Chapter

Insight into the Mechanism of Red Alga Reproduction. What Else Is Beyond Cystocarps Development?

Pilar Garcia-Jimenez and Rafael R. Robaina

Abstract

Volatile growth regulators play an important role in triggering aspects related to red seaweed reproduction. The last 10 years have brought clarification to how ethylene and methyl jasmonate work. Taking two reproductive stages of thalli of red seaweed—fertilised and fertile thalli—as benchmarks and a precise characterisation of the elicitation and disclosure periods of cystocarps, monitoring different gene expressions, namely candidate gene for reproduction and genes encoding proteins involved in biosynthesis pathways of both volatiles and reactive oxygen species, has enabled us to discern the differential behaviour of genes. These studies have also revealed that the volatile-mediated signal could affect cell wall loosening. All in all, studies have shown evidence of putative signalling pathways where volatile signal regulators form part of them at several levels, ranging from disclosure, development to the maturing of cystocarps. This signal information is crucial to determine the final response. The chapter also discusses whether signal transduction is related to different sensing for each volatile and whether this could be elicited in accordance with signal strength. This chapter compiles our current understanding of molecular mechanisms of algal reproduction and how volatile-mediated signals affect other developmental processes.

Keywords: ethylene, genes, methyl jasmonate, red seaweed, volatile growth regulators

1. Introduction

Carposporogenesis in red algae requires the disclosure and development of reproductive structures named cystocarps and cell wall weakness and also requires these reproductive structures to mature. Disclosure of cystocarps, in other words, the period in which the first cystocarps become visible, is elicited by external signals such as volatile growth regulators. On the other hand, controlling the elicitation period is essential for the proper development of cystocarps. If this period does not lead to the disclosure of cystocarps, cell wall loosening will not occur, and these structures will not mature either.

Once the elicitation period occurs, cystocarp development begins with the weakening and relaxation of the cell wall. In Floridophyceae, the accepted view is that the cell wall is made of well-organised layers, whilst the intracellular matrix is comprised of less organised material. Some of components of the cell wall and matrix are sulphated galactans, which have a physiological significance that varies

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according to the different life stages of the macroalgae [1]. Despite the relationship between the loosening and weakening of the cell wall and the different life stages, the biochemical and molecular mechanisms have not been fully discovered. Evidence suggests that reactive oxygen species, under growth regulator control, are able to cleave cell wall polysaccharide, causing the wall to loosen [2, 3] during reproductive events in seaweed.

The maturity stages of reproductive structures in red algae are complex processes (**Figure 1**), highly co-ordinated and, to a large extent, quite difficult to determine. Unlike some seaweeds where different stages of development of cystocarps are evident and can be recognised [4], in others, the maturity stages are assumed to occur from the beginning of the discloser of the reproductive structures to thalli. In these cases, the maturity process differentiates between two kinds of thalli, the fertilised thalli and fertile thalli. The fertilised thalli are the ones that have both non-visible cystocarps and incipient visible cystocarps. Meanwhile, fertile thalli range from thalli with well-developed cystocarps to those that have fully mature cystocarps (**Figure 2A**).

Changes related to the maturing of thalli are favoured by volatile growth regulators, which also lead to both cystocarps dehiscence with a marked reduction of the maturity period, and the presence of different reproductive structures in the same individual [5]. Moreover, other evidence such as sudden losses of seaweed mats and alternating life cycles could also give cues on how volatile compounds act as a signal to trigger the reproductive process. Actually, seaweeds have a defined reproductive period and are able to discern between volatile signals. The latter leads to the presence of 'putative' volatile receptors although they are not yet known and only a proposed ethylene receptor in red algae has been reported [6].

With this scenario, advances in gene studies have been made by combining different approaches—based on evidence of in vitro culture in the presence of volatile growth regulators and on algal physiology—and thus to decipher the network of interactions between different metabolic pathways that lead the transition from fertilised to fertile thalli. This path can lead to an understanding of a complex network of interacting genes and signal pathways that occurs. Hence one of the key questions is also to unveil how this process can be co-ordinated to work efficiently.

In recent years, great strides have been taken to gain understanding of molecular events in red seaweeds. These endeavours have allowed for a better understanding of the changes that occur during the transition from disclosure to the maturity of



Figure 1.

