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Reconstructing the germination pathway from the *Rafflesia* seed transcriptome

BY

VENKATA SIVA SANKAR MADDU

A MASTER'S THESIS SUBMITTED TO THE FACULTY OF

RICHARD L. CONOLLY COLLEGE, **LONG ISLAND UNIVERSITY**, IN PARTIAL

FULFILLMENT OF REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

June 2023

DEPARTMENT

Life sciences

MAJOR

Biology

CERTIFIED BY:

Dr. Joseph Morin

Chairman of the Department

Date June 28, 2023

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Reconstructing the germination pathway from the *Rafflesia* seed transcriptome

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VENKATA SIVA SANKAR MADDU

AN ABSTRACT OF A MASTER'S THESIS SUBMITTED TO THE FACULTY
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ABSTRACT

Rafflesia (Rafflesiaceae) produces the largest flowers in the world but has no stems, roots, or leaves. It is a holoparasitic angiosperm that derives all its nutrients from its host vine, *Tetrastigma*. All species are also threatened with extinction, but propagating it has been incredibly challenging. Its germination from seed has never been observed. The thesis aims to reconstruct the germination pathway of *Rafflesia* from its published seed transcriptome to gain insight into its molecular genetics and understand what germination genes can be stimulated to facilitate infection of its host for ex-situ propagation. The published seed transcriptome of *Rafflesia speciosa* was annotated. These were then bioinformatically compared to the seed germination pathway genes in the model photosynthetic plant *Arabidopsis* (Brassicaceae) and in another parasitic plant, *Striga* (Orobanchaceae), to determine if there are similar genes conserved, especially the Strigolactones-responsive gene which promotes *Striga* germination in response to hormones called strigolactones. The assembled *Rafflesia speciosa* seed transcriptome contained more than 123,000 transcripts. Out of more than 123,000 de novo assembled transcripts, 7025 with blast hits were mapped and annotated. Among these, genes involved in abscisic acid, auxin, brassinosteroids, cytokinin, and ethylene signalling were detected in *Rafflesia*, in common with those in *Arabidopsis*. Still, no Strigolactones-responsive genes were detected in *Rafflesia*. This study showed that some germination signalling genes are conserved between *Rafflesia* and *Arabidopsis* but not with another parasitic plant. This suggests that strigolactones will not be useful in stimulating *Rafflesia* germination for propagation attempts.

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INTRODUCTION

Holoparasitic angiosperms of the genus *Rafflesia* belong to the family Rafflesiaceae, order Malpighiales. In Java, this genus was discovered between 1791 and 1794, according to the French naturalist and surgeon Louis Deschamps. It differs from other flowering plants because of its dramatically reduced vegetative systems and yet still produces the largest flowers in the world (Nikolov et al., 2014). The largest flower ever measured was a *Rafflesia arnoldii*, which can reach a diameter of 100 cm (Barkman et al., 2008). *Rafflesia* appears as a uniseriate strand inside its host, as an endophyte, and with the right stimulation—which is unknown—it can fast transition into the blooming stage. (Nikolov et al., 2014; Wicaksono et al., 2020). *Rafflesia* has a blossoming phase that lasts just four to eight days after an extraordinarily extended life cycle of up to four years (Meijer et al., 1997; Meijer et al., 1958; Hidayati et al., 2000).

Holoparasitic *Rafflesia*, which lacks branches, roots, or leaves, receives all of its nutrition from the host vines of the genus *Tetrastigma* (Vitaceae; Nais et al., 2001; Davis et al., 2007). Their lack of stems, roots, and leaves has made it difficult to comprehend their evolutionary history, which was unknown until 20 years ago (Barkman et al., 2004; Davis et al., 2007). Phylogenetic evidence has placed Rafflesiaceae as a sister to the photosynthetic family Euphorbiaceae (spurge family), which surprisingly produces small flowers, 1/80th of *Rafflesia*'s size (Davis et al., 2007). Because of its ancient parasitic relationship, *Rafflesia* has lost its chloroplast genome (Molina et al. 2014). Since the parasite also closely associates itself with its host, information may be transferred genetically to the parasite (Davis & Wurdack, 2004; Xi et al., 2012, 2013).

