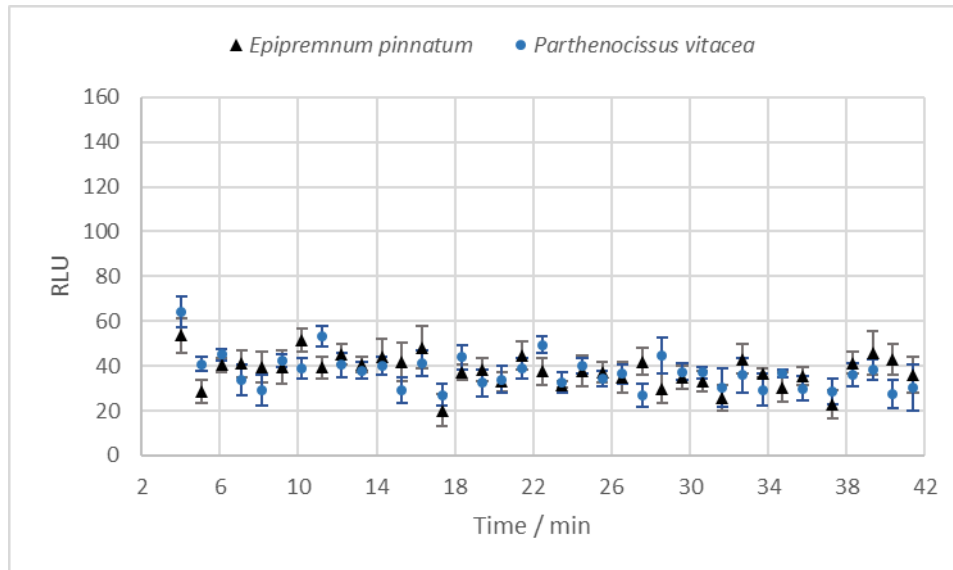
### 3.2.1 Typical kinetic course of ROS production after flg22

A typical positive reaction after flg22 treatment can be seen in Figure 4 . The positive reaction has to include a clear RLU peak which usually occurs around 10 minutes after the treatment. The peak corresponds to transient production of H<sub>2</sub>O<sub>2</sub>. It indicates a presence of the FLS2 or its ortholog in the plant because the plant reacts to flg22. Each positive result had to be repeated at least once to for a plant to have a final positive result shown in Figure 6.



**Figure 4:** A typical positive reaction after flg22 treatment demonstrated on *A. thaliana* and *Ceropegia woodii* Schltr.