Diagram of a tri-genetic life cycle in the red alga Grateloupia imbricata comprising the gametophyes (haploids), called carposporophyte, that develops on the female gametophyte after fertilisation, and the sporophyte (diploid).



Figure 2.

(A) Schematic showing the timeline for the periods of disclosure and maturity of cystocarps and the corresponding fertilised thalli within cystocarps, ranging from non-visible to visible incipient and fertile thalli from well-developed to fully developed cystocarps. (B) Timeline of gene expression of ODC for fertilised and fertile thalli of Grateloupia imbricata after ethylene and methyl jasmonate treatment. The sloping lines represent significant increase or decrease in gene expression with respect to absolute values (copies μl^{-1}). Horizontal line indicates no changes in gene expression relative to expression in control thalli. MeJa, methyl jasmonate.

cystocarps in response to growth regulator. In this chapter we present our research output in the carragenophytic red seaweed model *Grateloupia imbricata*, compiling our current understanding of molecular mechanisms of algal reproduction and how a volatile-mediated signal can affect other developmental processes. This work does not forget to review other articles, but it does focus on what the state of the art is concerning red seaweed reproduction based on (1) candidate genes, (2) genes that encode cell wall weakness and reactive oxygen species and (3) genes that encode biosynthesis of volatile growth regulators such as ethylene and methyl jasmonate.

2. Candidate gene of reproduction

Growth, development and reproduction of multicellular organisms require precise and multifunctional cell–cell communication events. This is even more necessary in marine seaweed, where changes in irradiation, salinity and temperature, due to the tidal period, affect sporulation and mean that these organisms have to handle and adapt environmental signals in an extremely precise manner to survive. Taking this into consideration, it is easy to understand that algae make quick acclimation reversible acclimation—and adaptation to the marine environment and that the control of some of these vegetative and reproductive processes is particularly based on short-range signalling.

With this complex net of intervening factors, in order to interpret what is occurring with a particular event, the election of a candidate gene, which represents the manifestation of a trait such as the development and maturity of cystocarps, has provided insights into the carposporogenesis of red seaweeds. Unlike unspecific genes that are overexpressed under a given condition and are assumed to be responsible for a particular event/trait/action [7–9], our candidate gene encodes the synthesis of the main protein ornithine decarboxylase (ODC, EC 4.1.1.17) responsible for the synthesis of polyamines and is related to the maturing process of cystocarps in seaweeds [10–12].

The differential behaviour of this gene (GiODC) and its integration with volatiles contribute to it being chosen as a candidate gene for several reasons. First, the inhibition of ODC enzyme synthesis by the inhibitor $DL-\alpha$ -difluoromethylornithine (DFMO) implies the lowest levels of polyamines. This inhibition also halts the maturity of cystocarps and the eventual release of spores from cystocarps [11]. Second, the enzyme activity of ODC is related to the endogenous levels of polyamines. The reduction in ODC enzyme activity and polyamine levels are also related to the presence of cystocarps [10, 12]. Third, reactive oxygen species are released through polyamine catabolism pathways and are under the control of ODC. During cystocarp development, spermine is accumulated, favouring the process of development and maturity of the reproductive structure. When it exerts an inductive effect, polyamine oxidase enzyme activity increases as the spermine degrades [12]. Fourth, GiODC is expressed differently in both the fertile thalli (with cystocarps), than in infertile thalli (vegetative thalli), and in the apical part of fertile thalli, against the basal part of these thalli, as reported using -time quantitative PCR and in situ hybridisation techniques [13]. Fifth, sequencing the upstream region of GiODC revealed transcription factors involved in regulation by jasmonate (Myc2, Myc3 and Myc4) and ethylene (RAV, SMZ and Abi4). This means that there is a relationship between volatiles and ODC expression [14]. Sixth, monitoring GiODC gene expression after treatment with volatiles during the well-defined periods of elicitation and disclosure of cystocarps reveals differential behaviour of this gene, depending on the development and maturity of the cystocarps [14].

