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Physiological responses of *Burkholderia phytofirmans* strain PsJN colonized plantlets of grapevine (*Vitis vinifera* L.) to low non-freezing temperatures

By

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This work is dedicated to

my partner, Eleni

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"The goal of education is to make people wiser, more knowledgeable, better informed, ethical, responsible, critical and capable of continuing to learn. Education also served society by providing a critical reflection on the world, especially its failings and injustices, and by promoting greater consciousness and awareness, exploring new visions and concepts, and inventing new techniques and tools. Education is also the means for disseminating knowledge and developing skills, for bringing about desired changes in behaviours, values and lifestyles, and for promoting public support for the continuing and fundamental changes that will be required if humanity is to alter its course, leaving the familiar path that is leading towards growing difficulties, and starting the uphill climb towards sustainability. Education, in short, is humanity's best hope and most effective means to the quest to achieve sustainable development"

United Nations Education, Scientific and Cultural Organization

Abstract

*Burkholderia phytofirma*ns strain PsJN has been well characterized as a Plant Growth Promoting Rhizobacteria (PGPR) that triggers induced resistance in grapevine against fungal pathogens. Recently, it has been demonstrated that *B. phytofirmans* may also enhance resistance to stress in low non-freezing temperatures. To better understand the interaction between grapevine and *B. phytofirmans* strain PsJN, changes in the expression pattern of different defence related genes were investigated in Chardonnay grapevine leaves after root inoculation with PsJN strain. *B. phytofirmans* induced a systemic spread of a signal from roots to leaves after root inoculation with bacteria, a phenomenon referred to as Induced Systemic Resistance (ISR). The expression pattern of well-characterized grapevine defence genes was also monitored in grapevine plantlets bacterized four weeks earlier, and subjected to low non-freezing temperature. Results report that PsJN induces earlier and/or higher transcript accumulation of defence genes in bacterized plantlets upon low non-freezing temperatures according to the phenomenon of priming. Further investigation of several biochemical parameters reveals that bacterized grapevine plantlets are in a primed physiological state able to increase their sugar, starch and proline levels upon low non-freezing temperatures while the analysis of membrane lipid peroxidation markers indicates a faster degradation of aldehydes, malonaldehyde and hydrogen peroxide beyond one week, addressing the better adaptation of bacterized plantlets than non-bacterized plantlets to low non-freezing temperature. In addition, nine combinations of non-radioactive digoxigenine labelled-*PstI* and *MseI* primers were used to generate differentially expressed genes by cDNA-AFLP technology for further investigation of primed physiological state induced by PsJN and isolation of over-expressed genes upon low non-freezing temperatures. In conclusion, it is suggested that PsJN strain is an ISR-inducing PGPR able to stimulate grapevine defence mechanism by priming physiological responses critical to acclimation under low non-freezing temperatures.

Résumé

*Burkholderia phytofirma*ns souche PsJN a été caractérisée comme une rhizobactérie promotrice de la croissance des plantes (PGPR, pour Plant Growth Promoting Rhizobacteria) capable d'induire la résistance de la vigne contre certains champignons pathogènes. Récemment, il a été démontré que *B. phytofirmans* pouvait également améliorer la résistance de la vigne aux basses températures. Pour mieux comprendre l'interaction entre la vigne et *B. phytofirmans* souche PsJN, le profil de l'expression de différents gènes de défenses a été analysé au niveau des feuilles de vitroplants de vigne (Chardonnay) après l'inoculation des racines par la bactérie. Les résultats obtenus montrent que la souche PsJN induit la propagation d'un signal systémique, des racines vers les feuilles, caractéristique d'une résistance systémique induite (ISR). L'expression de gènes de défenses préalablement caractérisés chez la vigne a également été analysée au niveau des vitroplants de vignes bactérisés 4 semaines avant leur traitement par les basses températures. Les résultats obtenus montrent une accumulation précoce et/ou intense des transcrits de gènes de défenses au niveau des vitroplants bactérisés et soumis aux basses températures selon le phénomène de potentialisation. Une analyse de différents paramètres biochimiques a révélé que cet état de potentialisation permet aux vitroplants bactérisés d'augmenter la teneur en sucres solubles, amidon et proline après le stress thermique. Parallèlement, l'analyse des marqueurs de peroxydation membranaire a montré une dégradation plus rapide des aldéhydes, du malondialdéhyde et du peroxyde d'hydrogène, une semaine après le début du stress froid, indiquant ainsi une meilleure adaptation des vitroplants bactérisés aux basses températures.

En complément, neuf paires d'amorces digoxigénine non radioactives marquées *PstI* et *MseI* ont été utilisées pour générer les gènes exprimés différemment par la technique cDNA-AFLP (polymorphisme de longueur des fragments amplifiés) pour mieux comprendre l'état de potentialisation induit par la souche PsJN et d'identifier les gènes surexprimés lorsque la plante est soumise aux basses températures.

En conclusion, nous avons suggéré que *B. phytofirma*ns souche PsJN est une PGPR inductrice de l'ISR qui est capable de stimuler les mécanismes de défense de la vigne *via* un état de potentialisation qui lui permettrait une acclimatation en condition de basses températures.

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1. LITERATURE REVIEW

1.1 General introduction

Sustainable development is a pattern of resource use that aims to meet human needs while preserving the environment so that these needs can be met not only in the present, but in the indefinite future (IISD, 1997). Sustainable development is both a goal and a concept (Kates *et al*., 2005). As a goal, it is an idea of a world where people protect the environment as they carry out their day-to-day activities. As a concept, sustainable development calls for conceptual probing about limits on natural resources, capacities of ecosystems, and interactions among social, economic, political and environmental systems. The central theme underlying this concept is working towards a sustainable quality of life, now and in the future. Sustainable development supports principles of equity and social responsibility. This includes equity among nations, equity within nations, equity between humans and other species, as well as equity between present and future generations. The concept of sustainable development also calls for particular skills, knowledge, values and attitudes regarding the environment, the economy and the well-being of people. Consequently, the nature of sustainable development is a decision-making process, a way of thinking, a philosophy, and an ethic (Williams, 1994).

1.1.1 Sustainability in agriculture

Sustainable agriculture is a model of social and economic organization based on an equitable and participatory vision of development that recognizes the environment and natural resources as the foundation of economic activity (ATTRA, 2003). Agriculture is sustainable when it is ecologically sound, economically viable, socially just, culturally appropriate and based on a holistic scientific approach. According to ATTRA (2003; 2005), sustainable agriculture preserves biodiversity, maintains soil

fertility and water purity, conserves and improves the chemical, physical and biological qualities of the soil, recycles natural resources and conserves energy, uses locally available renewable resources, appropriate and affordable technologies. Further, sustainable agriculture minimizes the use of external and purchased inputs, thereby increasing local independence and self sufficiency and insuring a source of stable income for farmer communities (ATTRA, 2005).

1.1.2 Beneficial microorganisms in sustainable agriculture

To significantly increase food production during the last decades according to sustainable concepts, it was essential to develop mainly crop cultivars with improved genetic capabilities (i.e. greater yield potential, disease resistance, and nutritional quality) and with a higher level of environmental competitiveness, particularly under stress conditions (i.e. low rainfall, high or low temperatures, nutrient deficiencies, and aggressive weed growth). Nowadays, it is well understood that crop growth and development are closely related to the nature of the soil microflora, especially those in close proximity to plant roots, i.e. the rhizosphere (Lynch & Whipps, 1991; Higa & Parr, 1994). Most biological activities are influenced by the state of these microscopic units of life. An area that appears to hold the greatest promises for technological advances in crop production, crop protection, and natural resource conservation is that of beneficial and effective microorganisms and their interaction with plants (Glick, 1995; van Loon, 2007). It is recognized that the best soil and crop management practices to achieve a more sustainable agriculture will also enhance the growth, numbers and activities of beneficial soil microorganisms that, in turn, can improve the growth, yield and quality of crops (Kloepper *et al*., 2004a,b). In essence, increased

activity of beneficial microorganisms in soil is the very foundation of a more sustainable agriculture (Parr *et al*., 1994).

Plant Growth Promoting Rhizobacteria (PGPR) are usually a group saprophytic bacterial microorganisms that live in the plant rhizosphere, able to colonize the root system. They are studied as plant growth-promoters for increasing agricultural production and as biocontrol agents against plant diseases (Kloepper *et al*., 1992; Chen *et al*., 2000; Saravanakumar *et al*., 2007a,b). Among them the genus of *Burkholderia*, probably the most diverse and environmentally adaptable plant-associated bacteria, contains species and strains that are beneficial to plants (Compant *et al*., 2008a). *Burkholderia phytofirma*ns strain PsJN was subsequently shown to be a highly effective plant-beneficial rhizobacterium, although originally isolated as a contaminant from *Glomus vesiculiferum*-infected onion roots (Nowak, 1998; Nowak & Shulaev, 2003). It is a non-sporulating, rod-shaped, motile bacterium, with a single polar flagellum strain PsJN (Frommel *et al.*, 1991). Based on various biochemical and physiological studies, strain PsJN was originally classified as beneficial microorganism representing non-fluorescent *Pseudomonas* sp. (Frommel *et al*., 1991). However, subsequent studies revealed that in fact it represents a member of the genus *Burkholderia* (Sessitch *et al*., 2005). Nowadays, it is known that the strain is able to establish rhizosphere and endophytic populations associated with various plants, where it stimulates plant growth and induces developmental changes leading to (i) better water management (Frommel *et al.*, 1991; Nowak *et al.*, 1995; Pillay & Nowak, 1997), (ii) increase in the resistance to heat (Bensalim *et al.*, 1998) and cold stress (Ait Barka *et al.*, 2006), and (iii) increased resistance of plants against pathogens (Nowak *et al.*, 1995; Sharma & Nowak, 1998; Ait Barka *et al.*, 2000; 2002).

1.1.3 Sustainable viticulture in cool climate

Vitis vinifera L. and other species of genus *Vitis*, are among the oldest cultures in the world (Box 1). *Vitis vinifera*, cultivated for premium quality wine production, is frequently exposed to low temperature (Gladstones, 1992). Although, it is a temperate-zone plant, it is not well-adapted to the cooler parts of the temperate zone, where growing seasons may be too short to allow the fruit to reach maturity, or where low winter or spring temperatures may damage its tissues and organs (Goffinet, 2004). In Europe, such cold events can exist in the important viticultural areas of Champagne in France, Chianti in Italy, and the Rhinegau in Germany (Pool, 2000). The minimum temperature for normal physiological activity for grapevine has been commonly estimated at 10°C (Winkler *et al.*, 1974**)**. Studies on grapevine physiology by Buttrose (1969) have revealed that grape shoot and root growth and fruiting yields are significantly reduced by exposure to low non- freezing temperatures. Grace (1988) states that in the absence of other limiting factors, a decrease of temperature by 1°C can decrease plant productivity by approximately 10% and that this correlation is strengthened as the altitudinal and latitudinal temperature limits of a species are approached.

Sustainable viticulture in cool climate requires the accommodation of those climatic factors near the limits of commercial grape production (Howell, 1988). Common viticultural practices have been related with specific selection of appropriate mesoclimate and development of specific microclimate, and the use of resistant cultivars to cold (Pool, 2000). In addition, the management of physiological functions, like bud initiation and differentiation, crop ripening, carbohydrate storage, wood and bud maturation, and acclimation to freezing temperature and maintenance of vine cold hardiness can protect grapevine productivity from low temperatures (Howell, 1988).

Box 1:

Taxonomy and origin of most important species of genus *Vitis*

Vitis is a genus of about 60 species of vining plants in the plant family Vitaceae (Galet, 1967). The genus is made up of species predominantly from the Northern hemisphere. Most *Vitis* species are found in the temperate regions of the Northern Hemisphere in Europe, North America and Asia with a few in the tropics. Most grapes come from cultivars of *Vitis vinifera* L., the European grapevine native to the Mediterranean and Central Asia (Galet, 2000). Minor amounts of fruit and wine come from American and Asian species (Mullins *et al.,* 1992) such as:

- *Vitis aestivalis*, native to eastern North America
- *Vitis rupestris*, native to North America
- *Vitis riparia*, the "river bank grape", native to north-eastern North America
- *Vitis amurensis*, the Asiatic grape variety, native to Siberia, and China
- *Vitis rotundifolia*, the muscadines, native to the southern half of the United States
- *Vitis labrusca*, native to northeastern North America

These species occur in widely different geographical areas and show a great diversity of form. However they are sufficiently closely related to allow easy interbreeding and the resultant interspecific hybrids between *V. vinifera* and one or more of *V. labrusca*, *V. riparia* or *V. aestivalis* better known as French hybrids (Galet, 1979).

Vitis vinifera

Over 9,600 cultivars are listed as *Vitis vinifera*, most of them having been selected for a specific region and purpose (Galet, 2000). Subsp. *vinifera* (ssp. *sativa* Hegi) has hermaphoditic flowers, and fruits 6–22 mm, ellipsoid to globose, green, yellow, red or purple-black, sweet, with 2 seeds which are pyriform with a rather long beak. Cultivated for wine making and for edible fruit in southern and central Europe and widely naturalized. In general, grapes are unsuitable to humid, steamy, hot tropics, as they need a cold period for resting and a dry sunshine climate for ripening fruit. The wild grape is often classified as *V. vinifera* ssp. *sylvestris* (in some classifications considered *Vitis sylvestris*), with *V. vinifera* ssp. *vinifera* restricted to cultivated forms. Domesticated vines have hermaphrodite flowers, but ssp. *sylvestris* is dioecious (male and female flowers on separate plants) and pollination is required for fruit to develop (Mullins *et al.,* 1992).

1.1.4 The "terroir" of northern European vineyard

Champagne wine region is a province in the northeast of France, famous for the history and the production of Champagne wines (Box 2). The distinctive natural components of the terroir in Champagne is a unique combination of soil (including microorganisms), subsoil, cool climate and grape varieties are the underlying factors which account for the uniqueness of the wines of the region (Dominé, 2001). The exceptional nature of the soil on which the Champagne vine is planted determines the unique flavour characterizing the wine of Champagne. Most vineyards in Champagne area are situated halfway up layer of limestone. This chalky subsoil provides the vine with nutrients and ensures perfect drainage, allowing excess water to seep through, yet preserving sufficient humidity in the soil. Furthermore, the chalk stores up and gently redistributes warmth, thus regulating the elements to the benefit of the ripening plants (Domine, 2001).

The climate of the Champagne area also plays a major role as far as the formation of top quality grapes is concerned. The vines must adapt to an annual average temperature of 10^{o}C (below an average of 9 ^{o}C, the grape cannot ripen) and live through the dangers of frost in spring time and poor weather during the flowering period. Although varieties like Pinot Noir, Pinot Meunier and Chardonnay, which grow in this region, have been specially selected to overcome these harsh weather conditions, low temperatures affect grapevine biology resulting to many physiological and biochemical changes.

1.2. The biology of plants in cold

Among various environmental stresses, a low temperature is one of the most important factors limiting the productivity and distribution of plants. Chilling and

Box 2:

The Champagne area

The region of Champagne lies at a crossroads of northern Europe – the river valleys leading south to the Mediterranean and north to Paris, the English Channel and Western Germany – and thus has been the setting of many dramatic events in the history of the French nation. As a convenient access point, it has been for hundreds of years, the chosen path of many invaders. The Hundred Years' War and the Thirty Years' War brought repeated destruction to the region as armies marched back and forth across its landscape. By the 17th century, the city of Reims has seen destruction seven times and Epernay no less than twenty-five times (Kladstrup & Kladstrup, 2005).

The history of Champagne wine

For centuries (and before the mid-1600's) the wines had still the typical style and were held in high regard by the nobility of Europe. But the cool climate of the region and its effect on the wine making process was to play an important part in changing all of that. Although Champagne was only about 10% of the region's output in the 18th century, it was enjoyed increasingly as the wine of English and French royalty and the lubricant of preference at aristocratic gatherings. Its popularity continued to grow until, in the 1800's, the sparkling wine industry was well established. By 1853 total sales of champagne reached 20 million bottles up from just 300,000 bottles at the turn of the century World War I again brought devastation to the region. The early months of the war saw a rapid German advance into northern France and during the fall of 1914, they were camped south of the river Marne. By 1915 they were driven back just north of the city of Reims. The enormous caves – Roman chalk quarries – beneath Reims that were used for the storage and production of champagne, now became shelters from the 1000 days of bombardment the city endured from 1914 to 1918. After the war, the city had to be completely rebuilt. The years after the Great War were difficult. The Bolshevik Revolution in Russia, Prohibition in the United States, and then the Great Depression saw the champagne market dry up. The champagne houses stopped buying grapes, so the growers formed the first champagne cooperatives at this time. With the ending of Prohibition in 1934, the industry began to turn around. The influential head of Moët & Chandon, Robert-Jean de Vougë, was most instrumental in securing its future. He proposed that the purchase price of champagne grapes be set at a level that ensured a decent living for the growers, and in 1941, during the German occupation of France, became the driving force in persuading the Germans to establish the very successful Comité Interprofessionel du Vin de Champagne – C.I.V.C. Since World War II champagne sales have climbed upwards, nearly quadrupling between 1945 and 1966. Champagne has trickled down the social scale and is no longer considered just a luxury (Kladstrup & Kladstrup, 2005).

cold, referring to low but not freezing temperatures (0°C - 15°C) frequently occurring in nature, damage many species of plants (Lyons, 1973; Wang, 1990). Plants of tropical and subtropical origin are sensitive to chilling stress and usually lack the capacity to survive in low non-freezing temperatures (Levitt, 1980). Many chilling sensitive crops such as rice, maize, and tomato are only marginally low temperature-adapted in regions, withstanding hardly to low non-freezing temperatures (Lyons, 1973). On the other hand, plants originating from temperate zones can continue to grow and develop at temperatures in the chilling range, but could not normally complete their life cycle if maintained continuously near temperatures above 0°C (Fig. 1.1). This might occur because some critical phase of the growth cycle, which in perennial plants is usually seasonally related, is susceptible to chilling damage with negative and unforeseeable effects on plant biomass and productivity (Raison & Lyons, 1986). These plants, however, should be generally classified as 'chilling-insensitive' unless the study is specifically directed toward the phase of the plant that is susceptible to chilling (Raison & Lyons, 1986). The main characteristic of the majority of insensitive plants is that they can increase freezing tolerance by being exposed to low non-freezing temperatures, a process that is known as cold acclimation (Tomashow, 1999).

Over the last decade, studies on plant responses to cold stress have been focused on the mechanisms of cold acclimation rather than how insensitive plants can resist to chilling temperatures. Nevertheless, recent evidence indicates that some of the molecular changes that occur during cold acclimation are also important for plant chilling tolerance (Gong *et al.*, 2002; Hsieh *et al.*, 2002**;** Dong *et al.*, 2006). According to this approach, it is concluded that chilling resistance exhibited by chilling insensitive plants is not entirely constitutive and at least part of it is developed

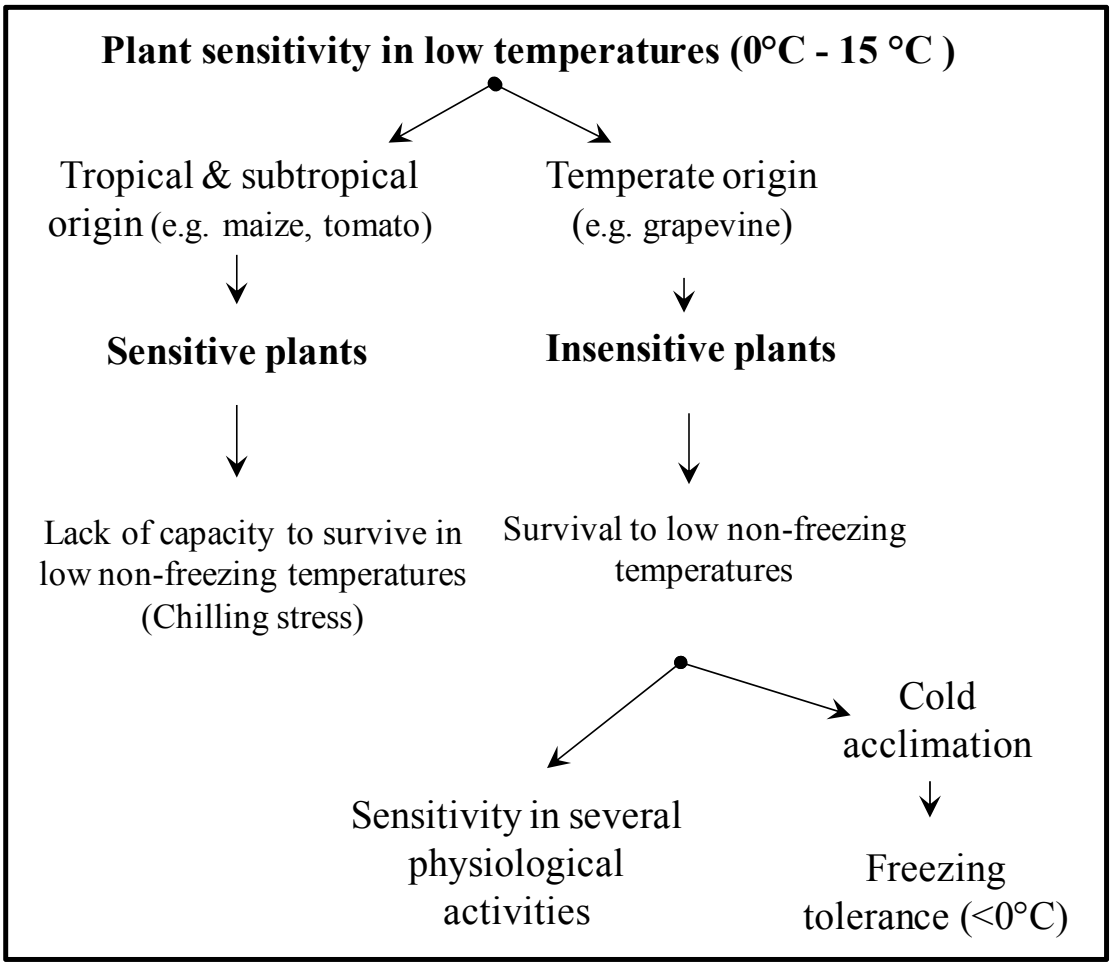

Fig. 1.1: Characterization of plants according to sensitivity in low non-freezing temperatures (adapted from Lyons, 1973; Raison & Lyons, 1986; Wang, 1990; Tomashow, 1999).

during exposure to low non-freezing temperatures. The changes in plant metabolism induced by the effects of low temperatures are a complex phenomenon. Plant response to cold temperatures appears to be both species- and tissue-specific. In addition, several parameters like the severity and the duration of stress, the rate of the cooling, and the concomitant presence of the other environmental conditions like air humidity, water soil availability, wind presence (causing dehydration), and the light intensity (causing photoinhibition), have a critical effect in plant sensitivity (Bracale & Coraggio, 2003). In spite of the complexity of the matter, different approaches in

genetics and genetic engineering and countless physiological, biochemical and molecular studies contribute a global view and a comprehension of the physiological changes developed in plants upon low non-freezing temperature. The phenomenon has experienced a burst of knowledge during the last decades due to the study of gene expression, the analysis of specific signal transduction factors, and the study of changes in proteins and enzymes in plants responding to low non-freezing temperatures.

1.2.1 How sensitive plants perceive cold

The term "chilling sensitivity" has been used to describe many types of physiological damage resulting from low, but non freezing temperatures (Raison & Lyons, 1986). Many physiological and biochemical cell dysfunctions have been correlated with visible (wilting, chlorosis, or necrosis) or not, symptoms in chilling sensitive plants (Lyons, 1973). These physiological and biochemical dysfunctions occurring upon chilling frequently include changes in cell membrane structure and lipid composition (Lyons & Raison, 1970), leakage of ions through cell membranes (Lyons & Raison, 1970), cellular leakage of electrolytes and amino acids, a diversion of electron flow to alternate pathways (Leopold & Musgrave, 1979), alteration in protoplasmic streaming (Lewis, 1956), redistribution of intracellular calcium ions (Bush, 1995), and phosphorylation of thylakoid proteins (Bannett, 1991). They also include several metabolic modifications (Sochanowicz & Kaniuga, 1979; Levitt, 1980; Trevanion *et al.*, 1995), changes in protein content (Marmiroli *et al*., 1986; Bredenkamp & Baker, 1994) and enzyme activities (Byrd *et al.*, 1995; Kumar & Triphathy, 1998), and ultrastructural changes in a wide range of cell components like plastids, thylakoid membranes and mitochondria (Wise *et al.*, 1983; Ishikawa 1996; Kratsch & Wise, 2000).

For more precise characterization of these plant dysfunctions, Raison and Lyons (1986) proposed distinction of primary and secondary stages of chilling injury. The primary stages might be a change in membrane lipid structure **(**Raison, 1974**)**, a conformational change in some regulatory enzyme or structural protein (Graham & Patterson, 1982) or an alteration in the cytoskeletal structure of the cell (Patterson *et al.*, 1979). Such event is more or less instantaneous and occurs at some critical or threshold temperature which correlates with the onset of chilling injury, and in the short term, it is reversible (Fig. 1.2). Secondary events, which follow, can incude loss of turgor, leakage of cytoplasmic solutes, lack of energy metabolism, disruption of the photosystems, accumulation of active oxygen species (AOS) or similar events that lead to visible symptoms of injury (Raison & Lyons, 1970). The secondary stages are both time and temperature dependent. In the short term they are reversible if the chilling stress is removed (Fig. 1.2). However, if the stress is maintained, the imbalance and/or loss of cellular integrity become excessive and the process turns irreversible. After this stage, warming to non-chilling temperatures exacerbates the symptoms of injury. The rate of development and magnitude of the visible symptom of injury depend, to a large extent, on the metabolic status of the tissue at the time the chilling stress is imposed (Lyons, 1973).

1.2.1.1 Chilling injuries to cell membrane

There is a strong correlation between plant chilling sensitivity by membrane damage and the degree of unsaturation of fatty acids (Nishida & Murata, 1996). A high degree of saturated fatty acids in phosphatidylglycerol in membranes has been observed in chilling sensitive plants (Lee *et al.*, 2005).

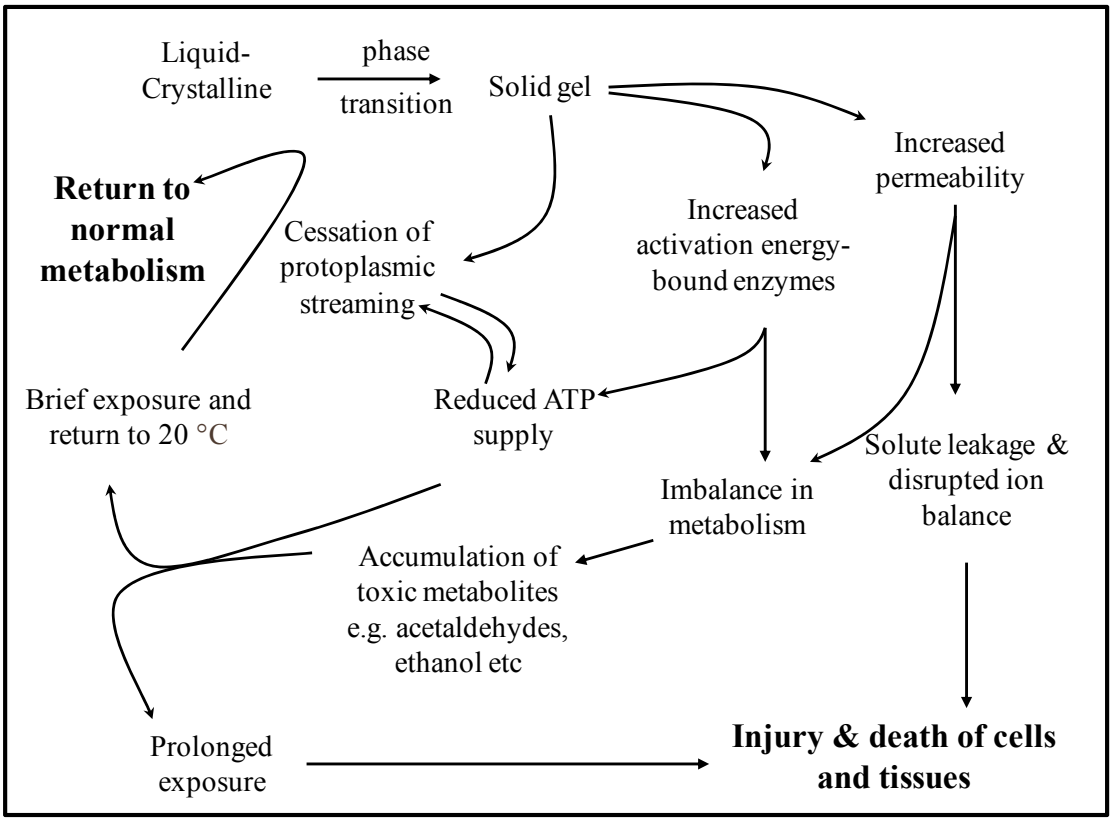

Fig. 1.2: An elegant model to explain many symptoms of chilling injury in chilling sensitive plants adapted from Raison and Lyons (1970) and in detail by Lyons (1973).

Since saturated fatty acids have a high melting point, membranes isolated from chilling sensitive plants can undergo a phase transition from the liquid crystalline phase to the gel phase even at room temperature (Fig. 1.2). The gel phase domains represent lipids with low kinetic motion and tight, rigid packing of the acyl chains. The presence of a gel phase domain in the membrane bilayer prevents proper functioning of integral membrane proteins, and will not maintain an effective permeability barrier (Murata *et al*., 1992; Kodama *et al*., 1994). Another factor of cold injury by membrane damage is the decrease in membrane fluidity and loss of function due to lipid peroxidation (Barclay & McKersie, 1994). Chilling enhances production of free radicals and peroxidized membranes. This causes the loss of unsaturated fatty acids, an increase in membrane rigidity due to the formation of covalent bonds among

lipid radicals, a higher lipid phase-transition temperature, and membrane degradation (Alonso *et al*., 1997). Through this primary effect on the phase properties of membrane lipids, low temperature was proposed to cause a cascade of the secondary events. On the contrary, chilling plants that contain high proportions of unsaturated fatty acids keep the phase transition temperature below the applied chilling temperature, avoiding the phase transition of the membrane (Murata *et al.,* 1992; Kodama *et al.,* 1994).

1.2.1.2 Increase in free cytosolic Ca^{2+} and AOS in cells upon chilling

The two recent hypotheses that have attracted attention of researchers about chilling are the induction of injury by a rapid increase in the concentration of free (ionized) cytosolic Ca^{2+} (Minorsky 1985; Jian *et al*., 1999; Holdaway-Clarke *et al*., 2000), and by development of oxidative stress upon cooling of chilling-sensitive tissues (Hariyadi *et al.*, 1993; Prasad *et al*., 1994).

Cytosolic Ca^{2+} regulates numerous physiological processes, like phytohormone signal transduction, protoplasmic streaming, changes in membrane potential, and transport of cations (Poovaiah & Reddy, 1987). The activation of Ca^{2+}-dependent proteins and enzymes (protein kinases, phosphatases) affects protein phosphorylation and protein functioning, as well as operation of transcription and translation systems **(**Saijo *et al.*, 2001) (Fig. 1.3). According to Lieberman and Wang (1982), the chilling-induced increase in cytosolic Ca^{2+} in cells of chilling-sensitive plants leads to a fast (within several minutes) cessation of cytoplasm streaming and to

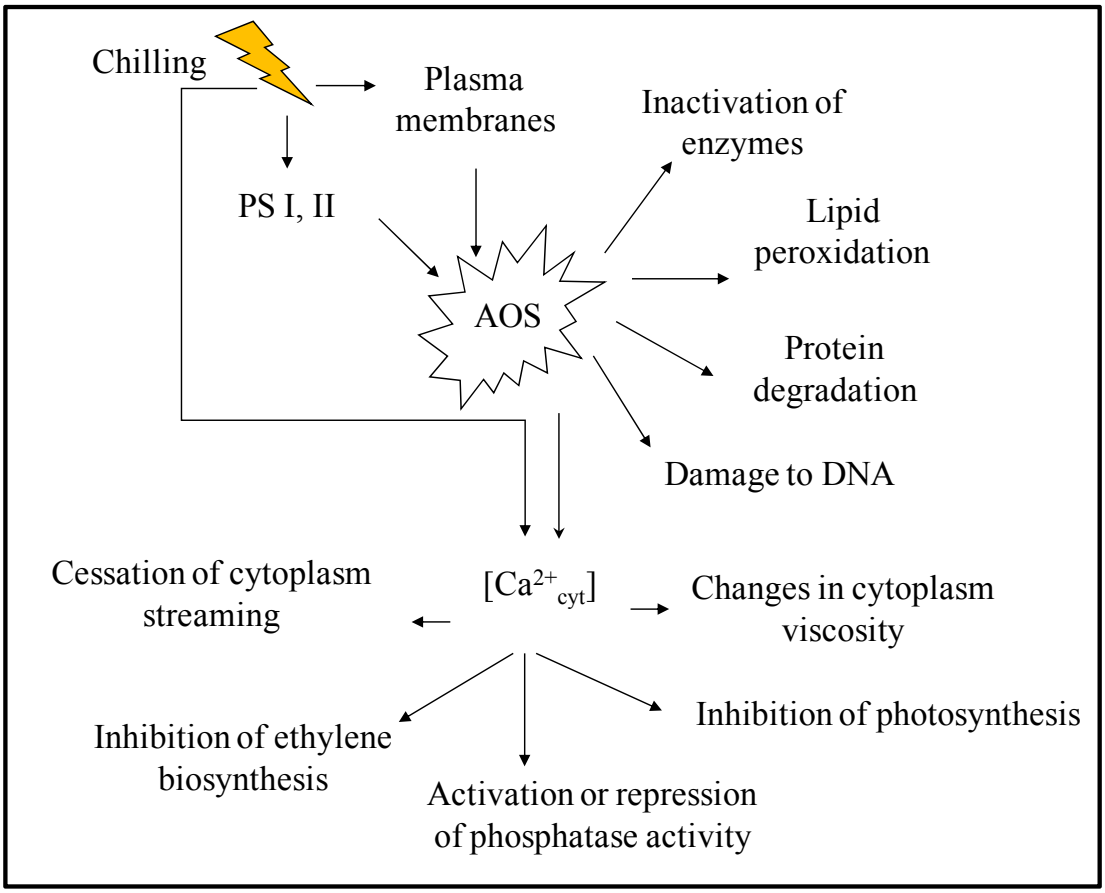

Fig. 1.3: Ca^{2+} and activated oxygen species (AOS) responses to chilling by sensitive plants (adapted from Raison & Lyons, 1986; McKersie & Lesham, 1994; Asada & Takahashi, 1987; Pei *et al*., 2000).

changes in cytoplasm viscosity resulting to several physiological changes, such as inhibition of ethylene biosynthesis, inhibition of photosynthesis and activation or repression of phosphatase activity leading to a change in the phosphorylation stature of another protein. These processes provide further signal transduction pathways for chilling responses (Fig. 1.3) (Hughes & Dunn, 1996).

Changes in cytosolic Ca^{2+} are intimately related to oxidative stress which also contributes significantly to chilling damage (Omran, 1980; Wise & Naylor, 1987; Prasad *et al*., 1994). Oxidative stress occurs in situations where the formation of activated oxygen species (AOS) exceeds their destruction and they begin to accumulate. Lyons (1973) proposed that altered membrane properties during chilling

stress, and following increased activation energy of membrane-bound enzymes, may lead to metabolic imbalance and accumulation of toxic metabolites. Activated oxygen species may form a significant proportion of these toxic molecules and it has been proposed that secondary chilling injuries in particular are mediated by them (Raison & Lyons, 1986). Prolonged presence of high levels of AOS, by activated formation of superoxide, singlet oxygen, hydrogen peroxide or hydroxyl radicals (McKersie & Lesham, 1994), is detrimental since they are highly reactive and may cause inactivation of enzymes, lipid peroxidation, protein degradation and damage to DNA (Asada & Takahashi, 1987). Several sites for subcellular production of AOS have been documented, the most well known being light reactions of photosystems I and II (PSI and PSII) in the chloroplast and mitochondrial electron transport (Elstner, 1991). In addition to spontaneous formation of AOS as a side-product of metabolic activities, active production of AOS takes place through NADPH oxidases in plasma membranes (McKersie & Lesham, 1994). Therefore, more serious damage is observed by AOS when plants are exposed to low temperature in combination with high light intensities (Inzé & van Montagu, 1995). Further evidence indicates that H_2O_2 and Ca^{2+} are both involved in a signaling cascade leading to the closure of stomata in *Arabidopsis* (Pei *et al*., 2000).

