SOFTER SHOCK

Protective compound choices: mechanism of action at low and high temperatures

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Acknowledgments

Finding innovative protection strategies against temperature stresses involves a deep understanding of the complex physiological processes involved. We are grateful for all the discussions we had with experts in the domain, which guided us on the path to finding the best approach possible!

Many thanks to Mr. Nicolas Aveline, Dr. Maya Bar Dolev, Dr Ido Braslavsky, Dr. Guillaume Charrier, Dr. Christian Huyghe and Dr. Camille Lenoir for taking the time to share their knowledge and giving enriching advices towards Softer Shock protecting strategies.

Highly affected by extreme temperature events, grapevine cultures are currently at risk in France and beyond. This report aims at summarizing all the potential compounds that Softer Shock micro-organisms can synthesize at the plant surface to perform their protective action. We will discuss several strategies and their corresponding compounds, and try to assess the best theoretical model.

Summary

I. Potential strategies for cold protection

A) Introduction

Beyond the global increase of temperatures, climate changes are also associated with larger gaps between temperature extremes, and more frequent variations. This lead to the appearance of sudden cold episodes in autumn and spring. Those events are particularly harmful for grapevines when happening late after winter, as the plants exposed to freezing stress have often already restarted their activity at this time. In those harsh conditions, young leaves and shoots can be destructed leading to severe losses in production. The recent gel episodes of last April had a huge economic impact as nearly 60% of total French vineyards were destroyed (www.vitisphere.com). Early autumn frosts are also problematic in their own way since they make grapevines more vulnerable to fungi attacks as botrytis (Snyder RL & de Melo-Abreu JP, 2004). To limit the potential impacts of sudden freezing stresses on grapevines, the microorganisms we are engineering will secrete a specific protectant as temperatures drop.

Figure 1: Frost damages on primary shoots of grapevines (Green A., 2015)

B) Physiological effects of frost damages on plants

The Softer Shock spraying solution will have a protective action on plants, and especially grapevines, against freezing injury. This term refers to all the damages caused by temperatures below 0°C, and indirectly by the consequent formation of ice. Despite the existence of defense mechanisms and adaptive strategies, an excessive stress can leave plants physiologically altered. To elaborate our protective strategy, we first had to understand how freezing stress was responsible of damaging vines.

1. What is frost?

Cold temperatures can induce various stresses at the plant level, depending on the temperature range concerned. Consequently, several terms are used when dealing with cold weather issues. Between 0°C and 4°C, we can speak of chilling stress. Chilling stress mostly affects grapevines on a long-term fashion, gradually slowing down carbon metabolism, but rarely causes severe damages that can result in production loss (Sawicki M et al, 2012). That is why we decided to focus on freezing stress, induced by negative temperatures, that can result in harsh negative consequences on grapevines. The term "frost" is commonly used when dealing with freezing injury in plants. It corresponds to a meteorological event during which temperatures fall below 0°C, and can subsequently induce the formation of ice crystals. Those may result from the freezing of dew, formed on exposed surfaces by air water vapor condensation, or form inside the plants causing severe damages.

Two frost types can be distinguished depending on the source of cold: radiation frost corresponds to a sudden drop in surface temperatures during cloudless nights while daytime temperatures are greater than 0°C, whereas advection frost results from a large-scale invasion of freezing air (Snyder RL & de Melo-Abreu JP, 2004). The first type mainly explains spring-time frost events, towards which we will focus our protection method. Apart from the global metabolism slowdown induced by complex physiological perturbations during freezing nights (Sawicki M et al, 2012), severe damages can also result from rapid ice crystal formation. This is the principal cause on which we decided to focus for our protection strategy.

2. Nucleation mechanism

What is nucleation ?

The main cause of injury during spring-time frosts is not the cold weather but rather the subsequent ice crystal formation outside and inside of the plant, called nucleation. As temperatures drop below 0°C, water molecules can either form a stable ice nucleus spontaneously (homogeneous nucleation), or by accumulating around another compound called a nucleator (heterogeneous nucleation). These compounds can be of several types, either produced by the plant itself (polysaccharides and other biological molecules), of bacterial origin (ice nucleation-active bacteria like the pathogenic *Pseudomonas Syringae*), but also organic or inorganic debris (Pearce R, 2001).

Where do ice crystals form?

Inside the plant organs and tissues, spontaneous nucleation of pure water remains a rare event at modest sub-zero temperatures due to its supercooled liquid state. However, the presence of nucleators inside but more largely on the leave surface can induce heterogeneous ice crystal formation in the presence of water. Studies have shown that the water freezing outside of the leave through heterogeneous nucleation could promote the ice growth within the whole leave (Figure 2).

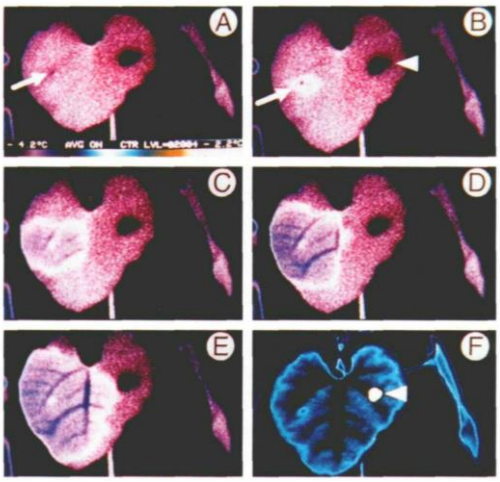


Figure 2: Ice nucleation and ice propagation in a bean (Wisniewski M et al, 1997).

The white arrow on the left indicates a 2µL droplet of a suspension of *Pseudomonas Syringae*, a bacterium producing ice nucleation active proteins. The expansion of ice observed by IR video-imaging clearly shows that this external droplet becomes the center from which the nucleation phenomenon happens inside of the tissues, and consequently triggers the freezing of the whole leave.

3. Ice propagation and freezing damages

Frost damages mostly result from ice crystal formation inside of the plant tissue. In this location, ice crystals can be formed either intracellularly or extracellularly, and then induce diverse types of negative consequences at the plant scale. Most damages are caused by extracellular ice crystal formation, more frequent, and deleterious as they induce dehydration but also mechanical damage to membranes and other tissues (Bar Dolev M et al, 2016).

First, secondary cellular dehydration is a leading cause of freezing-induced plant injury. The growth of extracellular ice crystals impels the water contained inside the cells to escape and reach the apoplast to bind the crystals under formation. This phenomenon, due to the difference of water potential between solid and liquid water, brings cells to collapse under dehydration stress. Membranes are the first tissues affected by this process, which makes them

vulnerable to structural damage and leakage (Snyder RL & de Melo-Abreu JP, 2004). Guillaume Charrier, researcher specialized in vegetal ecophysiology, gave us his own insights about this specific topic: in a way, dehydration is beneficial. It is a natural reaction of the plant to increase its cold tolerance by concentrating intracellular solutes. But this mechanism is advantageous until it reaches a critical point, from which it becomes detrimental and specifically for young leaves.

More global structural changes can also directly result from the formation of large ice masses inside and outside of the cells. The growth of these large ice crystals, called recrystallization, is particularly induced by prolonged exposures to freezing temperatures and follows the Ostwald ripening process. They can structurally affect the plant tissues and organ structures, causing a separation between cell layers and creating cavities (Pearce RS, 2001).

C) How spring frosts affect grapevines

Numerous factors influence the nature of freezing-induced damages and the parts attained: for short cold periods like spring-time frosts, the speed of cooling seems to be a major factor determining the critical damage temperature. Damages also depend on the growth stage of the plant, the physiological state of cells before the temperature drops and the number and repartition of nucleators (Snyder RL & de Melo-Abreu JP, 2004). It is then difficult to predict exactly the extent of damages caused by brief and sudden gel episodes as they depend upon the event localization and timing, but also on the variety of the grapevine attained.

During the intercrop winter season, their dormant state allows a protection of the most important organs for long cold periods. In this state, their metabolism favors the production of protective molecular compounds, comprising soluble sugars obtained by starch hydrolysis (Chen L.-J. et al, 2014). Buds are desiccated, since physically separated from the plant's vascular system. Thus, they are protected until any reproductive activity takes place (Martinson T. & Goffinet M., 2012). This period is critical for grapevines as it brings a certain amount and duration of chilling, required for an optimal subsequent growing (Moyer M. et al, 2011). Despite the possibility of winter freezing injury, our protective solution specifically targets sudden and transient frost episodes outside the winter period.

Grapevines are particularly subject to spring-time freezing damages as their protective mechanisms are not sufficient facing sudden cold episodes at this period. As temperatures rise, major changes occur at the physiological level, beginning with a redistribution of carbohydrates and water reserves into tissues. Buds are vascularized again and rehydrated. Their break, first sign of the restarting activity of the plant, is followed by the appearance of tiny shoots which

will carry the future flowers transforming into fruits (Martinson T & Goffinet M, 2012).

