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Characterization of molecular processes involved in the pearl formation in Pinctada margaritifera for a sustainable development of pearl farming industry in French Polynesia

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Abstract:

Tahiti's pearl farming industry plays a major socio-economic role in French Polynesia. In an increasingly competitive market where the production of high quality pearls becomes essential, research can help secure and ensure sustainable production. In that context, Ifremer, in close collaboration with the "direction des resources marines" (French Polynesian government agency) has developed research projects on the "sustainable development of pearl farming". This program is organized along 3 axes: (1) understanding the animal physiology and initiating a genetically selective breeding program of donor oysters; (2) understanding pearl oyster larvae dispersal and recruitment; (3) understanding the molecular mechanisms underlying biomineralization processes during shell and pearl formation. It is in this frame that we have developed a highthroughput Expressed Sequence Tags pyrosequencing program on the calcifying mantle, combined with proteomic analyses of the shell and pearl. We analyzed 276738 EST sequences, leading to the constitution of a *P. margaritifera* mantle transcripts catalogue of 82 sequences potentially involved in biomineralization. Our results provided direct evidence that our ESTs data set covers a large number of the matrix proteins of *P. margaritifera*. In addition, our proteomic analysis enabled us to retrieve, *in silico*, all the sequences from *P. margaritifera* involved in the biomineralization process already published on databases. Integration of these two methods allowed, for the first time, the global composition of calcifying tissue and calcified structures to be examined in tandem.

Keywords : pearl oyster ; biomineralization ; *Pinctada margaritifera*

INTRODUCTION

In French Polynesia, cultured pearls are the product of grafting and rearing of *Pinctada margaritifera* pearl oysters in their natural environment. Pearl culture includes several stages. To start with, the pearl oysters are collected and raised to serve either as donor or receiver. The grafting process then takes place following a surgical operation during which the graft, a small piece of mantle tissue, is inserted into the "pearl pocket" of the receiving oyster together with a nacre bead, the nucleus (Figure 1). Once inserted into the receiving oyster, the external epithelial-cells of the graft multiply to form a pearl sac around the nucleus (Cochennec et al, 2010). The pearl sac then starts to deposit calcium carbonate polymorphs layers onto the nucleus. This is the starting point for the future pearl. A rearing period of about 18 months is needed to finally produce a pearl with a sufficiently thick layer of nacre (0.8 mm) (Figure 2). Generally, with oysters that have produced a high quality pearl, a second graft can be carried out.

Pearl culture is a fundamental part of the polynesian economy. As the largest export industry, worth 66.2 million euros in 2009, it employs around 5000 people, and contributes both to land management and the reversal of human migration from the atolls to Tahiti. The increased availability of maritime leases from the start of the 1990s and the spread of grafting skills (techniques previously mastered only by Japanese guest grafters) among local people led to the multiplication of farms and rapid increases in production. Pearl quality suffered from that rapid/anarchical expansion of pearl production and the profitability of farms was affected, demonstrated by a fall of the mean price per gram of exported pearls from 2001 onwards. Additionally other countries like Australia, China, Indonesia and the Cook Islands entered the market, with the advantage of lower production costs. This strengthening of international trade led French Polynesia to increase the number of poor quality pearls on the market. On

average, only 5 % of pearls in a first harvest are of very high quality. An increase in the number of high quality pearls would therefore be a considerable advantage for the industry.

1- THE "SUSTAINABLE DEVELOPMENT OF PEARL FARMING" RESEARCH PROJECT

In the context of a more and more competitive market where the production of high quality pearls becomes essential, research can contribute to secure and ensure durable production. Ifremer in close collaboration with the "service de la perliculture" ("pearl farming office", a French Polynesian government agency) has developed a research project on the "sustainable development of pearl farming". Its aim, in accordance with pearl farmers concerns, is to propose methods and tools that will increase superior pearl quality production and intend to support the sustainable development of pearl farming in French Polynesia. Researches are organized along 3 axes: (1) understanding the animal physiology; (2) understanding pearl oyster larvae dispersal and recruitment; (3) understanding the molecular mechanisms underlying biomineralization processes during shell and pearl formation.