1.2.1.3 Photosynthesis and Chilling Temperatures

When low temperature events physically and metabolically restrict the demand for carbon, supply exceeds demand and photosynthesis is down regulated to correct this balance. This down regulation is affected by reduced RuBPC activity or reduced rate of RuBPC regeneration. The photosystems are the primary targets for chilling-induced photoinhibition (Cavaco *et al*., 2003). Slower enzymatic reactions of the

thylakoid and carbon metabolism at cool temperatures can lead to a down-regulation of the efficiency of PSII electron transport as a result of increased quenching of excitation energy by the xanthophyll cycle and other processes in the antennae (Allen & Ort, 2001). In some chilling-sensitive plant species inhibition of photosynthetic electron transport may occur, despite relatively minimal reductions in variable to maximal fluorescence ratio (Fv/Fm[1]) due to photoinactivation of PSI rather than PSII (Sonoike, 1999; Hendrickson *et al*., 2004) when leaf temperature drops to around 10°C. High light and low temperature may increase the net damage of PSII as well as slow down the repair processes responsible for recycling non-functional reaction centres (Krause, 1994; Long *et al*., 1994). Under most circumstances, the aggregate effect of these and other protective processes, coupled with a considerable capacity for repair, actually prevents chronic photoinhibition by excess irradiance. However, photoinhibition is frequently exacerbated by low temperature in plant species evolutionarily adapted for growth in warm climates (Long *et al*., 1994). In addition, highly chilling-sensitive herbaceous species such as tomato (*Lycopersicon esculentum*) experience dysfunction that is not attributable to PSII damage. The persistence of an inhibition of net CO_2 assimilation rate following the chill in tomato arises from the inability of the chilled plants to light-activate fructose 1,6-bisphosphatase and sedoheptulose 1,7-bisphosphatase, two key enzymes of the photosynthetic carbon reduction cycle (Hutchison *et al*., 2000). In addition to photosystems, chilling temperatures can limit photosynthesis *via* stomatal closure, inhibition of thylakoid electron transport and photophosphorylation, RuBPC inactivation, inhibition of key enzymes in sucrose and starch biosynthesis, and phloem loading (Allen & Ort, 2001; Huang & Guo, 2005).

[1] Fv/Fm is a parameter widely used to indicate the maximum quantum efficiency of PS II. Fv/Fm is presented as a ratio of variable fluorescence (Fv) over the maximum fluorescence value (Fm).

Low temperature-induced stress also limits the growth of grapevine (Buttrose, 1969). However grapevine leaves in the field remain relatively resistant to low temperature-induced net photoinactivation of PSII based on sustained, high variable chlorophyll fluorescence (Flexas *et al*., 2001; Hendrickson *et al*., 2003; 2004). This implies that one or more highly efficient energy dissipation mechanism(s) are induced in grapevine leaves by the combination of low temperature and high irradiance.

1.2.2 Mechanisms of acclimation to low non-freezing temperatures

Exposure of plants to low non-freezing temperatures leads to a number of transient biochemical processes, any of which could act as perception points for initiation of the signalling cascades also to elicit the stable developmental responses to chilling or freezing temperatures (Smallwood & Bowles, 2002; Zhu *et al.*, 2007). This phenomenon is known as acclimation. By chilling acclimation, plants of tropical or subtropical origin may increase chilling resistance by exposure to a moderate temperature such as 14°C for several days (Cabané *et al*., 1993; Prasad *et al*., 1994; Chang *et al*., 2001) (Fig. 1.4). Similarly, by cold acclimation, insensitive plants to low non-freezing temperatures may induce freezing tolerance after exposure at 4°C (Steponkus, 1984; Thomashow, 1999; Xin & Browse, 2000; Browse & Xin, 2001). Although it was suggested that chilling and cold acclimation are mechanisms with quantitative and temporal differences (Provart *et al.,* 2003), a few similarities can be observed.

The main mechanisms that have been extremely involved in the phenomenon of chilling or cold acclimation are related to physiological and molecular modifications in plant membranes, accumulation of cytosolic Ca^{2+}, accumulation of

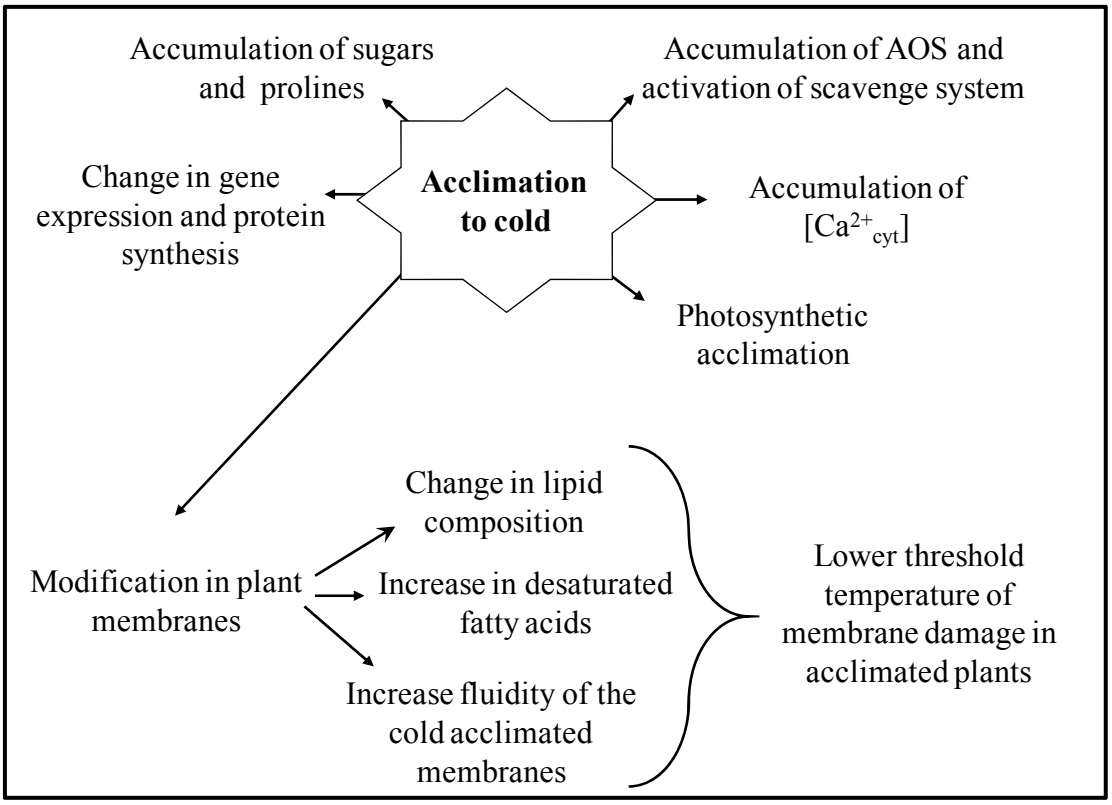

Fig. 1.4: Acclimation to cold induces changes in many different cellular processes (adapted from Hellergren *et al.,* 1983; Cabané *et al*., 1993; Prasad *et al*., 1994; Uemura & Steponkus, 1999; Thomashow, 1999; Xin & Browse, 2000; Chang *et al*., 2001; Browse & Xin, 2001).

AOS and activation of scavenge system, sugars and proline accumulation, biochemical alteration in photosynthesis, changes in cold related genes, transcription factors and alteration in protein synthesis (Fig. 1.4).

1.2.2.1 Modifications in plant cell membranes

During periods of chilling/cold acclimation, plant membranes undergo both qualitative and quantitative modifications (Uemura & Steponkus, 1999; Xin & Browse, 2000) (Fig. 1.4). The lipid composition of the plasma membranes and chloroplast envelopes changes in a way that the threshold temperature of membrane damage is lowered when compared to non-acclimated plants (Uemura & Steponkus,

1999). This is due to the increasing fluidity of the cold-adapted membranes, which results from a change in lipid composition towards an increase in desaturated fatty acids (Vogg *et al.,* 1998). Due to alterations in lipid membrane components, the protein fraction in these membanes also changes. The lipid-protein ratio of thylakoid membranes increases during adaptation to low temperatures (Vogg *et al.*, 1998), as well as the activity of plasma membrane H+-ATPase (Hellergren *et al.,* 1983). Lipid composition strongly influences the stability of the membrane in cold and the effect can be explained from the viewpoint of alterations in the lyotropic characteristics of the membrane (Steponkus *et al.,* 1993).

Stability of the plasma membrane is also affected by cytosolic and other membranous factors. For example because the occurrence of some of the freeze-induced lesions associated with the plasma membrane requires the participation of some endomembranes (Webb *et al.,* 1994), it is expected that the stability of the endomembranes also influence the stability of the plasma membrane in cold. In addition, since many studies have demonstrated a protective effect of sugars on the stability of membranes or lipid bilayers during freezing or dehydration (Heber *et al.,* 1981; Strauss & Hauser, 1986; Crowe *et al.,* 1988; Crowe & Crowe, 1993), it is suggested that an accumulation of cytosolic sugars during cold acclimation may act to increase the cryostability of the plasma membrane *in situ.* Furthermore *COR* genes, such as *COR15a* (see chapter 1.2.2.4.1), that are regulated by low temperatures and directly affect the freezing tolerance of plants may increase the cryostability of the plasma membrane (Artus *et al*., 1996).

1.2.2.2 Role of Cytosolic Ca^{2+} and AOS in acclimation to low temperatures

Cold stress evokes transient increases in intracellular Ca^{2+} level in plants (Knight *et al*., 1996; Polisensky & Braam, 1996; Lewis *et al*., 1997). These findings suggested that Ca^{2+} influx plays a major role in the cold stress response and also that an intracellular Ca^{2+} source might be involved in cold acclimation process. In addition, H_2O_2- activated Ca^{2+} channels mediated both the influx of Ca^{2+} in protoplasts and increases in cytosolic Ca^{2+} in intact guard cells (Pei *et al*., 2000). Recent studies on the regulation of cold responsive genes have investigated the role of Ca^{2+} in the signal transduction cascade. At the incidence of low temperature or oxidative stress, a mobilization of intracellular Ca^{2+} has been recorded (Price *et al*., 1994). Cytosolic Ca^{2+} signalling is also required for the induction of cold related genes during cold acclimation (Monroy & Dhindsa, 1995; Tähtiharju *et al*., 1997). In addition to serving as a link in a signalling cascade, the fluctuation of cytosolic Ca^{2+} could be one of the mechanisms that leads plants to remember what they have suffered (Knight *et al*., 1996). This inference comes from the observation that *Arabidopsis* treated with either sublethal cold or H_2O_2 modifies its calcium signature in response to subsequent cold stress (Knight *et al.*, 1996) (Fig 1.5).

Over the last decades, it has become evident that exposure of plants to low non-freezing temperatures increases tolerance in plants with an increase in antioxidant enzymes, protecting cellular membranes and organelles from damaging effects of AOS (Foyer *et al*., 1991; Anderson *et al*., 1995; Scebba *et al*., 1998; 1999; Lee & Lee, 2000; Kuk *et al*., 2003). The role of AOS in abiotic stress management has become a subject of considerable interest, given that relatively low levels have been implicated in processes leading to plant stress acclimation (Prasad *et al*., 1994; Doke *et al*., 1994; Foyer *et al*., 1997; Lopez-Delgado *et al*., 1998; Dat *et al.*, 1998; Karpinski *et al*.,

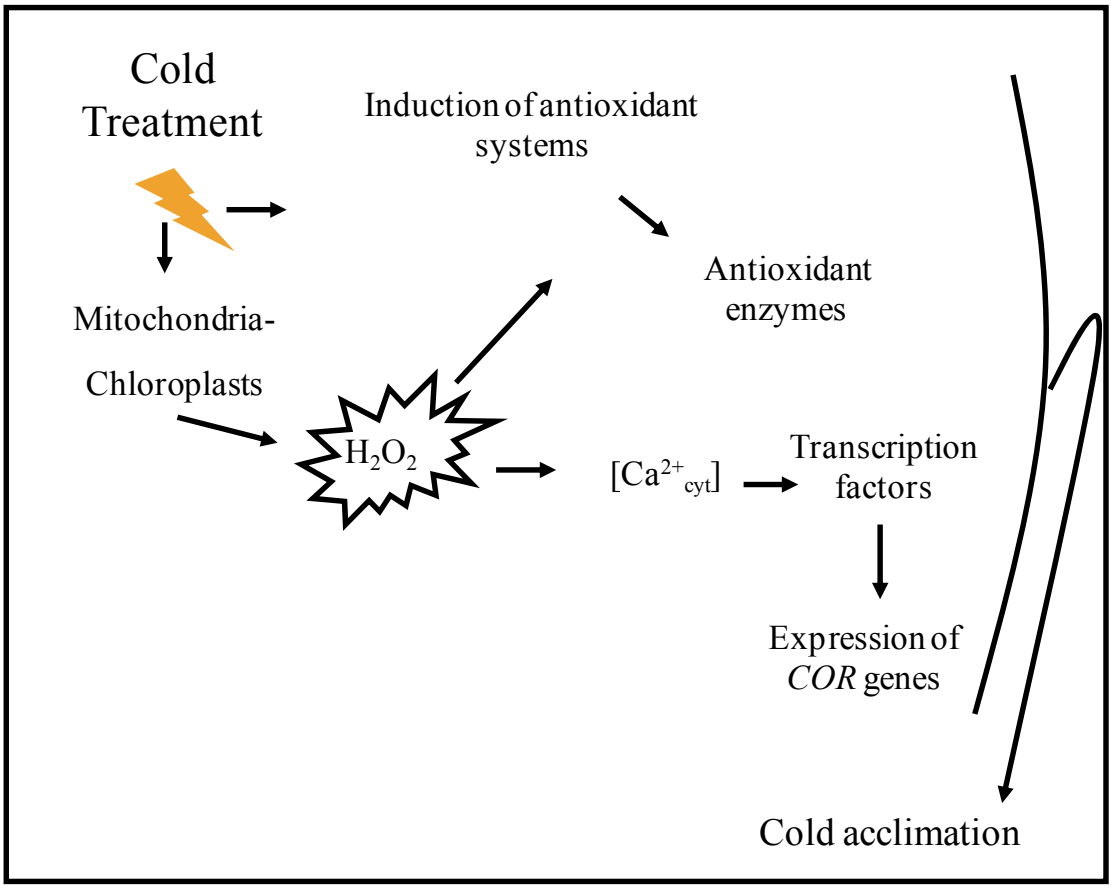

Fig. 1.5: Role of $[Ca^{2+}_{cyt}]$ and H_2O_2 in acclimation to low temperatures (adapted from Price *et al*., 1994; Monroy & Dhindsa 1995; Tähtiharju *et al*., 1997; Pei *et al*., 2000).

1999; Dat *et al*., 2000). This finding means that AOS are not simply toxic by-products of metabolism but also function as signalling molecules (Foyer *et al*., 1997; van Camp *et al*., 1998). Among AOS, H_2O_2 seems best suited to play the role of signalling molecule due to its higher stability and longer half-life. If H_2O_2 serves as a stress signal, the fluctuation of H_2O_2 levels in plants should spatially and temporally reflect changes in the environment. Indeed, an oxidative burst is a common response to both biotic and abiotic stresses (Desikan *et al*., 2004). AOS levels peaked during chilling of non-acclimated maize seedlings, while it is further reported that maize seedlings pretreated with H_2O_2 acquire additional chilling tolerance as compared with control

plants (Prasad *et al*., 1994). These results suggest that chilling-induced AOS appears as a message to induce antioxidative systems in cells. Nowadays, increasing evidences support the idea that AOS also function as signalling molecules that modulate the expression of various genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production (Neill *et al*., 2002).

1.2.2.3 Accumulation of cryoprotectants

During exposure to low non-freezing temperatures, accumulation of low molecular weight compounds is observed in numerous species and their distribution is species specific (Kushad & Yelenosky, 1987; Salerno & Pontis, 1989; Hekneby *et al*., 2005; Patton *et al*., 2007). Such compounds include the following:

a) proline

The accumulation of free proline is often associated with resistance of plants to numerous stresses and particularly low temperature (Ait-Barka & Audran, 1997; Dörffling *et al*., 1997; Wang *et al*., 2008). Proline has been suggested to play multiple roles in plant stress tolerance, as a mediator of osmotic adjustment (Yoshiba *et al*., 1997), a stabilizer of proteins and membranes (Chen & Li, 2002), and inducer of osmotic stress-related genes (Iyer & Caplan, 1998). Proline also acts as a scavenger of AOS (Saradhi *et al*., 1995), a readily available source of nitrogen and carbon (Brugière *et al*., 1999), and a source of reduction equivalents during recovery from stress (Hare & Cress, 1997). The positive correlations between the accumulation of endogenous proline and improved cold tolerance have been found mostly in chilling-insensitive plants, such as barley (Chu *et al*., 1978), rye (Koster & Lynch, 1992), winter wheat (Dörffling *et al*., 1997), grapevine (Ait-Barka & Audran, 1997), potato

(Swaaij *et al*., 1985; 1986) and *Arabidopsis thaliana* (Xin & Browse, 1998; Nanjo *et al*., 1999). Most chilling-sensitive plants, which also accumulate proline under chilling, did not acquire improved cold tolerance (Chu *et al*., 1978; Kushad & Yelenosky, 1987) unless a high concentration of proline was applied prior to stress (Duncan & Widholm, 1987; Xin & Li, 1993). It appears that proline may have the potential for alleviating chilling injury in chilling-sensitive plants, but for some reason this system fails under natural conditions.

Proline accumulation during osmotic stress like cold, is mainly due to increased synthesis and reduced degradation. Although proline transport certainly plays an important role in proline distribution, its role during stress has been poorly studied (Rentsch *et al.*, 1996). In plants, there are two different precursors for proline. The first pathway is from glutamate, which is converted to proline by two successive reductions catalyzed by pyrroline-5- carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), respectively (Hu *et al.*, 1992). An alternative precursor for proline biosynthesis is ornithine (Orn), which can be transaminated to P5C by Orn-d-aminotransferase (OAT), a mitochondrial located enzyme (Kavi Kishor *et al*., 2005). The proline degradation is the reverse process of proline biosynthesis and catalyzed by Pro dehydrogenase (PDH) and P5C dehydrogenase (P5CDH). Proline biosynthesis occurs in the cytosol and in the plastids (like chloroplasts in green tissues) while proline degradation takes place in mitochondria (Elthon & Stewart, 1981; Rayapati *et al*., 1989; Szoke *et al*., 1992).

b) carbohydrates

According to several studies, carbohydrate content also influences chilling sensitivity. For example, the decrease of sugars by dark treatment of cotton seedlings **(**Rikin *et*

al., 1981**)** and other chilling-sensitive species (King *et al*., 1982) prior to chilling increased the severity of chilling injury. Also, the reducing sugar content in grapefruit peel correlated with decreased chilling sensitivity of the fruits (Purvis *et al*., 1979; Purvis & Grierson 1982). Treatment of rice seedlings with fructose or glucose prior to chilling increased their resistance to chilling injury (Tajima & Kabaki, 1981), while cotton cotyledon discs floated on a sucrose solution in the dark were less injured by chilling temperatures (Rikin *et al*., 1981). On the other hand, chilling resistance of tomato seedlings decreased when starch and sugar content fell markedly during the dark period (King *et al*., 1988**)**. According to the authors, increased concentrations of sugars were detected 15 min after the start of the light period, suggesting that changes in chilling sensitivity over the diurnal period are regulated by the light cycle. It also suggests that increased sensitivity at the end of the dark period could be due to carbohydrate depletion, and that chilling tolerance following light exposure is likely due to carbohydrate accumulation or closely related events.

Carbohydrates are also easily detectable in cold tolerant species (Pollock & Lloyd, 1987). Among them, carbohydrates like sucrose, sorbitol and raffinose were the first protective substances described in plants (Levitt, 1980). The oligosaccharides raffinose and stachyose are especially associated with cold hardiness, low temperature and dormancy, but sucrose also enhances cold hardiness and desiccation tolerance of buds in woody plants (Stushnoff *et al.,* 1997). Sucrose is the most easily detectable sugar in cold-tolerant species, increasing several folds during exposure to low temperature (Salerno & Pontis, 1989). If its accumulation is impeded, cold tolerance is lost (Guy *et al*., 1980). The soluble carbohydrate content of grasses for example can undergo a 10-fold increase within 8 h of transfer from a warm to a cold environment (Pollock, 1984).

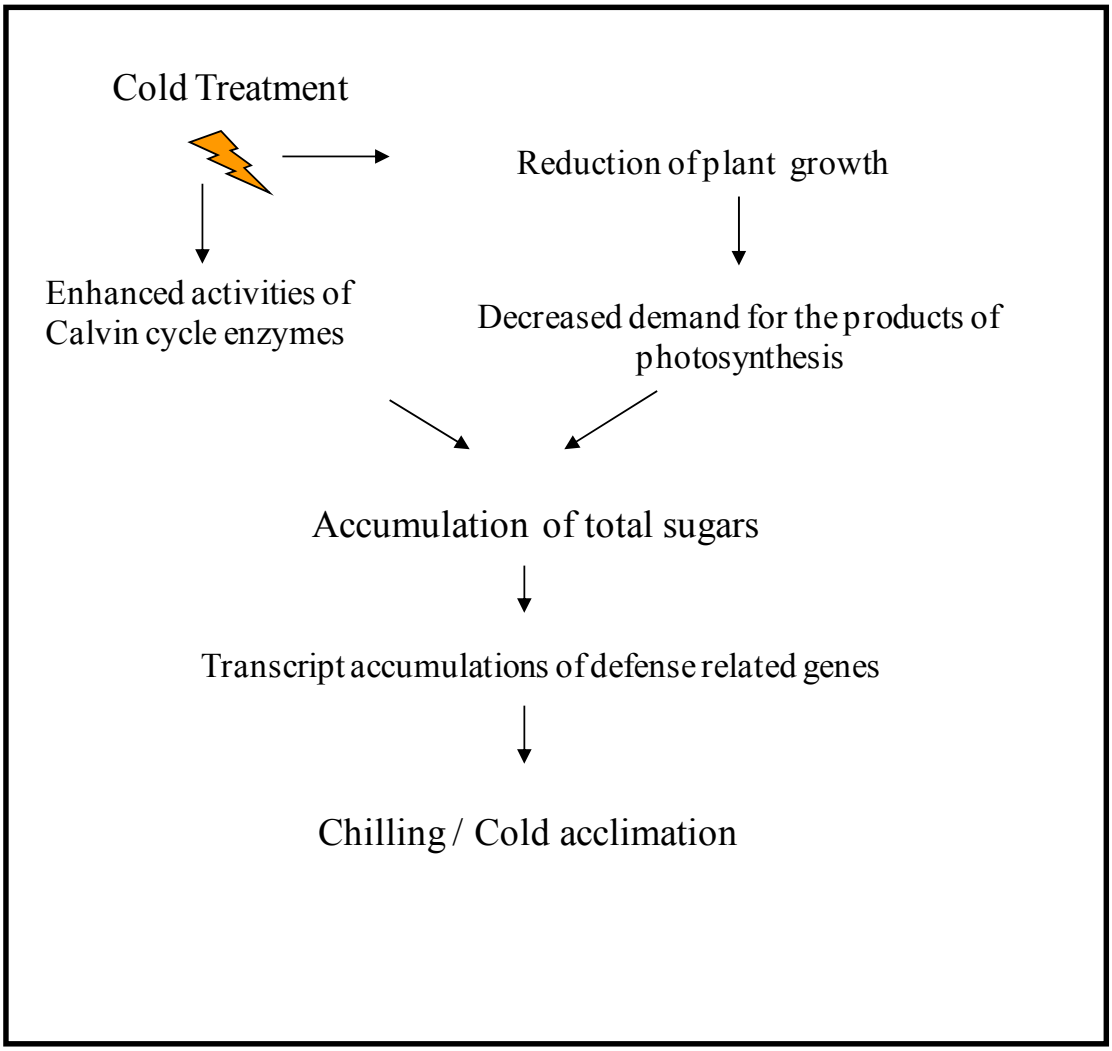

Fig. 1.6: Time scale of carbohydrates and transcript accumulations in cold acclimated plants (adapted from Fischer & Höll, 1991; Sasaki *et al*., 1996; Ögren *et al*., 1997; Greer *et al*., 2000).

In temperate perennials and many herbaceous species, when starch concentrations decrease, the concentrations of soluble sugars increase in cold acclimating tissues (Fischer & Höll, 1991; Ögren *et al.,* 1997; Greer *et al.,* 2000). During exposure to low-temperatures, starch content typically declines by hydrolysis, and free saccharides exhibit a direct quantitative increase (Sakai &Yoshida, 1968;

Pollock & Lloyd, 1987). Although some of these responses to low temperature could be viewed as incidental consequences that lead to shifts in carbohydrate status, the breakdown of starch in many species at precisely the same time with soluble sugars accumulation suggests a more important purpose.

However, an increase in both soluble sugars and starch has been observed during cold acclimation (Fig. 1.6). For example, in cabbage seedlings exposed to non-freezing low temperature (5°C), starch and all soluble sugars in leaves (except *myo*-inositol) increased gradually during cold acclimation (Sasaki *et al*., 1996). However, the induced freezing tolerance was lost after only 1 d of acclimation at control temperatures, and this change was associated with a large reduction in sugar content.

In grapevine, with the onset of cool autumn temperatures, carbohydrate metabolism shifts from production of disaccharides (sucrose) to monosaccharides (fructose and glucose) and raffinose (Hamman *et al.,* 1996; Ait Barka & Audran, 1996). The peak of raffinose production in the dormant vine occurs at midwinter, precisely when the vine is at its peak of cold acclimatization (Ait Barka & Audran, 1996). It appears that raffinose in water solution acts as a cryoprotectant or natural antifreeze inside the cell. It lowers the freezing point of the cellular water and prevents it from freezing at all but extreme temperatures (Hamman *et al*., 1996). In addition to their role as cryoprotective components for plants, carbohydrates may have a nutritional role during acclimation to low non-freezing temperatures (Trunova, 1982) and also during recovery from freezing-stress (Eagles *et al*., 1993).

Carbohydrate accumulation at low temperatures may be explained through the photosynthesis process (Fig. 1.6). At low temperatures, the photosynthetic energy capture is reduced to a lesser degree than the metabolic utilization processes and this leads to surplus-reducing potential in the form of pyridine nucleotides (NADH,

NADPH), and ATP production (Levitt, 1980), which can be channelled into CO_2 fixation processes. In this case, active growth is almost always reduced or suspended resulting in decreased demand for the products of photosynthesis and finally their accumulation. Alternatively, carbohydrate accumulation at low temperatures may be explained through the activation of specific enzymes (Hurry *et al*., 1995). According to this, even if low temperatures lead to the inhibition of sucrose synthesis and photosynthesis, the biochemical and physiological adaptation to low temperatures include the post-translational activation and increased expression of enzymes of the sucrose synthesis pathways and the changed expression of enhanced activities of Calvin cycle enzymes (Savitch *et al*., 1997*;* Stitt & Hurry, 2002) and, in particular, with enhanced activities of the cytosolic fructose-1,6-bisphosphatase, sucrose phosphate synthase and sucrose synthase (Hurry *et al*., 1994).

The time-scale of increase in freezing tolerance and stress-gene-expression in plants is much longer (Dunn *et al*., 1994; Pearce *et al*., 1996), indicating that changes in sugar supply may precede acclimation and cold-induced gene expression. In *Arabidopsis thaliana*, which acclimates much more rapidly than barley, sugar accumulation is detectable within 2 h from transfer to cold, when the increase in stress-gene expression is only just detectable (Fig. 1.6), and precedes measured increase in freezing tolerance (Wanner & Juntilla, 1999).

There could be a causal connection between the accumulation of sugars and freezing-tolerance, because providing soluble carbohydrates to plants or cultured cells induces freezing tolerance (Steponkus & Lanphear, 1967; Tumanov *et al*., 1968; Leborgne *et al*., 1995; Travert *et al*., 1997). Soluble carbohydrates could function directly to confer freezing tolerance through colligate and non-colligate effects; even

if it does not necessary indicate that they have a regulatory role (Levitt, 1980; Crowe *et al*., 1992; Travert *et al*., 1997).

1.2.2.4 Expression of cold/chilling related genes and the role of *CBF* transcription factors

Plant acclimation to low temperatures causes changes in the function of several cold/chilling related genes and proteins (Thomashow, 1999; 2001). The process can involve the modification of pre-existing proteins and the up- and down-regulation of gene expression or protein synthesis. New gene expression and protein synthesis has also been observed during acclimation. Many cold-induced proteins and genes have been studied in several plant species (Guy, 1990; Howarth & Ougham 1993; Hughes & Dunn 1996; Thomashow, 1999). According to these studies, it is suggested that cold/chilling induced gene activity may aid in the metabolic adjustment to low non-freezing temperatures or confer freezing tolerance to tissues (Guy, 1990). Stress-induced genes may also be involved in the signal transduction of the stress-response (Ingram & Bartels, 1996; Thomashow, 1999).

1.2.2.4.1 Changes in cold/chilling related gene expression

Nowadays, a wide variety of cold regulated genes (*COR*) has been isolated from cold-acclimated plants (Svensson *et al*., 2006). The cloned genes can be classified into those whose protein products function directly in protecting against environmental cold/chilling stress and in those which regulate gene expression during adaptation response (Shinozaki & Yamaguchi-Shinozaki 1996; Fowler & Thomashow, 2002) (Fig. 1.7). Further classification divides gene products into those which mediate

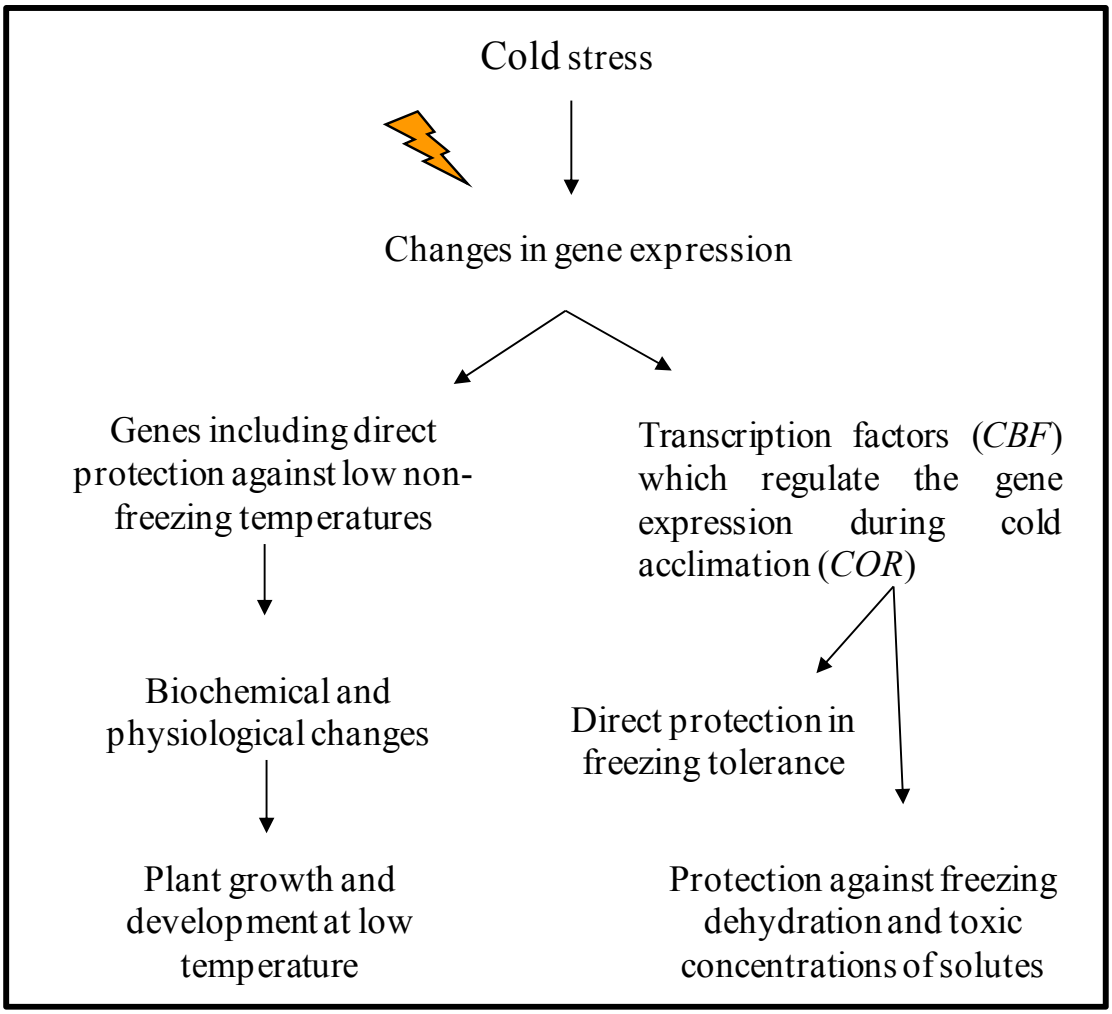

Fig. 1.7: Classification of cloned genes in chilling/cold acclimation (adapted from Shinozaki & Yamaguchi-Shinozaki, 1996; Thomashow, 2001; Fowler & Thomashow, 2002).

biochemical and physiological changes required for growth and development at low temperatures and those whose gene products have a direct role in chilling and freezing tolerance (Thomashow, 1998). During cold acclimation, the battery of *COR* gene products accumulates simultaneously with increased freezing tolerance. A common characteristic that these products share, is the property of being very hydrophilic and remaining soluble upon boiling (Thomashow, 1998). They also have relatively simple amino acid compositions and sequence motifs that are repeated several times.

However, the role of *COR* products in cold acclimation has been clarified only for a few genes. One of the most intensively studied cold-responsive genes is *COR15a*, the gene product (a small, plastid-targeted polypeptide that is processed to a mature form called *COR15am*) which is targeted to chloroplasts during cold acclimation (Thomashow, 1994). Over-expression of *COR15a* in transgenic plants resulted in increased cryostability of plasma membranes (Artus *et al.*, 1996) but had no effect on the freezing survival of whole plants (Jaglo-Ottosen *et al*., 1998).

In contrast to cold-acclimation and freezing tolerance, much less is known about molecular changes affecting regulatory and biochemical mechanisms triggered to optimize growth at low but above-freezing temperatures. Transcriptome of *Arabidopsis* under normal (22°C) and chilling (13°C) conditions by Provart *et al.* (2003) have surveyed the molecular responses of a chilling-resistant plant to acclimate to a moderate reduction in temperature. The mRNA accumulation of approximately 20% of about 8,000 genes analyzed was affected by chilling. In particular, a highly significant number of genes involved in protein biosynthesis displayed an increase in transcript abundance. The mRNA accumulation profiles for the chilling-lethal mutants were highly similar and included extensive chilling-induced and mutant-specific alterations in gene expression. The expression pattern of the mutants upon chilling suggests that the normal function of the mutated loci prevents a damaging widespread effect of chilling on transcriptional regulation. This reference gene list, including genes related to lipid metabolism, chloroplast function, carbohydrate metabolism and free radical detoxification, represents a potential source for genes with a critical role in plant acclimation to suboptimal temperatures (Provart *et al*., 2003).