Hot early springs encourage an early break of buds, therefore exposed to injuring frosts (Robinson J. & Harding J., 2015). If the first expanding bud called primary bud is damaged by ice formation, the secondary and tertiary buds subsequently activated are generally less fruitful than the former one (Figure 3). In 2012, 80% of the primary bud shoots were killed due to spring frosts, whereas secondary buds were not significantly altered. (Frioni T et al, 2017). Spring-time frost can also alter the vascular tissue inside of the canes and trunks, comprising xylem and phloem. The freezing of conducted water can further trigger trunk splitting (Figure 4) (Moyer M. et al, 2011). Ice can also form in inflorescences at the flowering stage, inducing a failure of the fruit to set. This can be a major cause of poor summer harvests. Beyond yield levels, spring frost effects can also be reflected in the fruit characteristics. In 2013, the absence of spring freeze events led to a 61% increase in yield and a better fruit quality (Frioni T et al, 2017).

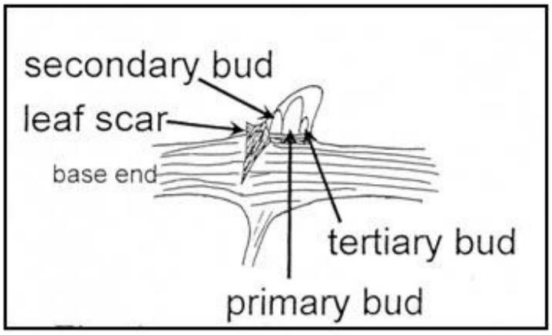
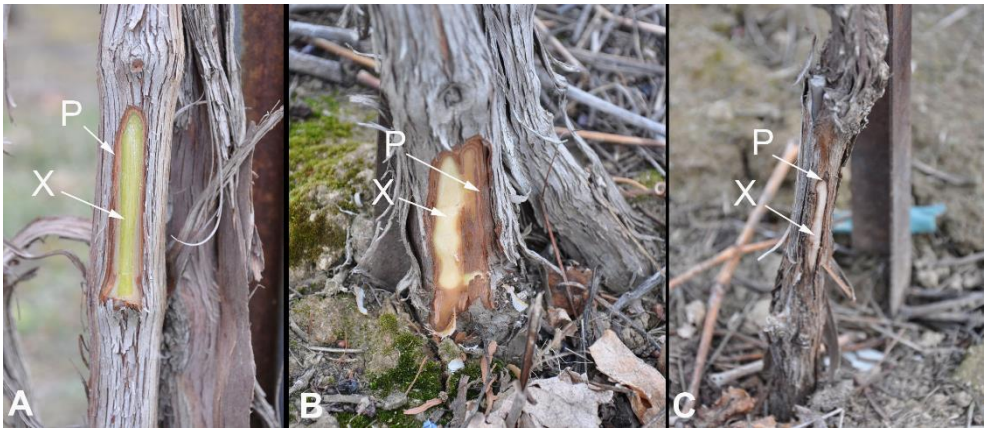


Figure 3: Schematic representation of a visible grapevine bud consisting of three internal buds (Moyer M. et al, 2011).



Dead phloem but healthy, green xylem

Dead phloem and damaged xylem

Both the phloem and xylem are dead

Figure 4: Trunk cold injury (P = phloem; X = xylem) (Moyer M. et al, 2011).

Autumn frosts can also cause damages to grapevines to a lesser extent. This period is characterized by the fruit ripening and the restoration of

carbohydrate reserves before winter. The eventual defoliation following frost events can be problematic if those events are not completed yet (Robinson J. & Harding J., 2015).

D. Our strategy: using ice-binding proteins as frost protectants

Ice-Binding proteins (IBPs) gather a protein family displaying very particular properties: the capacity to bind and interact with ice crystals. While antifreeze proteins (AFPs) encircle crystals to inhibit their growth, Ice-Nucleation Proteins (INPs) promote their formation (Bar Dolev M, 2016). We will see why those strategies, looking quite contradictory at first sight, both constitute promising strategies to help preserving plants from freezing damages in their own way.

1. Producing antifreeze proteins to inhibit ice crystal growth

a. Strategy relevance

Our ambition is to provide a protection for grapevines against frost damages. This will be achieved by the capacity of our engineered micro-organism to secrete a specific protectant when exposed to sudden plunges in temperatures. The first idea that came to our minds was to look for the natural protection mechanisms adopted by plants to resist frost. However, most of them are related to the protection of cell membranes as they represent the first elements exposed to frost injuries. Therefore, natural plant strategy mainly consists in the endogenous production of protective enzymes and other molecular compounds thanks to the action of complex networks of cold resistance related genes (Chen L.-J. et al, 2014). Yet, for safety and regulatory reasons, we are keen that our micro-organism remains at the surface of the plant only. It is then unlikely that the proteins secreted will penetrate the plant, and even less intracellularly. This way, we want to ensure that the grapevine varieties are respected and that no external compounds penetrate inside of the tissues.

We had to find a strategy to act against frost damages at the grapevine surface. We looked for an external process known to significantly trigger injuries at the whole plant scale, and thought about ice nucleation. Given the fact that the presence of ice crystals inside tissues partly results from the formation and growth of external ones, we thought that lowering the freezing temperature of the water present on the plant and inhibiting the growth of eventual already formed

crystals were both promising strategies. These two actions are allowed by a specific protein family known as "Antifreeze Proteins" (AFPs), participating to natural protection mechanisms in wintering plants (Gupta R & Deswal R, 2014). Another argument for not expressing antifreeze proteins endogenously is that extracellular ice nucleation, when controlled, can be of significant importance in plants. This process notably allows freeze-tolerant species to precisely control the location of ice crystal growth (Duman JG, 2015). Expressing AFPs internally could disturb the cold-tolerance mechanism in plants, especially when applied during winter, and provoke an opposite effect.

b. How do antifreeze proteins bind to ice?

It has been suggested that a specific site on the protein, called IBS (Ice Binding Site), bind water molecule with clathrates. Clathrates are molecular structures which can trap other molecules, for instance water. Then, AFPs organize these water molecules at the surface of ice crystal before freezing together. This model explains how AFPs can bind to ice through the thin water layer at the crystal surface.

c. How do antifreeze proteins inhibit crystal growth?

The Gibbs–Thomson Effect shows a correlation between the curvature of a surface and the equilibrium phase transition temperature. When IBPs bind the surface of a growing ice front, they confine water molecules to join the ice only between adsorbed IBP molecules. The ice continues to grow for several nanometers, starting from a flat surface with infinite radius of curvature that turns into a round surface as the ice grows, eventually reaching a critical radius at which growth becomes energetically unfavorable (Figure 5).

Two main actions result from the action of AFPs: Thermal Hysteresis activity (TH) and Recrystallisation Inhibition (RI). The first one designates the displacement of water freezing temperature below the normal equilibrium, until a certain point at which ice crystals start to form again. The basic mechanisms underlying this action remain uncertain, however it is suggested that the reversible adsorption of antifreeze proteins onto ice crystal surface disadvantages ice crystal expansion. When subfreezing temperatures are reached, this interaction would become irreversible through the protein "freezing" to the surface of the newly formed crystal plane, consequently lowering the freezing point (Kristiansen E. & Zachariassen KE, 2005). Inhibiting recrystallisation, as explained before, is important to reduce structural damages caused by large crystal growth propagating inside the cytoplasm (Duman JG,

2015). The balance between these two actions at the organism level depends on its own physiological properties and living needs.

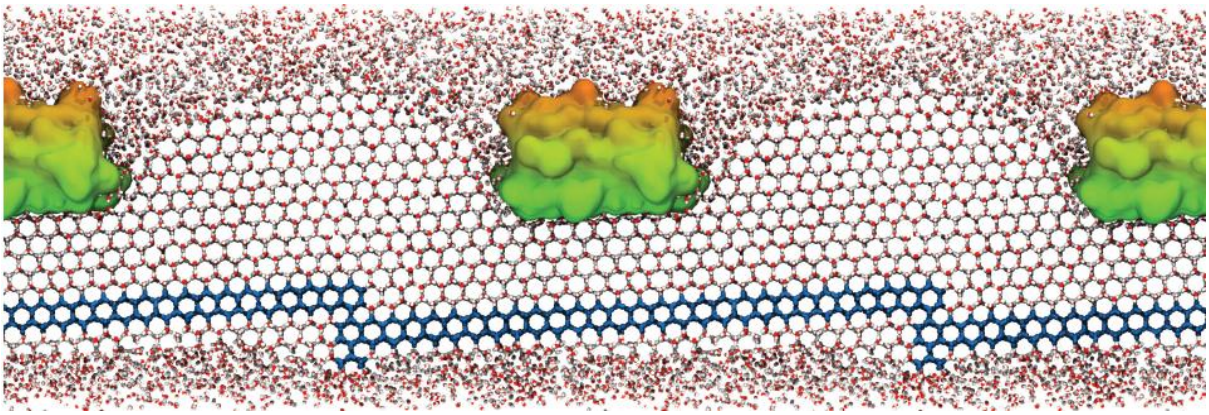


Figure 5: Simulation of spruce budworm antifreeze protein (sbwAFP) on an ice surface that illustrates the Gibbs–Thomson effect. *Three molecules of the sbwAFP are shown bound to the ice surface. The ice (hexagonal pattern) bulges out between the adsorbed AFPs as the temperature falls below the equilibrium freezing/melting temperature (Tm).*

d. Choice of the suitable antifreeze protein for protecting vineyards

Plants, animals, fungi, bacteria… There is no lack of examples of species naturally expressing AFPs. Based on the organism physiological properties and living needs, AFPs do not all act on the same manner on ice crystals and are not all equally efficient at reducing the freezing process. We tried to understand those distinct characteristics to assess which protein would best fit our protection objective on grapevines.