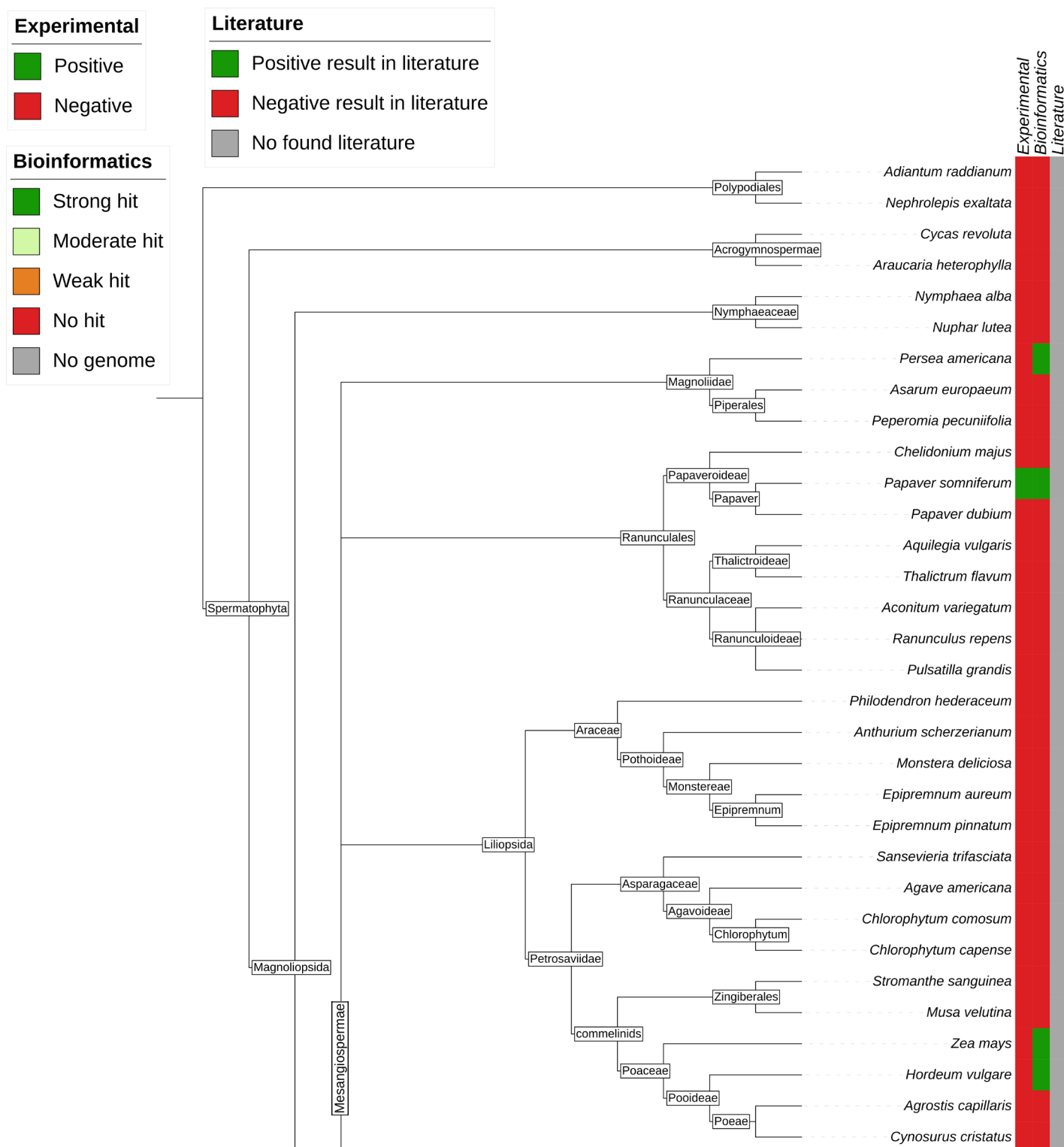
A typical negative response, as seen in Figure 5, lacks a RLU peak and is composed of random points bellow 100 RLU.