Putting all the data together suggests two important conclusions regarding the candidate gene: Expression is dependent on the existence of cystocarps and the kind of growth regulator used to elicit reproduction. Generally, down-expression of the gene candidate goes hand in hand with the presence of cystocarps and points to a quick transduction signal (**Figure 2B**) [15, 16]. Nonetheless, it is worth mentioning that there are two different gene expression patterns that occur when methyl jasmonate is used as an elicitor. Hence, in thalli without visible cystocarps, gene expression is upregulated due to methyl jasmonate signalling (fertilised thalli, **Figure 2B**). Moreover, in thalli containing fully mature cystocarps, other up-expressions are related to the stage of maturity of the cystocarp due to methyl jasmonate (fertile thalli, **Figure 2B**).

Far from being a mismatch for a candidate gene, it is understood that different signals are executed over the course of cystocarp development, and hence one can infer that thalli are able to discern between volatiles; they sense them in order to provide co-ordinated responses [14–16].

3. Genes encoding proteins related to oxidative stress and softening of thalli

In most organisms, factors including drastic changes in temperature, irradiation and desiccation are stressful and potentially destructive. Nonetheless reproduction in algae is also highly regulated by temperature and tidal periods, which has an impact on processes such as sporulation. The generation of reactive oxygen species in turn is triggered by these environmental factors, as can be expected. To ameliorate this situation, organisms display various physiological responses which

are often being associated with an increase in the production of proteins that scavenge free radicals and reactive oxygen species (ROSs) [17, 18]. Unlike what has been well studied in higher plants, where stress proteins can be synthesised as a key survival strategy, we know that similar processes can occur, but it remains unclear whether stress proteins are metabolically biosynthesised or whether free radicals can be eliminated by chemical scavenging. Consideration also has to be given to the fact that certain red algae render methylate halides using methyltransferases that use S-adenosyl methionine (SAM, pivotal compound for the synthesis of ethylene and methyl jasmonate) as the methyl donor. Methylation of halides is a mechanism eliminating halide and sulphide ions, both of which are known to be phototoxic [19, 20].

Beyond this, seaweeds develop strategies to signal events related to growth and development, including the biosynthesis of volatiles. These volatile signals appear to crosstalk with other growth regulators such as polyamines [21]. As an example, polyamines, ethylene and methyl jasmonate share the same precursor—SAM—for these biosynthesis routes. Moreover, ROSs can be also released through metabolic pathways of growth regulators. The contribution of these signal pathways to growth and development is difficult to appraise as volatiles can have synergistic effects on one or more of the other pathways involved in seaweed reproduction, and this combination of all the pathways might give rise to several responses. Ethylene and methyl jasmonate provoke changes in the oxidation state of intermediates during synthesis. These include jasmonates, which are compounds, resulting from lipid oxidation of the cell membrane. In particular, methyl jasmonate is derived from linolenic acid, via lipoxygenase, in which the synthesis of methyl jasmonate activates the oxidative metabolism of polyunsaturated fatty acids, generating ROSs (in the form of O₂, H₂O₂ or OH⁻) and oxidised derivatives of polyunsaturated fatty acids [22, 23]. Oxygenated volatile compounds have been shown to not necessarily involve photodamage of cell membranes. Meanwhile the reactivity of the ethylene double bond allows this olefin to be easily converted into a range of intermediates [24].

With this framework, ROSs also have the potential to interact with many cell components and can give rise to several physiological responses, such as when ROS acts as an important signal transduction molecule during growth [18]. Indeed, it has been inferred that ROSs play an important role in softening of thalli and therefore in the development of cystocarps in red seaweed. This is significantly important with the heat shock protein WD40 and cytochrome P450 which are responsible for reducing oxidative damage [25]. WD40 and cytochrome P450 are specifically related to ethylene and methyl jasmonate signalling [15, 16].

Furthermore, what is striking is that genes that encode WD40 and cytochrome P450 mirror their expressions depending on whether they are elicited by ethylene or methyl jasmonate signals [15, 16]. The synchronised behaviour of these genes based on their expressions seems to determine close co-ordination due to the elicitor. Our results with G. imbricata suggest that the expression of one gene can become activated and repressed without the assistance of another one, but expression is also linked to different signals related to both cystocarp disclosure and development. In G. *imbricata*, this means that WD40 gene expression responds to the ethylene signal when cystocarps are still non-visible, whilst this gene expression increases in the presence of the first cystocarps after methyl jasmonate treatment (disclosure period). Otherwise cytochrome P450 is expressed in the presence of the first cystocarps (developing cystocarps) when they are treated with ethylene. Conversely, after the methyl jasmonate elicitor, cytochrome P450 expression responds when cystocarps are still invisible. In both cases, as the cystocarps mature, expression holds over time without any significant changes between thalli with well-developed cystocarps and fully developed cystocarps [15, 16] (Figure 3).