AFP-expressing species can be divided into two main groups depending on their cold tolerance strategy: freeze-avoiding and freeze-tolerating organisms. The first ones get their name from their absolute need to prevent freezing. Consequently, they preferentially express AFPs displaying an important TH activity (Duman JG, 2015). It is notably the case of many insects and marine fishes which need to carefully control the lethal freezing of their body fluids (Bale JS, 2002). Once in the supercooled state, fluids remain liquid even when exposed to subzero temperatures (Wharton DA, 2012).

On the other hand, freeze-tolerant species favor the expression of AFPs with a high potential of RI and a lower TH activity. This can be explained by their frequent inability to physically run away from cold temperatures, as it is the case for plants, leading them to develop cold acclimation. Even some insects possess this impressive ability to survive freezing inside their bodies (Wharton DA, 2012). These species, instead of avoiding nucleation, restrict ice crystal formation at

precise extracellular locations using ice-nucleation proteins (INPs). A controlled balance between AFPs and INPs therefore allows them to minimize intracellular lethal freezing, and AFPs tend to lower the potential structural damages caused by large crystals on cellular membranes rather than lowering freezing temperature (Thomashow MF, 1998).

Based on all the above findings about grapevine frost damages and AFP properties, it is now the time to think of which protein would act as the most efficient protectant for grapevines. We made a table summarizing the main AFPs that could be of interest for us, along with their respective properties (Table 1). Based on all the data collected, we identified the most important criteria to consider on the road to the final choice.

Name	Size	Origin	Gene(s)	Thermal hysteresis activity	Biosynthesis	Post-translational modifications	Comments	Maximum activity
RiAFP	12.8 kDa	Rhagium inquisitor (Siberian cerambycid beetle)	afp-h4	High	Already done in E.Coli & previous iGEM competitions	Just one disulfide bond	Only a single disulfide bridge (compared to other beetle AFPs), implying an absence of tight folding	7°C
MpAFP	1,5 MDa	Marinomonas primoryensis, antarctic Gram-bacterium	Unknown (aa sequence known)	"Powerful" (lower freezing point by 2 °C). Hyperactive TH activity	Repeat tandem (active part) done in E.Coli	No PTM	Its activity is dependant on calcium and it is a membrane bound protein	0,8°C (TH activity)
ZeAFP	7-14 kDa	Zoarces elongatus	Unknown (aa sequence known)	Moderate	Done in E.Coli	No PTM	13 isoforms	1°C
TmAFP	9 kDa	Tenebrio molitor	Unknown (aa sequence known)	High (1µM 12h = 10µM 10min)	Done in E.Coli (bad folding !)	8 disulfide bonds	Threonin array are supposed responsible to ice binding. Crystal structure is fully know. Several isoforms.	5,5°C

Table 1: Overview of all antifreeze protein candidates for the Softer Shock project (Hakim A. et al, 2012; Gilbert J.A. et al, 2005; Marshall B. et al, 2002)

Which type of action are we looking for?

Our protective action will be performed at the plant surface only. Consequently, we will not be able to prevent intracellular ice formation nor inhibiting recrystallisation process directly, as freeze-tolerating species do by endogenously expressing AFPs. It will probably be more relevant to produce AFPs with high TH activity to bind ice crystals under formation and lower water freezing point. This way, we will operate a "micro freeze-avoiding strategy" at the leaf surface scale: by restricting ice growth, we limit the risk of ice penetration inside the plant. The challenge remains in the fact that AFPs produced by freeze-avoiding species do not act at extremely low temperatures, to which those

species are normally not submitted to. It will therefore be a sort of "temperature gamble" for us. However, we mostly target sudden cold spring episodes, during which temperatures generally do not fall below -5°C. Moreover, some exceptions of hyperactive AFPs remain functional even at highly low temperatures. We can for example turn to AFPs from the *Rhagium inquisitor* beetle, which body fluids have been reported to supercool to below -25°C (Kristiansen E et al, 2011). These hyperactive proteins of 13kDa, called RiAFPs, were already used in several past iGEM projects and are proven to significantly enhance TH. This could be a leading choice for us.

At which concentration should AFPs be produced on the plant?

Depending on the micro-organism choice, this latter will potentially act as a nucleator on the leaf which would be inconsistent with our antifreeze strategy. We will then have to carefully modulate the individual expression rate for the chosen host to reach an optimal proportion allowing optimal antifreeze activity without enhancing ice crystal formation at the plant surface. The results obtained by the 2015 Canadian Queens iGEM team on AFPs allowed us to anticipate the potential comportment of different AFP concentrations with ice crystals. The protein concentration has to be sufficient to allow a satisfying antifreeze activity. At the opposite, an overexpression of proteins could lead to tissue damage due to a needle-like ice structuring.

What would be the perfect protein size?

The protein size must be the smallest possible, otherwise the energy required might be too important and the expression rate will consequently be lowered.

Why are post-translational modifications considered as an important criterion?

It is known that prokaryotes perform post-translational modifications, but they are of different nature compared to those performed by eukaryotes. Our antifreeze proteins of choice all come from animals, and their functionality can therefore require some post-translational modifications that are not applicable to our chassis. In the case of RiAFP, only one disulfide bond is needed to make the protein functional. Prokaryotes commonly operate this type of modification (Hatahet F. et al, 2014), therefore this protein should be suitable for our application regardless of our chassis choice.

What should be the maximal temperature activity?

RiAFPs have a relatively high maximal temperature of activity of 7°C. This property is in accordance with our preventing mechanism, under which the

protectant starts to be synthesized at 15°C. This way, at the time ice crystals start to form, the protein will already be functional and ready to perform its inhibitory activity. This allows a quick response to brutal freezing events.

e. Enhancing the anti-freeze effect through protein engineering and cocktail mix

Antifreeze protein activity can be enhanced by different mechanisms that are linked directly to the protein's structure and molecular actions (Figure 6) (Bar Dolev M, 2016). Here will be reviewed all the possible ways AFP activity could be made more efficient and how we could optimize Softer Shock protective action. These methods could be crucial for us for many reasons, because they could permit to lower the number of microorganisms we use per application, hence lower the price of our product and increase its biosafety.

The first way to influence AFP activity, especially the TH activity, is through protein engineering. "Protein engineering is the design of new enzymes or proteins with new or desirable functions. It is based on the use of recombinant DNA technology to change amino acid sequences" (Turanli-Yildiz et al., 2012). There are several ways to engineer AFPs. One can increase their weight and size by simply adding a tag or by close interaction (usually with another protein like maltose binding protein or thoredoxin), which will increase the AFP size and modify its shape (DeLuca et al., 1998). Both shape and size of AFPs have been proven to influence the TH activity so this track is interesting (Bar Dolev M, 2016).

Fusion proteins of two AFPs or more have also been proven to function properly (Wisniewski et al., 2011). We must be careful however that the association does not occur around the Ice-binding site of the engineered protein.

AFPs proteins are usually made of many repeats of the same domain, and it has been shown as well that adding supplementary domains to existing AFPs increase their activity. For example, an AFP composed of 8 repeats of 11 amino acids could be enhanced by adding the same 11 amino acids 4 more times to the N-terminus. This is explained by the fact that each repeat technically represents an ice binding domain, and adding repeats hence increases the total surface of ice crystal the AFP can interact with (Marshall et al., 2004). Nevertheless, synthesizing a larger AFP will probably cost more time and energy to the host organism, especially at cold-temperatures so we might explore other strategies.