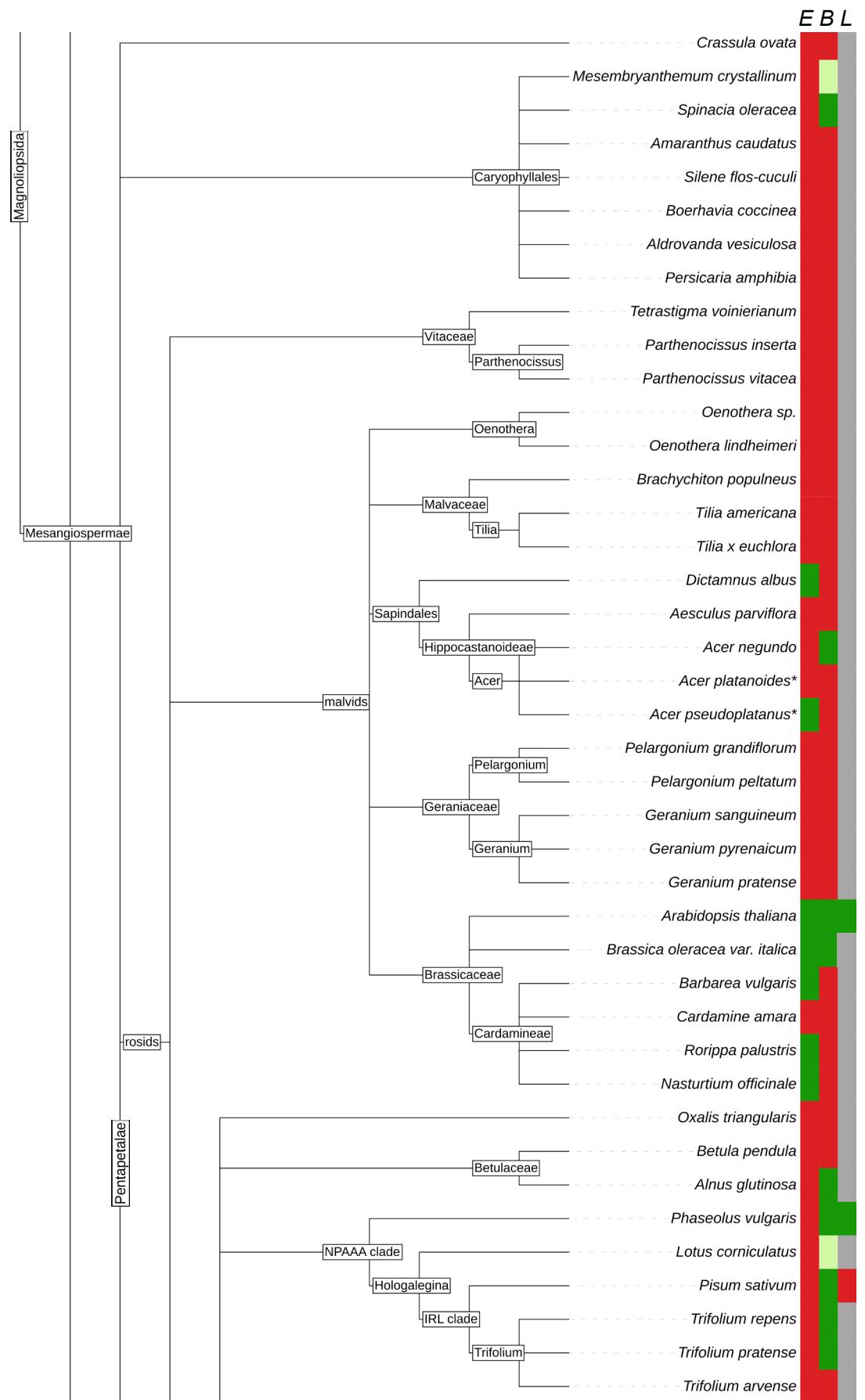
**Figure 5:** Typical negative reaction after flg22 treatment demonstrated on *Epipremnum pinnatum* and *Parthenocissus vitacea*