Rafflesia encompasses approximately 30 species unique to Southeast Asia's tropics and has earned the nickname "panda of the plant world" because of their charming characteristics, several of which are at risk of extinction. (Barcelona et al., 2009;

Wicaksono et al., 2016). *Rafflesia* species are becoming endangered because of human activities like logging, turning forests into vast agricultural fields, urbanization, and unsustainable ecotourism (Fauzan et al., 2021). The Philippines is the hub of *Rafflesia* diversity, with several new species recorded recently (Pelser et al., 2019).

Ex-situ propagation of *Rafflesia* has proved challenging--Western botanic gardens still need to grow the plant. Molina and coworkers have been transporting *Tetrastigma* cuttings infected with *Rafflesia* from the Philippines to the United States Botanic Garden (USBG) in Washington, D.C., for multiplication since 2015. The cuttings, however, have now died (Molina et al. 2017). However, since 2018 uninfected host plants have flourished and have been routinely inoculated with *Rafflesia speciosa* seeds. However, the appearance of a *Rafflesia* bud has yet to be seen (Molina et al., 2017). The seeds of *Rafflesia speciosa* have also been incubated with a range of plant growth regulators, including synthetic strigolactone GR24 and gibberellins, which encourage germination in unrelated parasites of the Orobanchaceae family and conventional autotrophic plants, respectively. In *Rafflesia*, however, no germination has been observed even after addition of these growth regulators (Molina et al., 2017).

The tiny flowering *Arabidopsis thaliana* (Brassicaceae) is often used as a model organism in plant biology. Although *Arabidopsis* does not impact agriculture much, it significantly benefits fundamental genetics and molecular biology research. Its seed germination pathway has been characterized and involves genes that respond to and regulate abscisic acid (ABA), auxin (AUX), brassinosteroids (BR), cytokinin (CK), ethylene (ETH), gibberellin (GA), and Karrikin (KR) (Carrera-Castano et al., 2020).

Generally, seed germination involves the antagonistic interactions between ABA and

GA, with GA pro-germination and ABA inhibitory/pro-dormancy. The other hormones regulate GA or ABA levels, with ETH, CK, and KR generally promoting germination, while AUX delays germination (Carrera-Castano et al., 2020).

Parasitic plants of the Orobanchaceae family are the source of devastating weed species, including *Striga*, *Orobanche*, and *Phelipanche*, which infest important crops and cause economic losses of more than \$1 billion globally. Strigolactones (SLs) are signaling molecules plants produce to promote mycorrhizal associations and increase nutrient uptake. However, parasitic plants of Orobanchaceae have evolved to exploit these hormones to detect the presence of their host to induce their seed germination. (Saeed et al., 2017).

The seed transcriptome of *Rafflesia* was sequenced and reconstructed to get better insight into the molecular genetics of the plant's development (Molina et al 2023). This thesis seeks to identify the seed germination genes that are common/homologous between the model plant *Arabidopsis thaliana* and *Rafflesia*, as well as determine if strigolactones-responsive genes in Orobanchaceae are present in *Rafflesia* to determine how seed germination in *Rafflesia* can be stimulated to benefit ex-situ propagation and conservation efforts.

MATERIALS & METHODS

Rafflesia speciosas seed transcriptome was extracted, sequenced, and annotated by (Molina et al., 2023), resulting in c. 123,000 transcripts. These transcripts were imported into Omics Box software (Biobam Bioinformatics, Valencia, Spain), blasted against Magnoliopsida non-redundant protein sequences using the blastx fast algorithm with e-100, and then annotated. *Arabidopsis* gene IDs (<https://www.arabidopsis.org>) for genes involved in the seed germination pathway of the model plant *Arabidopsis thaliana* (Carrera-Castano et al., 2020) and genes in the SLs signaling pathway (Wang & Li, 2017) were extracted and blasted against the *Rafflesia* seed transcriptome (blastn, e-val $1.0e^{-10}$) which was imported as a custom database in Geneious Prime (Biomatters Ltd). The resulting homologous *Rafflesia* sequences were blasted against the NCBI protein database (blastx, e-val $1.0e^{-50}$), and their functions were searched in TAIR (<https://www.arabidopsis.org>)

RESULTS

Seven thousand twenty-five transcripts (7025) were mapped and annotated out of more than 123,000 de novo assembled transcripts from the *Rafflesia* seed (Molina et al., 2023). Figure 1 shows the histogram with the length of *Rafflesia* seed transcripts plotted against the number of sequences. The shape of the graph is right-skewed, with the *Rafflesia* seed transcripts being 1392 bp on average long.