In addition, the ascorbate peroxidase gene, which encodes a protein involved in the response to oxidative stress [6, 15], is also associated with the disclosure and development of cystocarps rather than with their maturity process [16].

Alternatively, polyamines, which are nonvolatile molecules but do have an important role in the process of maturing of the cystocarps, are synthesised through the candidate gene known as ODC [10, 14–16]. The synthesis of the polyamine precursor, putrescine, renders downstream spermidine and spermine due to the addition of one or two aminopropyl groups from decarboxylated SAM. Endogenous levels of these three polyamines—that is, putrescine, spermidine and spermine—are balanced by amine oxidase and polyamine oxidase, whilest H_2O_2 is released as a by-product of this reaction.

Monitoring amine oxidase gene, whose gene expression was seen to depend on the disclosure and development period of cystocarps, but also that once cystocarps have developed, reported that this gene expression would help to maintain poly-amines levels (**Figure 3**) [16].

In short, our results confirm that genes encoding ROS proteins are related to physiological events. If we take the results as a whole, these behaviours of genes enable us to discern two action modes. Initially, WD40, cytochrome P450 and APX point to promoting the disclosure and development period of cystocarps, and they help to soften the thalli as up-expressions occurs. Meanwhile, amine oxidase expression shows a dual response. In other words, it helps cystocarp disclosure but it also balances ROS levels in order to fine-tune polyamine levels and prepare the thalli for the next time.



Figure 3.

Timeline of gene expression encoding stress proteins (WD40, cytochrome P450 and amine oxidase) for fertilised and fertile thalli of Grateloupia imbricata after ethylene and methyl jasmonate treatment. The sloping lines represent significant increase or decrease in gene expression with respect to absolute values (copies μ l⁻¹). Horizontal line indicates no changes in gene expression relative to expression in control thalli. CytP450, cytochrome P450; AAO, amine oxidase; MeJa, methyl jasmonate.

4. Genes encoding proteins involved in biosynthesis pathways of growth volatile regulators

Despite the commercial importance of red seaweed, we still lack information on reproductive events if 'our' interest is to be able to control what happens over the course of the development and maturity processes of the reproductive structures and consequently manage to produce a large number of individuals.

Unlike the amount of information based mainly on next generation sequencing data, little progress has been made on the temporal control of genes, which affect growth and development. These aspects are of critical importance from the point of view of farming them. It is worth to highlight seaweeds that had received little attention worldwide to elucidate gene functions and to delve into the development and progress of functional genomic. Particularly in this section and as a practical goal, it is expected that molecular mechanisms related to volatile biosynthesis during carposporogenesis will provide tools for control and regulation of growth and developmental process in seaweeds. Thus, insight might allow to initiate a genetic programme for macroalgae which is economically valuable, increasing its viability and value.

The molecular nature of the signal(s) that control development and maturity of cystocarps is unknown, although efforts have been made in recent years to accurately describe the elicitation and disclosure periods of cystocarps in the red alga *G. imbricata*. One of the most striking features is that alterations in gene expressions even start prior to the presence of visible cystocarps, which seems to suggest that communication through signal pathways is essential for the disclosure of cystocarps.

Our research team has focused on gene screening related to proteins specifically involved in biosynthesis pathways of volatile growth regulators instead of profiles of up- and downregulated genes reported in massive sequencing. Although it is obvious that any attempt is appropriate given the lack of molecular information in red seaweeds, we ought to bear in mind the existence of environmental acclimation of algae and the tremendous changes in the levels of expression of a large number of genes during the disclosure, development and maturity of cystocarps. Incidentally, we have to remember that factors such as salinity and sporulation are connected, and our aim is to be able to discern precisely what is happening.