1.2.2.4.2 Role of *CBF* trascription factors in cold acclimation

The signal transduction pathways leading to expression of *COR* involves a regulatory network, where a few regulatory genes control genes involved in the cold response (Shinozaki & Yamaguchi-Shinozaki, 2000; Fowler & Thomashow, 2002) (Fig. 1.8). An attempt to isolate a regulatory element responsible for the initiation of the *COR*-gene transcription at low temperatures has been performed mainly in *Arabidopsis* (Heather *et al*., 2006). *COR* genes are also expressed at warm temperature with the influence of the *CBF* (C-repeat Binding Factor) family of transcriptional activators (Stockinger *et al*., 1997; Gilmour *et al*., 1998; Skinner *et al*., 2005), also known as *DREB1* (Dehydration Response Element Binding) proteins (Liu *et al*., 1998; Shinwari *et al.,* 1998). CBF /DREB-regulated *COR* genes contain in their promoters a cold- and dehydration-responsive DNA regulatory element known as the *CBF/DRE* (Baker *et al*., 1994; Shinozaki &Yamaguchi-Shinozaki, 1996). The genes *CBF1*, *CBF2*, and *CBF3* (also known as *DREB1b*, *DREB1c*, and *DREB1a*, respectively), are located in tandem on chromosome 4 (Gilmour *et al*., 1998; Shinwari *et al*., 1998). Overexpression of *CBF1* in *Arabidopsis* was subsequently shown to activate expression of the entire battery of known *CBF/DREB* regulated *COR* genes and to enhance whole plant freezing survival without a low temperature stimulus (Jaglo-Ottosen *et al*., 1998). Additional studies have shown that *CBF1* is a member of a small gene family encoding nearly identical proteins (Gilmour *et al*., 1998; Shinwari *et al*., 1998). Overexpression of *CBF3* in *Arabidopsis*, like overexpression of *CBF1*, activates *COR* gene expression and enhances freezing tolerance at warm nonacclimating temperatures (Liu *et al*., 1998; Kasuga *et al*., 1999). Furthermore, the homolog of the CBF/DREB1 proteins *CBF4* (up-regulated by drought stress, but not by low temperature), overexpressed in transgenic *Arabidopsis* plants results

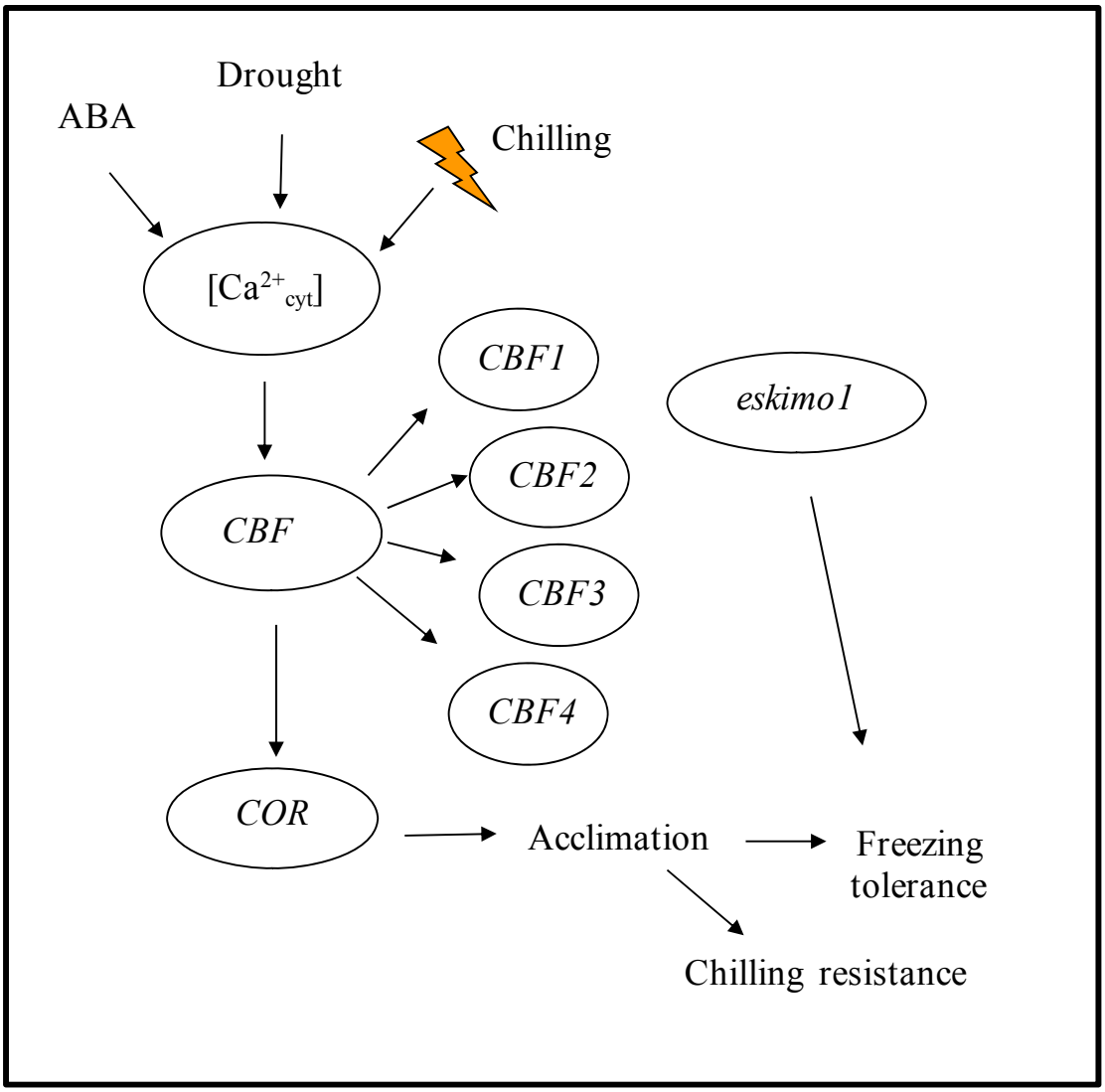

Fig. 1.8: Role of CBF trascription factors in cold/chilling acclimation (adapted from Liu *et al*., 1998; Gilmour *et al*., 1998; Shinwari *et al*., 1998; Kasuga *et al*., 1999; Shinozaki & Yamaguchi -Shinozaki, 2000; Fowler & Thomashow, 2002).

in the activation of C-repeat/dehydration-responsive element containing downstream genes that are involved in cold acclimation (Haake *et al*., 2002).

Although, it is currently unknown whether molecular mechanisms for cold acclimation and acclimation to chilling temperature are related, current data suggest that heterologous *CBF1* expression can also increase the resistance of plants to chilling **(**Hsieh *et al*., 2002). All three *CBF* genes are cold-induced. Indeed, *CBF*

transcript levels increase within 15 min after transferring plants to low temperature followed at approximately 2 h by accumulation of transcripts for the target *CBF/DRE*-regulated *COR* genes (Mantyla *et al*., 1995). The accurate mechanism whereby the *CBF* genes are activated by low temperatures does not involve autoregulation (Gilmour *et al*., 1998). A few years ago, it was shown that cold induction of the three *CBF* genes is controlled by a set of redundant and interacting transcription factors (Zarka *et al*., 2003; Chinnusamy *et al*., 2003). Some of these transcription factors cross-regulate each other (Novillo *et al*., 2004; Agarwal *et al*., 2006). The cold-induction of *CBFs* is also controlled by circadian clock (Fowler *et al*., 2005).

Many of these genes are also induced by abscisic acid (Knight *et al*, 2004) or by dehydration (Shinozaki & Yamaguchi-Shinozaki, 2000), which is consistent with the fact that these two processes may increase freezing tolerance when overexpressed in transgenic plants (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998) (Fig. 1.8). However, the existence of *CBF*-parallel pathways involved in cold-acclimation has been supported by transcription profiling of plants overexpressing the three members of the *CBF* family (Fowler & Thomashow, 2002). On the other hand, the *Arabidopsis* mutant *eskimo1* displays freezing tolerance in the absence of cold treatments without changes in expression of the components of the *CBF*-pathway but with high level of accumulated proline suggesting that distinct signalling pathways activate different aspects of cold acclimation and that activation of one pathway can result in considerable freezing tolerance without activation of other pathways (Xin & Browse, 1998).

Although all these studies have increased the knowledge of the molecular basis of cold acclimation and *CBF* pathway in herbaceous species, very little is known concerning woody plants. Published information on a *CBF* gene from woody plants

are the functional analysis of *PaCBF* from *Prunus avium* (Kitashiba *et al*., 2004) and the *EguCBF1a* and *EguCBF1b* isolated from *Eucalyptus gunnii* (El Kayal *et al*., 2006).

Three *CBF/DREB1*-like genes, *CBF 1-3*, have also been isolated from both freezing-tolerant wild grape (*V. riparia*) and freezing-sensitive cultivated grape (*V. vinifera*) (Xiao *et al*., 2006). Expression of the endogenous *CBF* genes was low at ambient temperature and enhanced upon low temperature (4°C) treatment, first for *CBF1*, followed by *CBF2*, and about 2 d later by *CBF3*. No obvious significant difference was observed between *V. riparia* and *V. vinifera* genes. The expression levels of all three *CBF* genes were higher in young tissues than in older tissues. *CBF1, 2* and *3* transcripts also accumulated in response to drought and exogenous abscisic acid (ABA) treatment. More recently, the isolation of another CBF*/DREB1*-like gene has been reported. *CBF4* has been isolated from both freezing-tolerant wild grape (*V. riparia*) and freezing-sensitive cultivated grape (*V. vinifera*) (Xiao *et al*., 2008). Expression of the endogenous *Vitis CBF4* genes was low at ambient temperature, but enhanced upon exposure to low temperature (4°C). Uncommon for *CBF* genes, this expression was maintained for several days. No significant difference in expression pattern was observed between *V. riparia* and *V. vinifera*. *Vitis CBF4* was expressed in both young and mature tissue, in contrast to the previously described *Vitis CBF1-3*. Altogether, these results suggest that *CBF4* represents a second type of *CBF* in grape that might be more important for the over-wintering of grapevine plants.

A question thus raised is whether the *CBF* transcription factors are limited to activating the expression of *COR* genes encoding cryoprotective polypeptides, or alternatively, have a role in activating multiple components of the cold acclimation response. Overexpression of *CBF3* in *Arabidopsis* results in multiple biochemical

changes associated with elevation of level of proline and total soluble sugars, including sucrose, raffinose, glucose, and fructose (Gilmour *et al*., 2000). Accumulation of proline and soluble sugars also occurred in non-acclimated transgenic plants that overexpressed the *CBF-1* and *CBF-2* (Gilmour *et al*., 2004). These results lead to conclusion that *CBF* integrates the activation of multiple components of the cold acclimation response.

1.2.2.5 Expression of homology to pathogenesis-related (PR) genes and synthesis of antifreeze proteins (AFPs)

Several cold-induced genes have been studied in several plant species encoding several proteins like cryoprotective proteins (Hincha *et al.,* 1990). In the last decades, research has been focused on specific proteins with antifreeze activity that accumulate in apoplast upon cold acclimation, offering plant resistance against freezing (Griffith *et al*. 1992; Yaish *et al*., 2006) (Fig. 1.9). These proteins have been found in many overwintering vascular plants (Urrutia *et al*., 1992; Duman & Olsen, 1993; Doucet *et al*., 2000; Zamani *et al*., 2003). Antifreeze activity is present in overwintering plants only after they have been exposed to low temperatures and only in plants that tolerate the presence of ice in their tissues (Griffith & Yaish, 2004).

AFPs have been isolated and characterized from the apoplast of winter rye leaves (Huang & Duman, 2001). These proteins were identified as β-1,3-glucanase-like proteins, chitinase-like proteins, and thaumatin-like proteins (Hon *et al*., 1995) and as polygalacturonase inhibitor proteins (Meyer *et al*., 1999; Worrall *et al*., 1998). Antifreeze activity has been observed in different parts of overwintering plants (Urrutia *et al*., 1992; Duman & Olsen, 1993; Doucet *et al*., 2000). AFPs are localized

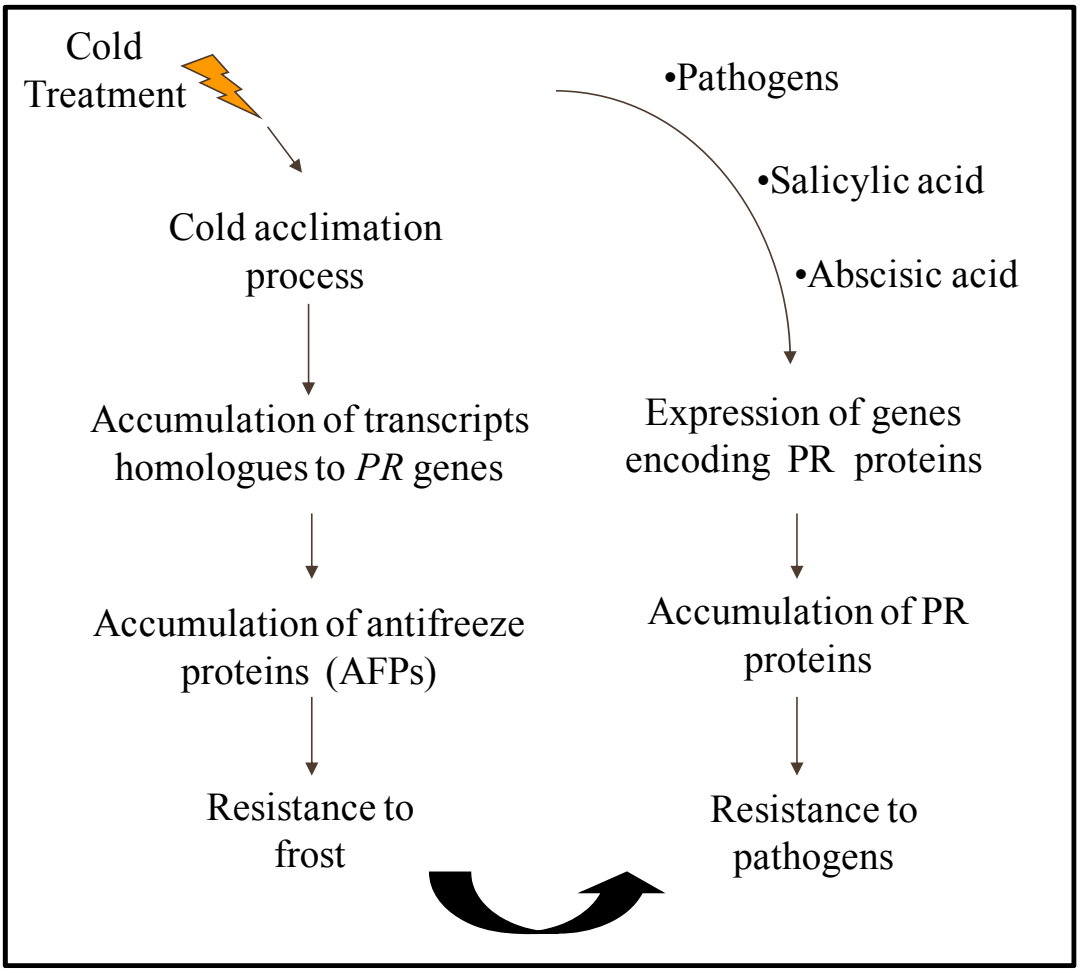

Fig. 1.9: Accumulation of antifreeze proteins (AFPs) in cold-acclimated plants with antifungal activity (adapted from Griffith *et al*., 1992; Urrutia *et al*., 1992; Hon *et al*., 1995; Yeh *et al*., 2000; Huang & Duman, 2001; Yaish *et al*., 2006).

in the epidermis and in cells surrounding intercellular spaces in cold-acclimated plants. Although they were present in non-acclimated plants, they were found in different locations and did not exhibit antifreeze activity, which suggests that different isoforms of pathogenesis-related proteins are produced at low temperature (Antikainen *et al*., 1996). Until now, no plant has been reported to have constitutive antifreeze activity. Rather all studies have shown that transcripts and translation products of *AFP* genes accumulate during cold acclimation (Griffith *et al*. 1992; Urrutia *et a*l., 1992; Hon *et al*., 1995; Yeh *et al*., 2000; Huang & Duman, 2001). The

conditions used for cold acclimation mimic autumn when days become shorter and colder. Therefore, low temperature and day length are important environmental cues for AFP production (Marentes *et al*., 1993).

Similar proteins are known to be related to the mechanism of plant disease resistance and are therefore classified as PR proteins (Stintzi *et al*., 1993; Ryals *et al*., 1996) (Fig 1.9). PR proteins are released into the apoplast in response to pathogen infection and act together to degrade fungal cell walls enzymatically and inhibit fungal enzymes. Psychrophilic pathogens such as snow molds prosper under snow cover where the temperatures are nearly constant, the humidity is high (Gaudet *et al*., 2000; Snider *et al*., 2000), and it is difficult for host plants to mount a *de novo* defence at subzero temperatures. By accumulating PR proteins during cold acclimation, overwintering grasses and cereals acquire a systemic, nonspecific, pre-emptive defence against these pathogens and exhibit greater disease resistance (Gaudet *et al*., 2000; Tronsmo, 1993a,b; Hiilovaara-Teijo *et al*., 1999). In winter rye, the AFPs exhibit antifungal, hydrolytic activities and ice-binding activity (Hiilovaara-Teijo *et al*. 1999). Therefore, cold-acclimated plants are more resistant to injury caused either by snow molds or by freezing (Hiilovaara-Teijo *et al*., 1999). Genetic studies have shown that there is genotypic correlation between freezing tolerance and snow mold resistance indicating that the same genetic traits are involved in these two physiological processes (Tronsmo, 1993b). Although, the PR proteins induced in winter rye plants infected by pathogens or treated with salicylic acid or abscisic acid in warm climate, lack antifreeze activity (Hiilovaara-Teijo *et al*., 1999; Yu & Griffith, 2001), the analysis of plant-microbe interaction could further clarify not only the phenomena about the responses of plants to cold, but also how this symbiotic relation could be beneficial for plant resistance to low temperatures.

1.3 Plant-microbe Interaction

In the field, plants interact with a large and diverse community of beneficial and deleterious microorganisms. This plant-microbe relationship very often leads to the establisment of a mutualistic or pathogenic interaction (Schippers *et al*., 1987). In general, plant-microbe interaction involves complex coordinated cellular processes that determine the beneficial or negative outcomes of this relationship. During the early stages of this association, there is a considerable communication between plant and microbe in which signal molecules play an essential role (Pozo *et al*., 2005).

In plants, 40% of carbon- and nitrogen-containing compounds produced by photosynthesis are released into the surrounding soil of roots (Lynch & Whipps, 1991). Microorganisms attracted by this nutritious environment, use the plant exudates and lysates for their growth and multiplication on the root surface and in adjacent rhizosphere soil (Lynch & Whipps, 1991). Many pathogenic fungi and bacteria can damage the plants, causing diseases that significantly contribute to the overall loss in crop yield worldwide (Strange & Scott, 2005; Savary *et al.,* 2006; Montesinos, 2007). On the other hand, beneficial microorganisms such as mycorrhizal fungi and many plant growth-promoting rhizobacteria (PGPR) can protect the plants against several adverse environmental stresses (Waller *et al*., 2005; Chandanie *et al*., 2006). In this extremely diverse rhizosphere microflora, a dynamic interplay exists between microorganisms mediated by synergistic and antagonistic interaction (Garbeva *et al*., 2004). These many different regulatory signals that are exchanged between fungi, bacteria and plant roots form effectively a highly dynamic below ground communication network (Hirsch *et al.*, 2003).

1.3.1 Plant-Pathogen Interaction

The large variety of sophisticated mechanisms involved in plant responses upon pathogen attack, can be divided in three classes according to distinct temporal and spatial expression patterns of different reactions observed in several systems (Kombrink & Somssich, 1995). The immediate response of plants occurring at the site of pathogen penetration is known as hypersensitive response (HR) (Kombrink & Schmelzer, 2001). The HR in directly invaded plant cells starts with recognition and transduction leading frequently to rapid cell death (Goodman & Novaky, 1994). Accompanied by a large set of biochemical changes (Atkinson, 1993), HR stimulates secondary metabolic pathways (Stoessl *et al*., 1976, Legrand, 1983), producing phytoalexins and phenolics, some of which are incorporated into the cell wall for its reinforcement (Dixon & Lamb, 1990). Many of these defence responses have been shown to result from transcriptional activation of defence genes (Lamb *et al*., 1989).

HR is accompanied by biochemical changes not only at the site of infection but also at distant sites in the plant (Madamanchi & Kuc, 1991; Sticher *et al*., 1997). The biochemical changes in the close vicinity of infection sites result in direct/indirect inhibition of pathogens. Indeed, the synthesis of numerous secondary products (Kombrink & Somssich, 1995) and the production of a broad range of defence related proteins with antimicrobial activity can be highly synthesized in the zone surrounding the pathogen infection (Stintzi *et al*., 1993). The phenomenon of induced resistance bordering pathogen infection zone is known as local acquired resistance (LAR) (Ross, 1961a).

In addition to HR and LAR, a systemic activation of genes encoding pathogenesis-related (PR) proteins is associated with a resistance beyond the LAR zone. This acquired resistance was called systemic acquired resistance (SAR) (Ryals

et al., 1996). Several studies have shown that SAR provides a significant level of resistance against a broad range of pathogens (Ryals *et al*., 1996; Jackson & Taylor, 1996; Durrant & Dong, 2004). Many of these plant defense responses have been extensively studied in elicitor-treated, cultured plant cells and have been found to be essentially the same in such simplified systems as in true plant/pathogen interaction (Hahlbrock *et al*., 1995; Somssich & Hahlbrock, 1998; Cordelier *et al*., 2003). A model in which pathogen restriction is achieved by the combined effects of HR, LAR and SAR, has been proposed by Dorey *et al.* (1997) under elicitation of tobacco leaves with glycoprotein elicitor (Fig. 1.10). According to the authors, the perception by plant cells of a pathogenic signal inducing an HR, leads to the death of those cells by accumulation of salicylic acid accumulating also the transcripts of early expressed genes but not those encoding PR proteins. SA production also occurs in zones surrounding HR cells. Because death is not induced in these cells, SA originated from the HR can accumulate high amounts of gene products, including PR proteins (Cordelier *et al.*, 2003). This endogenous signalling triggers the strong defense responses resulting in production of defense proteins and metabolites (LAR) (Dorey *et al*., 1998). This signal spreads systemically throughout the plant according to SAR. The synergistic reaction of HR, LAR and SAR induced in plant as defense reaction against pathogens are sufficiently effective to protect plants in many cases from deleterious organisms.

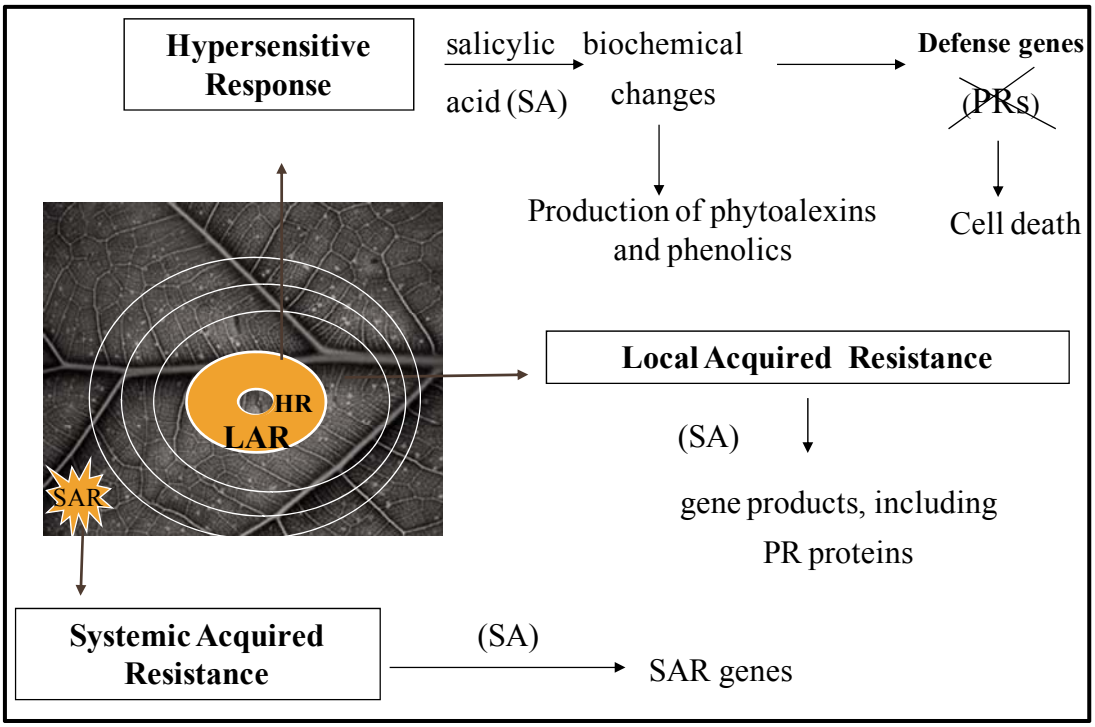

Fig. 1.10: Proposed spatio-temporal scheme of signaling events and metabolic alteration of hypersensitive reaction in tobacco leaves elicitated by glycoprotein (adapted from Dorey *et al*., 1997). The perception by plant cells of a pathogenic signal inducing an HR leads to the death of those cells. HR cells express defense genes leading to accumulation of SA. However, due to the timing of gene expression and cell death, sustained transcription and translation have time to proceed to some extent for early defense genes but not for others, such as *PR* genes. A similar sequence of defense gene induction and SA production also occurs in a narrow zone of cells surrounding the HR cells. Because cell death is not induced in narrow zone, the defense gene products can accumulate in high amounts. An endogenous signaling would originate from the HR cells and trigger the strong defense responses found in this zone: the resulting defense proteins and metabolites would contribute to LAR. According to the author, it is not clear whether LAR and SAR are mediated by different signals or different signal mechanisms. Indeed, a rapid decay of a single signal along the way of translocation could explain the occurrence of LAR and SAR. By this study, it is observed that elicitation by glycoprotein can stimulate the resistance of tobacco leaves with similar way as induced by infection with tobacco mosaic virus (TMV), proposing that a high degree of resistance occurred similarly in a zone surrounding to pathogen-induced HR, and that SAR occurs immediately beyond the LAR tissue (Ross, 1961a,b).

1.3.1.1 Systemic Acquired Resistance (SAR)

According to SAR, the infection of plants by pathogenic bacteria, viruses or fungi results in synthesis of a signal at the site of infection, that spreads systemically throughout the plant, leading to the expression of a broad-spectrum long lasting immunity in both infected and non-infected plant parts (Ryals *et al*., 1994; Jackson & Taylor, 1996). Initially, SAR was thought to be equally effective against many different pathogens, but few studies suggested intra-and interspecific variability about the type, the amount and the time course of the responses (Schneider & Ullrich, 1994; Botha *et al*., 1994; Schneider *et al*., 1996). SAR gene expression for example, was first detected in tobacco about six days after inoculation (Ryals *et al*., 1994), but as early as seven hours after a primary infection in cucumber (Schneider *et al*., 1996).

In both species, patterns in the activity of the various hydrolytic enzymes depended on the inducer showing that the response by SAR depends on the precise identity of both partners (Schneider & Ullrich, 1994). Variation in several ecotypes occurs particularly with respect to the efficacy of defense against pathogens. In *Arabidopsis thaliana,* accessions vary in their resistance to cauliflower mosaic virus (Callaway *et al*., 1996), while some lines of barley differ in their ability to produce chitinases and glucanases (Ignatius *et al*., 1994). Recent studies showed that SAR responses in *Arabidospis* can be age-dependent (Kus *et al*., 2000; Zeier, 2005), while several environmental parameters like light can influence the establishement of SAR (Zeier *et al*., 2004).

The unrelatedness between the induced defense and the inducing pathogens is another characteristic of SAR. Inoculation for example, of tomato plants with tobacco necrosis virus, can induce resistance against *Phytophthora infestans* (Anfoka & Buchenauer, 1997).

1.3.1.2 The Signal of SAR

The efficacy of SAR against a variety of pathogenic microorganisms has been convincingly demonstrated (Hoffland *et al*. 1996; Anfoka & Buchenauer, 1997; Jeum *et al*., 2000; Block *et al*., 2005). A characteristic of SAR is the accumulation of SA (Kessmann *et al*., 1994; Ryals *et al*., 1996; Kubota & Nishia, 2006). Since SA can indeed be synthesized in one leaf and translocated to the next (Shualev *et al*., 1995; Molders *et al*., 1996), this accumulation occurs both locally and at lower levels systemically. The reverse phenomenon occurs, with exogenous application of SA to induce SAR in several plant species (van Loon *et al*., 1982; Gaffney *et al*., 1993; Thulke & Conrath, 1998). Both pathogen- and SA-induced resistance are associated with the accompanying induction of several families of PR proteins. The induction of PR proteins is an important marker of SAR since they are invariably linked to the systemic induced state upon necrotizing infections (Ward *et al*., 1991; Kessmann *et al*., 1994; Nawrath & Métraux, 1999). According to Maleck *et al*. (2000), SAR involves the induction of genes encoding PR proteins, and the accumulation of PR proteins such as chitinase, 1,3-β-glucanases, lysozymes and permatins which may protect against further infections (Ryals *et al*., 1996). Therefore, the important contribution of these proteins to the increased defensive capacity of induced tissues has been extensively suggested.

The pathways leading from a first and locally restricted infection to induction of chitinase and 1,3-β-glucanases is not well understood but SA seems to be involved in this transduction (Malamy *et al*., 1990; Métraux *et al*., 1990; Delaney *et al*., 1994; Thulke & Conrath, 1998). The specific role of SA in SAR induction was shown using transgenic plants that expressed the bacterial salicylate hydroxylase (NahG) gene and thus, cannot accumulate SA. Transgenic NahG plants were incapable of developing

SAR and PR gene activation upon pathogen infection, indicating that SA is a necessary intermediate factor in signalling pathways of SAR (Gaffney *et al*., 1993; Delaney *et al.*, 1994; Kachroo *et al.*, 2000). Thus, SA is an important regulator of basal resistance against a wide spectrum of pathogens (Wildermuth *et al.*, 2001; Jagadeeswaran *et al.*, 2007). On the other hand, grafting studies between wild-type tobacco plants and plants that are unable to accumulate significant amounts of SA have shown that the large increase in SA accumulation in inoculated leaves is not necessary for SAR induction, suggesting that SA is not the primary systemic signal (Rasmussen *et al*., 1991; Willits & Ryals, 1998). Vernooij *et al*. (1994) suggested that most likely SA is not the distant signal that leads to induction of resistance in leaves systemically but instead, SA is required for the transduction of the perceived long-distance signal leading to the onset of SAR. Furthermore, SAR is dependent on the level and activity of NPR1, a protein that interacts with transcription factors that regulate the expression of defense-related genes (Zhang *et al*., 1999; Fan & Dong 2002; Wang *et al*., 2005). PR proteins produced in response to pathogens can also be induced by the plant hormone ethylene (Boller *et al*., 1983; Vierheilig *et al*., 1994; Verbeme, 2003). Although the induction of chitinase and 1,3-β-glucanases by the stress hormone ethylene has been shown in several plant species, non-responsive to ethylene plants showed normal sensitivity to the SAR-inducing chemicals SA and 2,6-dichloroisonicotinic acid with respect to SAR gene induction and pathogen resistance (Lawton *et al*., 1994). In addition to pathogen infection, SAR can be induced experimentally by certain chemicals, *i.e.* benzothiadiazole (Friedrich *et al*., 1996; Lawton *et al*., 1996; Yu & Muehlbauer, 2001), and by mechanical wounding (Ignatius *et al*., 1994; Kim *et al*., 2003).

1.3.1.3 Stimulation of grapevine defense mechanisms against pathogens

Grapevine has multiple defense systems against pathogenic microorganisms. A common defense system is the block of entrance door to pathogens, by stomata closure and/or secretion of substances such as callose (Donofrio & Delaney 2001; Gindro *et al*., 2003). The productions of toxic compounds, like phytoalexins, (Jeandet *et al*., 1991; Adrian *et al*., 1997; Bais *et al*., 2000) that accumulate at sites of successful infection can also slow down or stop the pathogen development. Responses to pathogen infection have been associated also with an expression of distinct reaction such as the accumulation of phenolic compounds (Kortekamp & Zyprian, 2003), and the deposition of lignins (Dai *et al*., 1995).

In addition, grapevines fight fungal infection by the synthesis of a number of PR proteins. In grapevine, PR proteins have been detected, analyzed and/ or cloned (cDNA), namely chitinases (Derkel *et al*.,1996; Busam *et al*., 1997; Robinson *et al*., 1997; Derkel *et al*., 1998; Bézier *et al*., 2002), 1,3-β-glucanases (Bézier *et al*., 2002; Kraeva *et al*., 1998; Derkel *et al*., 1998; 1999) and thaumatin-like proteins (Tattersall *et al*., 1997; Salzman *et al*., 1998). Derckel *et al*. (1996) have reported that six of the 13 chitinase isoforms detected in grapevine tissues were found in untreated leaves and four new acidic isoforms appeared in wounded leaves or leaves treated with SA. The mRNA accumulation of genes encoding chitinases, which may hydrolyze chitin in pathogen cell walls, has also been shown to be differentially regulated in grapevine when challenged with pathogens like *Erysiphe necator*, *Plasmopara viticola*, *Botrytis cinerea*, and *Pseudomonas syringae* pv. *pisi* (Busam *et al*., 1997; Jacobs *et al*. 1999; Robert *et al*. 2002). Basic class I (CHIT1b), acidic class III (CHIT3), and acidic class IV (CHIT4c) chitinase cDNA were cloned from grapevine cells (Busam *et al*., 1997) and in grapevine leaves and berries infected with *Botrytis cinerea* (Bézier *et al*.,

2002). The specific role of chitinases has also been analyzed in selected line of *in-vitro V. vinifera* 'Chardonnay' with resistance to *Elsinoe ampelina* (Jayasankar *et al.*, 2000). According to the authors, chitinase proteins were expressed in differentiated somatic embryos and also in the intercellular fluids of resistant plants regenarated from the selected lines.

The class I 1,3-β-glucanases are antifungal vacuolar proteins involved in plant defense which exhibit pathogenesis-related regulation. The antifungal activity of plant 1,3-β-glucanases is thought to hydrolyze the structural 1,3-β-glucanases glucan present in some fungal cell wall. cDNA clones corresponding to different 1,3-β-glucanase genes have been characterized in grapevine (Jacobs *et al*., 1999; Bézier *et al*., 2002).

Inhibitors of serine proteases (PIN) have emerged as a class of antifungal PR-6 proteins which have potent activity against plant and animal pathogens and are thought to interfere with fungal penetration by inhibiting the degradation of the plant cell wall by fungal endopolygalacturonases (PG) (van Loon & van Strien, 1999). Grapevine defenses were further characterized by cloning a polygalacturonase inhibitor protein (PGIP) gene, while investigating the grapevine defense responses against *B. cinerea* (Bézier *et al*., 2002). An increase of the expression of these genes was also monitored in detached leaves and grapevine cell after elicitation by laminarin elicitor (Aziz *et al*., 2003).

1.3.2 Plant interaction with plant growth-promoting rhizobacteria (PGPR)

The beneficial interaction between plants and microbes occurs frequently in nature (Lynch & Whipps, 1991). In many cases, this typical symbiosis can improve plant nutrition and help plants to resist biotic and abiotic stresses (De Weger *et al*., 1995;

Gerhardson, 2002; Postma *et al*., 2003; Welbaum *et al*., 2004; Mayak *et al*., 2004a,b; Waller *et al*., 2005**)**. In particular, microbial activity in the rhizospere is a major factor for availability of nutrients to plants and has a significant role on plant health and productivity (Rovira, 1965).