### 3.2.2 Response to flg22 across species

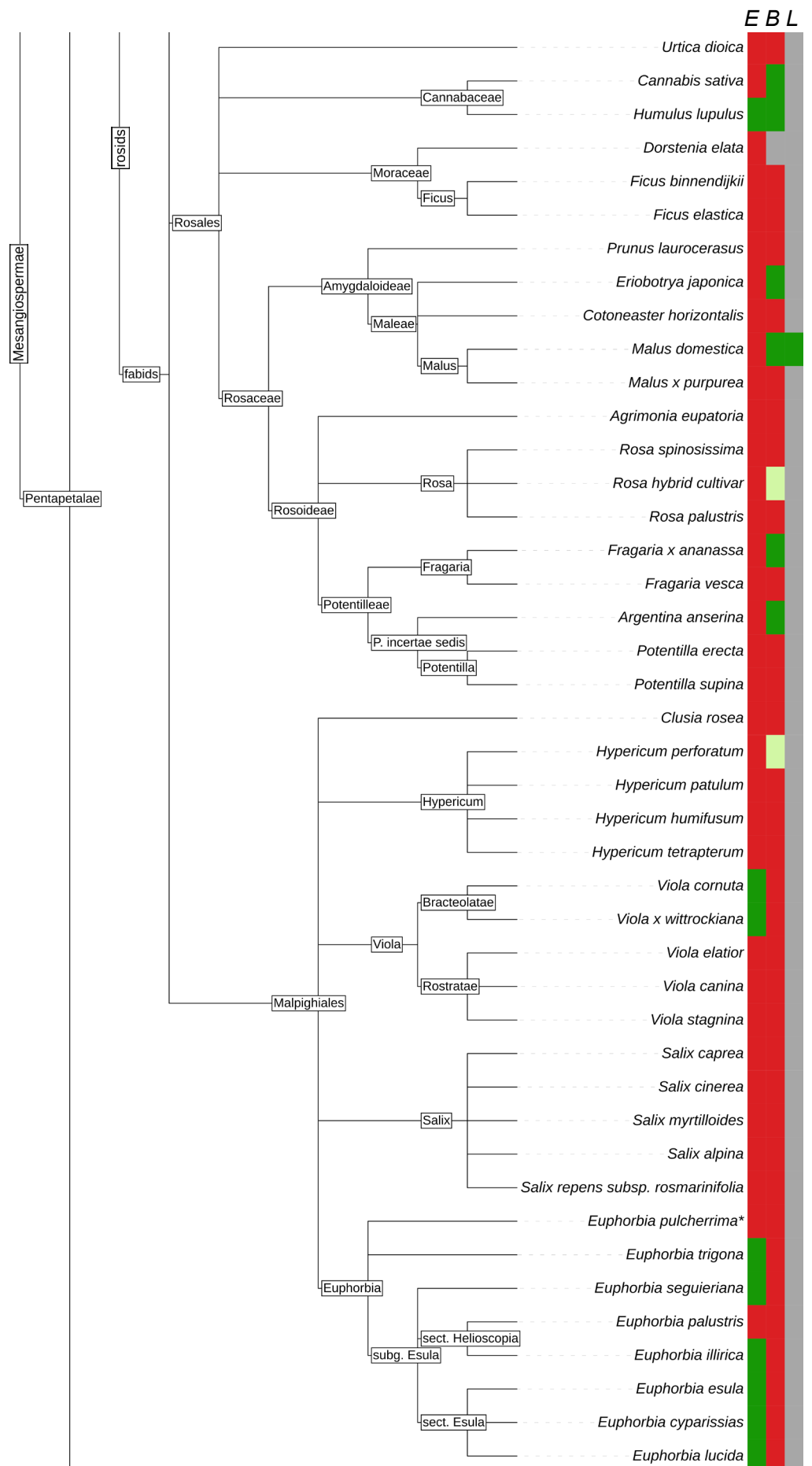
The search for alternative plant models to study flg22-induced immunity, particularly in our laboratory for cuticle research, led to this large-scale screening of ROS burst upon flg22 treatment. To investigate possible family correlations between the plants the experimental data is shown in a phylogenetic tree as seen in Figure 6. These results show only the well-developed leaves reactions. Overall, 29 species, that has not yet been tested, showed ROS burst upon flg22 elicitation. Five of which showed a strong indication of the FLS2 homolog in the bioinformatic assay.



**Figure 6.1:** Phylogenetic tree displaying results of all tested species, for plants with “\*” both well developed and young leaves were tested