1.1 Understanding the animal physiology with the aim to breed families of graft donor oysters selected for their capacity to produce pearls of particular colours and/or rapid growth

In the pearl oyster *P. margaritifera*, the pearl is subjected to the genetic determinism of the graft donor oyster. The graft donor oysters are the stake of a genetic selection (Arnaud-Haond et al., 2007). The aim of the selection program of *P. margaritifera*, developed by Ifremer and the "service de la perliculture" is to breed families of graft donor oysters selected for their capacity to produce pearls of quality and/or particular colours and/or rapid growth. The hatchery production of selected spat depends on the production of gametes and embryos from synchronous breeders conditioned in laboratory. Additionally, the control of the reproduction

is essential for the set up of a genetic improvement program and relies on knowledge of the underlying physiological mechanisms and the control factors. *P margaritifera* is a protandric species in which the sex ratio normally is biased toward males. There is a growing interest to control gender and find mechanism increasing the proportion of females in order to shorten the generation time of selected families. Current research seeks to determine the effect of the environmental factors on the sex ratio. Broodstock conditioning is also necessary to achieve a nutritional state before spawning. In pearl oyster, while gametogenesis is subjected to both biotic and abiotic factors, the influence of temperature and trophic resources on gametogenesis is not well known. Gametogenesis of *P. margaritifera* has been investigated (Thielley et al. 1993) and studies of the reproductive cycle, with qualitative and quantitative observations of gonadal changes, was completed in different areas in French polynesia (Pouvreau et al. 2000; Le Moullac et al. in press a).

Crossbreeding is imperfectly controlled because of the absence of knowledge on reproduction and spawns triggering (Chavez-Villaba et al. 2011). As a consequence, gamete cryopreservation could greatly improve the control level on crossbreeding and assist the pearl farming industry in preserving black pearl oyster lineages and selective breeding for desirable traits of pearl quality (Hui et al. 2011). In that context, several research projects on *P. margaritifera* physiology are investigated with the aim to breed families of graft donor oysters for genetic selection: (i) nutrition and reproduction, and broodstock conditioning, (ii) Spermatozoa cryoconservation and (iii) larval rearing in flowthrough system. The *in situ* studies ended to design experiments in laboratory to establish the relationship between food availability, reproductive effort and gametogenesis. Results allowed first to modelize the relationship between food availability and the ingestion rate. Then we showed that the reproductive effort of *P margaritifera* is positively related to the food availability while when

under-fed, the mitotic process of the germinal stem cells was altered (Le Moullac et al, in press b).

In order to preserve genetic diversity and selected patrimonies, works have been done to cryopreserve spermatozoa of the black-lip pearl oyster. These works allowed us to develop a cryopreservation technique and to test the hatchery applicability of such a method (Hui et al, 2011). The long-term development of the pearl industry will rely on the development of hatcheries, that can guarantee production of selected spat in the required quantities and whenever needed. Recently, we developed a larval rearing system for *P margaritifera* larvae based on a continuous water renewal combined with a continuous supply of microalgues allowing to obtain outputs of 25% of survival at eyed stage while prohibiting the use of antibiotics (Hui et al, 2011).

1.2 Understanding bivalve larvae dispersal and pearl oyster recruitment with the aim to optimize the spat collection strategy.

The sustainability of pearl-oyster culture in French Polynesia relies on spat collection and spat availability in the lagoons. Understanding the mechanisms which drive development and dispersion abilities of larval stages and lead to spat recruitment therefore represents a major challenge. Our studies have been carried out in Ahe, one of the main pearl culture and spat production atoll of French Polynesia. Spatial and temporal scales of variability in phyto- and bacterioplankton abundance were investigated and the deep lagoon waters showed a classical phytoplankton composition, but with higher picophytoplankton (*Prochlorococcus*, *Synechococcus* and picoeukaryote) concentrations than the mean for Polynesian atoll lagoons (Thomas *et al.*, 2010). Day-to-day fluctuation was the major source of temporal variation and appeared highly driven by vertical patterns and mixing events showing phytoplankton supply from deep layers. Distribution of bivalve larvae has been investigated over a range of spatial