Beneficial bacteria that colonize the plant roots have been characterized as plant growth-promoting bacteria (PGPR) by Kloepper and Schroth (1981). Bacteria that colonize the root system of plants are referred to as 'rhizobacteria'. Except for the ones that remain confined to the root surface (rhizoplan), some enter the root interior and behave as endophytes (Sturz *et al*., 2000). Since it has been extensively shown that PGPR can facilitate the growth of plants, they are increasingly used as inoculants for biocontrol, biofertilization and phytostimulation (Fig. 1.11). PGPR enhance plant growth by direct and indirect means (Glick, 1995; Hallman *et al*.**,** 1997; Sturz *et al*., 2000; Bloemberg & Lugtenberg, 2001; Lodewyckx *et al*., 2002; Dobbelaere *et al*., 2003; Bakker *et al*., 2003; Compant *et al*., 2005a; Ait Barka *et al*., 2006). Although, the specific mechanisms involved in plant-PGPR interaction are not well-characterized (Kloepper *et al*., 1993; Glick, 1995), many PGPR have been reported to enhance plant growth by a variety of mechanisms like fixation of atmospheric nitrogen that is transferred to the plant **(**Hansen, 1994; Schultze & Kondorosi, 1998; Gualtieri & Bisseling, 2000; Sessitsch *et al*., 2002), production of siderophores that chelate iron and make it available to the plant root (Bar-Ness *et al*., 1991; Wang *et al*., 1993), solubilization of minerals such as phosphorus (Richardson, 2001), and synthesis of phytohormones (Glick, 1995). Enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPR has also been reported. PGPR strains may use one or more of these mechanisms in the rhizosphere. Molecular approaches using microbial and plant mutants altered in their

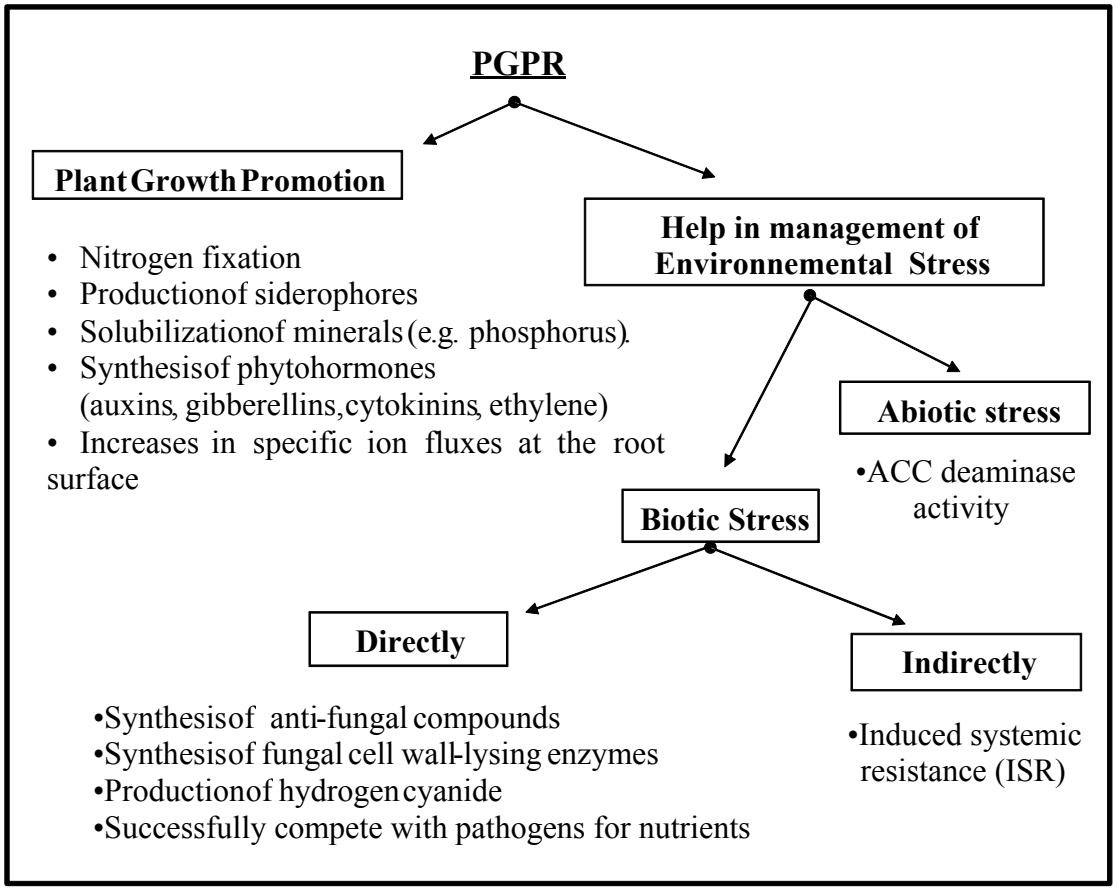

Fig. 1.11: Plant interaction with plant growth-promoting rhizobacteria (PGPR). Beneficial effects on plant growth promotion and help in management of environmental stresses (adapted from Koch *et al*., 1992; Schneider & Ullrich, 1994; van Loon, 1997; van Loon & Bakker, 2005; 2006).

ability to synthesize or respond to specific phytohormones, have increased our understanding of the role of phytohormone synthesis as a mechanism of plant growth enhancement by PGPR (Glick 1995, Cartieaux *et al*, 2003a). PGPR that synthesize auxins and cytokinins or that interfere with plant ethylene synthesis have been identified (Glick, 1995; Garcia de Salamone *et al*., 2001; Cartieaux *et al*, 2003a). PGPR help in management of abiotic stresses by showing high 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and is therefore able to lower the ethylene level in stressed plants. PGPR enhance plant growth also *via* suppression of phytopathogens directly or indirectly by a variety of mechanisms. Direct mechanisms

include (i) the ability to produce siderophores that chelate iron making it unavailable to pathogens; (ii) the ability to synthesize anti-fungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; (iii) the ability to successfully compete with pathogens for nutrients or specific niches on the root (Glick *et al.*, 1998; Glick, 2005; Compant *et al*., 2005a; Saleem *et al.*, 2007). Indirect mechanisms stimulated by PGPR are the ability of plants to induce systemic resistance (Bloemberg & Lugtenberg, 2001, Cartieaux *et al*., 2003a). Biochemical and molecular approaches provided new insight in the genetic basis of these traits, the biosynthetic pathways involved, their regulation, and importance of biological control in laboratory and field studies (Glick, 1995; Bowen, & Rovira, 1999; Bloemberg & Lugtenberg, 2001, Cartieaux *et al*, 2003b).

1.3.2.1 Plant Induced Systemic Resistance (ISR) by PGPR

Induced systemic resistance (ISR) has been defined by van Loon *et al.* (1997; 1998) as a state of increased defensive capacity developed by the plant upon appropriate stimulation by diverse agents, including rhizobacteria, through activation of latent resistance mechanisms. The nature of systemically induced resistance in plants has been characterized by van Loon (2007) according to the following criteria:

- the defensive capacity of the plant is enhanced through microbial stimulation or similar stresses,
- induced systemic resistance is active against fungi, bacteria viruses and, sometimes, nematodes and insects,
- the enhanced defensive capacity is expressed systemically throughout the plant,
- once induced systemic resistance is maintained for prolonged periods.

The PGPR-elicitation of ISR in plants has been shown in many studies (De Vleesschauwer *et al*., 2006; Saravanakumar *et al*. 2007a,b). Colonization of roots by *Pseudomonas* sp. strain WCS 417r can protect plants systemically against *Fusarium oxysporum* f. sp. *dianthi*, which is responsible for *Fusarium* wilt in carnation (van Peer *et al*., 1991). In another case, anthracnose disease caused by *Colletotrichum orbiculare* in cucumber can be reduced after treatment of seeds with PGPR (Wei *et al*., 1991; 1996). PGPR as seed-treatment alone or as seed-treatment plus soil-drenching can protect cucumber plants against anthracnose disease (Wei *et al*., 1996). Similarly, *P. putida* strain 89B-27 and *Serratia marcencens* strain 90-166 are PGPR that can reduce *Fusarium* wilt of cucumber incited by *F. oxysporum* f. sp. *cucumerinum* (Liu *et al*., 1995). In addition, application of *P. fluorescens* strains PF1 and and FP7 by seed-treatment followed by root dipping and a foliar spray in rice showed higher induction of ISR against the sheath blight pathogen, *Rhizoctonia solani* (Vidhyasekaran & Muthamilan, 1999).

PGPR can induce systemic protection agaisnt bacterial and viral diseases. Seed-treatment by *P. fluorescens* strain 97 can protect beans against halo blight disease caused by *P. syringae* pv. *phaseolicola* (Alstrom, 1991). Similarly, treated seeds with PGPR can decrease the incidence of bacterial wilt disease (Kloepper *et al*., 1993). Hoffland *et al*. (1996) confirmed the non specificity of ISR, after applying a rhizobacterial isolate to radish seeds, where they observed a reduced incidence of wilt plants by *F. oxysporum* f. sp. *raphani* as well as a reduction in the severity of attack by *Alternaria brassicicola* and *P. syringae* pv. *tomato.* The reduction of cucumber mosaic virus (CMV) infection in infected plants and the delay of development of symptoms in cucumber and tomato have been reported by seed-treatment with *P. fluorescens* strain 89B-27 and *Serattia marcescens* strain 90-166 (Raupach *et al*.,

1996). In another case, ISR has been established by application of *P. fluorescens* strain CHAO protecting against tobacco necrosis virus (TNV) in tobacco (Maurhofer *et al*., 1994; 1998). These experiments showed that PGPR strains initiate ISR against a wide array of plant pathogens causing fungal, bacterial and viral diseases.

The non-specificity against pathogenic fungi, bacteria, and viruses is one of the main characteristics of protection induced by PGPR. This non-specificity is an important advantage of ISR in comparison with classical methods of biological control of pathogens, in which the antagonist selected is normally active against only one or a few pathogens but not against a broad number of pathogens. The interaction of plants with rhizobacteria-mediated ISR has been documented in at least 15 species (van Loon & Bakker, 2006) involving the activation of different physiological mechanisms (van Loon, 2007). Non-pathogenic rhizobacteria may activate sometimes the plant defense mechanisms in a similar way to pathogenic microorganisms. ISR has been associated with a large number of enzymes, including peroxidase, phenylalanine amonia-lyase, lipoxygenase, 1,3-β-glucanases, and chitinase (Ye *et al*., 1990; Koch *et al*, 1992; Schneider & Ullrich, 1994; van Loon, 1997; van Loon & Bakker, 2005; 2006).

The development of molecular techniques has allowed the reaction of plants to rhizobacteria to be determined at the transcriptional level by analyzing differential gene expression in ISR. Timmusk and Wagner (1999) analyzed the changes in gene expression induced by inoculation with PGPR *Paenibacillus polymyxa* by RNA differential display. Cartieux *et al*. (2003) monitored gene expression by cDNA microarrays in both leaves and roots of axenic *Arabidopsis* plants infected by resistance-inducing *Pseudomonas thivervalensis* strain MLG45. Recently, the up and down regulated genes were also estimated by cDNA-AFLP in leaves of cucumber and

tomato plants, respectively, in response to root colonization by *Bacillus subtilis* strain M4 (Ongena *et al*., 2005). Several other studies have been performed in the last decade analyzing the induced systemic responses of bacterized plants by changes in gene expression (Park & Kloepper, 2000; Wang *et al.*, 2005; Sanchez *et al.*, 2005; Shuhegger *et al*., 2006).

1.3.2.2 Signalling pathways of systemically induced resistance

There are distinguished differences between ISR and systemic resistance elicited by avirulent pathogens that induce the hypersensitive resistance (HR) and the systemic acquired resistance (SAR). Expression analysis using *Arabidopsis* as a model plant showed that the mechanism is highly dependent on the bacteria partners. *P. flurescens* WCS417r elicits *via* a salicylic acid (SA)-independent pathways and *PR* gene activation (Pieterse *et al*., 1996; van Wees *et al*., 1997), since SA-non accumulating NahG plants developed normal levels of ISR against *Pst* DC3000 after root colonization (Pieterse *et al*., 1996; van Wees *et al*., 1997). Downstream of NPR1, *PR* genes are activated in the SAR pathways but not in the ISR pathways (Cao *et al*., 1994; Pieterse *et al*., 1998). Evidently, NPR1 differentially regulates ISR- and SAR-related gene expression, depending on the pathway that is activated upstream of it. Interestingly, ISR-inducing WCS417r bacteria elicited a substantial change in the expression of almost 100 genes (Verhagen *et al*., 2004; Léon-Kloosterziel *et al*., 2005), but no consistent alteration in gene expression was observed in systemic leaves (Verhagen *et al*., 2004). No alteration in the production of either JA or ET have also been detected in plants, suggesting that the mechanism of induced resistance is based on an enhanced sensitivity to these plant hormones than on an increase of their production (Pieterse *et al*., 2000).

SA and JA are two signalling molecules which play an important role in induced disease resistance pathways (Jayaraj *et al*., 2004). Between these defense signalling pathways the phenomenon of cross-talk has been demonstrated. On the one hand, signalling molecules such as JA and ethylene can act in conjunction to activate defense responses. On the other hand, SA can suppress JA-dependent responses (Reymond & Farmer, 1998; Pieterse & van Loon, 1999). Even if ISR and SAR are related to separated signalling pathways (Fig. 1.12), the two systemic systems share the regulatory factor NPR1 showing an interaction between JA-dependent ISR pathways and the SA-dependent SAR as referred by van Wees *et al.* (2000). According to the authors the activation of both pathways simultaneously resulted in an additive effect on the level of induced protection against *Pst* DC3000. However, such additive effect of both pathways was not evident in *Arabidopsis*. Furthermore, the level of *PR-1* gene expression, as SAR marker, was not altered in plants expressing both ISR and SAR compared to plants expressing solely SAR. This means that SAR and ISR pathways are compatible and that there is no significant cross-talk between these signaling pathways. Since plants expressing ISR and SAR simultaneously, did not show elevated levels of *NPR1* transcripts, it seems that the constitutive level of NPR1 is sufficient to facilitate simultaneous expression of both types of induced resistance.

Apart from the SA-independent pathway, a second SAR-type defense response associated with endogenous free SA accumulation and/or PR protein expression has been induced by nonpathogenic rhizobacteria. *Pseudomonas aeruginosa* 7NSK2 induced systemic resistance in beans in a SA-dependent manner (De Meyer *et al*.,

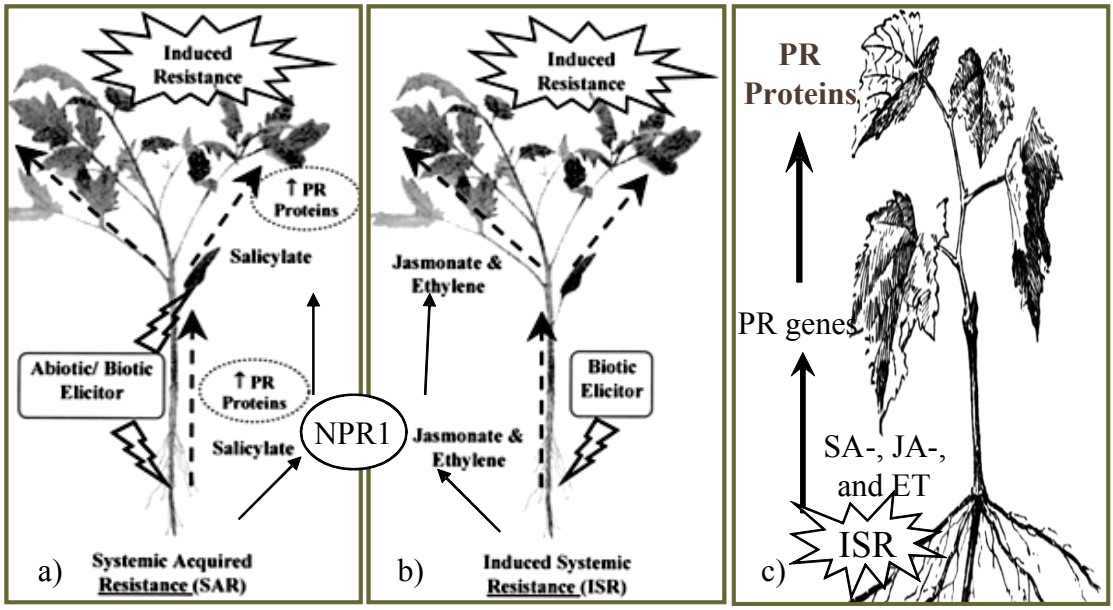

Fig. 1.12: A pictorial comparison of the three characterized forms of induced resistance in plants. (a,b) Systemic acquired resistance, induced by the exposure of root or foliar tissues to abiotic or biotic elicitors, is dependent of the phytohormone salicylate (salicylic acid), and associated with the accumulation of pathogenesis-related (PR) proteins. Induced systemic resistance, induced by the exposure of roots to specific strains of plant growth-promoting rhizobacteria, is dependent of the phytohormones ethylene and jasmonate (jasmonic acid), independent of salicylate, and is not associated with the accumulation of PR proteins (or transcripts). However, both responses are intertwined molecularly, as demonstrated by their reliance on a functional version of the gene *NPR1* in *Arabidopsis thaliana* ((Pieterse & van Loon, 1999; Vallad & Goodman, 2004). (c) Systemic resistance, induced by the exposure of roots to specific strains of plant growth-promoting rhizobacteria, is dependent of the phytohormones ethylene and jasmonate (jasmonic acid), dependent of salicylate, and associated with the accumulation of PR proteins or transcripts (De Meyer *et al*., 1999a; Timmusk & Wagner, 1999; Park & Kloepper, 2000; Tjamos *et al*., 2005; Magnin-Robert *et al*., 2007).

1999a). Resistance-inducing strain *Paenibacillus polymyxa* was associated with up-regulation of SA-, JA- and ET-responsive genes, including *PR-1*, in leaves, indicating an involvement of signalling through SA-, JA-, and ET in *P. polymyxa*- mediated systemic resistance (Timmusk & Wagner, 1999). Induction of PR-1 gene activity was assessed using transgenic tobacco plants expressing the β-glucuronidase (GUS) gene fused to the PR-1 gene promoter (Park & Kloepper, 2000). The results of this study support the conclusion that induction of PR-1a promoter activity and PGPR-mediated induced systemic disease resistance are linked events for the used PGPR strains. Other indications that some PGPR or rhizosphere bacteria may induce PR proteins have been reported. Maurhofer *et al*. (1994) indicated that induced tobacco protection against tobacco necrosis virus by PGPR strain *P. fluorescens* CHAO was associated with the induction of multiple PR proteins, including PR-1a, PR-1-b, and PR-1-c (Fig. 1.12). Similarly, Schneider and Ullrich (1991) reported that induction of tobacco protection against *P. syringae* pv. *tabaci* induced by culture filtrates of a *P. fluorescens* strain was associated with induction of chitinase, 1,3-β-glucanases, peroxidase, and lysozyme. These resistance-inducing PGPR induced defense reactions commonly associated with pathogen infection. Recently, it was shown by Magnin-Robert *et al*. (2007) that grapevine-associated bacteria can stimulate grapevine defense mechanisms. This was correlated to an accumulation of chitinase and 1,3-β-glucanase activities under field conditions in both leaves and berries.

1.3.2.3 Plant interaction with *Burkholderia phytofirmans* strain PsJN

As previously referred (paragraph 1.1.2)*, Burkholderia phytofirmans* strain PsJN, is a well-characterized PGPR able to establish rhizosphere and endophytic populations associated with various plants, where it stimulates plant growth and induces developmental changes leading to better adaptation in several environmental stress

continions. Usually, the plants inoculated with strain PsJN are characterized by a larger root system, with enhanced secondary roots and more root hairs, sturdier stems, and greater lignin deposition around the vascular system (Nowak, 1998). In addition, inoculated plants by strain PsJN were found to contain larger amounts of phenolics and chlorophyll (Nowak *et al*., 1997), as well as increased levels of cytokinins (Lazarovits & Nowak, 1997) and enhanced activity of phenylalanine ammonia lyase (Nowak *et al*., 1997). Isolated PsJN showed high 1-aminocyclopropane-1-carboxylate deaminase activity and is therefore able to lower the ethylene level in developing or stressed plants (Sessitsch *et al.*, 2005).

During the interaction between strain PsJN and grapevine, it was shown that grapevine plantlets *V. vinifera* co-cultured with bacteria grew faster and had significantly more secondary roots and root and leaf hairs. These effects were not observed when plantlets were inoculated with dead bacteria before their culture, meaning that the observed effect was not related to a fertility effect from lysed cells (Ait Barka *et al*. 2000). The response of plant towards bacterization was maintained and amplified after the second generation. Indeed, the PsJN bacterium is capable of establishing endophytic and epiphytic populations, allowing clonal multiplication of plantlets by nodal explants in *perpetuum* without the need for re-inoculation (Ait Barka *et al*. 2002). In addition, grapevine plantlets bacterized by PsJN appeared healthy and exhibited only small leaf surface necroses, when inoculated with *B. cinerea*, in contrast to control that they produced characteristic gray mold symptoms within 7 days (Ait Barka *et al*. 2000; 2002). Using *in vitro* grapevine plantlets, Compant *et al*. (2005b) showed that wild-type strain PsJN and genetically engineered derivatives of this strain tagged with *gfp* (PsJN::*gfp*2x) or *gusA* (PsJN::*gusA*11) genes

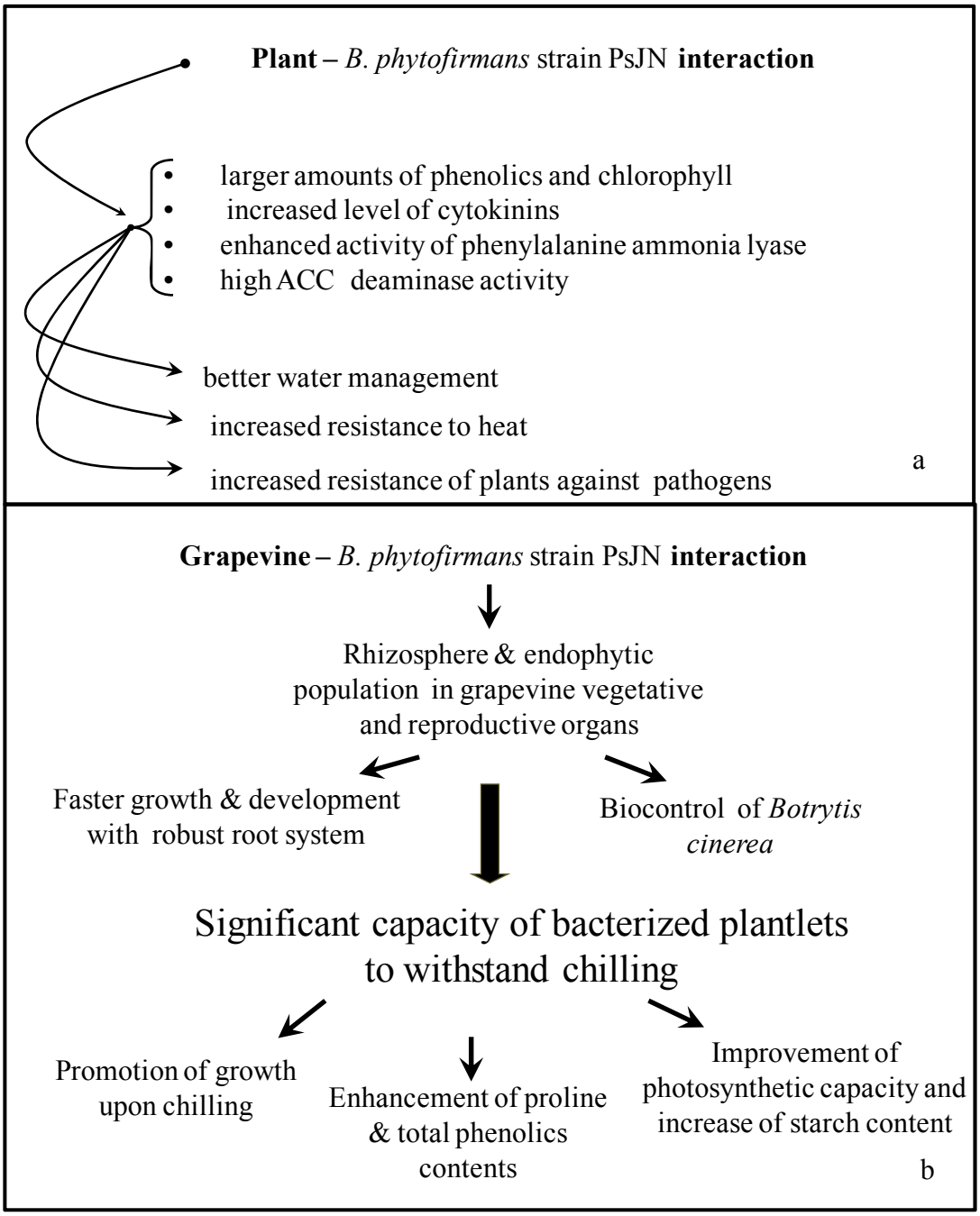

Fig. 1.13: Beneficial effects of *Burkholderia phytofirmans* strain PsJN in interaction with (a) plants and (b) grapevine (adapted from Nowak *et al*., 1997; Lazarovits & Nowak, 1997; Nowak, 1998; Sessitsch *et al*., 2005; Ait Barka *et al*., 2000; 2002; Compant *et al*., 2005b; Ait Barka *et al*., 2006; Compant *et al*., 2008b).

colonized the root surface and subsequently entered the endorhiza mainly through the 'root tip way', lateral root cracks or between rhizodermal cells *via* cell-wall-degrading enzyme secretions. Strain PsJN colonized stem and leaves through xylem vessels, before thriving as an endophyte inside substomatal chambers of leaves after using the plant transpiration stream. The colonization pattern of *V. vinifera* by strain PsJN::*gfp* 2x was also determined using grapevine fruiting cuttings with emphasis on putative inflorescence colonization under nonsterile conditions. Strain PsJN was chronologically detected on the root surfaces, in the endorhiza, inside grape inflorescence stalks, not inside preflower buds and flowers but rather as an endophyte inside young berries. Microscopic analysis revealed PsJN as a thriving endophyte in inflorescence organs after the colonization process. Strain PsJN was visualized colonizing the root surface, entering the endorhiza and spreading to grape inflorescence stalks, pedicels and then to immature berries through xylem vessels (Compant *et al*., 2008b). Data demonstrated low endophytic populations of strain PsJN in inflorescence organs, *i.e.* grape stalks and immature berries with inconsistency among plants for bacterial colonization of inflorescences. Nevertheless, endophytic colonization of inflorescences by strain PsJN was substantial for some plants.

Further investigation of grapevine-PsJN interaction and analysis of physiological responses of grapevine plantlets to chilling, showed that inoculated Chardonnay explants with strain PsJN, increased grapevine growth and physiological activity at low temperature. According to Ait Barka *et al.* (2006), there was a relationship between endophytic bacterial colonization of the grapevine plantlets, their growth at both ambient (26°C) and low (4°C) temperatures and their sensitivities to chilling with the major benefits of bacterization to be observed on root growth and

plantlet biomass. In this study, it was demonstrated for the first time that plant growth-promoting bacteria colonizing grape plantlets could significantly influence plantlets' resistance to chilling. Plantlet bacterization had a pronounced effect on grapevine growth, development, and responses to low temperatures, *i.e.* diminished rates of biomass reduction and electrolyte leakage during chilling and stimulated postchilling recovery. Bacterization significantly elevated also the level of proline and phenolics and enhanced the rate of photosynthesis and starch deposition. The inoculation with PsJN also significantly improved plantlet cold tolerance compared to that of the nonbacterized control, as indicated by their abilities to significantly increase starch content, proline accumulation and phenolic compound upon chilling.

1.3.3 The phenomenon of priming in plant-micobe interaction

There has been increasing evidence that activation of defense-related mechanisms by SAR- and ISR-stimulators has been related with a more efficient activation of plant cellular defense responses upon several biotic and abiotic stresses. This enhanced capacity of plants to express defense mechanism is known as priming, sensitization or potentiation, and has been related with an ability for rapid defense responses of plants to biotic and abiotic stresses (Conrath *et al*., 2002; 2006; Goellner & Conrath, 2008). Upon inoculation with necrosis-inducing pathogens, or various nonpathogenic root-colonizing rhizobacteria, and treatment with natural and synthetic compounds, plants react to biotic and abiotic stresses (Fig. 1.14). These reactions include the HR (Mittler *et al*., 1996), cell-wall strengthening (Hammerschmidt *et al*., 1982; Stumm & Gessler, 1986; Schmele & Kauss, 1990), the oxidative burst (Doke, 1996) and potentiated expression of various defense- related genes (Ryals *et al.*, 1996; Sticher *et al.*, 1997). Although the priming phenomenon has been known for years as a part of induced-

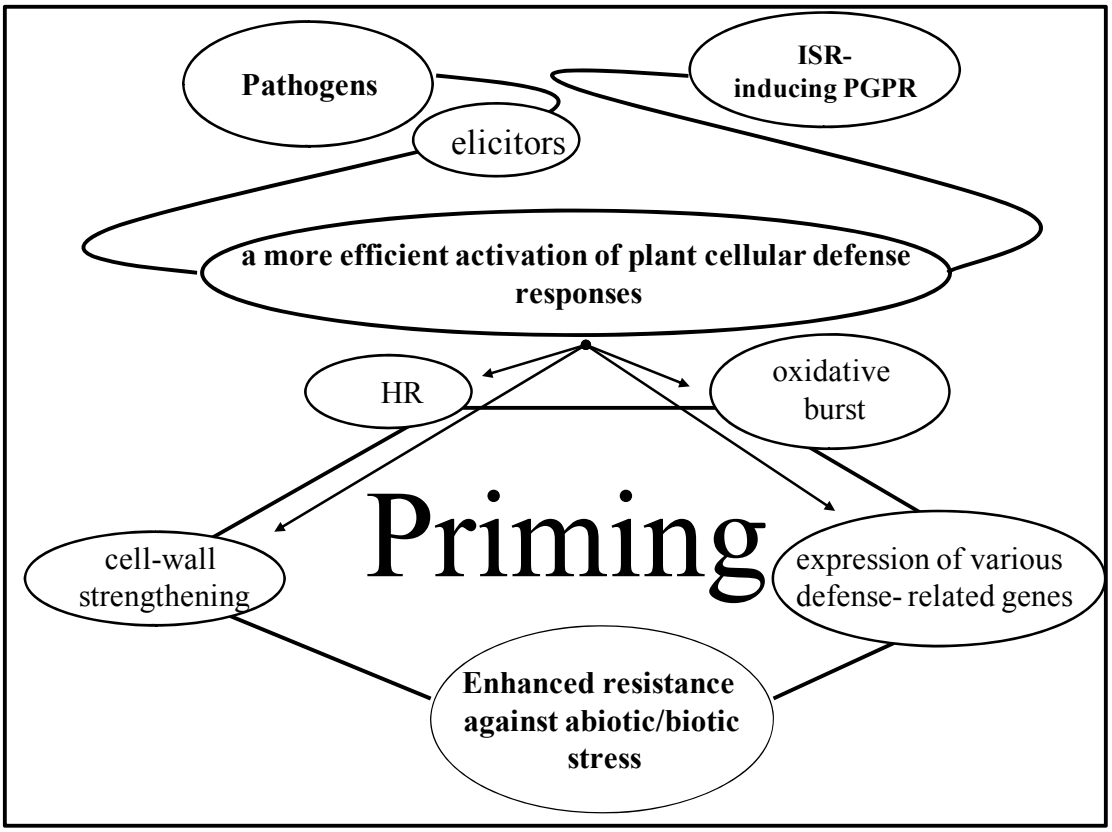

Fig. 1.14: Pictorial presentation of plant cellular defense responses induced according to priming (Schmele & Kauss, 1990; Mittler *et al*., 1996; Ryals *et al*., 1996; Doke, 1996; Sticher *et al*., 1997; Conrath *et al*., 2002; 2006; Goellner & Conrath, 2008).

resistance phenomenon (Hammerschmidt & Kuc, 1982; Stumm & Gessler, 1986; Kuc, 1987), it has mostly been overlooked in studies dealing with induced disease resistance of plants, because it only becomes apparent after challenge on the primed tissue. The molecular mechanism and genetic basis of priming and its role in induced disease resistance are still poorly understood. Hypothetically, the primed state is based on the accumulation, or post-translational modification of one or more signalling proteins that, after being expressed and/or modified, still remain inactive. Upon perception of a second pathogen-derived stress signal this enhanced defense

signalling capacity would enable a faster and stronger defense reaction (van der Ent, 2007).

1.3.3.1 Priming in resistance to pathogen

Since the potentiated induction of defense responses to pathogens seems discernible only after the challenge, the phenomenon of priming became apparent when studying SAR by accumulation of large set of *PR* genes (Cameron *et al*., 1999; van Wees *et al*., 1999; Kohler *et al*., 2002). Low doses of the SAR-inducer BTH (Benzothiadiazole) can prime the physiological state of *Arabidopsis* by potentiating the expression of *PAL* mRNA after inoculation with virulent *Pseudomonas syringae* pv. *tomato* DC3000 (Kohler *et al*., 2002). The ability of BABA (β -aminobutyric acid) to increase plant resistance against pathogens by priming has also been shown in *Arabidopsis*. *Arabidopsis* pretreated with BABA presented a rapid and high deposition of callose-containing papillae at the site of infection by *Peronospora paracitica* and potentiate mRNA accumulation of the SA-associated *PR-1* (Zimmerli *et al*., 2000). Moreover, BABA potentiated accumulation of *PR-1* transcripts, contributing thus to restrict *B. cinerea* infection in *Arabidopsis* (Zimmerli *et al*., 2001). Additionally, *Arabidopsis* infection with avirulent *P. syringae* pv. *tomato* DC3000 induces the potentiated activation of defense-related *PAL*, *PR-1, PR-2*, and *PR-5* genes, while tobacco plants which are SA-primed transgenic and carry the chimeric *Asparagus officinalis PR-1::uidA* and *PR-3::uidA* genes, displayed the potentiated activation of *PR-1* and *PAL* genes, after pathogen attack (Mur *et al*., 1996).

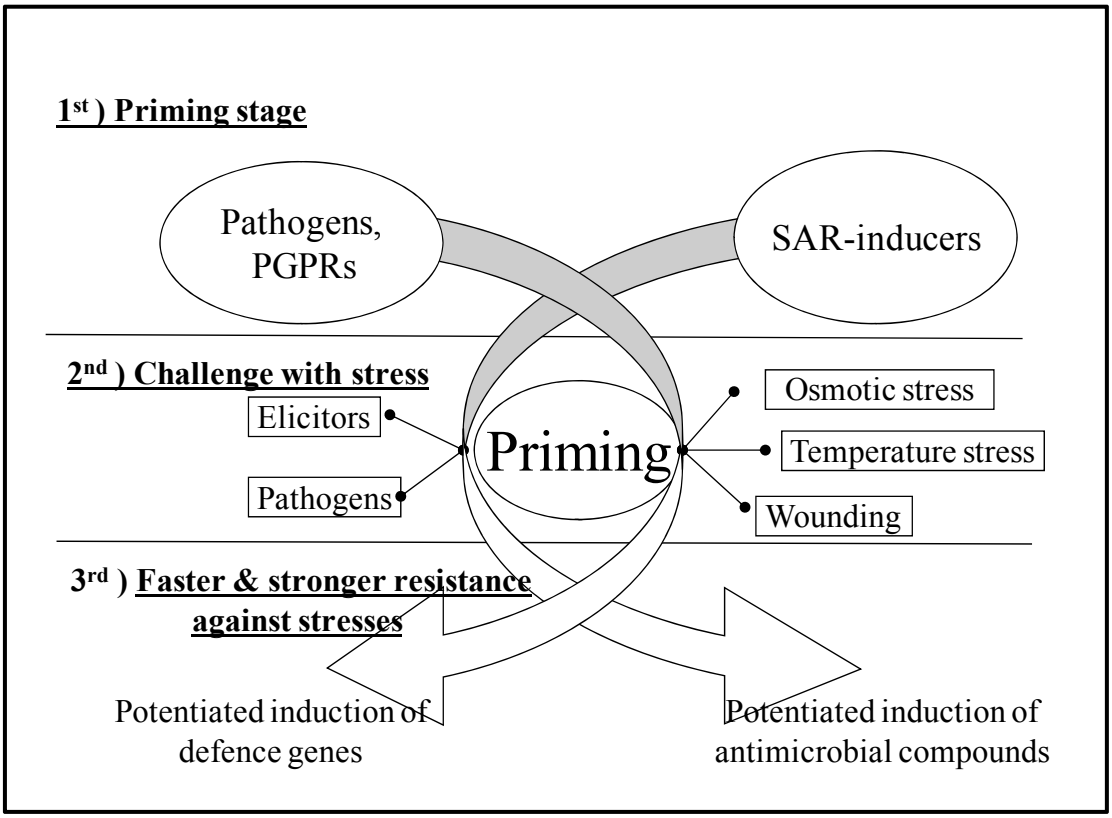

Fig. 1.15: The three steps of 'priming' phenomenon in plants (adapted from Conrath et al., 2002; 2006; Goellner & Conrath, 2008). In plants, a pretreatment with natural and synthetic compounds, or pre-infection with pathogens, or pre-inoculation by PGPR primes the cells to react more quickly and efficiently to subsequent elicitor treatment or pathogen attack or several abiotic stresses. (1st step) Priming step; (2nd step) Challenge with biotic or abiotic stress; (3rd step) Potentiated response.