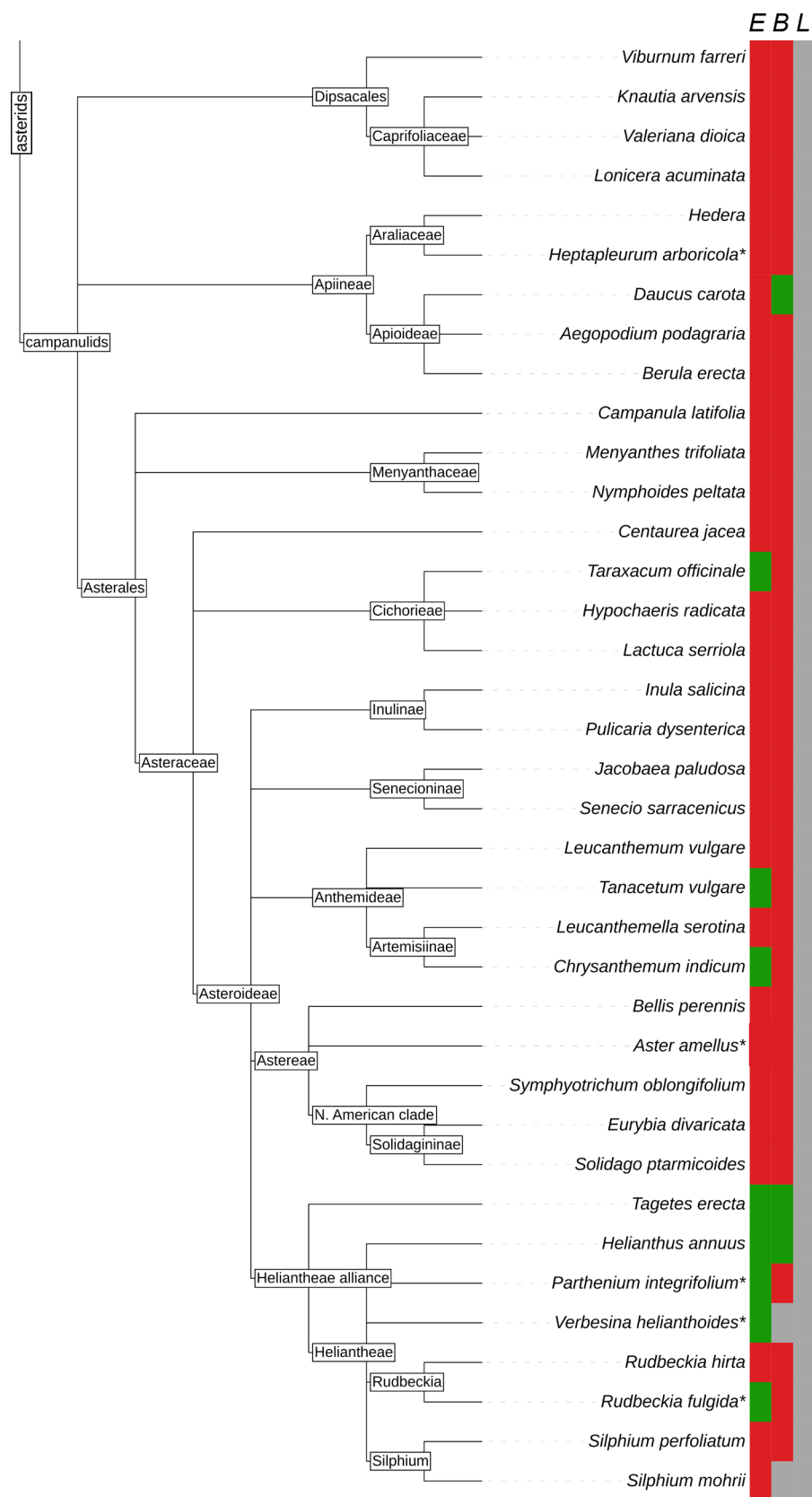


**Figure 6.2:** Phylogenetic tree displaying results of all tested species, for plants with “\*” both well developed and young leaves were tested



**Figure 6.3:** Phylogenetic tree displaying results of all tested species, for plants with “\*” both well developed and young leaves were tested





**Figure 6.5:** Phylogenetic tree displaying results of all tested species, for plants with “\*” both well developed and young leaves were tested

### 3.2.3 Different responses to flg22 between “young” and “well developed” leaves

To examine possible differences in immune responses based on age, visibly young and smaller leaves and well, fully developed leaves of the same species were collected and tested. The majority of tested species (8 out of 11) showed no difference between the age groups. The species that showed experimental differences are summarized in Table 3.