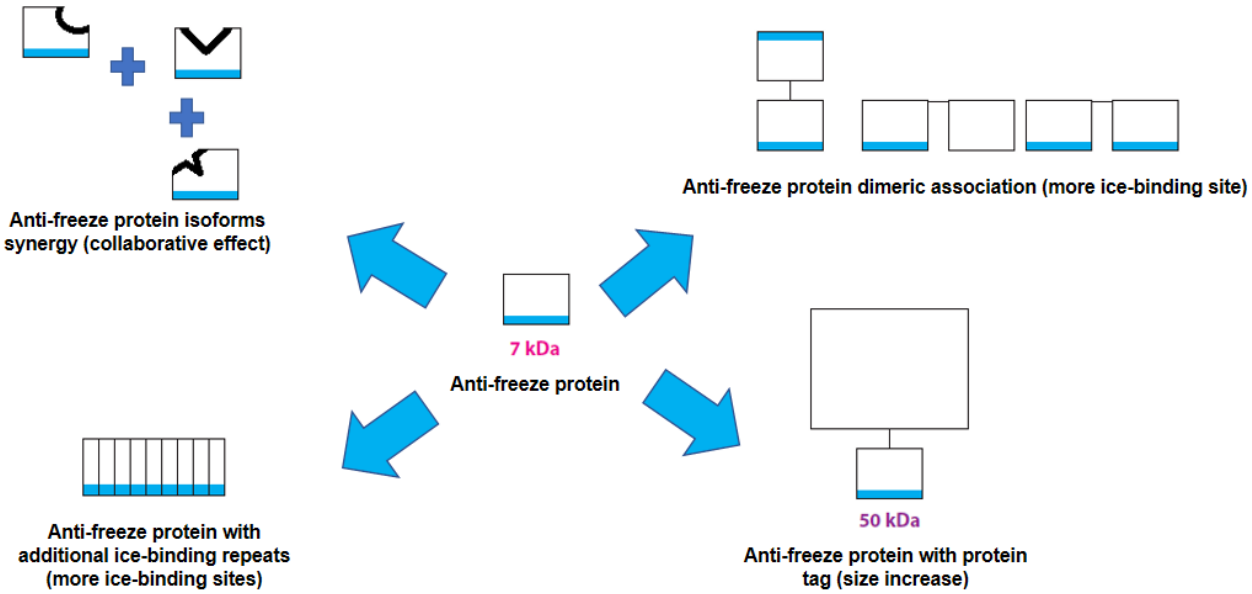


Figure 6: Different methods to increase the efficiency of Antifreeze Proteins, adapted from (Bar Dolev M, 2016)

Among other possibilities is included the promising synergic activity of different AFP isoforms. Isoforms are different versions of a single gene transcription pattern obtained through alternative splicing (Athena et al., 1987). Hence, two AFP isoforms will be expressed from the same gene but could have a different structure, size, and hence activity, and could demonstrate a collaborative effect. Nishimiya et al have shown for example that AFPs isoforms of the ZeAFP (*Zoarces elongatus)* are as numerous as 13, and that some of these isoforms displayed stronger ice-binding activity when in solution with other isoforms than alone (Nishimiya et al., 2005). This process of synergy, which means that the collaboration of two effects gives a better result than just adding them separately, is very interesting as well as the perspective of a multi-AFP producing organism for Softer Shock. We asked Dr. Maya Bar Dolev, expert in ice-binding protein function and structure, if this approach was relevant. This was her response:

"It is a good idea to combine two different AFPs that bind to a different set of planes, for instance, one from an insect that binds the basal plane and one from a fish that binds less of basal plane, or from a plant that may have different strategy of activity since it has high ice recrystallisation inhibition activity and low TH". *Bar Dolev M., PhD at Institute of Biochemistry Food Sciences and Nutrition*

The idea of expressing several complementary AFPs types represents an innovative approach to freeze protection with an exciting potential. We should perform some tests at a small scale first, and then in the field, to screen the

efficiency of different AFPs taken together and determine the best combination. This could be an interesting perspective for eventual Softer Shock successors!

2. Producing ice-nucleation proteins to enhance ice formation

a. Strategy relevance

Water sprinkling accounts for one of the most common existing techniques of crop protection from frost (Figure 7). This process consists in continuously spraying water to cover vines during frost periods. As water turns to ice, this solidification exothermic reaction releases latent heat. This heat release, in addition to the insulating property of the ice layer formed, maintains the plant tissue temperature at about 0°C.

Figure 7: Frost protection by sprinkling in apple orchard in England (Stanhill G., 1992)

The major limitations of this technique lie in the large quantity of water required, particularly when the frost lasts in time, as well as the high installation costs. Where water is limited, sprinkling is not a suitable technique for frost control (Stanhill G., 1992).

Ice-Nucleation Proteins (INPs) have the ability to promote ice formation. By using the air humidity as the water source for ice formation, or combining their action with sprinkling technique for lowering the amount of water needed, the use of INPs could bring significant economic and practical benefits for farmers.

b. How do INPs promote crystal growth?

Ice-Nucleating Proteins are large proteins that serve freezing-tolerant species to restrict ice crystal formation to precise locations and limit intracellular ice formation. The hypothesis underlying the ice binding mechanism is relatively similar between AFPs and INPs, and is named the Anchored Clathrate Water Hypothesis (ACW). The presence of a nucleating site in INPs seems to induce the ordering of water molecules into ice-like shapes. The newly formed structures can act as new centers from which ice can grow: heterogeneous nucleation is favored (Bar Dolev M., 2016).

c. The choice of the suitable INP for our project

INPs have a big disadvantage in comparison of AFPs. They are big proteins, so more complicated to produce and fold. The INP from *Pseudomonas Syringae* inaZ gene is well known, has proved its efficiency and is already commercialized by the snowmaking industry. Consequently, this protein could be a suitable candidate. The length of the protein is 1200 residues (http://www.uniprot.org/uniprot/P06620).

3. Efficiency tests in real conditions to determine the best strategy

Because of time limitations, we could not carry out the proof of concept of our project, but we imagined a possible lab strategy. Before modifying our micro-organisms to make them produce AFPs or INPs at low temperatures, it is necessary to determine the best strategy to protect grapevines. Experiments will be required to compare the effects of frost on grapevine leaves and buds with or without each protein type (Figure 8).

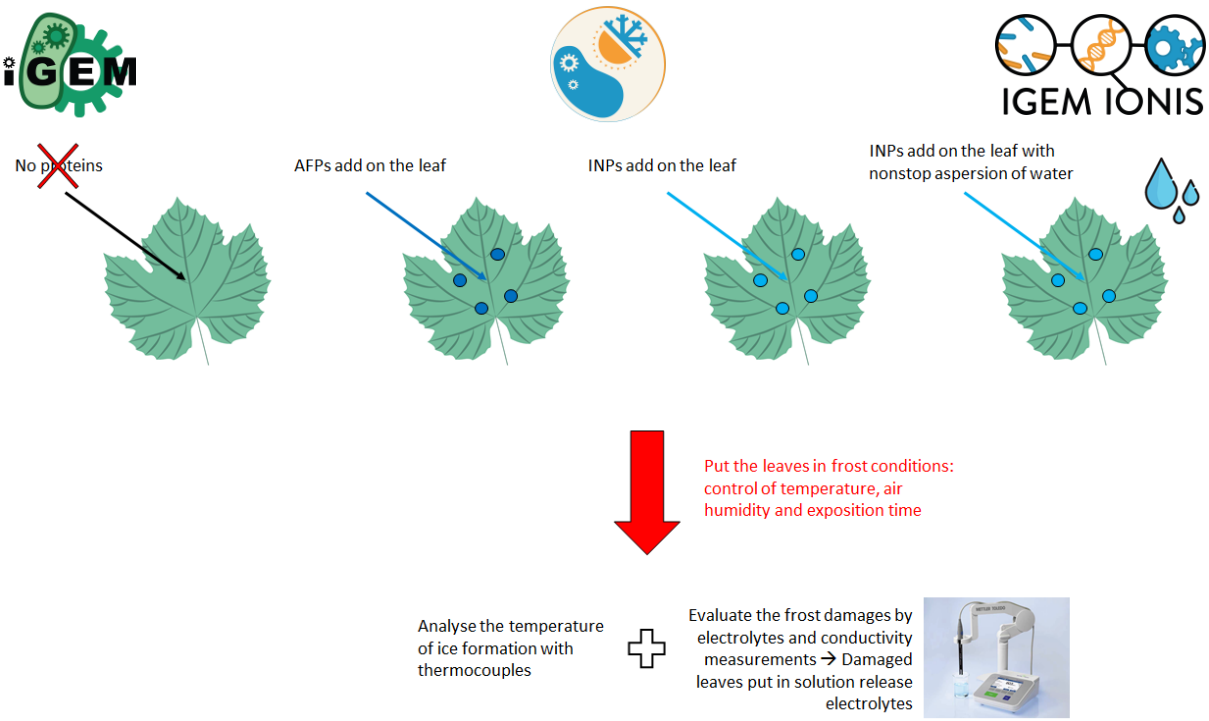


Figure 8: Laboratory strategy to evaluate the effects of AFPs and INPs on frost protection

If one protein seems to be more effective, we can then perform our cloning strategy to make our host express and secrete it (for AFPs only because INPs are transmembrane proteins). Then we could carry out the same experiments on the plant again, with the transformed micro-organism this time.

II. Potential strategies for heat protection

A. Introduction

In France and other countries around the world, summer heatwaves are common events, particularly because of the global warming. If irrigation is impossible or very limited, keeping water within plant and avoiding transpiration are essential for plant survival.