and temporal scales and scenarios were designed to explain the observed variation patterns according to physical or biological mechanisms. The large-scale wide range of larval size also suggested a more complex organization, possibly influencing the spatial structure of the adult communities (Thomas et al. 2012c). On a small time-scale, variations in larval abundance appeared to be driven by bivalve reproductive activity correlated with wind conditions. We developed a Dynamic Energy Budget (DEB) model for the larval phase of the pearl oyster (Thomas et al., 2012a) in order to evaluate the effect of spatio-temporal fluctuations of lagoon environment on its development success. The bivalve larvae dispersion in the Ahe atoll lagoon has been studied using a 3D transport model (MARS) integrating a larval vertical movement model (Thomas *et al.*, 2012b). The transport model has been validated with abundance data of an *in-situ* identified cohort monitored along time in the lagoon. Additionally, as it is difficult to distinguish morphologically pearl oyster larvae at a very early stage within the *Pinctada* genus, we have developed in the present study a whole-larvae *in situ* hybridization technique that allows the discrimination of close pearl oyster larvae species found in the French Polynesian atolls (Thomas et al., 2011). This result is a key-step required to develop the monitoring of *P. margaritifera* larval distribution in French Polynesian lagoons to increase spat collection efficiency and ensure a sustainable development of that activity.

1.3 Understanding the pearl formation by studying the grafting process and the biomineralization mechanisms with the aim to increase superior pearl quality production.

The objective of this axe is to study by a global approach the mechanisms underlying pearl formation and defects occurrence in *P. margaritifera*. Researches range from the complete assessment of the grafting process to the characterization of the cellular and molecular mechanisms of the pearl formation with a specific focus on pathology, grafting optimization and biomineralization. The aim, in accordance with pearl farmers concerns, is to propose

methods and tools that will increase superior pearl quality production. To fulfil the industry's expectations, a global research project (called ADEQUA) created in 2008, gathering 10 partner laboratories from French Polynesia and Metropolitan France, has been carried out as a global integrative approach (Figure 3). This approach integrates study of compartments involved in grafting processes, comparison of processes resulting in flawless or flawed pearls, study of the influence of external factors. For that, the ADEQUA research project is composed of six research actions:

(1) Analysis of the principal external factors known to play a role in determining the success of grafting and pearl quality.

(2) Improvement of nucleus quality to enhance grafting success and pearl quality.

(3) Detailed description and dynamics of molecular mechanisms of mineralization processes (from genes to proteins), analysing the first secretory activities of the pearl sac around the nucleus and the precise structural analysis of the mineral and protein make-up of the harvested pearls.

(4) Genetic improvement of graft donor oysters.

(5) Characterisation of shell and pearl colour by a combination of analyses on spectral qualities, pigmentation and genetics.

(6) The detailed description of all flaws and their statistical frequency in cultured pearls.

In a sector where the product prevalence cannot be maintained without a constant seek for maximum quality, this project will allow the French Polynesian territories to keep their actual leadership in terms of technological advance and to ensure the durable development of that industry. The results concerning the biomineralization mechanism are developed in the next paragraph.

2. MOLECULAR ANALYSES OF TISSUES INVOLVED IN PEARL FORMATION

The aim of this work was to investigate the molecular mechanisms underlying biomineralization processes during pearl formation in *P. margaritifera*. In pearl oysters, shells are subdivided in two calcium carbonate crystal structures: an outermost calcite prismatic layer and an inner nacreous aragonite layer. Both are embedded in an organic matrix framework. The mantle tissue is responsible for the secretion of the organic components necessary for mineralization of the shell as is the graft for the pearls. As central effectors of crystal development, previous studies have mainly focused on the purification of matrix proteins, identification of their primary structure and evaluation of their functions in shell formation. We still know however comparatively little about how the genes coding for these proteins are expressed in the mantle and how the grafting process affects gene expression through pearl formation.