**Table 3:** Plants showing differences in ROS production in young and well-developed leaves

| Plant species                   | Well developed leaf reaction | Young leaf reaction |
|---------------------------------|------------------------------|---------------------|
| <i>Parthenium integrifolium</i> | Yes                          | No                  |
| <i>Acer pseudoplatanus</i>      | Yes                          | No                  |
| <i>Euphorbia pulcherrima</i>    | No                           | Yes                 |

## 3.3 Discussion

### 3.3.1 Typical ROS response to flg22

A negative experimental reaction (Figure 5) does not necessarily imply the absence of FLS2 ortholog or other immune components necessary for ROS production. Since the plants were not grown in controlled conditions and are not fully genetically characterised there might be phenotypic differences stemming from natural mutations affecting the FLS2 receptor or any downstream component such as RBOHD [8,72]. To further demonstrate this, however would require genotypic validation using PCR techniques or any sequencing method.

Even though flg22 is a typical MAMP for recognition of bacterial flagellum, it represents only a small part of the flagellin protein. Plants may detect or be more sensitive to other flagellin derived epitopes such as flg15 or flgII-28 [73,74]. This would still provide immunity against bacteria with flagella without requiring the FLS2 ortholog. While flg22 is one of the most conserved regions of bacterial flagellum there is slight variation in the sequence depending on the bacterium which can lead to a different response [75]. For example, in *S. lycopersicum*, flg22 and flgII-28 peptides derived from different *Pseudomonas syringae* pv. *tomato* strains

(T1, Col338 and K40) led to quantitatively different ROS production compared to classical flg22 from *Pseudomonas aeruginosa* [76].

### 3.3.2 Response to flg22 across species

From the conducted experimental results, the flg22 recognition does not seem to be phylogenetically clustered, instead it appears as an independently acquired immunity trait across the plant kingdom. Despite focusing on a relatively small fraction of plants (194) the results indicate that flg22 recognition is a widespread trait across the plant kingdom. Certain families however show consistent positive results which could mean the FLS2 receptor gene plays a vital part of these families' immunity. Closely related plants within the same family are commonly attacked by the same type of pathogens which would explain the need for the same receptor and immunogenic response [77,78]. This is true in families like *Brassicaceae*, *Solanaceae* or *Euphorbiaceae* where most tested species showed positive experimental results. However, it is important to note that only a small subset of species was analyzed within these families. In contrast, families like *Asteraceae* that were represented by larger subset of species showed much higher variability in the experimental results. This raises a possibility that the species from consistently positive families may represent an exception compared to the rest of the family.

Certain plants have a negative bioinformatics result despite the experimental results being positive. This could suggest either an alternative receptor to FLS2 with enough differences in the sequence to go undetected in the analysis or limitations in the chosen bioinformatical method. For the bioinformatic analysis the full-length FLS2 sequence from *A. thaliana* was used, including the intracellular part. However, since the extracellular domain alone determines flg22 recognition, the intracellular region, which may vary across species due to differences in downstream signaling cascades, could have introduced unnecessary sequence. This might explain this difference in results.

As mentioned above the family *Brassicaceae* (Figure 6.2) showed relatively consistent results with the only outlier being *Cardamine amara*. Given that a model plant *A. thaliana* belongs to this family, it would be expected, that all plants from the family would show positive bioinformatical and experimental results. Nevertheless, among the tested plants, only *Brassica oleracea* var. *italica* (broccoli) produced a strong bioinformatic hit, while the remaining

species, showed no detectable FLS2 homologs. Despite the limitations of the bioinformatical method, mentioned earlier, it would have been expected that the closely related species such as *Barbarea vulgaris* or *Rorippa palustris* would contain a similar FLS2 ortholog. Apart from *A. thaliana* and broccoli the rest of the species from this family belongs to the genus of Cardamine that might have significantly diverged in the FLS2 ortholog sequence.