B. Physiological effects of heat damages on plants

1. Water stress

Plants are subjected to water stress due to a rapid drop in humidity or increase in temperatures. The cause can be a warm and dry air mass which moves into their environment. The result can be an increase in the vapor pressure gradient between the leaf and the surrounding air. Consequently, the transpiration rate increases (Hopkins, 2009). To keep water inside their tissues, plants close their stomata to match transpiration water loss through the leaf surfaces with the rate at which water can be resupplied by the roots (Hopkins, 2009).

But closure of the stomata cuts off access of the chloroplasts to the atmospheric supply of carbon dioxide and consequently stops the photosynthesis process. Water stress directly affects the structural integrity of the photosynthetic machinery as well. Damage resulting from water stress is related to the detrimental effects of desiccation on protoplasm. Removal of water, for example, leads to an increase in solute concentration as the protoplast volume diminishes (Hopkins, 2009).

2. Photoinhibition

In temperate countries, elevated temperatures are usually associated with an important amount of sunshine. At extreme hot temperatures, some plants are light saturated or photoinhibited by solar radiations (Figure 9) (Hopkins, 2009).

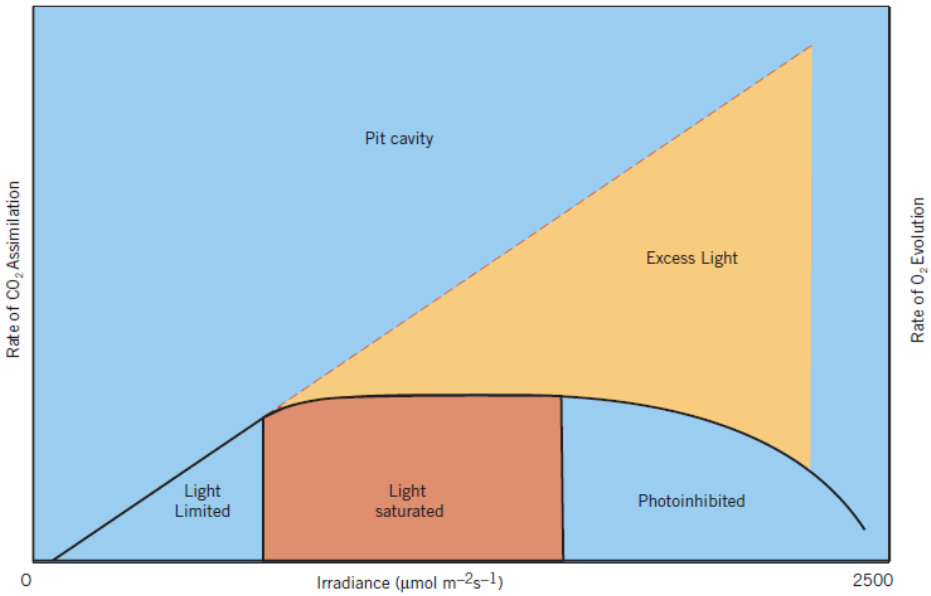


Figure 9: Schematic of the photosynthesis response to increasing irradiance (Hopkins, 2009).

3. Heat stress

All plants varieties do not have the same sensitivity to extreme temperatures, but all plants can be affected by high temperature stress starting from a certain temperature threshold. High temperature stress can cause irreversible protein denaturation and suppression of most protein synthesis including Photosystem 2 (PSII), essential in the photosynthesis process. High-temperature stress also induces the synthesis of the heat shock proteins (HSPs) family (Hopkins G, 2009). These chaperone proteins are able to repair 3D protein conformation.

4. Solar injury

An increase in the plant UV-B exposure results from the ozone layer depletion, due to human activities associated with natural thermic cycles. An overexposure to UV-B can severely alters plant DNA, proteins, lipids and membranes. The UV-A region of the spectrum is not dependent on the ozone layer reduction. Yet, both types can cause damages. Plants need sunlight to perform photosynthesis and develop, so they cannot avoid UV radiation and are therefore at risk (Stapleton A.E., 1992).

Grapevines are subject to sunburns, and particularly in the plant sections where leaves are absent. Removal of leaves has been used as a strategy by farmers to prevent the proliferation of parasites. Indeed, the resulting environment is unfavorable to fungi development (interview with Nicolas

Aveline). The consequence is the exposition of fruits to high temperatures and ultraviolet radiations, increasing the quantity of harmful free radicals inside of the cells. In time, cells are destructed giving shriveled fruits (Krasnow M.N. et al, 2010).

C. Our strategy: a reflective layer to modify the plant albedo

1. Light reflection: a strategy used by *Encellia farinosa*

The albedo is the property of surfaces to reflect or absorb light. The more the albedo increases, the more the surface reflects light. On the contrary, the more the albedo decreases the more the surface absorbs light. The absorption of light gives off heat and increases the temperature locally.

Leaf surface albedo is an important parameter for plant physiology since it directly impacts transpiration and internal temperature of the plant. The higher the plant albedo, the lowest the plant temperature. If the temperature increases at the leaf surface, the transpiration rate increases as well to cool down the plant. But the use of water for increasing transpiration and cool down the plant is not an efficient water use.

Encelia farinosa is a desertic plant withstanding dryness by exploiting seasonal leaf polymorphism. During dry seasons, the plant develops leaves covered with trichomes which give to the plant a white appearance (Figure 10). The white leaves reflect about 70% of solar radiations whereas the early green leaves reflect only 15%. “A reduced heat load serves to reduce transpiration and thus improves water-use efficiency. It will also help to maintain leaf temperature in the optimum range for photosynthesis.” (Hopkins, 2009). The natural capacity of certain plants to modify their surface albedo in response to heat brought us to the conclusion that this strategy appeared a promising research axis.

Figure 10: *Encelia farinose* expressing surface trichomes

Our strategy is to base on this property to make our microorganism produce a reflective film at the plant surface. The challenge of this idea is that we must find the right balance between reducing the UV light reaching the plant and the risk of subsequently compromising its photosynthetic activity. The ideal protective layer would selectively reflect damaging UV radiations but not Photosynthetically Active Radiations (P.A.R.) (Figure 11).

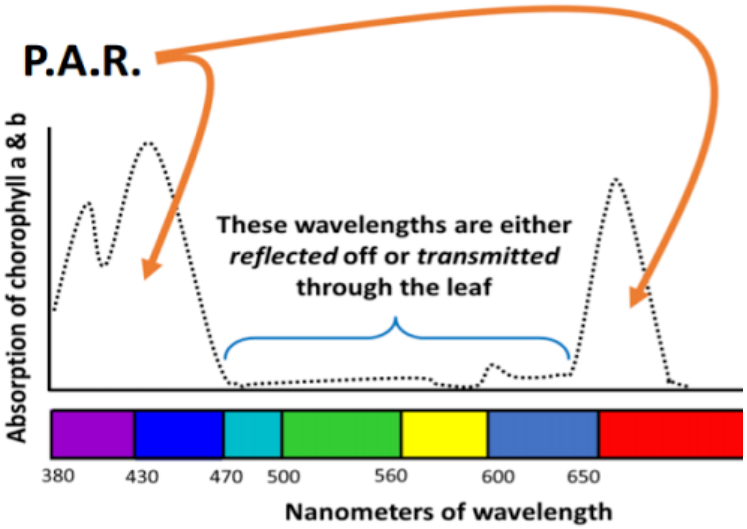


Figure 11: Photosynthetically active radiation wavelengths based on chlorophyll a & b absorption (Delage E., 2017)

UV radiations range from 280 to 400nm. The optimal protective film would therefore reflect radiations comprised between 280nm and 380nm not to alter the chlorophyll absorption capacities and subsequent plant activity.

Facing the difficulty of finding these very specific surface properties, we asked the question of the possible photosynthetic alteration to Christian Huyghe, deputy scientific director at the INRA. That was his response: “In your system,

albedo induction will be activated in response to high temperatures only (above 37°C). At this temperature, the plant enters a "survival" state and does not grow anymore, or very slowly. Indeed, a mechanism induces stomatal closure and the plant does not capture much light. So, in any case, photosynthesis is not really possible in those conditions. However, you have to make sure that the white coloration is reversible as temperatures come back to lower values." We thus decided that only finding a white compound would be simpler, as soon as its presence on the leaf surface remains transient and does not alter the plant activity on the long term.

2. Microbial calcium carbonate formation

Reflective particles such as kaolinite or calcium carbonate powder have shown their effectiveness in diminishing the thermal charge by reflecting solar radiations (Alvareza HL., 2015). Calcium carbonate crystals can be produced by microorganisms. Indeed, microbial mineralization is a natural process which can be carried out by urease producing bacteria (Figure 13) (Anbu P., 2016). Urease enzymes allow a local pH change (increase the alkalinity) and form carbonate ions (Phillips AJ., 2014). In the presence of sufficient calcium ion activity, saturation conditions become favorable for CaCO3 precipitation (Figure 12).