2.1 Functional characterization of mineralization proteins

This first approach allowed us to identify several gene families (*Pmarg-pearlin*, *Pmarg-nacrein* and *Pmarg-aspein*) representing the first homologues of calcium carbonate organic matrix genes found in *P. margaritifera* (Montagnani et al. 2011, Joubert et al, 2010). Pattern expression analyses of the first gene, called *Pmarg-pearlin*, and immunological detection of the protein in mineralized tissues seem to show its involvement in aragonite formation (Montagnani et al. 2011). The *Pmarg-aspein* genes exhibit an expression pattern specific to mantle cells thought to be the source of the prismatic layer matrix organic molecules. Indeed, our *in situ* analysis revealed that *Pmarg-pearlin* and *Pmarg-aspein* transcripts were specifically localized in the outer epithelium of the mantle known to be bearing mineralizing cells (Figure 4). These transcripts were localized in two distinct areas of the outer epithelium, the marginal zone for *pmarg-pearlin* and the central zone for *pmarg-aspein*. *Aspein* and *pearlin* genes are known to produce proteins specific to the nacre and prismatic layers of the shell, respectively (Joubert et al, 2010). Our spatial observations confirm the functional

subdivision within the pearl oyster mantle outer epithelium, *pmarg-pearlin* transcripts being specific to aragonitic nacre-forming cells and *pmarg-aspein* transcripts being specific to calcitic prism-forming cells.

We also isolated a new nacre matrix protein in *P. margaritifera* (Marie et al. 2011), called *Pmarg*-MRNP34 (methionine-rich nacre protein). MRNP34 is a remarkably methionine-rich protein that is specifically localised in mineralizing cells and nacre matrix. MRNP34 is an original protein and hence constitutes a novel feature of $CaCO_3$-associated matrix proteins offering new insights into molecular mechanisms of biomineralization. Finally, transcriptomic expression analyses of all these genes through the grafting process show a strong heterogeneity underlying the high level of complexity of pearl formation.

2.2 Genome-wide approach of biomineralization in P. margaritifera

We constructed and pyrosequenced a *P. margaritifera* mantle cDNA library, resulting in the production of 276 738 sequences of an average size of 234 bp (Joubert et al, 2010). Our *P. margaritifera* mantle EST collection thus contains 76 790 unique sequences. BlastX searches of the 76 790 unique sequences in the non-redundant protein databases (National Center for Biotechnology Information) revealed 29 479 (38.4 %) significant matches (E-value $\leq 10^{-3}$). Gene Ontology (GO) assignment was carried out on unique sequences in order to categorize transcripts from *P. margaritifera* mantle by putative function. In our study, 10004 unique sequences (13.0 %) were successfully assigned to one or more GO terms (Joubert et al, 2010). Among these, following the functional classification with the three unrelated GO ontologies, 59.7 % are involved in biological processes, 68.5 % have molecular functions and 57.3 % are cellular components. A significant proportion of sequences in our mantle EST collection are implicated in binding and particularly in ion binding. This result is consistent with observations from a previous study performed on the calcifying mantle of bivalve. We

therefore hypothesize that this classification could be a pattern typical of tissues of a secretory nature implicated in biomineralization processes.

To obtain an integrated view of the transcriptional events of the biomineralization process in *P. margaritifera* mantle, we made BlastX searches with our EST mantle library focusing on proteins known to be involved in these mechanisms. For this purpose, we first collected all available sequences regarding biomineralization in calcifying invertebrates from the literature or from public databases. BlastX searches of the 140 bivalves and 103 gastropods proteins in our EST database revealed 121 and 56 significant matches (E-value $\leq 10^{-3}$), respectively. In analyzing these 177 sequences together with sequences from our EST library, we identified 82 *P. margaritifera* non-redundant unique sequences potentially implicated in the biomineralization process. This study considerably increases the amount of transcriptomic data available in this field, making *P. margaritifera* one of the best documented marine protostomian with regard to biomineralization.