The next largely positively consistent family is *Euphorbiaceae* (Figure 6.3) with only two negative tested adult plants *Euphorbia palustris* and *Euphorbia pulcherrima*. Similarly to previously discussed family *Brassicaceae*, the species showed no detectable FLS2 homologs in the bioinformatical analysis. The eight tested species represent a broad ecological and geographical range from *Euphorbia esula*, a common weed in Europe and Asia, to *Euphorbia trigona*, a succulent originating in central Africa [79]. This shows the family's adaptability to various habitats for example, many species from *Euphorbiaceae* species produce toxic latex as a defense mechanism making them toxic to herbivores [79]. The consistent flg22 recognition across such varied species suggests a strong evolutionary conservation of this trait. The two exceptions may reflect a specific adaptation. *E. palustris* is a relatively rare plant native to Europe and western Asia. It can be found along river shores, lakes or swamps [80]. Its habitat might expose it to a distinct microbial community, which would lead to abolishment of flg22 perception. *E. pulcherrima*, on the other hand, is a widely cultivated species, due to its distinct red and green leaves, native to Mexico and Central America [81]. Its cultivation history might have altered its MAMP perception due to the controlled environment it is usually kept in [81].

Last family showing consistent positive experimental results is *Solanaceae* (Figure 6.4). Plants *Nicotiana tabacum* or *Solanum lycopersicum*, which are well studied in terms of flg22 response, show a clear FLS2 orthologs in the bioinformatical assay. Other plants from this family such as the wild relative of tomato *Solanum dulcamara*, demonstrate positive results of a FLS2 ortholog as well. This is likely due to Solanaceae's vulnerability to pathogens like *Pseudomonas*. In further research it would be beneficial to broaden this family to compare if these consistent results will last, particularly by including more wild species and additional subfamilies beyond *Solanoideae*.

As stated before the *Asteraceae* family (Figure 6.5) shows a wide variety of experimental and bioinformatical results. The positive results seem to be focussed most in the clade *Heliantheae*

*alliance* where five out of eight tested plants show a positive experimental result. The outliers being *Silphium perfoliatum* and *Silphium mohrii*, both from the genus *Silphium* and *Rudbeckia hirta* from the genus *Rudbeckia*. Given that both tested plants from the genus *Silphium* show negative experimental results, there might be a genus specific lack of FLS2 ortholog which is suggested by the negative bioinformatics results of *S. perfoliatum*. However, due to the lack a proper genomic sequence of *S. mohrii* it remains unclear. The example of *R. hirta* is more ambiguous since the two tested species from the *Rudbeckia* genus show opposing results. Apart from the *Heliantheae* alliance, positive experimental results were observed in species like *Centaurea jacea*, *Taraxacum officinale*, and *Leucanthemum vulgare*, though with less consistency across their respective genera.

In the *Rosaceae* family (Figure 6.3) there are no positive reactions despite the bioinformatical results for some of these species which show a possibility of a FLS2 ortholog. For example, *Malus domestica* exhibited a FLS2 ortholog and a previous study using a luminol-based assay successfully detected flg22-induced ROS in this species [82]. While the method to detect the ROS was the same, we have used higher concentrations of luminol and horseradish peroxide and 10 times lower concentration of flg22. Additionally, the tested leaf was a fully developed while the study used leaf buds. It has been showed that the ROS burst in *A. thaliana* is dependent on the flg22 concentration [57]. Additionally, immune responses can be age dependent (see 2.3.3), therefore both changes in the protocol might be the reason for the varying results.