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} NH_2COOH + NH_3$$

$$NH_2COOH + H_2O \xrightarrow{\text{Spontaneous}} NH_3 + H_2CO_3$$

$$2NH_3 + 2H_2O \longleftrightarrow 2NH_4^+ + 2OH^-$$

$$H_2CO_3 \longleftrightarrow HCO_3^- + H^+$$

$$HCO_3^- + H^+ + 2OH^- \longleftrightarrow CO_3^{2-} + 2H_2O$$

$$Ca^{2+} + CO_3^{2-} \longleftrightarrow CaCO_3(s)$$

Figure 12: reaction initiated by urease activity leading to calcium carbonate precipitation (Phillips AJ., 2014)

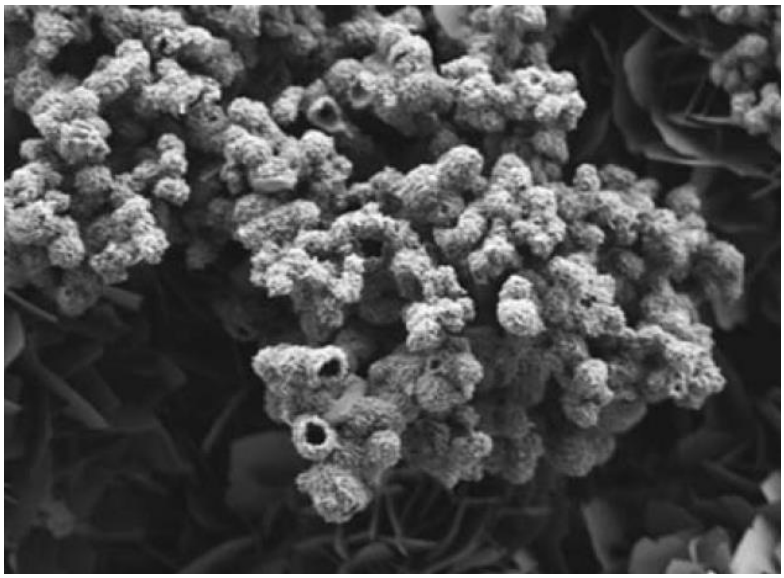


Figure 13: Scanning Electron Microscopy (SEM) image of tube-like calcium-containing minerals possibly entombing *S. pasteurii* shaped cells (Adrienne J. Phillips).

3. Biological compounds as reflectors

Besides minerals, another strategy is to make the bacteria directly secret a biological compound. To reflect sunlight radiations efficiently and without the need to display a very specific and complex structure, we thought that a white compound naturally reflecting the sunlight radiations would be interesting. Finding which biomolecule to express was not an easy task. We tried to find our inspiration in Nature, but only few biological compounds naturally display a white coloration. We were also constrained by the need for this molecule to be easily synthesized by our microorganism, and to maintain the regular plant activity as well as the foliar ecosystem at the time of expression. Which daily products are strongly white? What are the compounds responsible for this coloration? These were the questions that came into our minds at the time of our first reflections. Milk, egg white, beetle shell... We looked more into details what those products were composed of and came up with several ideas.

a. Casein

Why is milk white? The casein phosphoprotein is responsible. Majority protein in milk, its properties not only determine the white coloration, but also the texture, sensory and nutritional properties of most dairy products. Four main types of casein co-exist in milk: casein ∝-s1, casein ∝-s2, casein κ, and casein β. Based on their different physicochemical properties, those molecules aggregate together to create spherical structures called micelles. The exact structure of casein micelles has been highly discussed throughout the recent years. However,

it is suggested that self-association is made through weak interactions between calcium and phosphorus ions, creating a hydrophobic core with casein κ hydrophilic tails pointing outwards (Figure 14) (de Kruif C. et al, 2012). Suspended into the liquid phase, those structures reflect wide ranges of wavelengths partly explaining the white color of this colloid.

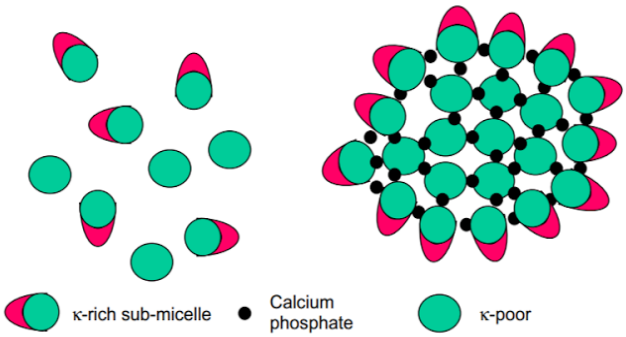


Figure 14: Schematic of the submicelle model of the casein micelle (Horne D, 2005)

The challenge is to find a way to make our microorganism able to synthesize casein. Casein bioproduction seems rare for the moment. Fortunately, a previous iGEM team had the amazing idea to make *Saccharomyces Cerevisiae* become a milk factory to produce "Real Vegan Cheese" (San Francisco Bay, 2014). In this aim, they isolated the sequences coding for each casein type and submitted two parts comprising the bovine beta casein or kappa casein respectively, associated with a secretion signal. They also included a sequence coding for a kinase allowing their phosphorylation (Figure 15).

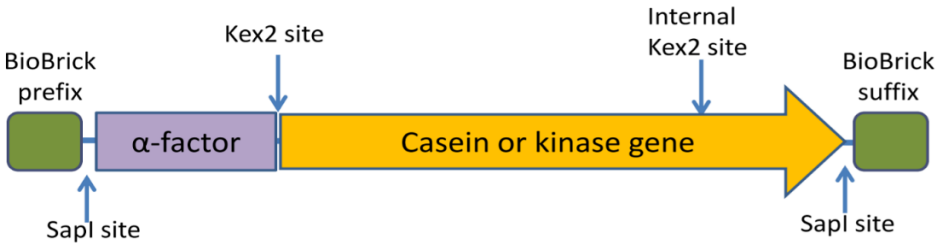


Figure 15: Representative part insert (iGEM team SF Bay, 2014)

The question is, do we need all those genes since our objective is only to display a white color?

Producing casein to meet current needs would not be totally pioneering: beyond the production of dairy products and food supplements, casein found a lot more applications in the non-edible industry: murals, glue, paint binder... It has even been recently shown that plastic could be crafted from solid casein or individual protein molecules! (Arney K, 2017). In agriculture, hydrolyzed casein is currently used to enhance biotic and abiotic stress tolerance in plants by activating some specific protection pathways. This is the case of

Constantino, which commercialized a Casein Protein CM Hydrolysate (Figure 16). The success of this product shows its lack of toxicity for the environment as well as humans and animals, but also its benefits for the plant itself against diseases and pest.

Figure 16: Example of hydrolyzed casein commercialized as a food supplement

Casein hydrolysis is the process enabling some bacteria and other microorganisms to use the amino acids produced. But this is also one of the aspects that constitute a limitation to this strategy. Indeed, the casein synthesized in case of a heat shock will be a nutritive element for all the microorganisms present on the leaf surface. It is in fact commonly used as a nitrogen source in microbial research and especially for fungi growth (Wang Y. et al, 2016). In the context of our application, bringing additional nutritional elements could alter their global allocation at the plant surface and therefore have an impact on the existing phyllosphere microbiota. This effect would contradict our approach to act against meteorological events without compromising the regular plant activity. The researcher Christian Huyghe was the first one to highlight this point as we suggested this alternative. He encouraged us to focus our research on a neutral compound, that would not have any influence on the ecosystem at the plant level.

Moreover, the breakdown of casein into small peptides and amino acids alters its original white coloration to produce uncoloured compounds (Reynolds J., 2011). This is a reaction we do not want to happen at the plant level, as it would inhibit the property of interest to reflect sunlight radiations. One way to counteract those problematics would be to identify the precise site of hydrolysis on casein, and inhibit it through protein engineering. This way, the protein will

not be able to split into small peptides and amino acids, and will not be taken up by the surrounding species.

b. Chitin and derivatives

We also turned to the animal kingdom to find our inspiration. A white beetle named *Cyphochilus Insulanus* displays a strong white coloration on its shell (Figure 17). This shade, reported as whiter than paper, was suggested to serve as a camouflage technique allowing this species to hide on white fungi (Dasi Espuig M., 2014). What is responsible for this "super-whiteness" ?

Figure 17: Cyphochilus Insulanus beetle (Horstman J., 2013)

The scales present at its surface are mostly composed of chitin filaments. This white polysaccharide polymer, widely found in Nature among arthropods but also yeast and fungi, has a protective action on living organisms. Two types of chitin co-exist in nature, the most common being the α-chitin. Their structure consists in chains organized in sheets, linked by hydrogen bonds (Figure 18). Chitin is already being used for industrial applications, ranging from food industry to biosensors or even drug carriers. One of the main characteristics of chitin is its insolubility in water and most other solvents. This is an interesting property for our application, favoring the polymer long-lasting on the leaf regardless of the air humidity and eventual rainy conditions (Rinaudo M., 2006).