Using the *P. margaritifera* EST mantle library, identification of shell matrix proteins was attempted by a complementary proteomic approach. The shell matrix proteins, extracted from decalcified shell powder, were digested with trypsin and the resulting peptides were analyzed by MS/MS mode mass spectrometry (Joubert et al, 2010). This proteomic approach allowed us to identify more than 30 contigs, but only 13 proteins presented homologies to previously characterized mollusk shell proteins. This list of proteins contains almost all of the shell proteins previously described before from shell matrix protein analysis of the *Pinctada* genus (Marin et al; 2008). Our proteomic analysis enabled us to retrieve *in silico* all the sequences from *P. margaritifera* involved in the biomineralization process already published on databases in our peptide library, and we were also able to find a match in our database for all proteins experimentally found from *P. margaritifera* shell in our EST library.

The integration of these two methods allowed the global composition of biomineralizing tissue and calcified structures to be examined in tandem for the first time. Our results provide direct evidence that our EST data set covers most of the diversity of the matrix protein of *P. margaritifera* shell, but also that the mantle transcripts encode proteins present in *P. margaritifera* shell, hence demonstrating their implication in shell formation. Combining transcriptomic and proteomic approaches is therefore a powerful way to identify proteins involved in biomineralization.

CONCLUSION

Pearl farming holds an essential place in French Polynesian economy. Ifremer collaborates with the "Service de la Perliculture" in Tahiti in order to deepen the knowledge concerning *P. margaritifera* physiology, the mechanisms underlying pearl formation and larval dispersion. This work is therefore closely linked to the concerns of the professionals and our aim is to propose methods and tools that will increase superior pearl quality production, sustain donor oyster genetic selection and support the sustainable development of pearl farming in French Polynesia. In the frame of this work, the POLYPERL project ("Integrated management and adaptation of pearl culture in French Polynesia in the context of global change: an environmental, economic and social approach") funded by the French National Research Agency (ANR) will start in 2012. POLYPERL project will develop an integrated and participative action research of the pearl oyster culture system, focusing on the environmental, technological, economical and societal dimensions impacting the industry. Its goal is to improve our knowledge of the pearl oyster and of pearl aquaculture, at the scale of French Polynesia, through an interdisciplinary and multi-stakeholders approach favouring the development of new knowledge on ecology, physiology, animal husbandry, genetics, physics, modelling, economics, social sciences, epidemiology, etc. The project offers the dual interests

of scientific advancement and applied innovations: on one hand it will enable knowledge of the species and its culture to be broadened; on the other it will provide innovations and decision-making tools in view of sustainable and integrated management of pearl culture in French Polynesia. This project, bringing together 10 partners (institutional, industrial and non governmental organization) partners, is scheduled to run for a period of 3 years.

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LEGEND OF FIGURES

Figure 1: **The different steps of the grafting process and pearl formation in the pearl oyster *Pinctada margaritifera*.** During the grafting process, a small piece of mantle tissue from the donor oyster (the graft), is inserted into the "pearl pocket" of the receiving oyster together with a nacre bead, the nucleus. Once inserted into the receiving oyster, the external epithelial of the graft multiplies and forms a pearl sac around the nucleus. The pearl sac then starts to deposit nacre (aragonite) layers onto the nucleus. This is the starting point for the future pearl. A rearing period of about 18 months is needed to finally produce a pearl with a sufficiently thick layer of nacre (illustration C. Montagnani).

Figure 2: **Harvest of colored pearls produced by the pearl oyster *Pinctada margaritifera*.** The quality of a pearl is determined at its harvest using several criteria: diameter, colour, lustre, shape and surface quality (present or absence of flaws). Surface defects are common in pearls. These correspond to visible modifications of the surface, either in terms of shape, substance or colour (photo Y. Gueguen).