Hence in order to gain a better and more accurate insight into the control mechanisms underlying the reproduction of red seaweeds, the monitoring of specific genes, that in turn are also related to growth regulators and their biosynthesis, has been successful (Figure 4). In particular, gene-encoding enzymes needed for the synthesis of ethylene, such as SAM synthase (SAMS) and ACC synthase (1-aminocyclopropane-1-carboxylate synthase), genes that encode proteins of polyamine metabolism (spermidine synthase (Spd synthase); amine oxidase), genes encoding proteins of methyl jasmonate synthesis, such as jasmonic acid carboxyl methyltransferase (JMT) and putative methyltransferase (MT); and a gene that encodes a transcription factor involved in controlling responses to stress, growth and development (MYB, [26]), have been monitored. These gene expressions have provided valuable information and helped to shed light on the complex process of red seaweed reproduction. As for genes related to ethylene biosynthesis, these are directly involved in cystocarp development, that is, SAMS, Spd synthase and ACC synthase. Otherwise, all genes studied in relation to methyl jasmonate are indiscriminately induced in the absence of cystocarps (Figure 5).

In general, we can indicate that methyl jasmonate and ethylene signalling occurs either immediately after the elicitation period or during the disclosure period, respectively (**Figure 5**). The time course of different gene expressions indicates a temporal regulation of algal reproduction. As part of this temporal regulation, the



Figure 4.

Biosynthetic pathway for polyamines and connections with the pathways for the biosynthesis of ethylene and jasmonate. SAMS, S-adenosyl methionine synthase; d-SAM, decarboxylated SAM; MT, putative methyl transferase; JMT, jasmonic acid carboxyl methyl transferase.



Figure 5.

Timeline of gene expression encoding biosynthesis proteins of ethylene (Spd synt, spermidine synthase; SAMS, S-adenosyl methionine synthase; ACCS, 1-aminocyclopropane-1-carboxylate synthase) and methyl jasmonate (JMT, jasmonic acid carboxyl methyl transferase; MT, methyl transferase) for fertilised and fertile thalli of Grateloupia imbricata after ethylene and methyl jasmonate treatment. The sloping lines represent significant increase or decrease in gene expression with respect to absolute values (copies μl^{-1}). Horizontal line indicates no changes in gene expression relative to expression in control thalli.

differential gene expressions represent the ability of seaweeds to sense ethylene and methyl jasmonate separately [15, 16].

Signal transduction—like the presence of cystocarps—brings up the question of whether the sensing of both volatiles could be elicited in accordance with the signal strength. The latter is within the bounds of possibility since (i) ethylene, which is the smallest volatile molecule, can easily cross through cell membranes and (ii) the

hypothetical model of ethylene receptor for algae is a simpler structure than the one reported in higher plants. A priori, although both volatiles require membrane receptors, the fact is that the ethylene signal of the candidate gene elicited 12-fold the expression of methyl jasmonate despite the period where gene expression is reported [14]. This could be important for the fine regulation of disclosure and development of cystocarps.

To make this more difficult, something else caught our attention. We have also wondered whether signal strength can be interpreted as a differential response between elicitor signal and signal transduction. Signal transduction is assumed to be the responsibility of a complex and integrated molecular network. The network for one or another volatile could overlap in such a manner that this overlapping simplifies signal channelling. Contrary to what some may think, we do not rule out separate signalling networks. Nevertheless, there could also be a signal output modulation 'mechanism' that regulates the disclosure and development of cystocarps [16]. We are a long way from knowing what is happening—in other words, the differential perception of volatiles, the separate and overlapping signal pathways and signal strength. Nonetheless, we realise that gene knockout studies will be advantageous to confirm these issues. Although we have accomplished the primary goal of revealing the molecular mechanisms underlying red seaweed reproduction, further studies are required to identify and explore other factors involved in the regulation of gene expression.

5. Conclusions

This chapter has summarised our insight into the complexity of gene regulation during red seaweed reproduction. There are grounds to believe that temporal patterns of gene expression are orchestrated under the control of volatile growth regulators signalling during the disclosure and development of cystocarps. Progress is being made in understanding how thalli transduce these volatile signals.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Author details

Pilar Garcia-Jimenez^{*} and Rafael R. Robaina Department of Biology, Faculty of Marine Sciences, Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Canary Islands, Spain

*Address all correspondence to: pilar.garcia@ulpgc.es

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