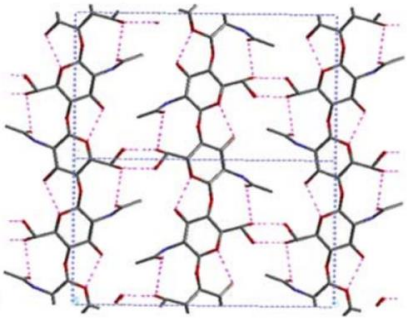


Figure 18: Structure of α-chitin arranged in sheets (Rinaudo M., 2006)

Other particularities make this polymer attractive for a possible plant application: displaying a low toxicity for the ecosystem, it is also biodegradable thanks to the action of the numerous chitinases present in nature. If chitin ever finds itself away from the plant, it will therefore be easily degraded and do not represent a danger for the surrounding species nor the environment. However, this particularity also raises a risk for the polysaccharide to be degraded on the plant by the bacteria present at that time, through their natural defense mechanisms, before any protective action is performed. We not only need to ensure that our microbial host does not express chitinase by knocking-out the responsible gene, but we also need to check if the quantity of chitin expressed is sufficient to balance an eventual degradation.

We studied the chitin synthesis pathway, to make it feasible at the micro-organism level. Chitin is synthesized thanks to the chitin synthase enzyme (CHS), coded by three genes in yeast (CHS1, CHS2 and CHS3). Each gene is responsible for a complementary aspect of chitin, but CHS3 appears essential for chitin synthesis in *S. Cerevisiae.* However, its post-translational modifications make its bacterial synthesis highly challenging. The bacterial homologous gene NodC originates from the gram-negative *Rhizobium leguminosarum* bacterium. The Darmstadt iGEM team 2017, through their project ChiTUcare, showed that the gene successfully induced functional enzyme expression leading to the production of chitin oligomers (Figure 19) (www.2017.igem.org/Team:TU_Darmstadt/).

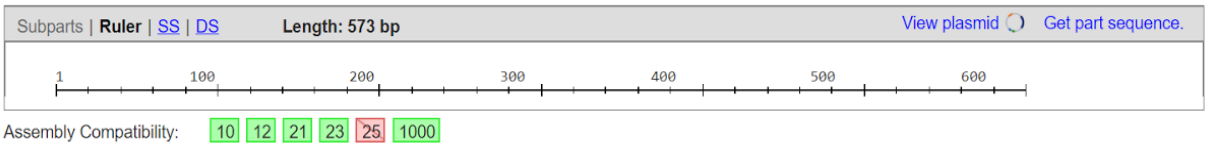


Figure 19: Part BBa_K2380000 coding for NodC gene from Rhizobium leguminosarum (http://parts.igem.org/Part:BBa_K2380000:Design)

The nod genes have also been used for industrial chitooligosaccharides production through the expression of nodC or nodBC genes in *E.Coli* (Samain E., 1997). The pathway chosen for the application on the plant will depend on the host choice.

The principal challenge remains in the efficiency of using chitin. We did not find any example of chitin use for sunlight reflection, so this method seems quite pioneering. We first have to assess how the produced chitin oligomers will structurally organize on the plant, and if those elements alone are sufficient to display satisfying reflective properties. Indeed, studies on insects suggested that the reflectiveness in *Cyphochilus* beetle depended on the three-dimensional

structure of its scales in addition to their white components itself (Vukusic P. et al, 2007).

Another candidate could be the chitin deacetylation product named chitosan (Figure 20). The deacetylation level as well as the acetyl group distribution directly influences its comportment in solution. Globally, chitosan shows a better solubility in aqueous solutions compared to chitin. This particularity makes it especially suitable for gels, films and fibers applications. It could be interesting for us to induce the formation of a film on the leaf, since we seek a large surface covering. However, chitosan hydrophilic character is also associated with a lower material stability despite its better easiness of processing compared to chitin. Chitosan has notably been used in agriculture for plant growth stimulation and seed coating, due to its capacity to enhance plant natural defenses and inhibit bacterial growth and infection (Rinaudo M., 2006). Using chitosan would therefore, in addition to providing heat protection, bring additional value for plant survival and growth!

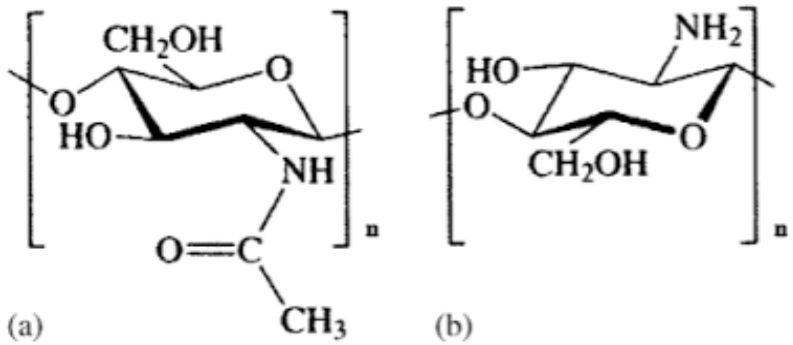


Figure 20: Chemical structure of (a) chitin and (b) chitosan repeat units (Rinaudo M., 2006)

Finally, we must cautiously take care of the agronomical traits of the plant. For this reason, we will study the duration of the polymer on the plant and its impact on the plant activity on the long term.

4. Efficiency tests in real conditions to determine the best strategy

Experiments on vines on the field during heatwaves are required to compare the efficiency of each candidate compound to increase the albedo and decrease leaf temperature. As heatwaves only occur during summer in temperate countries, reproducing heatwave conditions in a laboratory or in a greenhouse could be an alternative solution. In first place we could test the most easily available compounds: talc as control, calcium carbonate and casein (Figure 21).

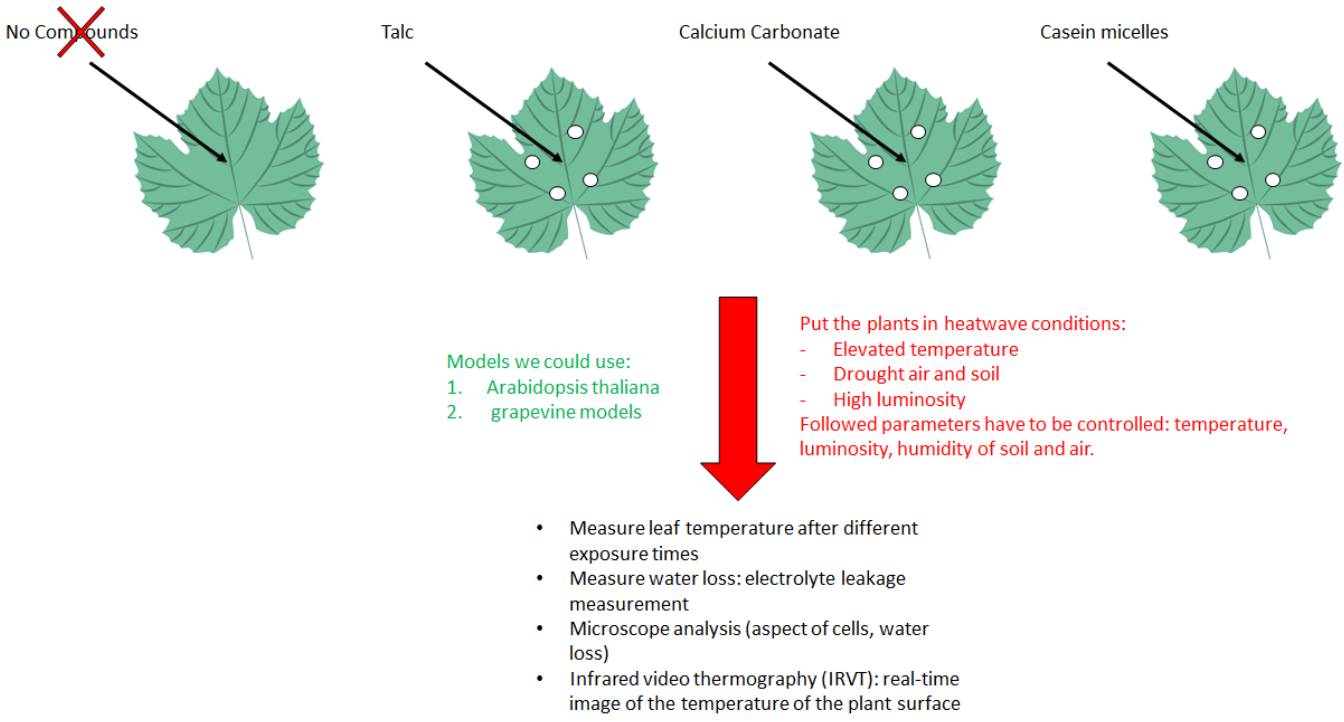


Figure 21: Laboratory strategy to evaluate the effects of reflective compounds for heat protection

III. Existing products used against temperature stress

A. *Frostban*: GMOs and Ice-nucleation proteins to prevent frost injuries

In the late 1980's, the engineering of a product called Frostban, developed by Advanced Genetic Sciences (AGS), caused many societal concerns. This product relied on the use of Genetically Modified Organisms (GMOs) to prevent frost injuries, which is relatively similar to our project. The main difference between Frostban and Softer Shock remains in the molecular mechanisms implied, both at the leaf surface and the genetic construction point of view.