In grapevine, Aziz *et al*. (2003) reported that the laminarin was an efficient elicitor of defense response in grapevine cells and plants against *Botrytis cinerea* and *Plasmopara viticola* and it acted through priming by over-expression of several *PR* genes. The non-protein amino acid BABA can prime also the cells to react more quickly and efficiently to *P. viticola* either by priming of callose deposition and lignification and by potentiated expression patterns of markers genes for SA and JA pathways (Hamiduzzaman *et al*., 2005), or by higher accumulation of stilbenes &

specific phytoalexins and the rapid increase in transcript levels of three genes involved in the phenylpropanoid pathway (Slaughter *et al*. 2008) (Fig. 1.16).

1.3.3.2 Priming in plant-beneficial microbe interaction

Priming is a common feature of the resistance responses induced by beneficial microorganisms (Conrath *et al*., 2002, 2006; Goellner & Conrath, 2008). Colonized roots by mycorrhizal fungi for example, can systemically protect the plants of tomato against *Phytophthora parasitica* infection. Non-direct accumulation of PR proteins exists under colonization; however upon pathogen attack, mycorrhized plants accumulate more PR-1a and basic BGL proteins than nonmycorrhized plants (Cordier *et al*., 1998; Pozo *et al*., 1999, 2002). Similarly to plant-mycorrhizal fungi association, plant growth-promoting fungi (PGPF) can also induce priming in plants. For example, plants that had been pre-inoculated with (PGPF) *Trichoderma asperellum* T203 can express a higher level of *PR* genes after infection with the leaf pathogen (Shoresh *et al*., 2005).

The phenomenon of priming in plant-microbe interaction has most extensively been studied under plant-ISR-inducing PGPR interaction. The first evidence with which potentiated plant defense responses are involved in PGPR-mediated ISR, was studied under inoculation of carnation (*Dianthus caryophyllus*) with *Fusarium oxysporum* f. sp. *dianthi*. The ISR-expressing carnation presented a faster rise in phytoalexin levels than uninoculated plants (van Peer *et al*., 1991). ISR induced by *Bacillus pumillus* SE34, protected beans against the root-rot fungus *F. oxysporum* f. sp. *pisi*, and confirms that endophytic bacteria may function as potential inducers of plant disease resistance (Benhamou *et al*., 1996). The typical host reactions in pre-bacterized roots included rapid strengthening at the site of attemped fungal

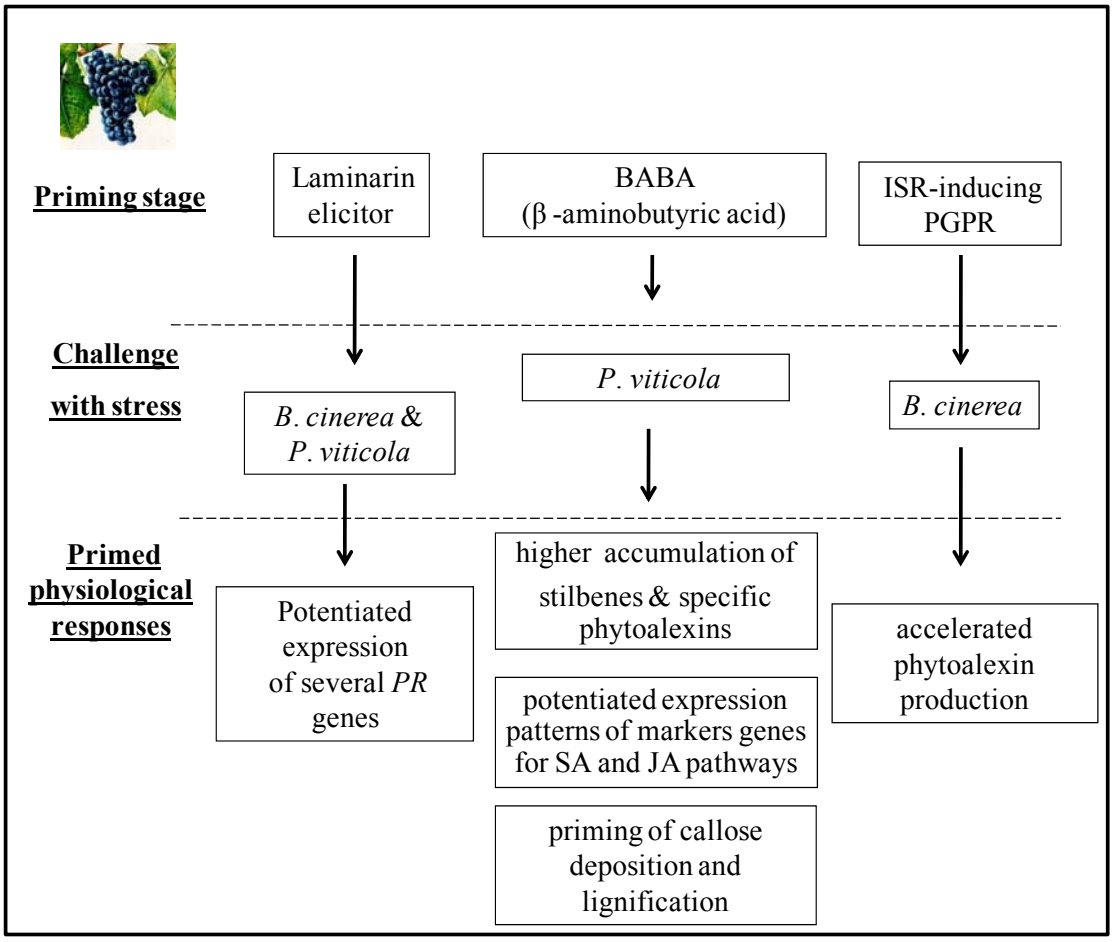

Fig. 1.16: The three steps of 'priming' phenomenon in grapevine (adapted from Aziz *et al.,* 2003; Hamiduzzaman *et al*., 2005; Slaughter *et al*., 2008; Verhagen *et al*., 2010).

penetration, through appositions that contained callose and phenolic material which prevent the fungal progress (Benhamou *et al*., 1996).

Other ISR-inducing PGPR have also been demonstrated to enhance plant's defense capacity by priming for potentiated expression of defense genes, strongly suggesting that priming is a common feature of PGPR-mediated ISR. Tobacco plants inoculated by selected strain of nonpathogenic rhizobacteria EXTN-1, capable of eliciting broad-spectrum induced systemic resistance (ISR) in several crops, showed an augmented, rapid transcript accumulation of defense-related genes including *PR*-

1a, *PAL*, and 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*) following inoculation with PMMoV (Ahn *et al*., 2002). The expression patterns of six distinct genes isolated after root colonization by the non-pathogenic rhizobacteria *Pseudomonas chlororaphis* O6, were related not only to induced systemic resistance in cucumber, but also to significantly faster and stronger transcript accumulation of these genes after challenge infection (Kim *et al*., 2004). Similarly, the bacterium *Paenibacillus alvei* K165 has the ability to protect *A. thaliana* against *Verticillium dahliae* by inducing resistance in the host, and concominant activation and increased transient accumulation of the *PR-1*, *PR-2*, and *PR-5* genes were observed in treatments where both the inducing bacterial strain and the challenging pathogen were present in the rhizosphere of the *A. thaliana* plants (Tjamos *et al*., 2005). De Meyer *et al*. (1999b) showed that ISR-*Pseudomonas aeruginosa* 7NSK2 can potentiate defense gene expression in systemic tissue, but is not associated with *PR1a* expression at the time of challenge with tobacco mosaic virus. During ISR induced by *P. syringae* pv. *tomato* DC3000, *Arabidopsis* leaves showed 81 over expressed genes, indicating that plants were primed to respond faster and/or strongly to pathogen attack (Verhagen *et al*., 2004). JA and ET have been predicted to be the regulators of the majority of genes with potentiated expression, showing that colonization of the roots by WCS417r-primed *Arabidopsis* plants augmented expression of JA- or ET-responsive, or both genes (van Wees *et al*., 1999; Hase *et al*., 2003). Recently, ISR-inducing PGPR with enhanced defense capacity by priming have also been demonstrated in grapevine. *Pseudomonas fluorescens* CHA0 and *Pseudomonas aeruginosa* 7NSK2 have the ability to induce resistance in grapevine against *Botrytis cinerea* by oxidative burst and phytoalexin (*i.e.* resveratrol and viniferin) accumulation in grape cells, while they can prime the physiological response in grapevine leaves by

accelerated phytoalexin production upon challenge with *B. cinerea* (Verhagen *et al*., 2010) (Fig. 1.16).

1.3.3.3 Priming in abiotic stress: connection between biotic and abiotic factors

Responses of plants to drought, cold or high salinity and other abiotic stresses involve the induction of a large number of genes (Bray, 1997; Fowler & Thomashow, 2002). Distinct mechanisms have been suggested to be involved in the regulation of stress-responsive genes. Some of these responses are modulated by hormones such as JA, SA and ABA signalling or by molecules like calcium (Bray, 1997; Fowler & Thomashow, 2002).

Induced stress resistance by many chemicals has been described in several plants (Sticher *et al*., 1997) either by induction of direct responses in absence of pathogens or by potentiation of physiological responses as inducers of priming. BABA as discussed above is an effective inducer of resistance against biotic stress in many plants (Zimmerli *et al,* 2000; 2001). However, recently it became apparent that it can also affect the defence capability of plants against abiotic stress by priming. Tolerance of plants to drought and salt stress has been increased in *Arabidopsis* by BABA (Jakab *et al*., 2005). SA-inducible *PR-1* and *PR-5* genes and the ABA-dependent *RAB-18* and *RD-29A* genes have been correlated with the protection of plants against salt and drought stress. SA-deficient plants showed a reaction that was similar to the one in wild-type plants while mutants impaired in ABA signalling could not be protected by BABA application. However, pretreatment with BABA did not induce ABA accumulation directly, but accelerated ABA production following osmotic stress. The augmented ABA production resulted in augmented ABA-inducible gene expression and accelerated stomatal closure (Jakab *et al*., 2005). These

finding demonstrates that BABA-induced tolerance to osmotic stress is based on priming for enhanced adaptation responses rather than on the direct activation of these responses. The potential of BABA to prime the physiological state of plants, which can react faster and more efficiently to biotic and abiotic stress, reveals a connection between these two types of stresses at the molecular level. This conclusion is supported by the fact that plant-growth promoting bacteria induce alteration in plant gene expression that can be correlated to resistance against abiotic and biotic stresses (Timmusk & Wagner, 1999).

As it was indicated in the previous chapter, cold-acclimated plants show an increased ability to survive much lower temperatures than non-acclimated ones (Thomashow, 2001). There is direct evidence that changes in gene expression occurring during the primed physiological state of cold acclimation are responsible for several biochemical and physiological changes, which contribute to an increase of the plant tolerance to extreme temperatures (Thomashow, 2001). In a similar way, acclimated plants strongly increase the resistance to snow moulds and other pathogen fungi (Tronsmo, 1984a,b; Tronsmo *et al*., 1993; Bryngelsson *et al*., 1994). The analysis of the effect of cold hardening and *Microdochium nivale* infection on expression of pathogenesis-related genes in winter wheat showed that the induction of PR-proteins was stronger and more rapid in plants that have been hardened prior to inoculation, according to the phenomenon of priming (Ergon *et al*., 1998). These results suggest that cold treatment can prime the physiological state of plants increasing their resistance not only to further cold stress but also against pathogens.

1.4 Objectives

Bulkholderia phytofirmans strain PsJN and grapevine, have developed a beneficial interaction with the bacteria improving grapevine growth and inducing the tolerance to some pathogens such as *B. cinerea*. Furthermore, it was also observed that the bacteria improve several grapevine physiological parameters that regulate growth and adjust grapevine responses to cold (Ait Barka *et al*., 2006). Despite the available information by previous studies, several questions remained regarding the beneficial interaction between grapevine and PsJN strain. How does grapevine sense the root colonization by bacteria and what are the molecular and physiological changes that occur in grapevine by this interaction? Which grapevine defense mechanisms can be activated by these changes and how could they help grapevine to better tolerate "cool" climate? To answer these questions, the three objectives of this study may be decomposed as follows:

I) Study of systemic response of grapevine plantlets after root inoculation by *Bulkholderia phytofirmans* strain PsJN

The effects of rhizobacteria have been demonstrated in different plant species, *e.g*. bean, carnation, cucumber, radish, tobacco, tomato, and in the model plant *Arabidopsis thaliana* (van Loon et al., 1998), and recently in grapevine (Magnin-Robert *et al*., 2007; Verhagen *et al*., 2010). Colonization of roots with plant growth-promoting rhizobacteria (PGPR) leads to induced systemic resistance in parts of plants that are spatially separated from the inducing microorganism (van Loon 1997; 1998). This protection is typically manifested as both a reduction in disease symptoms and inhibition of pathogen growth (van Loon, 2007). ISR is phenotypically similar to SAR that is triggered by necrotizing pathogens. These two types of resistance have been

reported as synonymous (Hammerschmidt *et al.,* 2001; Tuzun, 2006). Nevertheless, the signal transduction pathway and the molecular basis underlying ISR differ in many aspects from the pathogen-induced SAR. For instance, it was reported that pathogen induced SAR requires salicylic acid, whereas rhizobacteria-mediated ISR is almost always dependent on JA and ET signalling (van Loon & Bakker, 2005). Recently a second SAR-type defense response associated with endogenous free SA accumulation and *PR* gene expression and/or PR proteins accumulation has been proposed (Timmusk & Wagner, 1999; Park & Kloepper, 2000; Magnin-Robert *et al*., 2007). In order to further understand the physiological changes that occur in grapevine after root colonization, the first objective of this study is to investigate whether the PsJN strain is able to stimulate the defense mechanism in grapevine plantlets by induction of ISR, studying the changes in pattern of defense gene expression encoding enzymes of phenylopropanoid, pathogenesis-related proteins and octadecanoid pathways in leaves of grapevine plantlets after root inoculation (Fig 1.17a).

II) Characterization of grapevine physiological responses to cold in fully bacterized plantlets with *Burkholderia phytofirmans* strain PsJN

Very recently, the analysis of interaction between PGPR and plants has focused on the establishment of the primed physiological state by which plants are able to better or more rapidly mount defense responses, or both to stress (Conrath *et al*., 2002; Goellner & Conrath, 2008). In several studies, it has been reported that priming is a defense mechanism able to protect plants systemically from several environmental stresses by the potentiated activation of the various cellular defense responses. Nowadays, the event that ISR-inducing PGPR may prime the physiological state

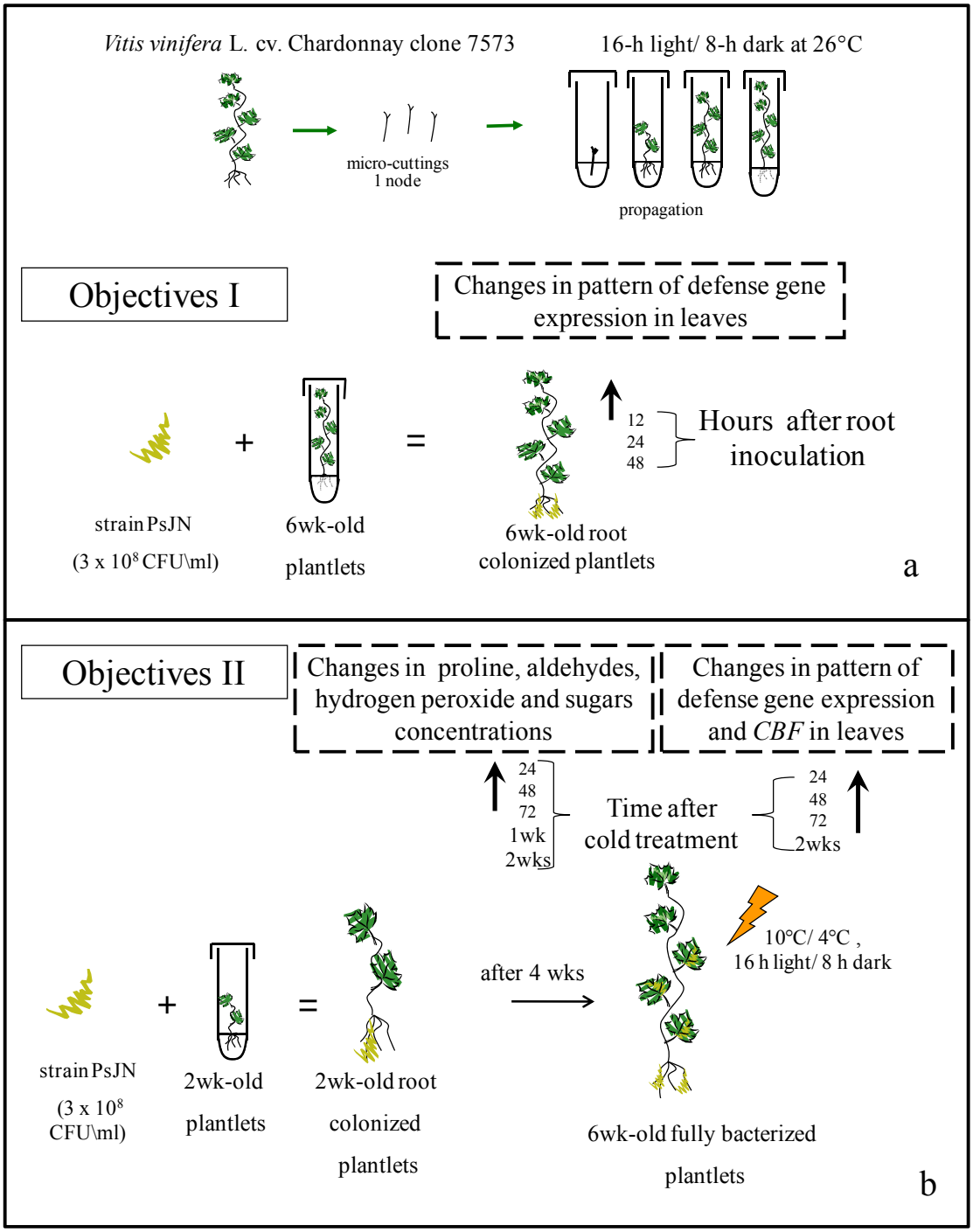

Fig. 1.17: The first and second objective of this study: (a) Study of systemic response of grapevine plantlets after root inoculation by *Bulkholderia phytofirmans* strain PsJN and (b) Characterization of grapevine physiological responses to cold in fully bacterized plantlets with *B. phytofirmans* strain PsJN.

protecting plants from several pathogens is a well-studied phenomenon in plant potentiation (Conrath *et al*., 2006). Although the understanding of several mechanisms of priming in model plants like *Arabidopsis* has progressed, priming induced by ISR-inducing PGPR is a poorly studied area for several plants including grapevine, especially in response to cold.

In order to characterize the defense mechanisms that have been activated in fully bacterized plantlets upon exposure to low non-freezing temperature, the second objective of this project was to check whether this beneficial effect is mediated by the grapevine primed physiological state induced by strain PsJN. Therefore, stress-related expression of genes encoding pathogenesis-related proteins, enzymes of phenylopropanoid and octadecanoid pathways, as well as cold specific transcription factors (*CBF*), and stress-related metabolites such as proline, aldehydes or hydrogen peroxide were monitored in order to characterize the defense mechanism induced in bacterized grapevine plantlets upon low non-freezing temperatures (Fig 1.17b). Additionally, the accumulation of several sugar markers with cryoprotective effects in cold-resistant plants were analysed, in order to further understand potentiated responses of plantlets bacterized by strain PsJN subjected to low non-freezing temperatures.

III) Transcript analysis by cDNA-AFLP technique of primed- physiological state induced by *Burkholderia phytofirmans* strain PsJN in grapevine plantlets

One of the cornerstones of modern molecular biology for analysis of plant physiological responses is the isolation of differentially expressed genes. cDNA-AFLP is a RNA fingerprinting technique that has been extensively used in recent years to display differentially expressed patterns in several plants (Yang *et al*., 2003;

Mao *et al*., 2004; Burger & Botha, 2004). Since the molecular mechanism and the genetic basis of priming are still poorly understood we used non-radioactive cDNA-AFLP technology for fingerprinting of grapevine mRNAs to (i) further investigate of primed physiological state in grapevine plantlets induced by strain PsJN and (ii) isolate and identify unknown over-expressed genes related to cold acclimation in bacterized plantlets after exposure to low non-freezing temperatures (Fig 1.18).

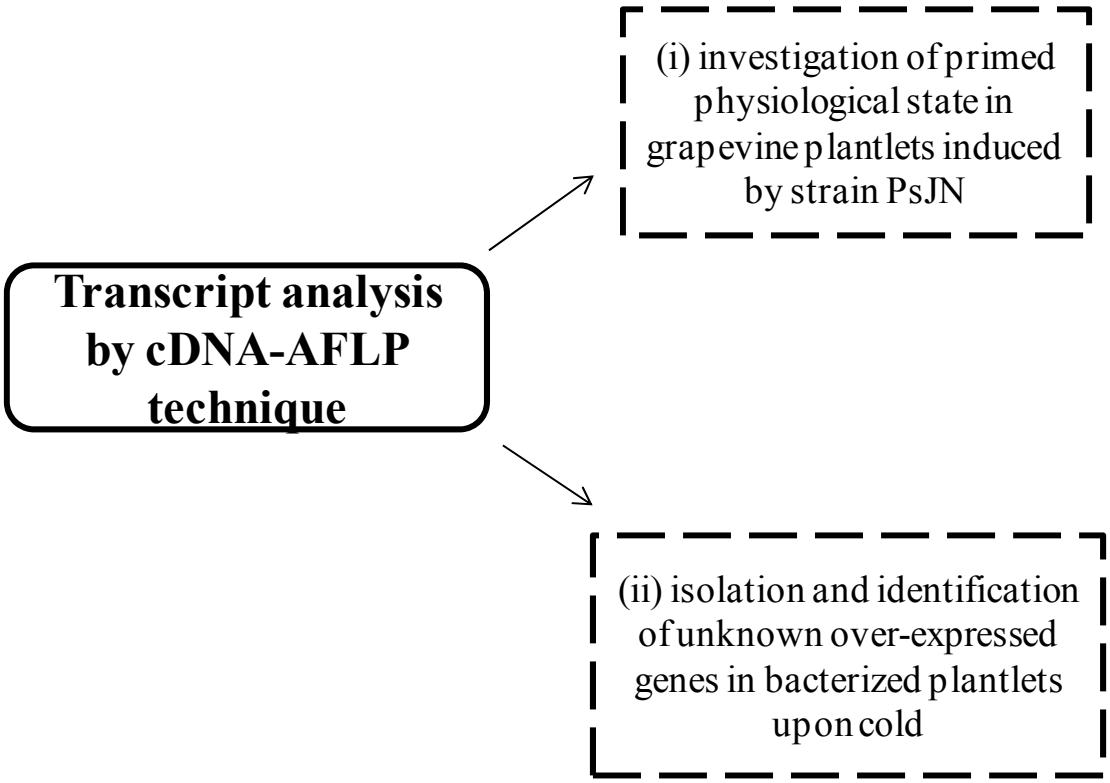

Fig. 1.18: The third objective of this study: Transcript analysis by cDNA-AFLP technique of primed- physiological state induced by *Burkholderia phytofirmans* strain PsJN in grapevine plantlets.

2. RESULTS & DISCUSSION

Induction of systemic resistance in grapevine plantlets by plant growth promoting rhizobacteria, *Burkholderia phytofirmans* strain PsJN

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Running title: PGPR induced defense in grapevine

Abstract

Burkholderia phytofirmans strain PsJN is a plant growth-promoting rhizobacterium (PGPR) able to establish rhizosphere and endophytic populations in grapevine. This bacterium was able to induce resistance against *Botrytis cinerea* and to low temperatures. To further investigate the interaction between grapevine and strain PsJN, changes in the expression pattern of several defense-related genes were analyzed in Chardonnay grapevine plantlets following root inoculation with bacteria. Results showed significant increase in the expression of defense related genes in leaves, although the bacteria were not present int the upper parts of the grapevine plantlets. The observed pattern of defense related-genes was typical for induced systemic resistance.

Introduction

Several biologically induced systemic defence responses have been well characterised. They include systemic acquired response (SAR), which is triggered by pathogens causing limited infection, such as hypersensitive necrosis (Durrant & Dong, 2004); and induced systemic resistance (ISR), which is activated upon colonization of roots by selected strains of non-pathogenic rhizobacteria (van Loon *et al.*, 1998; van Loon, 2007; van Wees, 2008) or mycorrhizal fungi (Pozo & Azcon-Aguilar, 2007).

Over the past decade several plant growth-promoting rhizobacteria (PGPR) have been used as inoculants to improve plant nutrition and as biological agents to control plant pathogens (Bakker *et al.*, 2003; Bloemberg *et al.*, 2001**;** Dobbelaere *et al.*, 2003; Compant *et al.*, 2005a). Part of effect of PGPR on growth promotion is due to their ability to antagonize deleterious microorganisms on the basis of various mechanisms such as competition for nutrients, siderophore-mediated competition for iron, or production of antibiotics or lytic enzymes (Compant *et al.*, 2005a; de Vleesschauwer & Höfte, 2009).

In addition to their direct antimicrobial proprieties, selected strains of rhizobacteria are also able to activate ISR. This phenomenon has been demonstrated in many different plant species (van Loon *et al.*, 1998, de Vleesschauwer & Höfte, 2009), and is effective against a broad range of plant pathogens, including fungi, bacteria and viruses (van Loon *et al.*, 1998).

The signal(s) induced by the PGPR can spread systemically through the plants, leading to a reduction of disease symptoms even if rhizobacteria and pathogens are spatially separated (van Loon *et al.*, 1998; van Loon & Bakker, 2005; 2006; Pieterse & van Loon, 2009).

The mechanisms involved in SAR are well documented in many plant species. They include several well-characterized defense reactions such as hypersensitive reaction (HR), oxidative burst, reinforcement of cell wall structures through lignification or callose deposition, accumulation of antimicrobial phytoalexins and induction of defense-related proteins with antifungal properties (Pieterse & van Loon, 2009). By contrast, mechanisms involved in ISR are less understood. They include, the reinforcement of cell wall structures through lignification or callose deposition (Benhamou *et al.*, 1996), the accumulation of antimicrobial phytoalexins (Ongena *et al.,* 1999) and the induction of genes encoding defense-related proteins (Bloemberg & Lugtenberg, 2001; Cartieaux *et al*, 2003; Verhagen *et al.*, 2004).

Endophytic PGPR*, Burkholderia phytofirmans* strain PsJN (Sessitsch *et al.,* 2005), has been isolated from surface-sterilized onion roots (Frommel *et al*., 1991). The bacterium is able to (i) establish rhizosphere and endophytic populations in various plants, (ii) stimulate plant growth, and (iii) induce developmental changes leading to better plant adaptation to environmental stresses (Bensalim *et al.*, 1998, Sharma & Nowak, 1998). Moreover, the strain PsJN also showed biocontrol activity since it protects effectively *in vitro* and *in vivo* against *Botrytis cinerea* (Ait Barka *et al.,* 2002, Compant *et al*., personal data).

After inoculation of grapevine plantlets with a *gfp* derivative of strain PsJN, the root interior is colonized within 3 h post inoculation (p.i.) (Compant *et al.*, 2005b). Nevertheless, the first PsJN::*gfp*2x cells were detected in the fifth leaf only after 72 h p.i. with a stationary-phase level that occurred 84 h p.i. (Compant *et al.*, 2005b).

Non-pathogenic rhizobacteria can stimulate ISR response in the host plant. Nevertheless molecular events underlying ISR are less well understood than in the

case of SAR. The present study investigated the ISR character of *B. phytofirmans* strain PsJN by monitoring the expression of several defense related-genes after colonization of grapevine by the bacterium.

Materials and Methods

Plant material and *in vitro* growth conditions.

Plantlets of *V. vinifera* cv. Chardonnay clone 7535 were micropropagated by nodal explants grown on 15 ml semisolid medium in 25 mm culture tubes as described by Ait Barka *et al.* (2006). The cultures were grown in a growth chamber under white fluoresent light (200 μE $m^2 \cdot s^{-1}$) with 16 h light at 26°C (constant temperature).

Bacterial inoculum

The bacterial inoculum was produced by transferring two loops of *B. phytofirmans* PsJN to 100 ml of King's B liquid medium in 250 ml Erlenmeyer flasks incubated at 20 °C at 150 rpm for 48 h. Bacteria were collected by centrifugation (3,000 x g for 15 min) and washed twice with phosphate-buffer saline (PBS) (10 mM, pH 6.5). The pellet was re-suspended in PBS and used as inoculum. The bacterium concentration was estimated by spectrophotometry (600 nm) and adjusted to 3 x 10^8 $CFU.ml^{-1}$ with PBS.

Plant bacterization.

Six-week-old plantlets, with six developed leaves, were gently removed from agar medium, inoculated by root immersion in 2 ml of *B. phytofirmans* strain PsJN (3 x 10^8 $CFU.ml^{-1}$). Roots of control plantlets were immersed in PBS. Plant leaves were sampled 12, 24 and 48 h post inoculation to analyze gene expression. At that time it

has been shown previously that bacteria were still in the rhizosphere and root internal tissues and did not colonize the aerial parts (Compant *et al.*, 2005b). Leaves, close to roots of plantlets were avoided to insure the absence of bacteria in the samples.

Analysis of gene expression

Sampling, DNase treatment, RNA extraction and synthesis of cDNA. Leaf samples were frozen in liquid nitrogen and stored at - 80°C until use. Leaves were ground in liquid nitrogen to a fine powder and total RNAs were extracted from 100 mg powder following the RNA Plant Purification Reagent protocol, according to the manufacturer's instruction (Invitrogen, France). The RNA pellet was re-suspended in 20 µl of RNase-free water. Genomic DNA was removed with RNase-free DNase treatment (Promega, ref 9PIM610). Five µl of total RNA were treated with 1 U of enzyme according to manufacturer's instructions. RNA purity and concentration were assessed by determining the spectrophotometric absorbance of the samples at 260 and 280 nm and A260/A280 ratios. RNA integrity was evaluated from the 28S and 18S rRNA bands on 1 % agarose gel after electrophoresis in 0.5xTAE (Tris-Acetate-EDTA), stained with ethidium bromide and visualised under UV light.

Reverse transcription of RNA was performed with 200 ng of total RNA, using M-MLV reverse transcriptase (Invitrogen, France) following the manufacturer's protocol.

Real time RT-PCR analysis: PCR reactions were carried out in duplicates in 96-well plates (25 µl per well) in a reaction buffer containing 1xSYBR Green I mix (PE Biosystems; including Taq polymerase, dNTPs, SYBR Green dye), 300 nM primers (forward and reverse) and a 1:50 dilution of reverse transcribed RNA. PCR

Table 1: Genes analyzed by real-time RT-PCR

Genes	Primer sequences
Ef1α	Forward 5′ GAA CTG GGT GCT TGA TAG GC 3′ Reverse 5′ AAC CAA AAT ATC CGG AGT AAA AGA 3′
Phenylalanine ammonia-lyase (VvPAL)	Forward 5′ TCC TCC CGG AAA ACA GCT G 3′ Reverse 5′ TCC TCC AAA TGC CTC AAA TCA 3′
Stilbene synthase (VvStSy)	Forward 5′ AGG AAG CAG CAT TGA AGG CTC 3′ Reverse 5′ TGC ACC AGG CAT TTC TAC ACC 3′
Lipoxygenase (VvLOX)	Forward 5′ CTG GGT GGC TTC TGC TCT C 3′ Reverse 5′ GAT AAG CCG CAG ATT CAT GC 3′
β-1,3-glucanase (VvGluc)	Forward 5′ AAT TTG ATC CGC CAC GTC AA 3′ Reverse 5′ TGC GGC TCC TTC TTG TTC TC 3′
Chitinase 4c (VvChit4c)	Forwrad 5′ GCA ACC GAT GTT GAC ATA TCA 3′ Reverse 5′ CTC ACT TGC TAG GGC GAC G 3′
Protease inhibitor (VvPIN)	Forwrad 5′ AGT TCA GGG AGA GGT TGC TG 3′ Reverse 5′ GCA CTA GGG TCC GTG TTT GGG TCG ACG 3′

conditions were 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension) for 40 cycles on a GeneAmp 5700 sequence Detection System (Applied Biosystems, France). Transcript level was calculated using the standard curve method and normalized against *Eflα* gene as an internal control (Terrier *et al.*, 2005). Non bacterized plantlets grown at 26°C were used as a reference sample (X1 expression).

The genes and the specific primers used in this study are listed in Table 1. They correspond to genes encoding enzymes of the phenylopropanoid pathway (phenylalanine ammonia lyase (*VvPAL*) and stilbene synthase (*VvStSy*)), three pathogenesis-related proteins (*VvGluc, VvChit4c,* and *VvPIN)* which encode an acidic PR-3, basic PR-2 and a PR-6 respectively), and lipoxygenase for the octadecanoid pathway (*VvLOX*).

Results

An induction of expression was observed for all genes after the inoculation of plants with the strain PsJN (Fig. 1). The pattern of *VvStSy, VvPAL, VvChit4c* and *VvLOX*

expression showed a peak 12 h after root inoculation with an increase between 7 to 30 fold higher than basal level in inoculated plantlets compared to the control. Transcript accumulation then decreased and reach the basal level 48 h after root inoculation (Fig. 1a,b,c,f). The expression of *VvGluc* increased after 12 h with a maximum 24 h (Fig. 1d) after inoculation (16-fold), then decreased to basal level for non-bacterized plantlets. The expression of *VvPIN* was induced 170-fold more than control starting from 12 h to 24 h after root inoculation (Fig.1e), then returned to the same level as control at 48 h.

Discussion

The root colonization by strain PsJN resulted in a significant increase in the expression of different defense related-genes in leaves. Because bacteria were still present in the rhizoplane and only began to penetrate internal root tissues (Compant *et al.*, 2005b), we suggest that the strain PsJN may induce a systemic spread of a signal from roots to leaves within 12 h after root inoculation. A similar phenomenon, referred to as ISR response, has been reported in several studies. For instance, ISR was described in *Arabidopsis thaliana* roots after colonization by *Paenibacillus polymyxa* (Timmusk & Wagner, 1999). The non-pathogenic rhizobacterium *Bacillus amyloliquefaciens*, was also able to elicit ISR by transcript accumulation of defense-related genes in tobacco leaves (Ahn *et al.*, 2002).