Table 1. Distribution of *Rafflesia* seed transcript sizes. The average transcript length was 1392 bp.

Length Range (bp)	Count (Contig range)	Percentage
300-600	146 (1 to 142)	2.07
601-1000	1800 (143 to 1943)	25.63
1001-2000	4136 (1944 to 6080)	58.88
2001-3000	701 (6081 to 6782)	9.98
3001-4000	174 (6783 to 6957)	2.48
4000+	67 (6958 to 7025)	0.96
Total	7025	100

Figure 1. Histogram of *Rafflesia* seed transcript length.

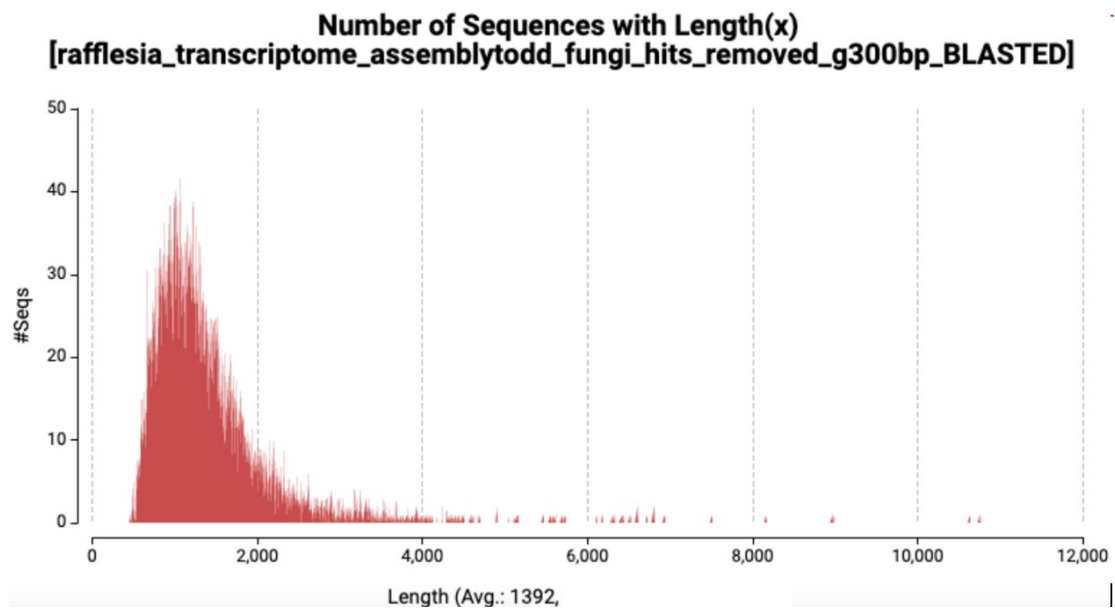


Fig. 2 Seed germination pathway of *Arabidopsis thaliana* adapted from (CarreraCastano et al., 2020). Green indicates the promotion of germination, and red inhibition (=promotion of dormancy). Yellow circles indicate seed genes that were homologous and detected in the *Rafflesia* seed transcriptome. Blue indicates plant hormones. White represent genes not detected in the *Rafflesia* seed transcriptome