Figure 3: Presentation of the ADEQUA research group "Improvement of pearl quality in *Pinctada margaritifera* in French Polynesia". ADEQUA is a multidisciplinary research project aiming to improve pearl quality by providing a precise description of the biological mechanisms of the pearl graft and the mechanisms underlying pearl mineralization or its failure. this original, multidisciplinary approach combines complementary expertise offered by the group of 10 participating laboratories. Using both pearl culture techniques and technological innovations (*e.g.*transcriptomics, proteomics, genetics and crystallography...) in an integrated approach, the project is the first in which all rearing stages from grafting to harvest, plus pearl structure analysis, have been taken into account simultaneously. It will provide dynamic converging data on the complete process of pearl formation (illustration C. Joubert).

Figure 4: Localization of pmarg-*pearlin* and *pmarg-aspein* gene transcripts in *P. margaritifera* mantle tissue by *in situ* hybridization. Paraffin-embedded sections of oyster tissues were hybridized with antisense or sense single stranded cDNA probes labeled with digoxigenin and revealed using alkaline phosphatase-conjugated antibodies. Positive cells are stained in dark blue, sense probes showed no hybridization (data not shown). Stained cells

enlargements are shown in A and B insets where scale bars are indicated. The expression partition limit is symbolized by a *. if: inner fold; mf: middle fold; of: outer fold; pg: periostracal groove; oe: outer epithelium; ie: inner epithelium; oec: outer epithelial cell (extracted from Joubert et al., 2010).

Figure 1

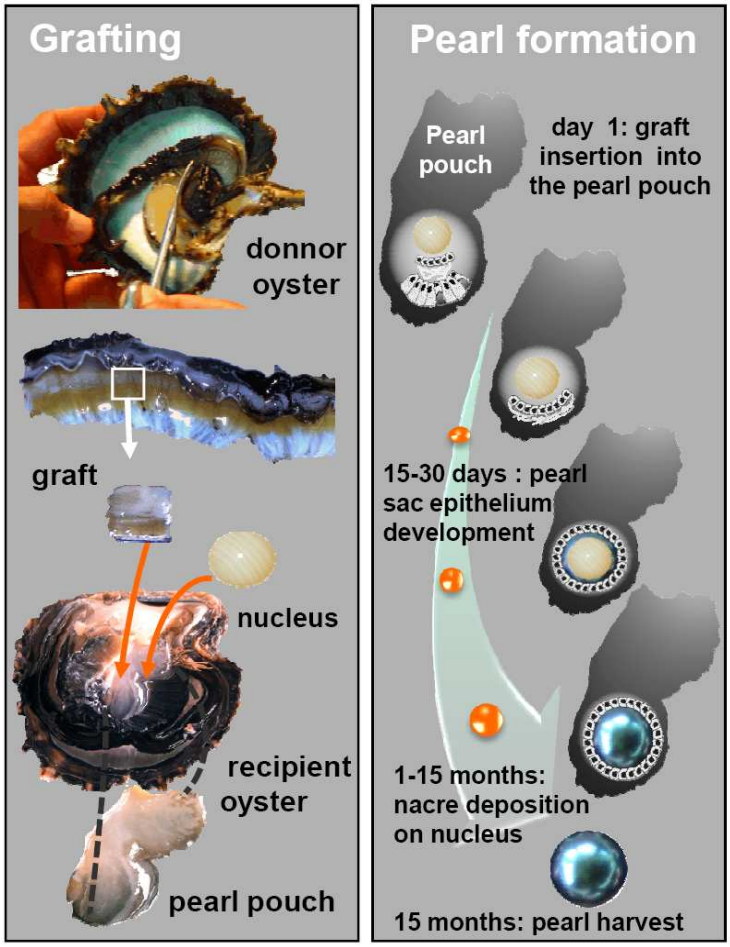

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Figure 2

Figure 2: **Harvest of colored pearls produced by the pearl oyster *Pinctada margaritifera.*** The quality of a pearl is determined at its harvest using several criteria: diameter, colour, lustre, shape and surface quality (present or absence of flaws). Surface defects are common in pearls. These correspond to visible modifications of the surface, either in terms of shape, substance or colour (photo Y. Gueguen).