Research on molecular mechanisms of rhizobacteria-mediated ISR was initially focused on the role of PR-proteins, as the accumulation of these proteins was considered to be strictly correlated with induced disease resistance. According to Pieterse and van Loon (2009) the onset of ISR, unlike SAR, is not accompanied by

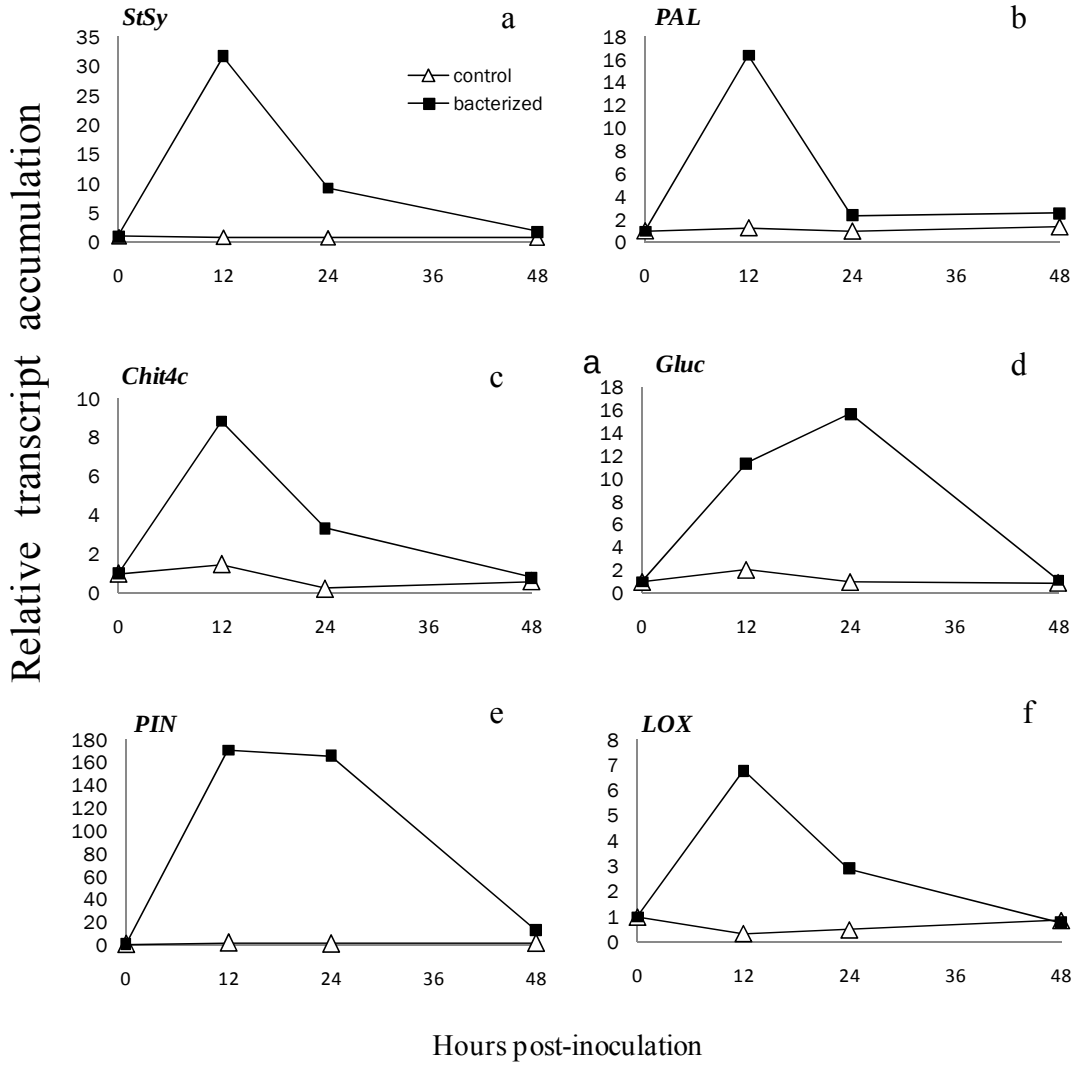

Fig. 1: Defense gene expression of bacterized and non-bacterized grapevine plantlets leaves. Transcript accumulation of *VvStSy* (a), *VvPAL* (b), *VvChit4c* (c), *VvGluc* (d), *VvPIN* (e), and *VvLOX* (f), within 12, 24 and 48 hours post-inoculation, in response to strain PsJN treatment. Level of transcripts was calculated using the standard curve method from duplicate data, with grapevine *EF-1α* gene as internal control and non-treated plantlets as reference sample.

the concomitant activation of *PR* genes. These authors reported that accumulation of PR-proteins was detectable only after pathogen assault. In the same line, radish plants of which roots were treated with ISR-inducing *P. fluorescens* WCS417r did not accumulate PR proteins, although these plants clearly showed enhanced resistance against fusarium wilt disease (Hoffland *et al.*, 1995). Interestingly, *AtTLP1* encoding a thaumatin-like protein that belongs to the PR-5 family was expressed in the root vascular bundle of *Arabidopsis* upon colonization by WCS417r (Leon-Kloosterzield *et al.,* 2005). These authors indicate that induction of *AtTLP1* is a local response of *Arabidopsis* roots to colonization by non-pathogenic fluorescent *Pseudomonas* spp. and is unlikely to play a role in systemic resistance.

In fact, according to the literature, it appears that the induction and accumulation of PR-proteins seems dependant to the host-ISR-inducting agent interaction.

Nevertheless, in accordance with our results showing an induction of genes encoding PR-proteins as response of root inoculation by strain PsJN, different reports have demonstrated that PGPR are able to induce the expression of genes encoding PR-proteins and/or accumulation of PR-proteins in plants (Timmusk & Wagner, 1999; Park & Kloepper, 2000; Ahn *et al.*, 2002), including grapevine (Magnin-Robert *et al.* 2007).

In most cases, ISR is suggested to be controlled by a SA-independent pathway (JA/ET dependant pathway) (van Wees *et al.* 2000; Pieterse and van Loon, 2009). Nevertheless, analysis of JA and ET levels in leaves of ISR-expressing plants revealed no changes in the production of these signal molecules (Pieterse *et al.*, 2000; Hase *et al.*, 2003). Therefore, it had to be assumed that the JA and ET dependency of ISR is based on an enhanced sensitivity to these hormones, rather than on an increase

in their production. Recently, Conn *et al.* (2008) reported that culture filtrate of *Micromonospora* sp. strain EN43 grown in a minimal medium resulted in the induction of the SAR pathway; however, when grown in a complex medium, the JA/ET pathway was activated. In this line, Maurhofer *et al.* (1994) showed that inoculation of plants by the root-invading *P. fluorescens* strain CHA0 induced the synthesis of PR-proteins. The increased resistance in tobacco as response to bacterium might be fully explained by the bacterial production of SA, which could elicit a SAR response leading to SA-inducible PR-proteins in the leaves.

Timmusk and Wagner, (1999) reported that *P. polymyxa* also induced gene expression of *PR-1*, a SA-dependent gene, suggesting that this PGPR induced a mild biotic stress. This effect initiated a systemic response that resulted in partial protection against *Erwinia carotovora*.

Later on, Wang *et al.* (2005) report the up-regulation of genes encoding PR proteins including β-1,3-glucanase (PR-2) and hevein-like protein precursor (PR-4) as response of *Arabidopsis* to *P. fluorescens* FPT9601-T5 colonization. It was also reported that the protection of *Arabidopsis* against CMV by strain 90-166 follows a signalling pathway for virus protection that is not dependent of SA and NPR1, but dependent on jasmonic acid (Ryu *et al.,* 2004).

Chitinases are up-regulated by a variety of stress conditions and by phytohormones such as ethylene, jasmonic acid, and salicylic acid (Busam *et al.* 1997). Like other PR proteins, chitinases play a role in plant resistance against distinct pathogens. Inhibitors of serine proteases (PIN), belonging to the class of antifungal PR-6 proteins, have a potent activity against plant and animal pathogens (van loon & van Strien, 1999). The *PIN* genes have been extensively characterized as a marker-gene in the jasmonate-induced transduction cascade. Our results showed an induction

of *Chit4c* and *PIN* expression after root inoculation. In accordance**,** Belhadj *et al.* (2007) reported an induction of several mechanisms of defense including genes encoding PR proteins such as *Chit4c* and *PIN*, up-regulation of *PAL* and *StSy* genes when grapevine cell suspensions were treated with MeJA in presence of carbohydrates.

The resistance induced by selected grapevine-associated bacteria was correlated with some plant defense responses such as chitinase and ß-1,3-glucanase activities in both leaves and berries (Magnin-Robert *et al.*, 2007).

In accordance with our result, the activation of systemic resistance by nonpathogenic rhizobacteria has also been associated with the induction of lipoxygenase (LOX) activity in bean and tomato (Akram et al;, 2008; Ongena *et al.*, 2004, Ongena *et al.*, 2007; Sailaja *et al.*, 1998).

By ensuring spatial separation between *B. phytofirmans* and the systemic response observed in leaves, the PGPR was able to induce the systemic response of several defense genes coding for enzymes of phenylopropanoid pathway, octadecanoid pathway and pathogenesis-related proteins may be involved in JA/ET pathway in grapevine plantlets after root inoculation. Although modification of the gene expression profiles caused by PGPR inoculation has been mostly reported with *Arabidopsis* (Cartieaux *et al.*, 2003; Wang *et al.*, 2005), our study indicates that the strain PsJN could possibly trigger the ISR in grapevine.

However, the results of this study showed that the PsJN the induce the expression of either SA and JA/ET pathways. This suggests that interaction between grapevine and strain PsJN may act through ISR and SAR by overlaping both mechanisms. Pieterse and van Loon (2009) noted that global expression profiling of various *Arabidopsis*-attacker interactions revealed substantial crosstalk between SA-,

JA- and ET-dependent defense pathways that provide a powerful regulatory potential allowing the plant to fine-tune its defense responses.

To complete this study, multiple ways should be followed under ISR condition:

i) Investigation of early events following root inoculation with the bacteria (ROS, MAPK, Ca^{+}) ;

ii) Analysis of SA and JA levels in plants to discriminate the pathway(s) involved in the establishment of ISR;

iii) Study of physiological changes occurring after the perception of PGPR (photosynthesis; phytoalexines);

iv) The ISR character could be independent from the protection /resistance proprieties toward phytopathogens. Consequently, it will be interesting to analyse the impact of the presence of the strain PsJN on the development of gray mold disease.

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Priming *Vitis vinifera* L. with *Burkholderia phytofirmans* strain PsJN enhances its acclimation to low non-freezing temperatures

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Abstract

Burkholderia phytofirmans strain PsJN is a plant growth-promoting rhizobacterium that colonizes all grapevine (*Vitis vinifera* L.) organs and confers resistance to low non-freezing temperatures. The goal of the paper was to highlight the mechanisms by which the bacterium favours grapevine acclimation to cold, especially whether the beneficial effect of the bacterium is mediated through the priming of plant responses. In order to investigate the putative primed state of bacterized plantlets, stress-related gene expression and stress-related metabolite accumulation were monitored in bacterized grapevine plantlets submitted to low non-freezing temperatures. When plantlets were grown at 26°C, bacterization had no significant effect on tested parameters. By contrast, both stress-related gene transcripts and metabolite levels increased to a higher extent in bacterized plantlets than in non-bacterized ones at 4°C. Results indicate that the presence of *B. phytofirmans* strain PsJN within grapevine tissues primes responses to cold stress. Moreover, after one week of cold exposure, the content of stress-related metabolites such as aldehydes and hydrogen peroxide decreased faster in bacterized than in non-bacterized plantlets, suggesting that the presence of the bacteria in plant tissues favours the cold acclimation process.

Introduction

During the course of evolution, plant species have developed mechanisms that enable them to acclimate to cold by minimizing the deleterious effects of cold stress (Gilmour *et al.*, 2000; Xin & Browse, 2000). Under low, non-freezing temperatures, cell membranes of cold sensitive plants rigidify, leading to a disturbance of membrane-related processes such as opening of ion channels or membrane-associated electron transfer reactions (Alonso *et al.*, 1997; Uemura & Steponkus, 1999). As a consequence, the whole plant physiology is affected as revealed by a decrease of photosynthesis and subsequent growth alteration (Ait Barka *et al.*, 2006). By contrast, plants may adapt to these adverse conditions and thus maintain their physiological activity.

Numbers of physiological changes have been identified related to cold acclimation (Thomashow, 1999; Chinnusamy *et al.*, 2006; Nakashima & Yamaguchi-Shinozaki, 2006), including general stress-related responses such as accumulation of reactive oxygen species (ROS) (Neill *et al.*, 2002), but also cold specific traits. Cold acclimation has been shown to be correlated to (i) accumulation of cryoprotective compounds such as sugars and proline (Hekneby *et al.*, 2006; Patton *et al.*, 2007), (ii) regulation of specific gene expression (Hughes & Dunn, 1996; Chinnusamy *et al.*, 2006) and (iii) synthesis of cold-stress related proteins (Hughes & Dunn, 1996; Thomashow, 1999).

The analysis of cold acclimation reveals a complex process resulting in a coordinated up- or down-regulation of hundreds of genes. For example, transcript levels of *COR* (cold regulated), *LTI* (low-temperature induced), or *KIN* (cold induced) genes greatly increased within a few hours following exposure to cold (Thomashow, 1999). The signal transduction pathways leading to expression of cold-regulated

genes in *Arabidopsis thaliana* involve a regular network in which *CBF* (C-repeat binding factor) transcription factors (*CBF1-3*) control many *COR* genes during cold acclimation (Fowler & Thomashow, 2002; Xiao *et al.*, 2006; Tattersall *et al.*, 2007; Xiao *et al.*, 2008).

In grapevine, low temperatures decrease both growth and photosynthesis (Flexas *et al.*, 1999; Hendrickson *et al.*, 2003; Hendrickson *et al.*, 2004; Bertamini *et al.*, 2005; Ait Barka *et al.*, 2006) but stimulate both carbohydrate metabolism (Hamman *et al.*, 1996) and proline accumulation (Ait Barka & Audran, 1997; Ait Barka *et al.*, 2006). Recently, a *Vitis vinifera CBF4* gene, homologue to *A. thaliana CBF1,* has been characterized upon exposure to low non-freezing temperature (4°C) and might be preponderant for the over-wintering of grape plants (Xiao *et al.*, 2008).

Burkholderia phytofirmans strain PsJN is a plant growth-promoting rhizobacterium (PGPR) able to establish rhizospheric and endophytic populations in various plants (Nowak & Shulaev, 2003). This bacterium stimulates plant growth and induces physiological changes leading to a better plant adaptation to environmental stresses (Nowak *et al.*, 1995; Pillay & Nowak, 1997; Bensalim *et al.*, 1998; Sharma & Nowak, 1998). In grapevine, it was recently established that *B. phytofirmans* strain PsJN colonizes the rhizosphere, penetrates roots and thus migrates into all plant tissues (Compant *et al.*, 2005). Furthermore, the presence of the bacterium in the plant causes a better acclimation to cold non freezing temperatures, as revealed by (i) lower cell damages, (ii) higher photosynthetic activity and (iii) accumulation of cold stress related metabolites such as starch, proline and phenolic compounds (Ait Barka *et al.*, 2006).

Following colonization of roots by beneficial microbes, infection by necrotizing pathogens, or after treatment with various chemicals, plants can establish a

unique physiological situation so called ‘‘primed’’ state (Conrath, 2009). Primed plants respond by activating defense responses faster and/or more strongly when subsequently challenged by microbial pathogens, herbivorous insects, or abiotic stresses (Conrath *et al.*, 2002; Conrath *et al.*, 2007; Goellner & Conrath, 2008; Conrath, 2009).

The beneficial interaction between grapevine and *B. phytofirmans* strain PsJN remains to be further elucidated, especially how the presence of this strain helps to withstand cold. We hypothesized that bacterized plants might be primed to respond quicker or more efficiently to cold conditions. For this purpose, we analyzed the ability of the bacterium to potentiate (i) expression of defense- and cold-related genes and (ii) changes in concentrations of several stress-related metabolites such as proline, stress-related aldehydes and hydrogen peroxide.

Materials and Methods

Plant material and *in vitro* growth conditions

Plantlets of *Vitis vinifera* cv. Chardonnay clone 7535 were micro-propagated by nodal explants grown on 15 ml agar medium in 25 mm culture tubes as described earlier (Ait Barka *et al.*, 2006). Cultures were performed in a growth chamber under white fluorescent light (200 $\mu E.m^{-2}.s^{-1}$) with 16 h light per day at constant 26°C.

Bacterial inoculum

The bacterial inoculum was produced by transferring two loops of *B. phytofirmans* PsJN to 100 ml of King’s B liquid medium in 250 ml Erlenmeyer flask incubated at 20°C at 150 rpm for 48 h. Bacteria were collected by centrifugation (3,000 g for 15 min) and washed twice with phosphate-buffer saline (PBS) (10 mM, pH 6.5). The

pellet was re-suspended in PBS and used as inoculum. The bacterium concentration was estimated by spectrophotometry (600 nm) and adjusted to 3 x 10^8 CFU.ml^{-1} with PBS (Pillay & Nowak, 1997).

Plant bacterization

Roots of two week-old plantlets were immersed in *B. phytofirmans* strain PsJN (3 x 10^8 CFU.ml^{-1}) for 10 s. Roots of control plantlets were immersed in PBS. After immersion, plantlets were grown as described above for four weeks before cold treatment. At that time, it is established that bacteria have colonized roots, stem and leaves of grapevine plantlets (Compant *et al.*, 2005).

Cold treatment

Six week-old (bacterized four weeks earlier) and non-bacterized plantlets were transferred to a cold growth chamber maintained at 10°C under 16 h light (white fluorescent light, 200 µE $m^{-2}.s^{-1}$) and at 4°C for 8 h dark, whereas the control plants were at 26°C. Each treatment was replicated three times and each replicate consisted of six plantlets.

Analysis of gene expression

Sampling, DNase treatment, RNA extraction and synthesis of cDNA. Leaf samples were frozen in liquid nitrogen and stored at - 80°C until use. Leaves were ground in liquid nitrogen to a fine powder and total RNA was extracted from 100 mg powder following the RNA Plant Purification Reagent protocol, according to the manufacturer's instruction (Invitrogen, France). The RNA pellet was re-suspended in 20 µl of RNase-free water. Genomic DNA was removed with RNase-free DNase treatment (Promega, ref 9PIM610). Five µl of total RNA were treated with 1 U of

enzyme according to manufacturer's instructions. RNA purity and concentration were assessed by determining the spectrophotometric absorbance of the samples at 260 and 280 nm and A260/A280 ratios. RNA integrity was evaluated from the 28S and 18S rRNA bands on 1 % agarose gel after electrophoresis in 0.5xTAE (Tris-Acetate-EDTA), stained with ethidium bromide and visualised under UV light.

Reverse transcription of RNA was performed with 200 ng of total RNA, using M-MLV reverse transcriptase (Invitrogen, France) following the manufacturer's protocol.

Real time RT-PCR analysis. PCR reactions were carried out in duplicates in 96-well plates (25 µl per well) in a reaction buffer containing 1xSYBR Green I mix (PE Biosystems; including Taq polymerase, dNTPs, SYBR Green dye), 280 nM primers (forward and reverse) and a 1:50 dilution of reverse transcribed RNA. PCR conditions were 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension) for 40 cycles on a GeneAmp 5700 sequence Detection System (Applied Biosystems, France). Transcript level was calculated using the standard curve method and normalized against *Eflα* gene as an internal control (Terrier *et al.*, 2005). Non-bacterized plantlets grown at 26°C were used as a reference sample.

The genes and the specific primers used in this study were listed in Table 1. They corresponded to genes encoding a phenylalanine ammonia-lyase (*PAL*), a stilbene synthase (*StSy*), three pathogenesis-related (PR) proteins (*Gluc, Chit4c,* and *Chit1b*), a lipoxygenase (*LOX*) and the transcription factor *CBF4*.

Analysis of stress-related metabolites

Table 1: Genes analyzed by real-time RT-PCR

Genes and accession numbers	Primer sequences
Elongation factor 1α (Ef1α) BQ799343	Forward 5′ GAA CTG GGT GCT TGA TAG GC 3′ Reverse 5′ AAC CAA AAT ATC CGG AGT AAA AGA 3′
Phenylalanine ammonia-lyase (PAL) X75967	Forward 5′ TCC TCC CGG AAA ACA GCT G 3′ Reverse 5′ TCC TCC AAA TGC CTC AAA TCA 3′
Stilbene synthase (StSy) AF274281	Forward 5′ AGG AAG CAG CAT TGA AGG CTC 3′ Reverse 5′ TGC ACC AGG CAT TTC TAC ACC 3′
Lipoxygenase (LOX) AY159556	Forward 5′ CTG GGT GGC TTC TGC TCT C 3′ Reverse 5′ GAT AAG CCG CAG ATT CAT GC 3′
β-1,3-glucanase (Gluc) AF239617	Forward 5′ AAT TTG ATC CGC CAC GTC AA 3′ Reverse 5′ TGC GGC TCC TTC TTG TTC TC 3′
Chitinase 4c (Chit4c) AY137377	Forwrad 5′ GCA ACC GAT GTT GAC ATA TCA 3′ Reverse 5′ CTC ACT TGC TAG GGC GAC G 3′
Chitinase 1b (Chit1b) Z54234	Forward 5′ ATG CTG CAG CAA GTT TGG TT 3′ Reverse 5′ CAT CCT CCT GTG ATG ACA TT 3′
C-repeat binding factor (*CBF4*) DQ497624	Forward 5'-ACC CTC ACC CGC TCG TTA TG- 3' Reverse 5'-CCG CGT CTC CCC GAA ACT T- 3'

Free proline. Two hundred µl of the potassium phosphate extract were mixed with 800 µl ninhydrin reagent which contained 1 % (w/v) ninhydrin in 60 % acetic acid (Ait Barka & Audran, 1997). The mixture was heated at 100°C for 20 min and then cooled in ice. One ml toluene was added and the sample was vigorously shaken for 15 s. The samples were placed in darkness at room temperature for at least 4 h. The absorbance of the upper phase was then read spectrophotometrically at 520 nm. Proline concentration was determined by using a calibration curve and expressed as µM proline.g^{-1} dry weight (DW).

Lipid peroxidation (LP) markers. The lipid peroxidation was evaluated by assaying the concentration of thiobarbituric acid reactive substances according to Heath & Packer (1968). Fresh leaves were ground in Fontainebleau sand and trichloroacetic acid (TCA) (0.1 % w/v). The homogenate was centrifuged at 4°C for 10 min at

12,000 g. One volume supernatant was mixed with 4 volumes of 20 % TCA containing 0.5 % (w/v) 2-thiobarbituric acid. The mixture was heated at 95°C for 30 min, quickly cooled in ice and centrifuged at 10,000 g for 5 min. The non-specific absorbance of supernatant at 600 nm was substracted from the maximum absorbance at 532 nm for malondialdehyde (MDA) measurement (Kumar & Knowles, 1993) and 455 nm for aldehydes (Meir *et al.*, 1992). For MDA and aldehyde calculation, an extinction coefficient (E) of 1.56×10^5 $M^{-1}.cm^{-1}$ was used for MDA at 532 nm, and an E of 0.457×10^5 $M^{-1}.cm^{-1}$ was used at 455 nm as average of E obtained for aldehydes (propanal, butanal, hexanal, heptanal, and propanal-dimethylacetal) according to Meir *et al.* (1992). Results were expressed in $nmol.g^{-1}$ DW.

Hydrogen peroxide. H_2O_2 content was evaluated according to the method of (Mondal & Choudhuri, 1981), with some modifications. Two hundred and fifty mg of leaf powder was homogenised in 1 ml of ice-cold acetone and was centrifuged at 13,500 g for 10 min. Two hundred and fifty µl of cold water and 100 µl of 5 % titanyl sulfate were added to supernatant. To this mixture, 500 µl of 1N NH_4OH solution were added to precipitate the peroxide-titanium complex. After centrifugation at 6,000 g for 5 min, the supernatant was discarded and the pellet was washed with cold acetone. The precipitate was then dissolved in 1.5 ml of 2N H_2SO_4 and the final volume adjusted to 2 ml with cold water. The absorbance of the solution was read at 415 nm. H_2O_2 content was calculated from a standard curve.

Statistical analysis

Metabolite and gene expression studies corresponded to 3 independent experiments. Each replicate corresponded to 6 plantlets. Reported data are means ± standard error

(SE) of the 3 independent experiments, except *CBF4*, for which reported data are means ± SE of a duplicate of one representative experiment out of 3 independent ones. Standard analysis of the variance (*t* test) was used to assess the significance of the treatment means at $P<0.05$ level.

Results

Gene expression

Whatever gene considered, our results showed that basal levels of gene expression were similar in bacterized and non-bacterized plantlets grown at 26ºC (Fig. 1). When plantlets were exposed to low-non freezing temperature (4ºC), transcripts of all genes accumulated in both non-bacterized and bacterized plantlets, except for *LOX*. Nevertheless, the accumulation profiles in bacterized and non-bacterized plantlets were significantly different with higher levels measured in the formers (Fig. 1).

After 24 h of cold exposure, *StSy* and *PAL* expression was enhanced by 460 and 40 respectively in bacterized plantlets, whereas it increased only by 150 and 9 in the non-bacterized ones (Figs. 1a, b). After 48h of cold exposure, transcript levels decreased but remained higher in bacterized plantlets. After two weeks of treatment, no differences were noticed between bacterized and non-bacterized plantlets.

Considering PR proteins, *Chit4c* transcripts accumulated gradually in bacterized plantlets, reaching a peak (40 fold) after 72 h of cold shift (Fig. 1c). After two weeks of treatment, transcript accumulation remained 15 fold above the basal level. In non-bacterized plantlets, the pattern was similar but less amplified. *Chit1b* expression was induced by 12 after 24h of cold exposure in bacterized plantlets and then decreased gradually until the end of experiment (Fig. 1d). In non-bacterized plantlets, expression was only enhanced by 4 after 24 h. Similarly *Gluc* expression

was also stimulated following cold treatment (peak at 48 h), but in a higher extent in bacterized plantlets (Fig. 1e).

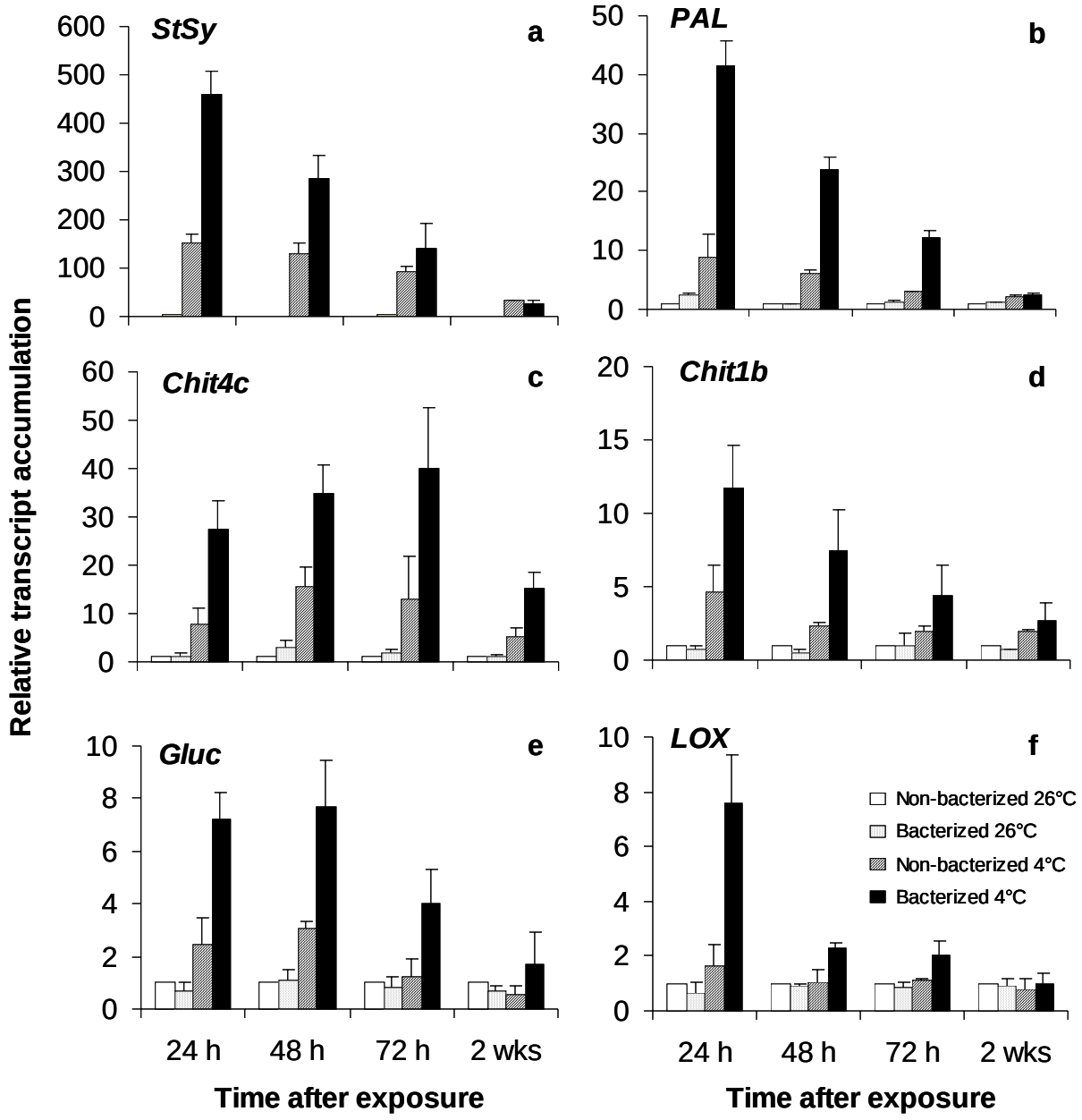

Fig. 1: Defense gene expression of *StSy* (a), *PAL* (b), *Chit4c* (c), *Chit1b* (d), *Gluc* (e) and *LOX* (f) in leaves of non-bacterized and bacterized grapevine plantlets after 24, 48, 72 h, 1 and 2 weeks of cold treatment. Results represent the mean fold increase of

mRNA level over control plantlets (non-bacterized, 26°C) referred as 1 x expression level and correspond to means of 3 independent experiments ± SD

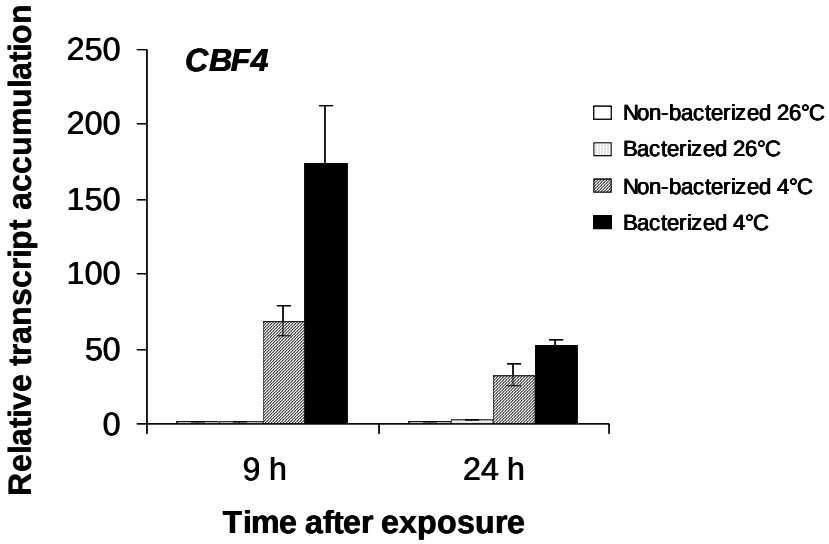

Fig. 2: *CBF4* expression in leaves of non-bacterized and bacterized grapevine plantlets after 9 and 24 h of cold treatment. Results represent the mean fold increase of mRNA level over control plantlets (non-bacterized, 26°C) referred as 1 x expression level and correspond to means ± SD of a duplicate of one representative experiment out of 3 independent ones.

In contrast to the other tested genes, *LOX* expression was not induced in non-bacterized plantlets (Fig. 1f). Oppositely, bacterization resulted in a transient peak of expression after 24 h of cold treatment (7.5 fold).

To analyze transcription factor *CBF4* expression, leaves were sampled earlier because it was reported that the peak of *Vitis CBF4* expression following cold stress occurred after 9 h (Xiao *et al.*, 2008). Transcript level was actually up-regulated in plantlets within 9 h after cold exposure (Fig. 2). Gene expression peaked to 60 and 160 fold in non- and bacterized plants, respectively. Interestingly, *CBF(1-3)* gene expression, known for their cold responsiveness in grapevine (Xiao *et al.*, 2006), was not induced in this study (data not shown).

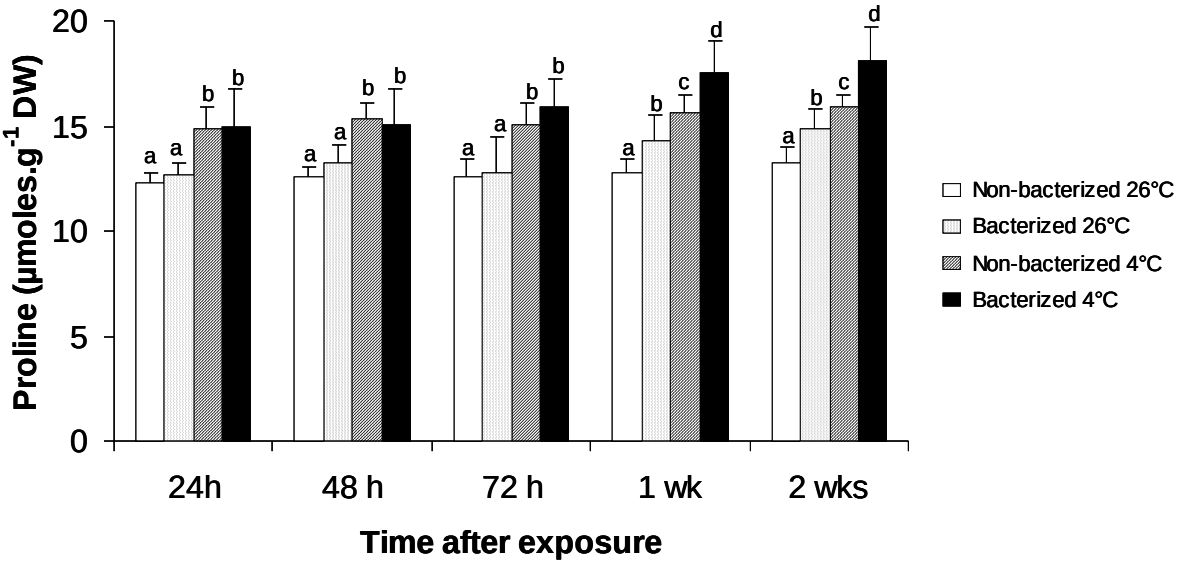

Fig. 3: Accumulation of proline in non-bacterized and bacterized grapevine plantlets after 24, 48, 72 h, 1 and 2 weeks of cold treatment. Means indicated with different letters are significantly different ($P<0.05$).

Proline content

At 26°C, bacterization of grapevine plantlets did not induce significant increase of proline level during the first 3 days (Fig. 3). After 1 and 2 weeks, proline content was slightly higher in bacterized plantlets. When bacterized or non-bacterized plantlets were subjected to low non-freezing temperature, they both accumulated proline in a greater extent than plantlets grown at 26°C. But proline over-accumulation was significantly higher in bacterized plantlets after one week of cold exposure.

Aldehyde and malondialdehyde content

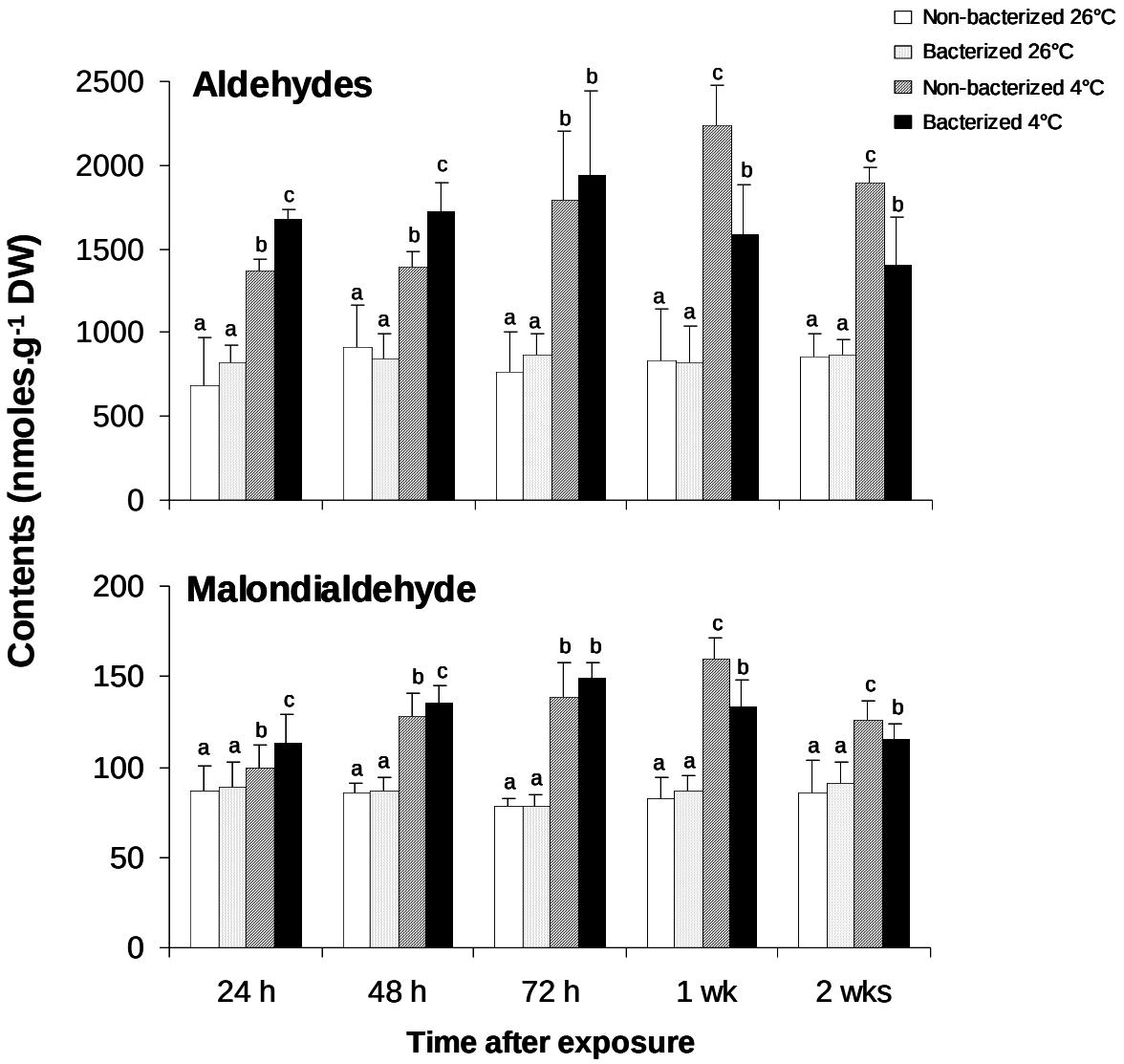

Fig. 4: Accumulation of aldehydes and malondialdehyde in non-bacterized and bacterized grapevine plantlets after 24, 48, 72 h, 1 and 2 weeks of cold treatment. Means indicated with different letters are significantly different ($P<0.05$).