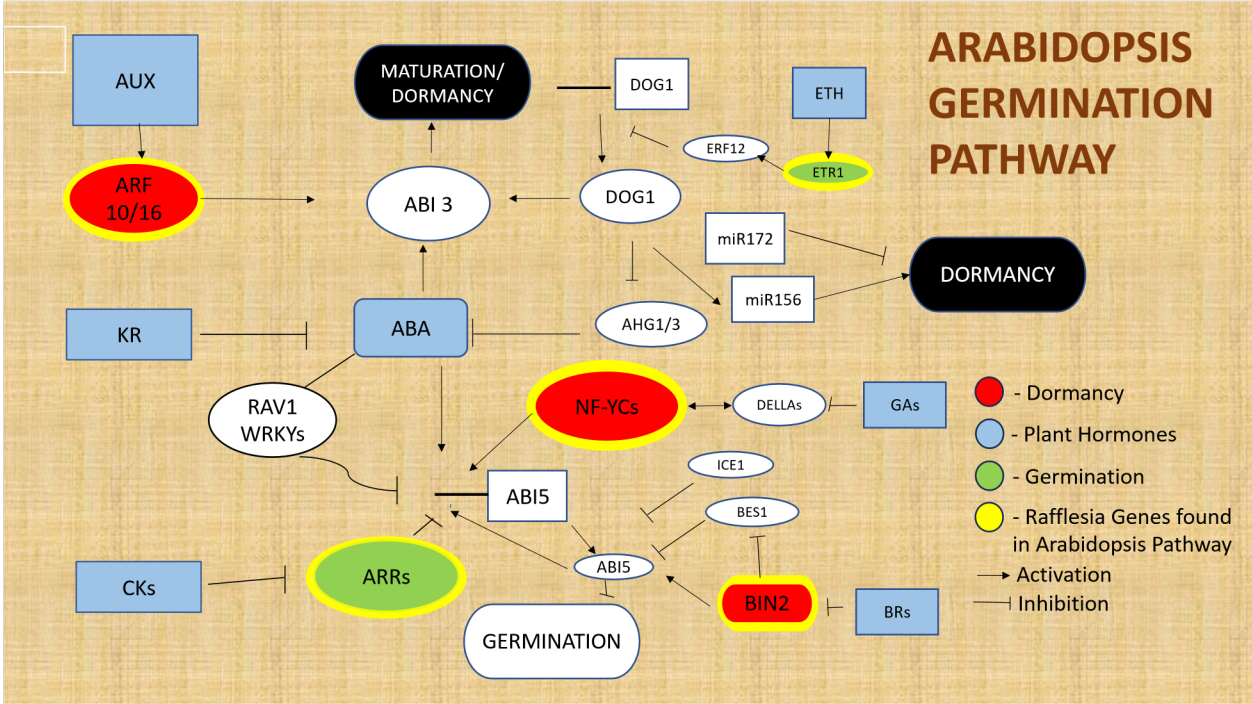
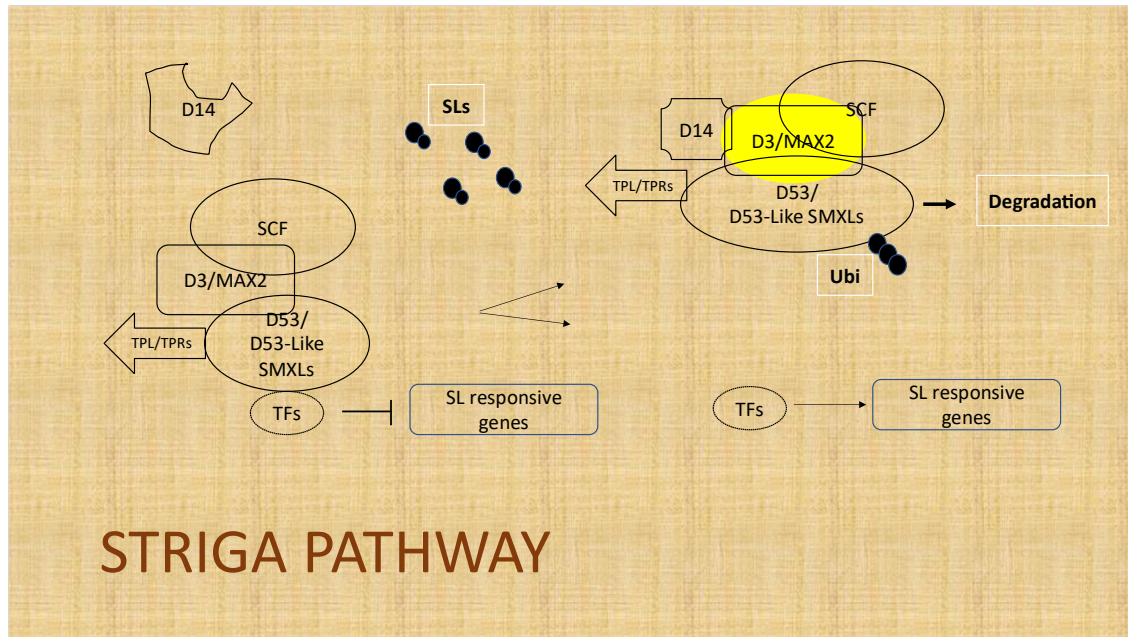