NPAAA clade (Figure 6.2) is a group of plants largely showing positive results in the bioinformatical analysis however there is not a single plant exhibiting a positive experimental result in ROS measurement. For example, in *Phaseolus vulgaris* bioinformatic analysis predicts the presence of the FLS2 ortholog, and published literature confirms the ability of flg22 to trigger ROS burst despite our negative experimental result [37]. In contrast with the discussed case of *M. domestica*, there are no differences in the luminol assay protocol itself but in the conditions under which the plant was allowed to grow. The *P. vulgaris* from the study was grown *in vitro* in fully controlled conditions, however we used a plant which grew outside in semi-controlled condition. Our plant might have been an ecotype lacking the FLS2 ortholog, as mentioned earlier, which would prevent the plant from producing ROS. Another example would be *Pisum sativum* which was found to lack ROS burst upon flg22 treatment

[41]. This would correspond to our experimental results. However, the flg22 used in the study originate from *R. solanacearum* (flg22<sup>Rso</sup>) and was used in a 10 times lower concentration compared to the one we used in our protocol. As stated before the ROS production is affected by the flg22 concentration, and the type of flg22. Moreover, in this same study *P. vulgaris* was treated with flg22<sup>Rso</sup> as well, and showed no ROS burst, in contrast with the above-mentioned study [37,41]. Which just further suggests that the immune response is dependent on the origin of the flg22.

Beyond the major families discussed, isolated species like *Humulus lupulus*, *Acer pseudoplatanus*, *Dictamnus albus*, and *Papaver somniferum* also exhibited ROS burst upon flg22 treatment. These scattered positives reinforce that flg22 recognition is phylogenetically widespread. Even though for some of the positive experimental results, no FLS2 ortholog has been found. It can be expected that these plants possess some form of a flg22 receptor.

### 3.3.3 Effects of the age of the leaves

*Parthenium integrifolium* and *Acer pseudoplatanus* exhibited ROS production in fully grown leaves but not in young leaves, suggesting that this type of immunity may develop with leaf maturation. It has been shown that FLS2 transcription in *A. thaliana* increases with seedling growth [83]. Therefore, the flg22 responses such as callose deposition increase with age of the plant [83]. The absence of ROS in young leaves could reflect a prioritization of growth over defense during early developmental stages, as young tissues may allocate resources to growth rather than immune activation [84].

Conversely, *E. pulcherrima* showed ROS production only in young leaver but not in fully grown leaves. Two different plants were tested twice and the results stayed consistent. This could indicate that young leaves are more susceptible to pathogens and therefore require a stronger initial immune response.

### 3.3.4 Effects of temperature

Another factor affecting immune responses is the ambient temperature. It was demonstrated that in *A. thaliana*, even six hours of exposure to elevated temperature (32 °C) significantly reduces the ROS burst after flg22 treatment [85]. Since a significant portion of the tested plants (see Table) were collected from the Botanical Garden in Třeboň during the summer months

(June-August), they were exposed to transient high temperatures ( $>30^{\circ}\text{C}$ ) before collecting and partly during transport. Furthermore, the used Luminometer was heating up with every measurement cycle reaching an average temperature of  $31^{\circ}\text{C}$ . These elevated temperatures may have reduced possible immune responses[85]. However, our positive control *A. thaliana* always worked fine.

Due to these conditions the sample collection system had to be improved. Initially, 3-4 plates of plants not normally available to us were collected in Botanical Garden in Třeboň. However, upon recognition of potential inhibitory effects of the temperature, all later collections were limited to only 1 plate of plants from the area of USB campus. The adjustment reduced the number of cycles from 8 (for 4 plates) to 2 (for 1 plate) thereby reducing the average temperature during the measurement to  $28.5^{\circ}\text{C}$ . Recently, we have a possibility to use another luminometer located at Biology Centre which can control temperature in measuring cell. This instrument could be good to use in further study especially in connection with the effect of temperature.

## 4. Conclusion

- In total 194 plants have been screened for ROS burst upon flg22 treatment
- ROS burst was detected for the first time in 29 species
- From the positively reacted species 7 showed a strong suggestion of FLS2 ortholog in the bioinformatical assay

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