During the sampling period, no significant difference in aldehyde and MDA levels was observed between plantlets when grown at 26°C (Fig. 4). However, after exposure to 4°C, both aldehyde and MDA levels increased since 24 h, with a stronger effect in the bacterized plantlets. After 72 h of treatment, aldehyde and MDA contents became similar in both bacterized and non-bacterized plantlets.

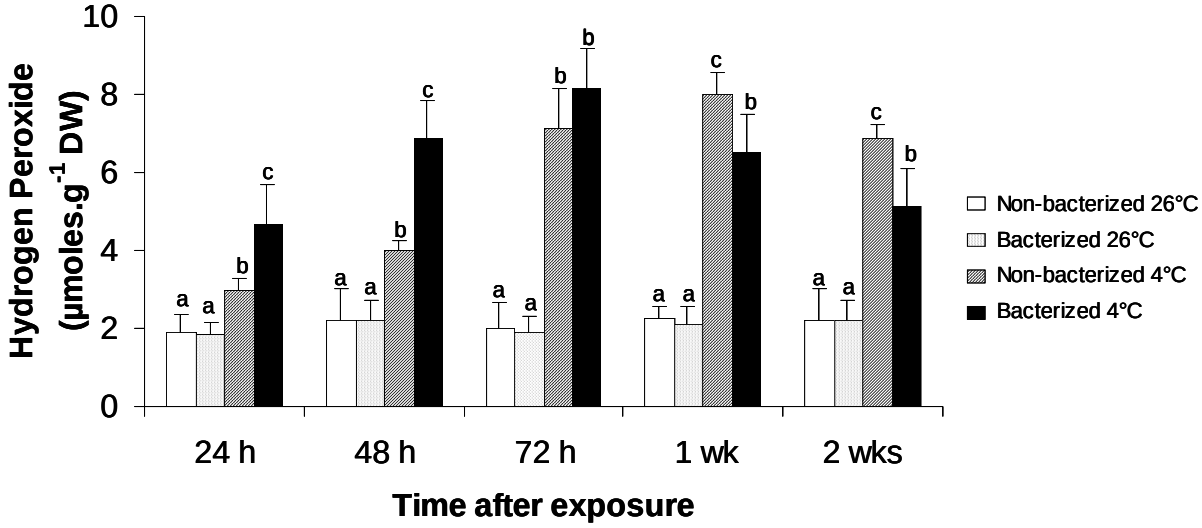

Fig. 5: Accumulation of hydrogen peroxide in non-bacterized and bacterized grapevine plantlets after 24, 48, 72 h, 1 and 2 weeks of cold treatment. Means indicated with different letters are significantly different ($P<0.05$).

Interestingly, afterwards aldehyde and MDA concentrations significantly decreased in higher proportions in bacterized plantlets.

Hydrogen peroxide accumulation

No H_2O_2 accumulated in bacterized plantlets when grown at 26°C (Fig. 5). By contrast, cold stress induced H_2O_2 accumulation in both bacterized and non-bacterized grapevine plantlets within the first 72 h of treatment, but in a higher extent in the formers. Here again, after 1 week of treatment H_2O_2 content was significantly lower in bacterized plantlets.

Discussion

In grapevine plantlets, low temperatures induced the transcription of the cold specific transcription factor *CBF4* after 9 h and later on (24 h) the accumulation of both

defense-related gene transcripts and stress-related metabolites. These reactions were stronger in bacterized plantlets. This may be correlated with previous results showing that leaf cells of bacterized plantlets are less affected by deleterious effects of cold (Ait Barka *et al.*, 2006), and further indicates that *B. phytofirmans* strain PsJN may improve grapevine resistance to low non-freezing temperatures according to the phenomenon of priming (Goellner & Conrath, 2008; Conrath, 2009). Priming induced by PGPR has been reported several times (De Meyer *et al.*, 1999; Hase *et al.*, 2003; Kim *et al.*, 2004; Verhagen *et al.*, 2004; Tjamos *et al.*, 2005; van Wees *et al.*, 2008; Verhagen *et al.*, 2010) when studying induced systemic resistance (ISR) after challenge with pathogens. Our results demonstrate for the first time priming induced by PGPR upon cold stress.

Induction of defense responses upon cold treatment

The induction of defense mechanisms following biotic or abiotic stresses has been well documented. For example, expression of *StSy* genes is often induced in grapevine when submitted to various forms of stress, including pathogen infection (Preisig-Muller *et al.*, 1999), ozone treatment (Brehm *et al.*, 1999) and UV-light application (Versari *et al.*, 2001). But only poor information is available about grapevine defense mechanisms triggered during cold acclimation. In this work, we showed that *StSy* expression was stimulated after 24 h, as reported previously (Sanchez-Ballesta *et al.*, 2007). Similarly, the induction of *PAL* following cold exposure is in accordance with previous results obtained in other plants using either transcriptomics (Christie *et al.*, 1994; Leyva *et al.*, 1995; Gaudet *et al.*, 2003) or proteomics (Cui *et al.*, 2005).

Chitinases and glucanases are of special interest when studying plant responses to cold since they exhibit both antifreeze and antifungal activities and have thus been

extensively analyzed in plants submitted to cold or pathogen attack (Hon *et al.*, 1995; van Loon & van Strien, 1999; Yeh *et al.*, 2000; Huang & Duman, 2002). Depending on the stress, they may be classified either as antifreezing (AFPs; Griffith & Yaish, 2004) or pathogenesis-related (van Loon & van Strien, 1999) proteins.

In *A. thaliana*, the expression of transcription factors *CBF*s is quickly stimulated (2 h) after cold treatment (Liu *et al.*, 1998; Gilmour *et al.*, 2000). More recently, four *CBF*-like genes, *CBF 1–4*, have been isolated from *Vitis* sp. and were shown to be induced within few hours after exposure to low temperatures, in particular *CBF4* (Xiao *et al.*, 2008). Our data showing a clear induction of *CBF4* expression after 9 h of cold treatment are therefore in accordance with these results.

Apart from gene expression, plant responses to cold also result from fluctuations of stress-related metabolites such as proline, hydrogen peroxide or aldehydes. Proline is the best characterized stress responsive molecule and its accumulation is often associated with plant resistance to low temperature, acting as membrane stabilizer, osmo-protector, regulator of enzymes, or scavenger of ROS (Brugiere *et al.*, 1999; Chen & Li, 2002; Wang *et al.*, 2008). It is therefore not amazing that in grapevine, accumulation of proline appeared as a response to cold applied either as a shock (Ait Barka & Audran, 1997) or for acclimation (Ait Barka *et al.*, 2006). Besides, chilling temperatures are known to induce the synthesis of ROS, which stimulate LP (Pinhero *et al.*, 1997). Among ROS, H_2O_2 has opposite effects on plant submitted to low temperatures depending on the process of application (Prasad *et al.*, 1994). Under cold shock, H_2O_2 accumulates to damaging levels in plant tissues because of low levels of antioxidant enzymes. By contrast, in cold-acclimated plants, H_2O_2 triggers the synthesis of antioxidant enzymes such as catalase or peroxidase that scavenge ROS and help the plant to overcome cold

conditions. In our case, the presence of *B. phytofirmans* in plantlets provokes stronger H_2O_2 accumulation within the first 3 days of treatment but also speeds up the decrease of H_2O_2 level after 1 week. Under cold conditions, aldehydes and MDA are produced by the peroxidation of polyunsaturated fatty acids and affect cell membrane fluidity and functions (Barclay & McKersie, 1994). In our work, fluctuations of both aldehydes and MDA levels are similar to those reported for H_2O_2, confirming that *B. phytofirmans* speeds up grapevine reaction to cold shift and later favours the acclimation process to cold temperatures.

The priming effect of *B. phytofirmans* strain PsJN

The presence of the bacterium results in a greater capacity to withstand damage provoked by cold stress (Ait Barka *et al.*, 2006). Our result showed for the first time that the PGPR *B. phytofirmans* strain PsJN acts as a priming agent of grapevine plantlets when submitted to low non-freezing temperatures. Indeed, at 26°C, the level of both stress-related transcripts and metabolites were globally identical in bacterized and non-bacterized plantlets but reactions were stronger in the former after a cold shift. In grapevine primed expression of defense mechanisms resulting in pathogen resistance has already been demonstrated in plants pretreated with β-aminobutyric acid (BABA) and challenged with downy mildew (Hamiduzzaman *et al.*, 2005; Slaughter *et al.*, 2008). Recently grapevine physiological responses have been potentiated against *Botrytis cinerea* by non pathogenic rhizobacteria *Pseudomonas spp* (Verhagen *et al.*, 2010).

PAL appears to be a reliable marker of the priming state. It was previously shown that either gene expression or enzymatic activity was higher when (i) tobacco is challenged with tobacco mosaic virus following pretreatment with chemicals (Conrath

et al., 1995), (ii) parsley cell suspensions are elicited after benzothiadiazole application (Katz *et al.*, 1998; Thulke & Conrath, 1998) or (iii) asparagus is challenged with *Fusarium oxysporum* f. sp. *asparagi* following salicylic acid treatment (He & Wolyn, 2005).

Among the tested genes, *LOX* has a particular behaviour since its expression is not induced in non-bacterized plantlets submitted to cold but follows the same pattern of expression than the other tested genes when plantlets are bacterized. Similarly, it was demonstrated that *LOX* was strongly induced in grapevine following infection with *Plasmopara viticola*, only when infection was preceded by a BABA priming treatment (Hamiduzzaman *et al.*, 2005). The authors also reported that *LOX* was jasmonic acid (JA)-regulated. The clear potentiated expression of *LOX* in bacterized plantlets after cold shift suggests that JA signal transduction pathway could be involved in the process of cold acclimation induced by *B. phytofirmans*.

The results obtained in this study about oxidative metabolism may appear incoherent. LOX generates peroxidative damages in plasma membrane through lipid peroxidation (Lee *et al.*, 2005), which is deleterious to plant cells. This is contradictory with the suspected beneficial effect of priming that may facilitate plant reaction to stress. However, one week after cold exposure, the LP markers and H_2O_2 decreased faster in bacterized than in non-bacterized plantlets, suggesting the occurrence of undefined mechanism that promotes ROS elimination. This may represent a pathway of plant acclimation to cold stress when bacterized.

In this paper, we report for the first time that grapevine priming was induced by a PGPR, significantly helping the plant to withstand cold stress. The state of priming offers a cost-efficient resistance strategy, usually characterized as enabling the plant to react more efficiently to biotic or abiotic stresses by boosting cell defense

responses (Conrath *et al.*, 2007; Conrath, 2009). Apart from facilitating cold acclimation, it was also shown that *B. phytofirmans* strain PsJN also protects grapevine plantlets against *Botrytis cinerea* (Ait Barka *et al.*, 2000). There is thus some probability that the priming effect induced by *B. phytofirmans* strain PsJN may also participate in the resistance of grapevine bacterized plantlets to grey mould.

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Sugar changes in grapevine plantlets (*Vitis vinifera* L.) induced by *Burkholderia phytofirmans* strain PsJN upon low non-freezing temperatures

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Keywords: cold tolerance, grapevine, priming, sugars.

Running title: Sugars and cold acclimation in PGPR-colonized grapevine.

Abstract

Burkholderia phytofirmans strain PsJN is a plant growth promoting rhizobacterium (PGPR) that enhances grapevine tolerance to low non-freezing temperatures by changing several physiological parameters. In order to further understand the mechanisms involved in the responses of *in vitro* bacterized grapevine plantlets to cold, the accumulation of carbohydrates was analyzed in leaves during cold acclimation. The results showed that sugars of the primary metabolism including sucrose, glucose, fructose and starch increased after exposure to low non-freezing temperatures. More precisely, bacterized plantlets reacted faster and stronger than non-bacterized ones to cold by accumulating higher levels of carbohydrates, suggesting that *B. phytofirmans* strain PsJN primed grapevine reactions to cold temperatures. Since sugar accumulation is known to participate in plant tolerance to cold, our results indicate that grapevine bacterization promotes plantlet acclimation to low non-freezing temperatures.

Introduction

Low temperatures cause significant stress to cold-sensitive plants affecting their physiological processes thus reducing crop quality and productivity (Levitt, 1980; Raison & Lyons, 1986). On the other hand, insensitive plants exposed to low non-freezing temperatures may become more resistant to cold, a phenomenon known as cold acclimation (Xin & Browse, 2000; Heknedy *et al*., 2006). The physiological, biochemical and metabolic alterations upon cold acclimation have been correlated with (i) biochemical changes in membrane lipid composition (Alonso *et al*., 1997; Uemura & Steponkus, 1999), (ii) changes in gene expression and accumulation of cold stress-related proteins (Hughes & Dunn, 1996; Thomashow, 1999), (iii) accumulation of proline contents (Hekneby *et al*., 2006; Patton *et al*., 2007) and (iv) increases in antioxidant enzymes (Lee & Lee, 2000; Kuk *et al*., 2003). Besides, the accumulation of cryoprotective carbohydrates is one of the best characterized phenomena that occur during the cold acclimation process (Strause & Hauser, 1986; Pollock & Lloyd, 1987; King *et al*., 1988; Leborgne *et al*., 1995; Ait Barka & Audran, 1996; Travert *et al*., 1997; Stushnoff *et al*., 1997).

In grapevine, the effects of low temperatures have already been correlated with fluctuations in growth (Hendrickson *et al*., 2003, 2004; Bertamini *et al*., 2005; Ait Barka *et al*., 2006), carbohydrate metabolism (Ait Barka & Audran, 1996; Hamman *et al.,* 1996), proline contents (Ait Barka & Audran, 1997; Ait Barka *et al*., 2006), gene expression and signal transduction (Thomashow, *et al*., 1998; 1999), as well as accumulation of CBF transcription factors (Xiao *et al*., 2006; 2008). Recently, it was revealed that *B. phytofirmans* strain PsJN is able to colonize all grapevine organs (Compant *et al.*, 2005; 2008). The colonization was concomitant with enhanced changes in stress-related gene expression, proline and phenolic compounds, as well as

hydrogen peroxide and membrane lipid peroxidation (LP) markers upon exposure to low non-freezing temperatures (Ait Barka *et al*., 2006; Theocharis, personal communication). These physiological changes have been proven to be stronger and faster in bacterized plantlets, suggesting that *B. phytofirmans* strain PsJN primes grapevine plantlet reactions after cold shift, resulting in a better acclimation to cold conditions. Moreover, focusing on carbohydrate metabolism, the presence of this bacterium in the whole plantlet also contributed to maintain the same level of photosynthesis in bacterized plantlet grown at 4°C than in non-bacterized ones maintained at 26°C (Ait Barka *et al.*, 2006).

In order to further understand the implication of carbohydrate metabolism in the process of cold acclimation in bacterized grapevine plantlets, we followed the fluctuations of soluble sugars with cryoprotective effects such as sucrose, glucose and fructose and starch within the first 2 weeks of cold exposure.

Materials and methods

Plant material

Plantlets of *Vitis vinifera* L. cv. Chardonnay clone 7535 were micro-propagated by nodal explants grown on 15 ml of semisolid medium in 25 mm diameter culture tubes under gnobiotic system as described by Ait Barka *et al.* (2006). Briefly, the cultures were grown in a growth chamber under white fluorescent light (200 μE $m^{-2}.s^{-1}$) with 16 h photoperiod at 26°C.

Plant bacterization

The bacterial inoculum was produced by transferring two loops of PsJN to 100 ml of King's B liquid medium in 250 ml Erlenmeyer flask incubated at 20°C at 150 rpm for

48 h. Bacteria were collected by centrifugation (3,000 g for 15 min) and washed twice with phosphate-buffer saline (PBS) (10 mM, pH 6.5). The pellet was re-suspended in PBS and used as inoculum. The bacterium concentration was estimated by spectrophotometry (600 nm) and adjusted to 10^6 CFU/ml with PBS. Roots of two week-old plantlets were immersed in *B. phytofirmans* strain PsJN (3×10^8 CFU.ml^{-1}) for 10 s, while roots of control plantlets were immersed in PBS. After immersion, plantlets were cultivated in tubes in growth chambers as described above for 4 weeks before cold treatment. At this moment, bacteria had fully colonized the roots, stem and leaves (Compant *et al.*, 2005).

Cold treatment

Six weeks-old, bacterized (four weeks before) and non-bacterized grapevine plantlets, were divided into two sets. The first set was transferred to a cold growth chamber maintained at 10°C under a 16 h photoperiod with light provided by white fluorescent lamps at 200 µEl m^{-2}.s^{-1}, and 4°C under dark for 8 h, whereas the second set (control) was left at constant temperature (26°C). Sampling was performed after 24, 48, 72 h and 1 and 2 weeks of cold treatment.

Total soluble carbohydrates

Two hundred and fifty mg of leaves were ground in 2 ml of 0.1M potassium phosphate buffer (pH 7.5). The homogenates were centrifuged at 12,000 *g* at 4°C for 15 min and the supernatants were used for each analysis. An aliquot of the potassium phosphate extract (200 µl) was mixed with 1 ml of anthrone-sulphuric reagent (0.1% anthrone and 0.1% thiourea in 12.5 N sulphuric acid) and incubated for 10 min at 100°C. After

cooling, the absorbance was read at 625 nm and results were expressed in $mg.g^{-1}$ dry weight (DW).

Sucrose, Glucose and Fructose

An aliquot of the initial potassium phosphate extract (200 µl) was used to measure the concentration of sucrose, glucose and fructose. The analysis was performed using enzymatic kits (Scil Diagnostic GmbH, Viernheim, Germany) according to manufacturer's protocol. Results were expressed in $mg.g^{-1}$ DW.

Starch extraction and analysis

The pellets from soluble sugar extraction were used for starch analysis. The collected pellets were re-suspended in dimethyl sulfoxide-8 M hydrochloric acid (4:1 v/v). Starch was dissolved over 30 min at 60°C with constant agitation (60 rpm). After centrifugation for 15 min at 12,000 *g*, 100 µl supernatant were mixed with 100 µl iodine-HCl solution (0.06% KI and 0.003% I_2 in 0.05 M HCl) and 1 ml distilled water. The absorbance was read at 600 nm after 15 min incubation at room temperature. Results were expressed in $mg.g^{-1}$ DW.

Statistical analysis

Each treatment was repeated three times and each replicate consisted of six plantlets. Reported data are means ± standard error (SE). Standard analysis of the variance (*t* test) was used to assess the significance of the treatment means at $P<0.05$ level.

Results

At the beginning of the experiment, bacterized and non-bacterized plantlets do not

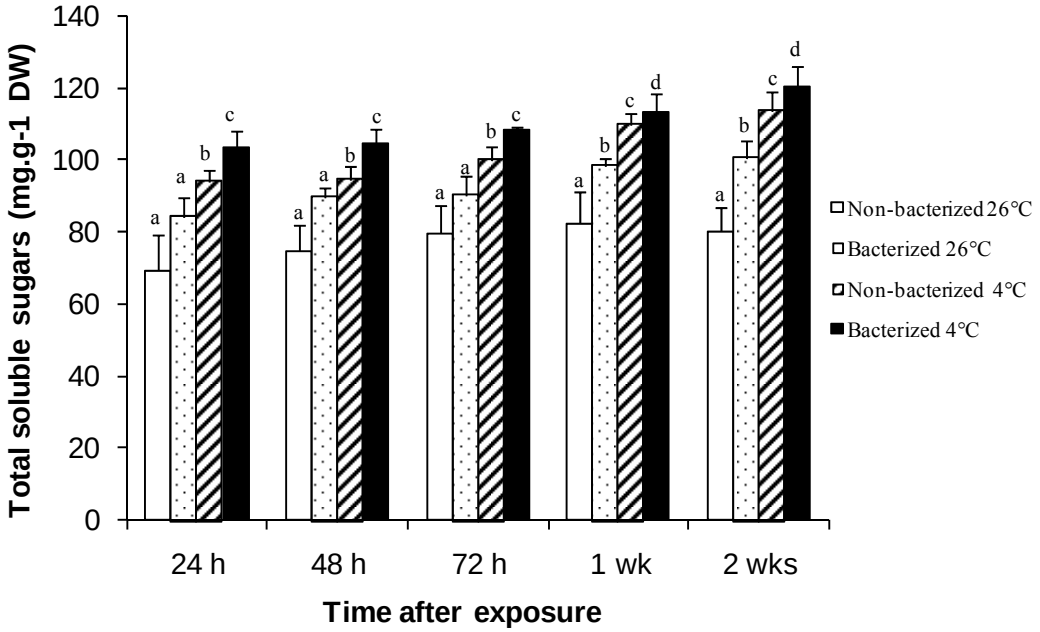

Fig. 1: Accumulation of total soluble sugars in non-bacterized and bacterized grapevine plantlets after 24, 48, 72 h, 1 and 2 weeks of cold treatment. Means indicated with different letters are significantly different ($P<0.05$).

exhibit similar physiological situations regarding the concentration of soluble sugars and starch in leaves, although both bacterized and non-bacterized plantlets were grown at 26°C. The presence of *B. phytofirmans* strain PsJN in the plantlet did not induce any significant change in soluble sugar concentration within the first 3 days of experiment when cultivated at 26°C (Figs. 1, and 2). Afterwards, leaf contents in total soluble sugars (Fig. 1), as well as glucose and fructose (Fig. 2) tended to be higher in bacterized plantlets after 1 and 2 weeks respectively but without significant differences. However, starch content was significantly higher in bacterized plantlets cultivated at 26°C, from the beginning and during the whole experiment at 26°C (Fig. 3).

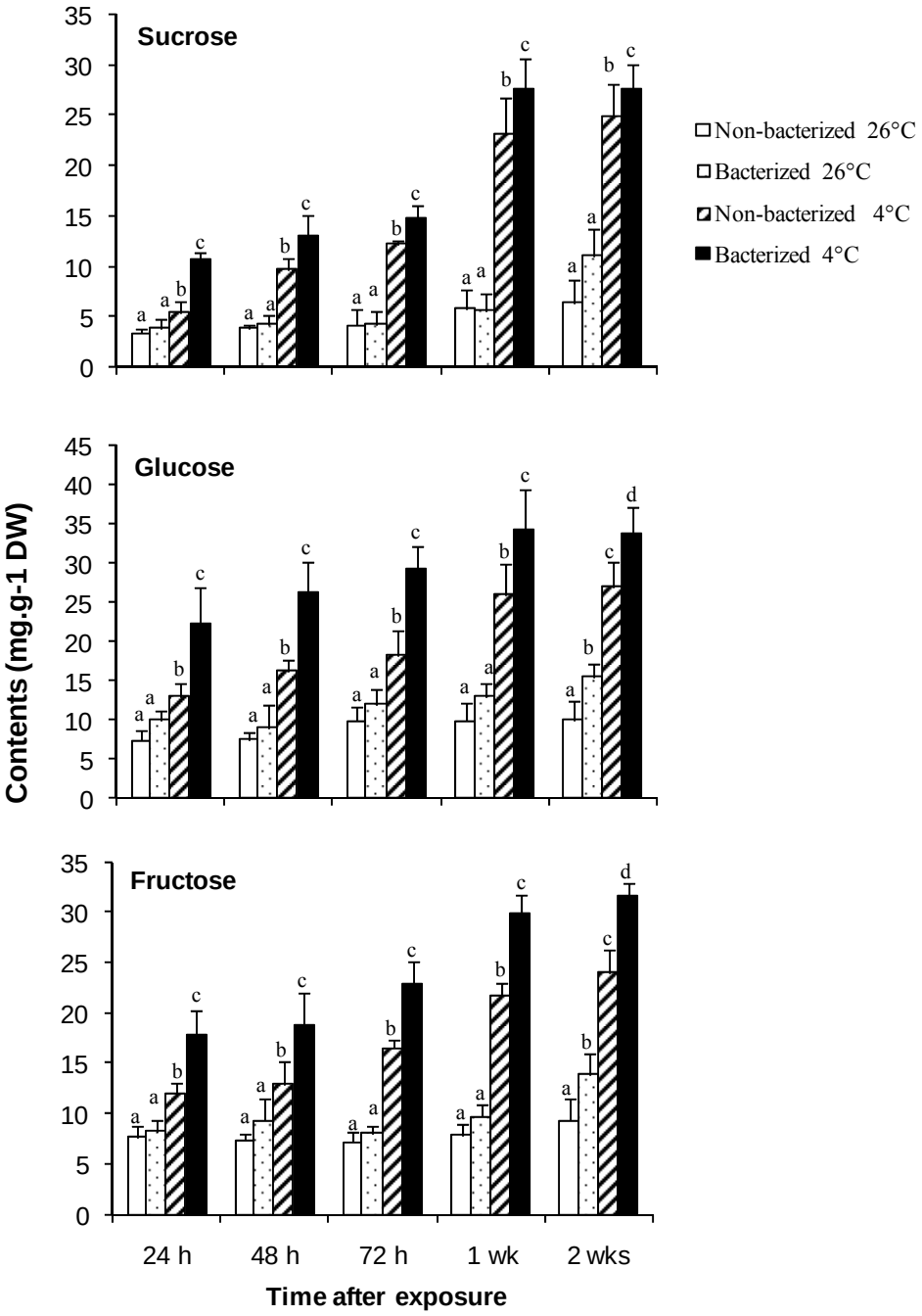

Fig. 2: Accumulation of sucrose, glucose and fructose in non-bacterized and bacterized grapevine plantlets after 24, 48, 72 h, 1 and 2 weeks of cold treatment. Means indicated with different letters are significantly different ($P<0.05$).

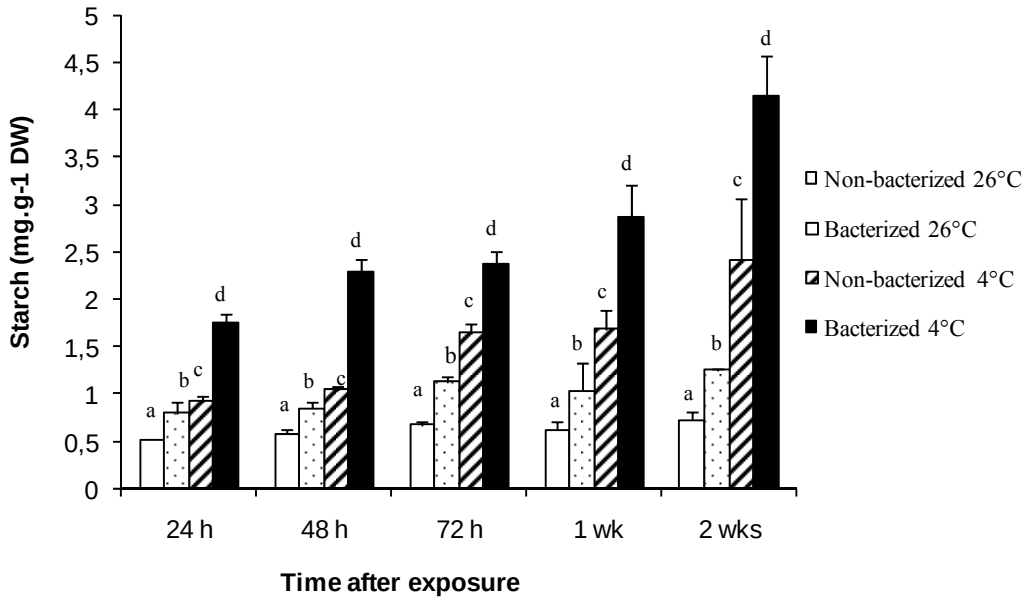

Fig. 3: Accumulation of starch in non-bacterized and bacterized grapevine plantlets after 24, 48, 72 h, 1 and 2 weeks of cold treatment. Means indicated with different letters are significantly different

In both bacterized and non-bacterized plantlets (Figs. 1, 2), the exposure to low non-freezing temperature induced a gradual increase of total soluble sugars, including sucrose, glucose and fructose. The increase was faster and stronger in bacterized plantlets, reaching a peak 1 week after cold treatment. Similarly, cold treatment induced a significant accumulation of starch in plantlets grown at 4°C (Fig. 3), but in a higher extent in bacterized ones.

Discussion

Grapevine reactions to cold temperatures

In woody plants, the correlation between cold tolerance and sugar accumulation has already been established (Leborgne *et al*., 1995; Travert *et al*., 1997). Under

temperate or continental climates, soluble sugars increase at the onset of winter when plants are submitted to low temperatures and next decrease at bud burst when winter dormancy is over (Ashworth *et al*., 1993; Rinne *et al*., 1994; Barbarox & Breda, 2002; Bhowmik & Matsui, 2003). Although the precise function of soluble sugars is not yet determined, their accumulation in cold-acclimated plants suggests a role in osmoregulation, cryoprotection or as signaling molecules (Wanner & Juntilla, 1999; Annikki & Palva, 2006). Moreover, carbohydrates may also act as reactive oxygen species scavengers and contribute to increases of membrane stabilization (Bohnert & Jensen, 1996).

Depending on the plant species, various forms of soluble sugars are involved in physiological reactions to cold stress. For example, sucrose is the most easily detectable sugar in cold-tolerant plants (Guy *et al*., 1980; Pollock, 1984; Salerno & Pontis, 1989). It enhances cold hardiness and desiccation tolerance of buds in woody plants (Stushnoff *et al.,* 1997). The accumulation of sucrose in sugar cane exposed to chilling stress supports the well-established role of this sugar as an osmoprotectant that stabilizes cell membranes and maintains turgor (Jouve *et al.*, 2004; Whittaker *et al.*, 2001). Also, the oligosaccharides raffinose and stachyose are closely associated with season-long cold hardiness and dormancy (Stushnoff *et al*. 1993; Ashworth *et al*. 1993; Flinn & Ashworth 1995; Imanishi *et al*. 1998). In grapevine, the onset of cool autumn temperatures induces significant modifications of carbohydrate metabolism. It shifts from production of sucrose to monosaccharides (fructose and glucose) and raffinose (Hamman *et al.,* 1996; Ait Barka & Audran, 1996), with a peak at midwinter (Ait Barka & Audran, 1996). This seasonal modification in soluble sugar accumulation of vineyard grown plants is quite similar with changes

reported here in bacterized and non-bacterized plantlets submitted to low temperatures.

Starch variation has been also demonstrated to be strongly correlated with cold acclimation following various pathways of fluctuations. In some plants such as cabbage seedlings (Sasaki *et al*., 1996), spinach (Guy *et al*., 1992) or grapevine grown in the vineyard (Ait Barka & Audran, 1996), cold acclimation induces an increase of both soluble sugars and starch. Oppositely, starch may be converted into soluble saccharides during cold exposure in some species (Sakai &Yoshida, 1968; Pollock & Lloyd, 1987; Fischer & Höll, 1991; Ögren *et al.,* 1997; Greer *et al.,* 2000).

The behaviour of *in vitro* grown grapevine plantlets is different. Whether plants are bacterized or not, starch content is significantly increased after only 24 h or treatment and continues to accumulate during the 2 weeks of cold exposure as also reported earlier (Ait Barka *et al*., 2006). Sucrose uptake is known to be stimulated under stressing conditions (Arbona *et al.*, 2005). Therefore, the difference of reaction between vineyard and *in vitro* grown plants upon low temperatures may be due to the presence of sucrose in the culture medium.

Effects of the bacterization on sugar fluctuations

At 26°C, bacterized plantlets accumulate soluble sugars and starch 6 weeks after the onset of inoculation with the PGPR *B. phytofirmans* strain PsJN. Recently, Conrath (2009) reports that induced resistance is associated with elevated levels of soluble carbohydrates resulting from alterations in primary metabolism that confers resistance to a variety of biotic challenges. Under stress conditions, grapevine is known to accumulate various forms of carbohydrates (Saladin *et al.*, 2003; Ait Barka *et al.*, 2006). It means that the bacterium affects carbohydrate metabolism in grapevine

plantlets that may be mediated through at least two different ways. First, it was demonstrated that bacterization with this bacterium causes a stimulation of net photosynthesis (Ait Barka *et al*., 2006), which may contribute to sugar accumulation. Second, the complete colonization of grapevine plantlets may provoke some stress and thus leads to sugar accumulation. Whatever, bacterium originating stress is weak since stress markers are not induced (Theocharis, personal observation). Such reaction may further be enhanced by the presence of high sucrose concentrations in the artificial culture medium.

In accordance with our results, various studies reported that application of sugar to various plant tissues, or inducing the accumulation of sugar in transgenic plants, can lead to activation of various *PR* genes (Herbers *et al.*, 1996a,b; Johnson and Ryan, 1990). Therefore, the potentiated responses of soluble sugars and starch accumulation induced by strain PsJN in grapevine plantlets may contribute to grape plant protection to low non-freezing temperatures. Here we demonstrate that bacterized and non-bacterized grapevine plantlets react to low non-freezing temperature by rapid accumulation of both soluble sugars and starch, which is faster and stronger in bacterized plantlets. Such reaction is a characteristic trait of a primed physiological response and is in accordance with the variations of stress-related markers reported earlier (Theocharis, personal observation). Therefore, we could address that PsJN is a PGPR that primes several physiological responses of grapevine plantlets under cold stress including the accumulation of soluble sugars and starch. Similarly, a correlation between elevated sucrose levels and primed defence responses was reported also for rice over-expressing the *PRms* gene from maize, which encodes a PR-1 type protein (Casacuberta *et al.*, 1991).

In conclusion, this study confirms the protective action of *B*. *phytofirmans* strain PsJN to grapevine plantlets against the negative effects of low non-freezing temperatures. This beneficial effect consists in (i) stimulating carbon metabolism including photosynthesis (Ait Barka *et al.*, 2006) and sugar accumulation and (ii) priming stress responses whether they are related to carbon metabolism (this work) or defence responses (Theocharis, personal observation). Thus the presence of *B. phytofirmans* strain PsJN into the whole grapevine plantlets may speed up the process of cold acclimation to low non freezing temperature.

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Differential gene expression analysis for investigation of primed physiological state stimulated by *Burkholderia phytofirmans* strain PsJN in grapevine upon low non-freezing temperatures

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Abstract

cDNA-AFLP analysis is a RNA fingerprinting technique that has been used extensively in recent years to display differentially expressed genes in plants. In the present study, the differential gene expression patterns were analysed in grapevine plantlets by cDNA-AFLP technique to further investigate the primed- physiological state of *Vitis vinifera* L. induced by ISR-inducing PGPR *Burkholderia phytofirmans* strain PsJN, and to isolate and identify unknown over-expressed genes after exposure to low non-freezing temperatures. Nine combinations of non-radioactive digoxigenine labelled-*PstI and MseI* primers were used to generate differentially expressed fragments for non-bacterized and bacterized plantlets at 26°C, and after a 24 hour-exposure to low non-freezing temperatures (10°C/16 h photoperiod, and 4°C/ 8 h dark). From forty up- and- down regulated cDNA fragments that were detected, the 75% were isolated successfully and re-amplified by PCR. The investigation of expressed bands in non-bacterized and bacterized grapevine plantlets by cDNA-AFLP technique has revealed that strain PsJN prime the physiological state in grapevine plantlets by stimulating the expression of about 50% of detected genes, while the identification of three isolated bands showed that bacterized plantlets can react to cold by over-expressing genes responsible for abiotic stress Although the analysis of isolated genes needs furher investigation, a part of transcript accumulation in priming phenomenon induced by strain PsJN upon low non-freezing temperature was detected.