Figure 3

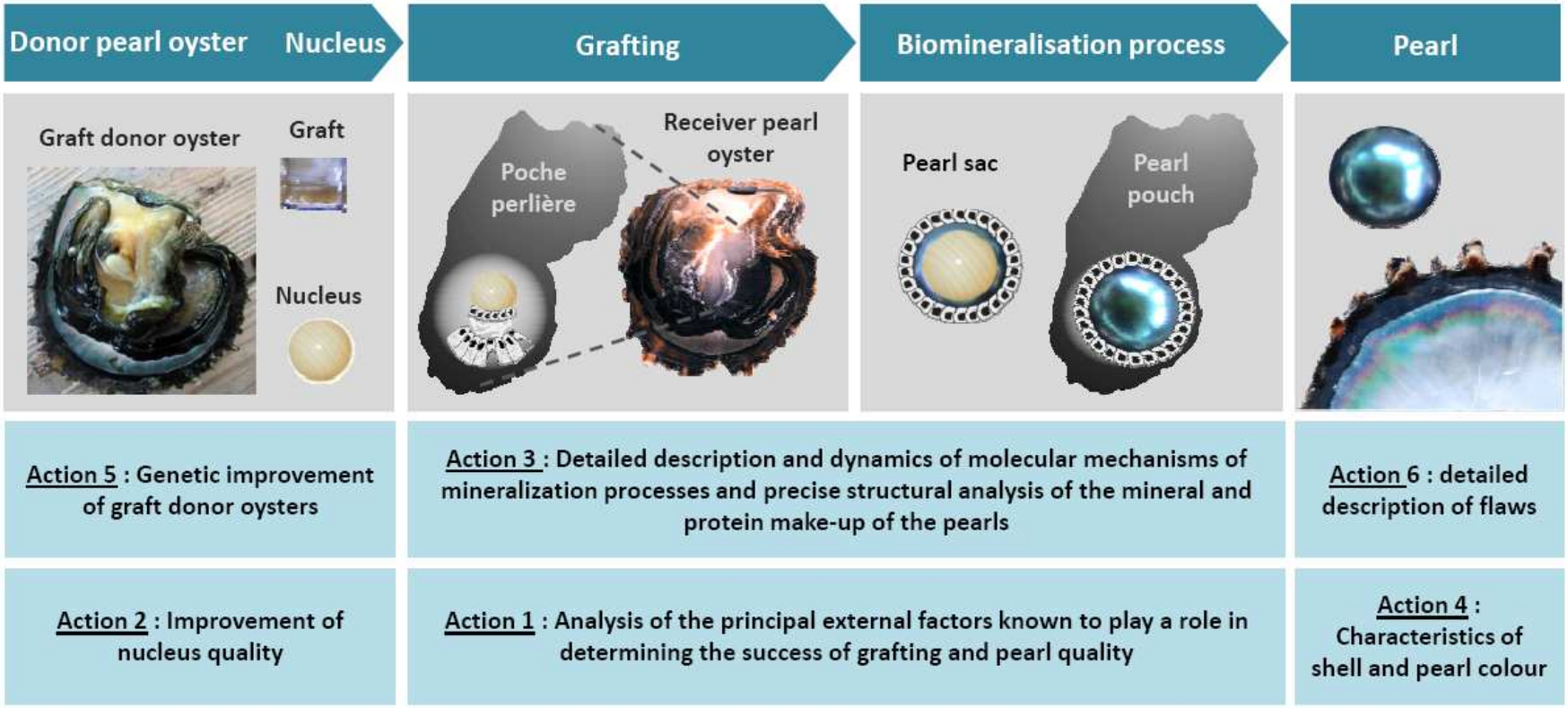

Figure 3: Presentation of the ADEQUA research group "Improvement of pearl quality in *Pinctada margaritifera* in French Polynesia". ADEQUA is a multidisciplinary research project aiming to improve pearl quality by providing a precise description of the biological mechanisms of the pearl graft and the mechanisms underlying pearl mineralization or its failure. this original, multidisciplinary approach combines complementary expertise offered by the group of 10 participating laboratories. Using both pearl culture techniques and technological innovations (*e.g.*transcriptomics, proteomics, genetics and crystallography...) in an integrated approach, the project is the first in which all rearing stages from grafting to harvest, plus pearl structure analysis, have been taken into account simultaneously. It will provide dynamic converging data on the complete process of pearl formation (illustration C. Joubert).