Softer Shock relies on the secretion of a given protein, whether INPs or AFPs, to induce or inhibit the formation of ice crystals. Frostban, on the other hand, relied on the knock-out of the INP gene from naturally occurring ice-nucleating bacteria *Pseudomonas Syringae* and *Pseudomonas Fluorescens*. The goal was to spray the modified bacteria that were unable to produce INPs on the target crops so that they can compete with natural ice-nucleating *P.syringae* and *P.Fluorescens* (Margaritis et al., 1991).

Overall apart from many controversies, several field studies made by Frostban, had, according to AGS, proven very interesting results (around 20% less reported frost damages) and show that other possibilities could have been thought for Softer Shock through the use of INPs genes (BLR, 1988). However, in the context of the iGEM and the use of Biobricks, knocking-out a gene to fight frost damages would have been problematic, as much as the potential scars we could have reanimated.

The potential of the product did not lead to a total abandon however. The idea of GMOs was left aside but the use of competition against ice-nucleating bacteria of the phyllosphere was kept. From this was born the product Blightban, using naturally ice-nucleating incompetent *P.fluorescens* to treat both blight and frost damages on crops (Skirvin et al., 2000). This product is still used and registered in several states, highlighting the potential of microorganisms in treating frost and other damages for future agricultural biotechnology applications.

If you wish to know more about the context of Frostban and all the controversies raised by this project, you can look at the case study we did on our wiki.

B. *Invelop*: using talc powder to fight heat damages

As discussed earlier in this report, our strategy for sunlight protection is to modify the albedo of our target surface to increase its ability to reflect sunlight radiations and protect it from heat damages. Such strategy is not new. Sunlight has been proven to, additionally to induce water loss, cause sunburn on the surface of fruits and leaves (Zhang et al, 2015).

Technologies aiming at increasing fruit and leave albedo have then been developed to protect the exposed parts from such damages, as they decrease the quality of the final product and its overall visual aspect (Figure 22).

Figure 22: Sunburns on apples and grapes (Compo Expert's Invelop sheet, 2016)

To counter the sunburns damages on fruits, a company called Compo Expert developed a product called Invelop. This product is based on a specific formulation of talc, a clay mineral with hydrophobic properties and known for its softness. The company specified that their formulation was necessary since talc is originally not adapted to foliar application (CE, 2016). The overall goal of the product is very similar to ours : applying a layer of white compounds to reflect sunlights and protect fruits and leaves (apple, apricots, grapes) by increasing their albedo (up to 82%, when an albedo of 100% reflects 100% of sunlight) (Figures 23 & 24) (CE,2016).

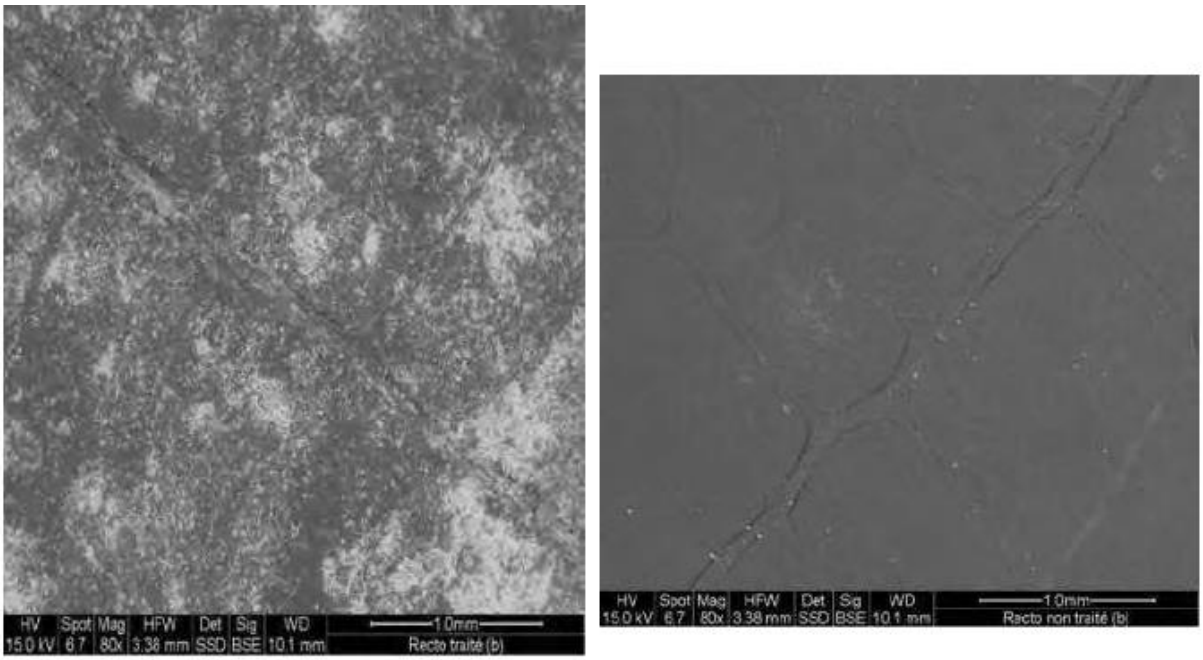


Figure 23: A scanning electron microscopy of *Invelop* covered walnut tree leaf as compared to an uncovered one, showing relatively homogeneous covering

.

This product is interesting for us because it provides data on the efficiency of our planned strategy. Indeed, Compo Expert did field tests on apples and apricots and reported an average of a 2°C decrease of surface temperature of the fruits covered by *Invelop* as compared to uncovered ones (CE,2016).

Figure 24: Invelop covered apricots (CE, 2016)

A variation of the thermal spectrum of protected fruits in comparison with untreated ones was also reported (Figure 25).

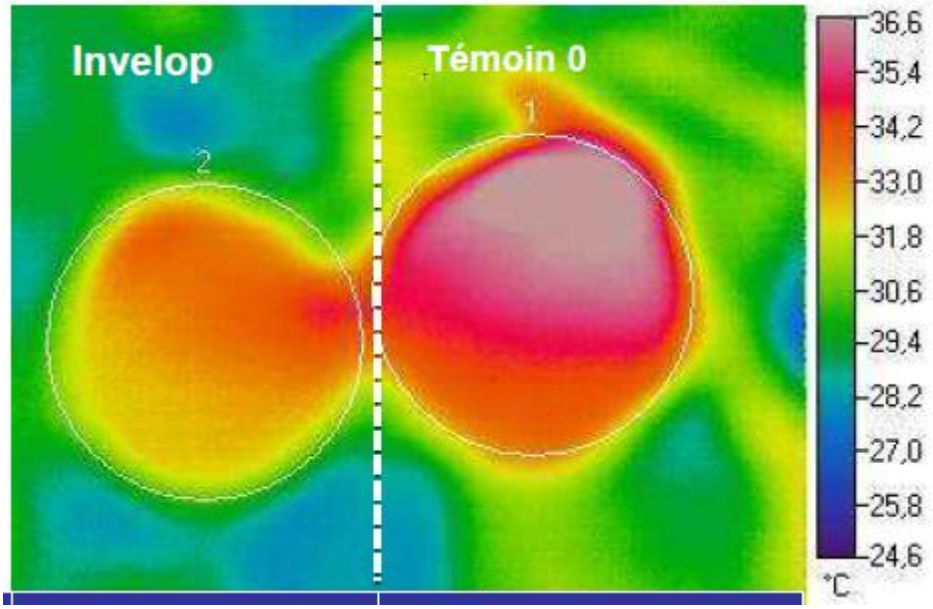


Figure 25: Two apples, one treated with Invelop and the other untreated, displaying different thermal spectrum and surface temperatures (CE, 2016)

This product furnishes precious data and shows that our strategy of protection has a great potential for plant care. Softer Shock can have several advantages as compared to Invelop. Indeed, talc, as other mineral compounds, will most likely be washed off by rainfalls (even if few rainfalls usually occur when the product is applied in summer, especially in France). It therefore needs regular applications, whereas our micro-organisms is designed to remain lastingly on the applied area and synthesize the protectants while the programmed death is not activated by the farmer. Our product should have a longer lifespan than *Invelop* and require only one application (although there will always be a need to sustain our organism with its synthetic amino acid). Furthermore, our organism could provide additional benefits to both leaves and fruits, including pathogen protection and plant growth stimulation. The 2 in 1 property of Softer Shock could also be seen as an advantage as compared to *Invelop* because our product will be multivalent.

The main flaws of Softer Shock compared to products like *Invelop* will be of course the cost, the environmental hazards (even though we tried to maximize its biosafety), and the storage. Indeed, storing talc is very easy as it is a mineral, but storing an organism can be very complicated (not if it can sporulate though) (Satinder et al., 2006). For the moment in any case, products like *Invelop* outmatch ours because of the societal context we are in, especially in France. That is why we tried to develop the bioreactor strategy. You can learn more about in the dedicated section in our wiki.

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