Figure 3. The strigolactone/SL signaling pathway model was adapted from Wang & Li (2017).

The yellow circle indicates the seed gene that was homologous with *Striga's* and detected in the *Rafflesia* seed transcriptome.



DISCUSSION

The seed transcriptome of *Rafflesia* was sequenced and reconstructed to get better insight into its molecular genetics and determine which of its genes are homologous/ conserved in the model photosynthetic plant *Arabidopsis* and the unrelated parasitic plant *Striga*.

The seed germination pathway of *Arabidopsis* has been characterized. It involves genes that respond to and regulate abscisic acid (ABA), auxin (AUX), brassinosteroids (BR), cytokinin (CK), ethylene (ETH), gibberellin (GA), and Karrikin (KR) (CarreraCastano et al., 2020). Generally, seed germination involves the antagonistic interactions between ABA and GA, with GA pro-germination and ABA inhibitory. The other hormones regulate GA or ABA levels, with ETH, CK, and KR generally promoting GA levels, thus germination. At the same time, AUX increases ABI, consequently increasing ABA, thus delaying germination (Carrera-Castano et al. 2020).

Five genes in the *Rafflesia* seed transcriptome were homologous to those in *Arabidopsis*, including ETR1, ARR, NF-YCs, ARF 10/16, and BIN2. ETR1 is an ethylene receptor activated by the presence of ethylene. Its presence in *Rafflesia* indicates that the *Rafflesia* seed produced ethylene when sampled. Activation of ETR1 inhibits DOG1, which, when repressed, promotes germination. Additionally, ARR was detected, which inhibits ABI. However, germination-inhibitory genes were also found, including ARF 10/16, which is involved in the AUX pathway, increases ABI, thus inhibiting germination. NF-YC was also detected in *Rafflesia* seed which also activates ABI. Additionally, BIN2 was also detected, that also increases ABI. The presence of

ARF10/16, NF-YC, and BIN2 seem to suggest that though some factors seem to promote germination in the *Rafflesia* seed, the actual germination—protrusion of the radicle (or, in the case of *Rafflesia*, something similar) has yet to occur, and perhaps still pending as it waits for host stimulation.

In the strigolactones pathway, the strigolactones-responsive gene D14 was not recovered. In *Striga*, when strigolactones are present, as secreted by its host plant, D14 docks and bind to D3/MAX2, which eventually begins a series of events that stimulate seed germination. However, D14 was not detected in *Rafflesia*, though D3/MAX2 was recovered in the *Rafflesia* seed transcriptome. This gene is also involved in Karrikin signaling. Karrikin is a smoke-derived molecule that induces germination in other plants. In addition, KAI2, the Karrikin-responsive gene, was found in *Rafflesia*, which supports that Karrikin may induce *Rafflesia* seed germination. This study showed that some germination signalling genes are conserved between *Rafflesia* and *Arabidopsis* but not with another parasitic plant. This suggests that strigolactones will not be useful in stimulating *Rafflesia* germination for propagation attempts. This study provides insights on hormones/ molecules that can be applied to benefit ex-situ propagation and conservation efforts of *Rafflesia*, the world's largest flower.

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