Figure 4

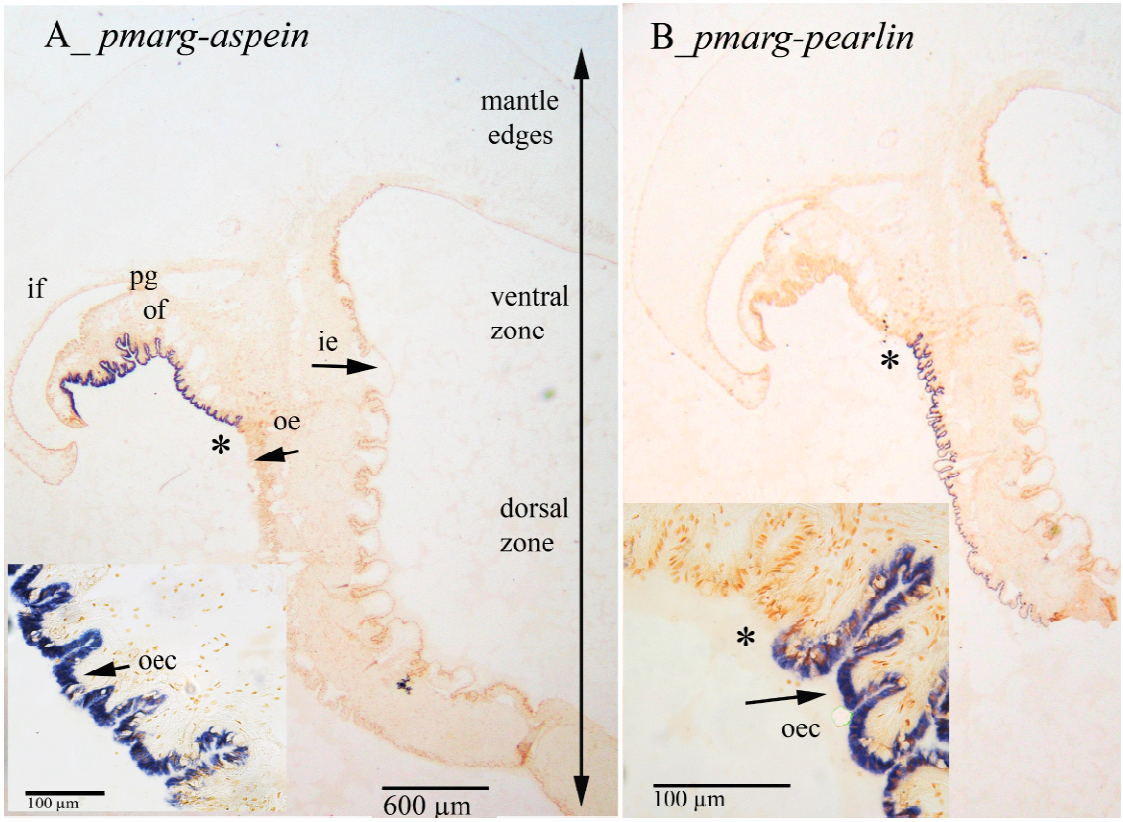

Figure 4: Localization of pmarg-*pearlin* and *pmarg-aspein* gene transcripts in *P. margaritifera* mantle tissue by *in situ* hybridization. Paraffin-embedded sections of oyster tissues were hybridized with antisense or sense single stranded cDNA probes labeled with digoxigenin and revealed using alkaline phosphatase-conjugated antibodies. Positive cells are stained in dark blue, sense probes showed no hybridization (data not shown). Stained cells enlargements are shown in A and B insets where scale bars are indicated. The expression partition limit is symbolized by a *. if: inner fold; mf: middle fold; of: outer fold; pg: periostracal groove; oe: outer epithelium; ie: inner epithelium; oec: outer epithelial cell (extracted from Joubert et al., 2010).