Introduction

The isolation of differentially expressed genes is one of the cornerstones in modern molecular biology. The several techniques to analyze genes that are expressed differently include the now classic approaches of differential and subtractive hybridisation, and the polymerase chain reaction (PCR)-based approaches for selective amplification of complementary DNA (cDNA) **(**Gray *et al*., 1992; Woodhead *et al*., 1998; Davies & Robinson, 2000; Burger & Botha, 2004).

Differential display reverse transcription PCR (DDRT-PCR) was the first *in vitro* technique developed for the determination of transcript patterns (Liang & Pardee, 1992). It has been widely applied to identify and clone a large number of genes that are differentially expressed (Wilkinson *et al*., 1995; Baldwin *et al*., 1999; Bézier *et al*., 2002; Iqbal *et al*., 2008). Nevertheless, this technique tends to give high rates of false positives (Debouck, 1995), which are primarily attributed to the presence of multiple DNA fragments in one particular band (McClelland *et al*., 1995; Men & Gresshoff, 1998). To counteract this high rate of false positives, several improved PCR-based methods have been developed (Kawamoto *et al*., 1999; Shimkets *et al*., 1999; Sutcliffe *et al*., 2000). A method that has widely been used to analyze expression of multigene families was developed by Fischer *et al*. (1995), as a combination of DDRT-PCR and amplified fragment length polymorphism (AFLP) (Vos *et al.,* 1995). This technique termed cDNA-AFLP analysis, is a RNA fingerprinting technique **(**Bachem *et al.,* 1996**)** that has been used extensively in recent years to display differentially expressed genes in plants (Yang *et al*., 2003; Mao *et al*., 2004; Burger & Botha, 2004; Ongena *et al*., 2005**)**. The method is based on digestion of cDNAs by two restriction enzymes and oligonucleotide adapters ligated to the resulting restriction fragments to generate template DNA for polymerase

chain reaction (PCR). PCR primers complementary to the adapter sequences with additional selective nucleotides at the 3′ end allow the amplification of a limited number of cDNA fragments (Vos *et al.,* 1995**)**. Unlike differential display methods that make use of small random primers **(**Liang & Pardee, 1992**)**, relatively high annealing temperatures can be used and, hence, cDNA-AFLP is more stringent and reproducible. In contrast to hybridization-based techniques, such as cDNA microarrays, cDNA-AFLP can distinguish between highly homologous genes from individual gene families (Agarwal *et al.,* 2008**)**. In addition, cDNA-AFLP does not need any pre-existing sequence information, which makes it an excellent tool to identify novel genes (Mao *et al*., 2004; Burger & Botha, 2004**)**.

Analysis of changes in gene expression by RT-PCR, has shown that grapevine plantlets, bacterized by ISR-inducing *Burkholderia phytofirmas* strain PsJN, can prime the accumulation of transcripts of several well-characterized defense genes under cold stress according to phenomenon of priming (Theocharis, personal observation). Using non-radioactive digoxigenine labelled-*PstI* and *MseI*-primers for fingerprinting of grapevine mRNA by cDNA-AFLP techology, the objectives of the present work are (i) the further investigation of primed physiological state in grapevine plantlets induced by strain PsJN, and (ii) the isolation and identification of unknown over-expressed genes of cold response in bacterized plantlets in low non-freezing temperatures.

Materials and Methods

Plant material and *in vitro* growth conditions.

Plantlets of *V. vinifera* cv. Chardonnay clone 7535 were micropropagated by nodal explants grown on 15 ml semisolid medium in 25 mm culture tubes as described by

Ait Barka *et al.* (2006). Briefly, the cultures were grown in a growth chamber under white fluorescent light (200 µE $m^2 \cdot s^{-1}$) with 16 h light at 26°C (constant temperature).

Plant bacterization

The bacterial inoculum was produced by transferring two loops of PsJN to 100 ml of King's B liquid medium in 250 ml Erlenmeyer flask incubated at 20°C at 150 rpm for 48 h. Bacteria were collected by centrifugation (3,000 × g for 15 min) and washed twice with phosphate-buffer saline (PBS) (10 mM, pH 6.5). The pellet was re-suspended in PBS and used as inoculum. The bacterium concentration was estimated by spectrophotometry (600 nm) and adjusted to 3×10^8 $CFU.ml^{-1}$ with PBS. Roots of two week-old plantlets were immersed in *B. phytofirmans* strain PsJN (3×10^8 $CFU.ml^{-1}$) for 10 s. Roots of control plantlets were immersed in PBS. After immersion, plantlets were cultivated in tubes in growth chambers as described above for four weeks before cold treatment.

Cold treatment

Six weeks-old, bacterized for four weeks, and non-bacterized plantlets were divided into two subsamples: the first was transferred to a cold growth chamber maintained at 10°C under 16 h light (white fluorescent light, 200 µE $m^2 \cdot s^{-1}$) and at 4°C for 8 h dark . The second subsample (control) was left at 26°C and 16 L: 8 D. Analyses were conducted 24 h after treatment.

Sampling, RNA extraction & synthesis of cDNA

Leaf samples were frozen in liquid nitrogen and stored at -80°C until use. Messenger RNA was extracted from 50-100 mg leaf material following the RNA Plant

Purification Regent protocol (Invitrogen, France). Leaves were ground in liquid nitrogen to a fine powder and homogenized in RNA purification extraction regent. The suspension was purified by two-phenol/chloroform/isomyl alcohol (25/24/1, v/v/v) extraction, and the RNA was then purified by an equal amount of isopropanol. After centrifugation, the RNA pellet was resuspended in 20 µl of RNase-free water and quantified by absorbance at 260 nm. Reverse Transcription of RNA was performed with 600ng of total RNA using M-MLV reverse transcriptase (Invitrogen, France) following the manufacturer's protocol.

cDNA-AFLP detected with non-radioactive digoxigenine labeled primers

AFLP associated-procedure was carried out according to modified cDNA-AFLP methods decribed by Venter *et al*. (2001) (Fig 1). The protocol with a non radiocative digoxigenine labelling used visualization of cDNA-AFLP fragments, according to Vrieling *et al*. (1997) was the following: 1) cDNA quantification: cDNA was quantitated by PicoGreen dsDNA Quantitation Reagent using Hoechst (bisbenzimide) dyes. 2) Digestion with restriction endonucleases: cDNA were digested with 2.5 U of both *Mse*I and *Pst*I restriction enzymes at 37°C for 3 hours incubated at 70°C for 15 min to inactivate the restriction enzymes. 3) Ligation of oligonucleotide adaptors: Non-phosphorylated adaptor sequences were ligated to the restriction fragments at 20°C overnight. 4) Pre-amplification reaction: The restriction ligation products were subjected to 30 cycles of pre amplification (94°C denaturation, 30 s; 56°C annealing, 1 min; 72°C polymerization, 1 min) using primers with no selective nucleotides to obtain a sufficient ammount of template. A second pre-amplification was followed using the products of the first one. The final products

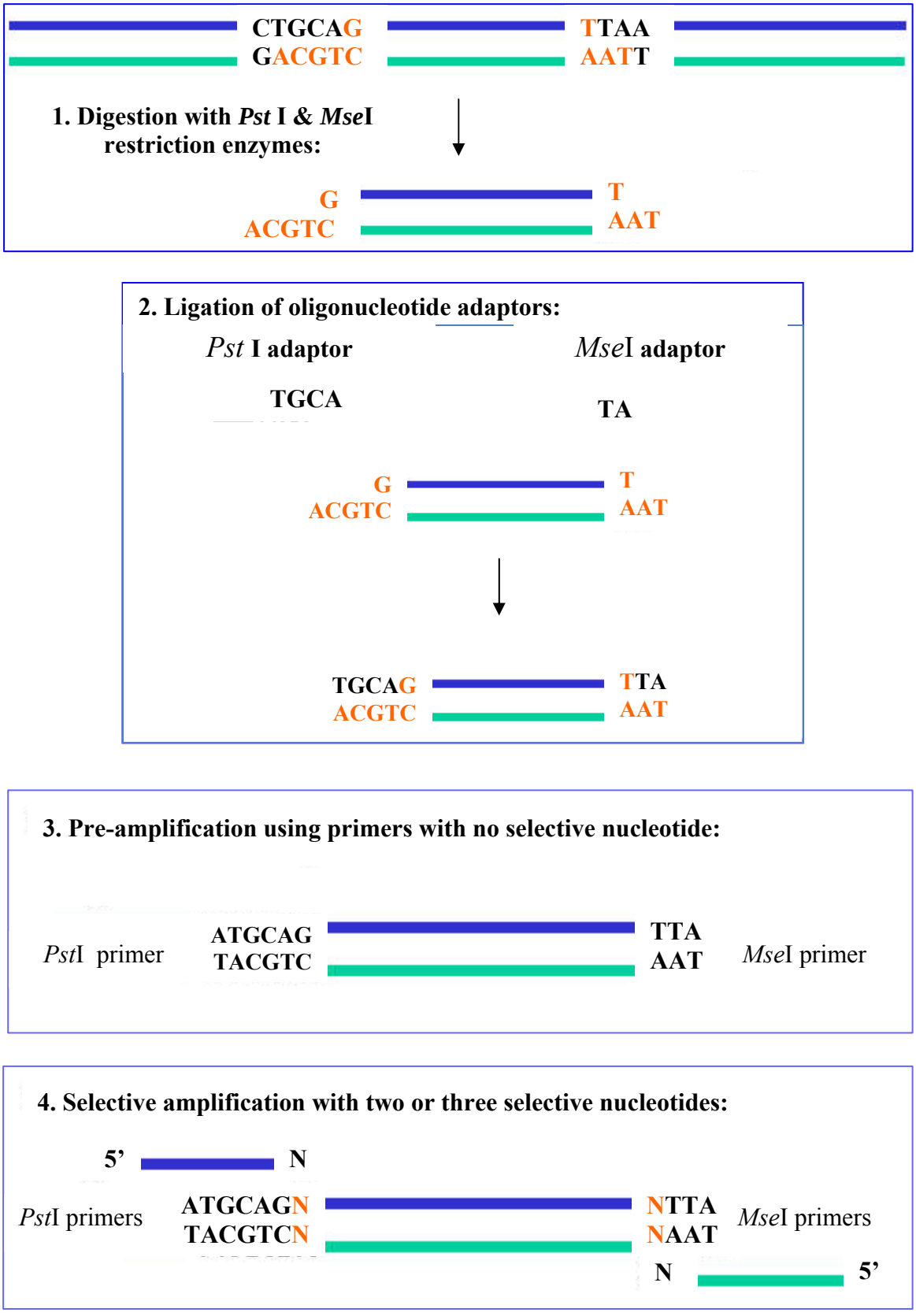

Fig 1: AFLP accociated procedure with 4 steps for development of cDNA amplified fragments.

were quantified by Pigogreen Quantification Reagent, stained with Syb Green asymmetrical cyanine dye and than visualized in on a 1% agarose gel. The expected sizes ranging from 100 bp to 500 bp. 5) Selective amplification with non-radioactive digoxigenine labeled primers (Touchdown PCR): Selective ampification was performed with 9 compination of *Pst*I primer (5′-GAC TGC GTA CAT GCA G+N-3′) and *Mse*I primer (5′-GAT GAG TCC TGA GTA A+N-3′) extensions where 'N' represent two or three selective nucleotides **(**Table 1**)**. The *Pst*I forward primers were digoxigenine labeled. Thirty six cycles of amplification (12 cycles: 94°C denaturation, 30 s; 65°C annealing, 30 s; 72°C polymerization; 1 min then 24 cycles 94°C denaturation, 30 s; 56°C annealing, 30 s; 72°C polymerization, 1 min) were carried out where the annealing temperature was lowered gradually from 65°C to 56°C at which efficient primer binding occurs. Thermocycling was started at 65°C annealing temperature for optimal primer selectivity. The final products of selective amplification were quantified by PigoGreen Quantification Reagent securing the equal sample cDNA concentration loaded in gel electrophoresis (concentration of loaded samples: 8.82ng/ 5µl)**.**

Table 1. Nine *Pst*I and *Mse*I primer combinations with two or three selective nucleotides used for cDNA-AFLP analysis.

*Pst*I / *Mse*I primer extensions		
1. CT/ CAA	4. GT/ CAA	7. GTA/ CAA
2. CT/ CAC	5. GT/ CAC,	8. GTA/ CAC
3. CT/ CAG	6. GT/ CAG	9. GTA/ CAG

6) Gel loading and analysis: Amplified products were heated at 95°C for 5 min after addition of an equal amount of formamide dye (98% (v/v) formamide, 10 mM EDTA pH 8.0 and 0.1% bromophenol blue and xylene cyanol) and immediately chilled on ice. Fragments were separated in 6% (m/v) denaturating polyacrylamide gels poured inside a glass sandwich (Nucleic Acid Electrophoresis Cell Bio-Rab) for polymerization of the gel. The two gels were developed in 80 W for about 100 min (gels were pre-run at 100W for about 1 h). 7) Gel blotting (dry blot transfer): Pre-soaked (in 0.5 X TBE) non-charged nylon membrane was placed over the gel, avoiding air bubbles. The membrane was rinsed in 0.5 X TBE and try for 15 min at 65°C, then base at 95°C for 30 min. The digoxigenine labeled amplified cDNA fragments were visualized on BioMAX MR film (Eastman Kodak Company, Rochester, New York) after exposure times to ranging between 24 and 48 hours. 8) cDNA recovery: Bands that appeared to be differentially expressed, were scored and removed by cutting the membrane. cDNA was recovered from each band after heat treatment at 95°C in 30 µl distilled water for 10 min. Fragments were re-amplified using the selective and non-selective primers and PCR conditions as used in the initial pre-amplification procedures and the succesfully re-amplified cDNA fragments were visualized by SybGreen asymmetrical cyanine dye in on a 1% agarose gel. 9) Cloning and Sequencing PCR products: For sequencing of amplified cDNA fragments, Invitrogen TOPO TA Cloning kit was used following the manufacturer's instruction. Due to the possibility of comigration associated with cDNA-AFLP (Lang *et al*. 2005), five clones were examined from each band on the gel. 10) Identification of sequences (BLAST): Homology search were performed using the Basic Local Alignment Search Tool (Blast) algorithm (Altchul *et al*., 1990). All ambiguities and vector and primer

sequences were removed before nucleotide sequences were submitted to search entries in the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and in DFCI Grape Gene Index (http://compbio.dfci.harvard.edu/tgi/cgibin/tgi/gimain.pl?gudb=grape). The significance of the homology was based on the Score and E-value.

Results

The amplified fragments by cDNA-AFLP analysis were separated by gel electrophoresis according to their length. Amplified fragments that showed the same mobility between the four samples (non-bacterized 26°C/4°C and bacterized 26°C/4°C) were considered as identical cDNA fragments. The brightest band among fragments with the same lenght in acrylamide gel electrophoresis was considered as the most overexpressed band (band B in gel Fig.2). In similar way, present bands are concidered as expressed in comparison to absent bands (band A and C in gel Fig.2). According to the gels' analysis**,** the size of amplified products ranged from 50 bp to 350 bp. The part of the gels which presented the majority of the amplified bands, ranged between 100 bp and 300 bp. The higher number of bands was obtained by primer combination N° 1 (*Pst*I +CT/ *Mse*I +CAA), N° 4 (*Pst*I +GT/ *Mse*I +CAA), and N° 9 (*Pst*I + GTA/ *Mse*I +CAG). Only a few bands were amplified by the primer combination N° 6 (*Pst*I + GT/ *Mse*I + CAG) while no amplified bands were obtained by primer combination N° 7 (*Pst*I+GTA/ *Mse*I +CAA) and 8 (*Pst*I+GTA / *Mse*I +CAC). From the forty well-developed differentially expressed bands scored in gel, the 50% were expressed in bacterized grapevine plantlets at 26°C, while 22,5% were expressed in bacterized grapevine plantlets at 4°C (Table 2). On the other hand, the 25% expressed at 26°C in non-bacterized plantlets, while

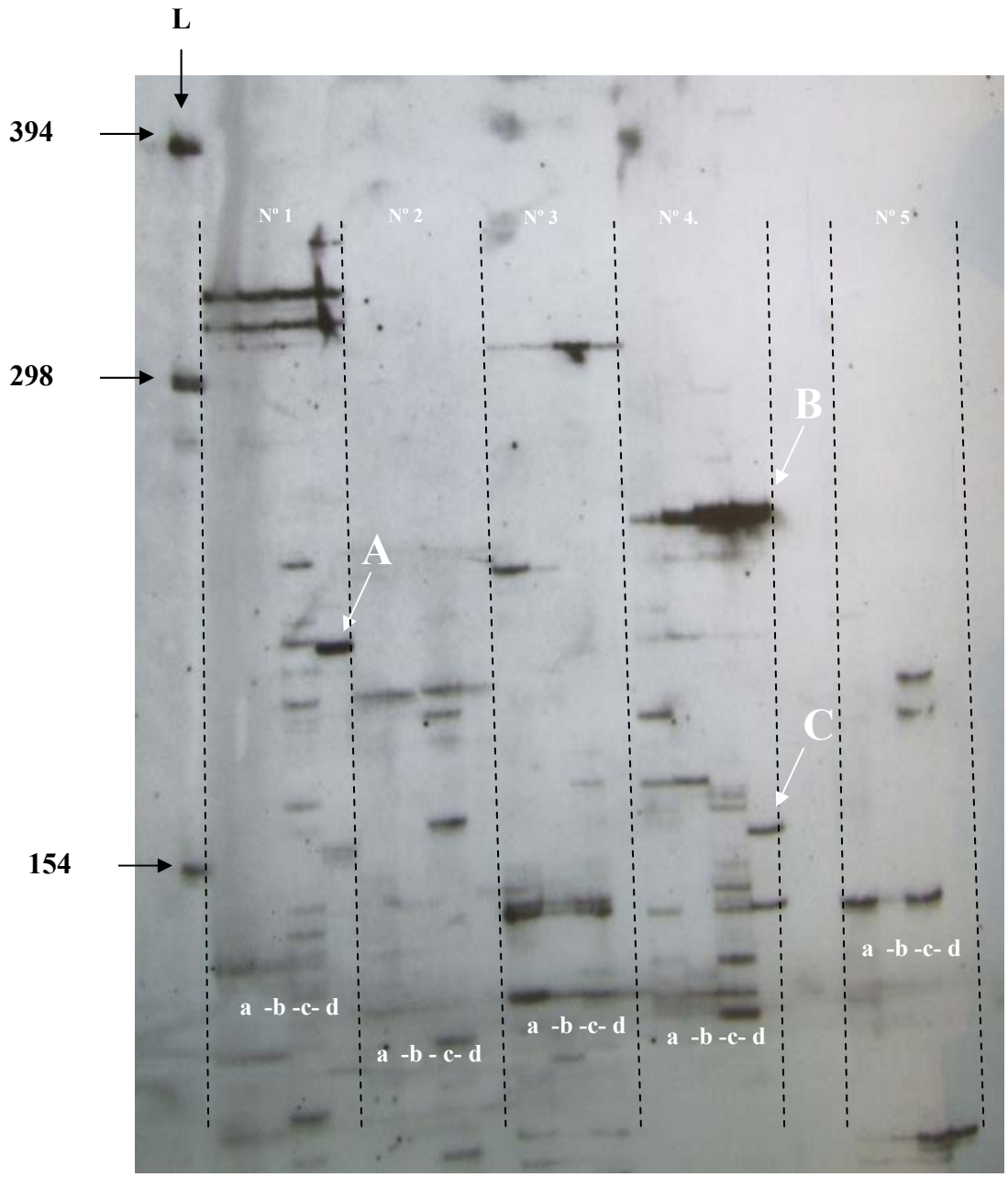

Fig 2: cDNA- AFLP analysis of differential expressed fragments by five primers (Table 1) together with a ladder (L) labelled by digoxigenine combination separated in 6% (m/v) denaturating polyacrylamide gel. (N^{o} 1,2,3,4,5): the primer compinations for selective amplification. (a): non-bacterized 26°C, (b): non-bacterized 4°C, (c): bacterized 26°C, (d): bacterized 4°C. (A, B and C): three differential expressed fragments which analysed further by cloning and RT-PCR.

Table 2: Differential expression of forty amplified fragments by cDNA-AFLPs. A, B and C are overexpressed bands in bacterized plantlets upon cold analysed by RT-PCR.

Bands	Non-bact 26°C (a)	Non-bact 4°C (b)	Bact 26°C (c)	Bact 4°C (d)	Compination of *Pst*I /*Mse*I (N°)
●	-	-	-	++	1
●	++	+	+	+	1
●	++	+	+	+	6
●	-	-	++	-	1
A	-	-	++	+++++	**1**
●	-	-	++	-	1
●	+++++	+	+	+	1
●	-	-	++	-	1
●	-	-	++		1
●	+++++	+	+	++++++	1
●	-	-	+++++	-	2
●	-	-	+++++	-	2
●	-	-	+++++	-	2
●	+	+	+++++	++	3
●	+++++	+	-	-	3
●	+++++	++	++	+	3
●	++	+	+++	+	3
●	+++	+	+	+	3
●	++		+++++	-	3
B	++	+++	++++	++++++	**4**
●	+++++	-	-	-	4
●	++	+++++	-	-	4
C	-	-	-	+++++	4
●	++		++	+++++	4
●	-	-	+++++	-	4
●	-	-	+++++	-	5
●	-	-	+++++	-	5
●	+++++	+	++++	+	5
●	+	-	++++++	-	6
●	+	++	++++	+++++	5
●	-	-	++++++	-	9
●	+++++	+		+	9
●	++	+++++	++++	+++++	9
●	-	++	+++++	++	9
●	+	++		+++++	9
●	-	-	+++++	-	9
●	++	-	+++++	-	9
●	++	+	+++++	+	9
●	-	-	+++++	-	9
●	++++++	+++++	-	-	9

Absence of band(-), faint band(+), presence of band (++), overexpressed band (+++++), most over-expressed bands(++++++).

significantly lower (10%) were the expressed fragments in non-bacterized plantlets at 4°C **(**Fig 3**)**.

From the forty differential expressed isolated bands, twenty four were re-amplified succesfully by non-selective primers showing a single amplified band, while five of them showed douple or smeared bands (Fig 4). The cloning and sequence analysis of three selective bands (A, B and C) overexpressed in bacterized plantlets showed that the three bands were homologous to grapevine genes induced in abiotic stress in leaves (A & C) and in berries (B) **(**Table 3**)**. For each band, the five clones resulted in identical sequences.

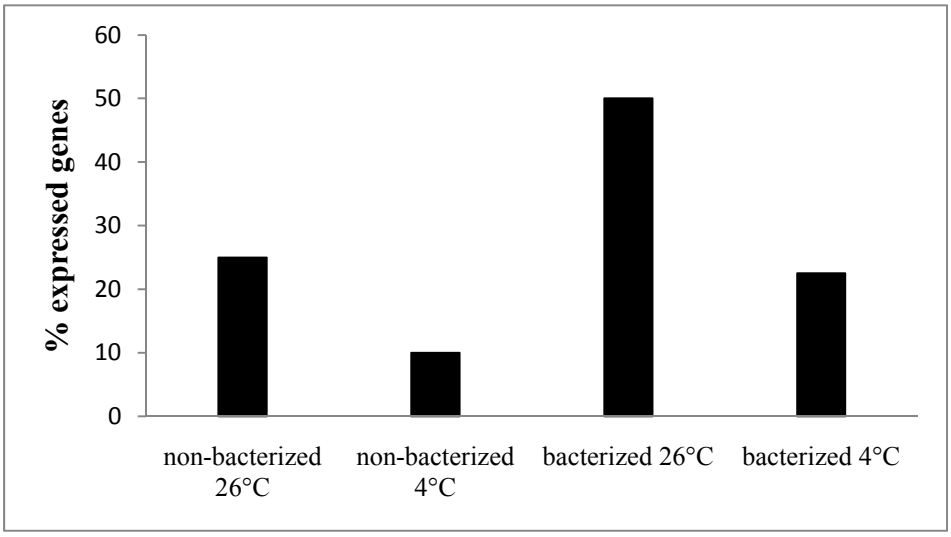

Fig 3: Percentage of up regulated genes in non-bacterized and bacterized grapevine plantlets at 26°C/4°C analysed by cDNA-AFLP with specific primers.

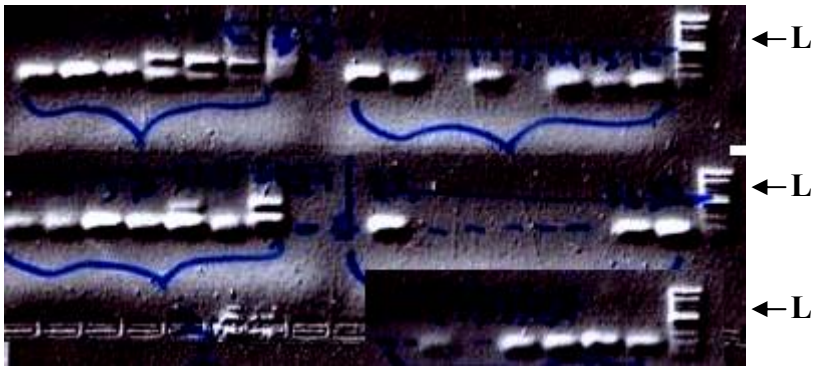

Fig. 4: Successfully re-amplified differential expressed bands by non-selective primers isolated from membrane and ladder (L) labelled by digoxigenine visualized by SybGreen asymmetrical cyanine dye in on a 1% agarose gel.

Table 3: Characterization of the 3 cDNA sequences identified from the single bands excised from cDNA-AFLP polyacrylamide gel. The ambiguities and vector and primer sequences were removed.

Clone (base pairs)	**Homology sequences**	Significance of Identity (Max Ident. and E-value)
Band A (209 bp)	An expressed sequence tag database for abiotic stressed leaves of *Vitis vinifera* var. Chardonnay cDNA clone VVB160G05 5, mRNA sequence (Cramer,G.R. and Cushman,J.C. 2002)	1e-100, 99%
Band B (63 bp)	An expressed sequence tag database for abiotic stressed berries of *Vitis vinifera* var. Chardonnay cDNA clone VVD103F10 5, mRNA sequence (Cushman,J.C., 2002)	3e-24, 100%
Band C (309 bp)	An expressed sequence tag database for abiotic stressed leaves of *Vitis vinifera* var. Chardonnay cDNA clone VVB179G05 5, mRNA sequence(Cushman,J.C., 2002)	5e-31, 100%

Discussion

cDNA-AFLP detected with non-radioactive digoxigenine labeled primers

The main advantage of PCR-based approaches for detection of differentially expressed genes is the ability to rapidly and simultaneously display mRNAs that are expressed in various eukaryotic cells or tissues, in different stages of development or under altered conditions. For detection of AFLP applications, radioactive and non-radioactive fluorescent-labelled primers were usually used (Cervera *et al*., 2000; Vignani *et al*., 2002; Theocharis *et al*. 2010b). However, radioactive labelling is not feasible in every laboratory, while the detection of fluorescent labels requires expensive equipment. Alternatively, the method based on the digoxigenine labelling of AFLP primers has proved reliable and cheap and in addition no major re-arrangements of original protocols are required (Vrieling *et al*., 1997; Hanada *et al*., 2003). Concerning the well-detected bands in our gel and the equal quantity (ng) of samples loaded in polyacrylamide gel-walls, it is addressed that non-radioactive digoxigenine method can succesfully reveal the differential expression of up and down regulated genes after grapevine plantlets exposure to low temperatures. In addition, the high percentage of re-amplified bands isolated from membranes and the fact that the cloning (five times for each band) resulted in identical sequences, allows the conclusion that digoxigenine labelling of AFLP primers is a reliable method for analysis of changes in gene expression at stress conditions like cold treatment.

Trsansription analysis

Changes in gene regulation induced by plant responses to cold have been addressed by several studies in the last decade (Jaglo *et al*., 1998, 2001; Kreps *et al*., 2002). For example, exposure of chinese cabbage to cold altered the expression of approximately

9% of genes (257 up-regulated and 311 down-regulated genes), while 4 weeks of cold changed the expression of approximately 7% of genes (181 up-regulated and 246 down-regulated), in comparison to untreated seedlings (Yang *et al*., 2005). To further understand the gene network controlling tolerance to cold stress in *Arabidopsis*, many studies have reported a large number of early cold-responsive genes which encode transcription factors that likely control late-responsive genes, suggesting a multitude of transcriptional cascades (Fowler & Thomashow, 2002; Lee *et al*., 2005; Oono *et al*., 2006). In grapevine, chilling after 8 h seems to cause changes in transcript abundance, with 78% of transcripts that changed at least two-fold in response to chilling to be increased in abundance, indicating a larger and more complex response in the acclimation process of a gradual long-term stress (Tattersall *et al*., 2007).

cDNA-AFLP was succesfully used to study gene expression of plants in response to low temperatures and to further understand cold acclimation process. According to Meng *et al*. (2008**),** 13 differentially expressed cDNA fragments were cloned, sequenced and further analysed from *Poncirus trifoliata* after 10, 24 and 55 h of low temperature treatment (4°C). Further study by cDNA-AFLP, revealed six up-regulated and two down regulated genes identified successfully based on their amino acid sequences, understanding cold tolerance mechanism of *Citrus unshiu* (Lang *et al*., 2005). Similarly, cDNA-AFLP analysis in our study reveals interesting conclusions about the forty up- and down- regulated genes which are expressed differentially. By these nine specific primer combinations, it is shown that the percentage of expressed regulated genes in non-bacterized plantlets was decreased from 25% (at 26°C) to 10% (at 4°C), while in bacterized plantlets, in the same conditions, from 50% to 22.5%. This characteristic down-regulation of genes reported by cDNA analysis, could be related with several physiological activities that are

suppressed in plants after cold treatment. Such suppression of essential genes by cold in insensitive plants can hinder the normal plant growth in cold, while it may lead to death in sensitive plants (Yoshida *et al*., 1996). Therefore the increased number of genes expressed in bacterized plantlets under cold stress can reveal a better adaptation of bacterized plantlets to low non-freezing temperature, in comparison to non-bacterized plantlets, although, the percentage of reduced genes under cold are similar in both of them (about 50% reduction).

Significant differences in gene expression between bacterized and non-bacterized plantlets were also addressed under normal growth conditions (26°C). The higher percentage (50%) of overexpressed bands in bacterized plantlets at 26°C, in comparison with non-bacterized plantlets (25%), can be related with the physiological state induced by strain PsJN stimulating several physiological activities of grapevine plantlets such as growth and photosynthesis (Nowak *et al.*, 1995; Ait Barka, *et al*., 2006), accumulation of proline (Ait Barka, *et al*., 2006; Theocharis, personal observation) and carbohydrates (unpublished results).

On the other hand, the overexpressed genes (homologous to genes in grapevine induced in abiotic stress in leaves and berries) in bacterized plantlets upon cold revealed that, in contrast to several physiological activities, grapevine defense mechanism was activated upon low non freezing temperatures, confiming the potentiated expression of defense genes in bacterized plantlets which were addressed in our previous work according to phenomenon of priming (Theocharis, personal observation).

Although further analysis of isolated bands and study of expression pattern by RT-PCR is necessary to confirm AFLP analysis, the investigation of gene expression in non-bacterized and bacterized grapevine plantlets by cDNA-AFLP technique can

reveal that strain PsJN prime the physiological state in grapevine plantlets by stimulating the expression of about 50% of detected genes, while the identification of three isolated bands showed that bacterized plantlets can adapt to cold stimulating grapevine defense mechanism by over-expression of genes responsible for abiotic stress.

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3. GENERAL CONCLUSIONS

Beneficial effects of PGPR

Plant growth-promoting bacteria are free-living soil bacteria that can directly or indirectly facilitate rooting and growth of plants (Mayak *et al*., 2004a,b; Glick, 1995; Compant *et al*., 2005a). An indirect stimulation of plant growth includes a variety of mechanisms by which bacteria prevent environmental stresses from inhibiting plant growth and development by induction of resistance (Glick & Bashan, 1997). Beneficial rhizobacteria induce plant resistance without damaging visually their host neither causing any localized necrosis. Although, the eliciting factors produced by ISR-triggering rhizobacteria must be different from elicitors or pathogens (van Loon, 2007), in several cases PGPR induce defense mechanism similarly to pathogens by accumulation of PR proteins (Timmusk & Wagner, 1999; Park & Kloepper, 2000; Tjamos *et al*., 2005; Magnin-Robert *et al*., 2007).

Expression of ISR may take different forms, depending on the activity of the inducing rhizobacterium and the nature of the interaction between the plant and the pathogen (Chester, 1993). A common feature of the resistance responses induced by ISR-inducing beneficial bacteria is the phenomenon of priming. The physiological condition in which plants are able to better and/or more rapidly increase defense responses to biotic or abiotic stress is called the "primed state" The primed-caused potentiation of plant defense responses has frequently been associated with enhanced resistance to various biotic and abiotic stresses (Goellner & Cornath, 2008).

***Burkholderia phytofirmans* strain PsJN beneficial effects in grapevine plantlets**

B. phytofirmans strain PsJN is a plant growth promoting rhizobacterium (PGPR) able to establish rhizosphere and endophytic populations in various crops, including grapevine (Nowak, 1998; Compant *et al*., 2005b, 2008). Stimulation of plant growth

and defense responses by PsJN in grapevine plantlets has been previously reported (Ait Barka *et al*. 2000; 2002), while the ability of bacterized plantlets to withstand cold has already been demonstrated by analysis of several biochemical and physiological aspects (Ait Barka *et al*., 2006). By analysis of interaction between grapevine and PsJN and the characterization of defense mechanism induced in bacterized plantlets, the beneficial role of bacteria in grapevine was further revealed in this study by:

i) Analysis of ISR-type response of grapevine plantlets bacterized with *Bulkholderia phytofirmans* strain PsJN

The analysis of gene expression patterns in leaves of grapevine plantlets showed that strain PsJN induced a systemic spread of a signal, from roots to leaves after root inoculation when bacteria are still present in rhizoplane and began penetrating root internal tissues. Similar modifications in gene expression profiles have been reported as characteristic reactions of defense mechanism induced by PGPR against pathogens, known as (ISR) (Ahn *et al*., 2002; Cartieaux, *et al*., 2003; Wang *et al*., 2005; van Loon *et al.*, 2007). Although change in gene expression profiles caused by ISR has been mostly reported in *Arabidopsis*, our study proposes that *B. phytofirmans* may induce an ISR-type mechanism by transcript accumulation of grapevine defense genes, including those encoding PR proteins (Fig. 1).

ii) Study of grapevine primed physiological responses with *Burkholderia phytofirmans* strain PsJN upon cold stress

In vitro inoculation of *V. vinifera* L. cv. Chardonnay explants with *B. phytofirmans* strain PsJN, increased physiological activity at low temperature, demonstrating a

better adaptation of bacterized plantlets to cold (Ait Barka *et al*., 2006). Changes in expression of well characterized defense genes and *CBF4* transcription factor behind the documented grapevine cold adaptation were investigated in our study. Compared to the non-bacterized control, PsJN treatment elevated the level of the response to low temperature stress causing earlier and higher expression of defense-related genes and *CBF4*, all crucial to plant adaptation and survival under stress. The grapevine plantlet responses to *B. phytofirmans* strain PsJN inoculation are consistent with the priming phenomenon as documented by potentiated accumulation of transcripts, addressing that primed physiological responses against cold may be induced by interaction with a microorganism (Fig. 1).

Several parameters were analysed to further understand the role of strain PsJN in grapevine to low non-freezing temperatures and the phenomenon of priming. Results showed that bacterized plantlets reacted to cold stress faster and/or stronger by accumulating carbohydrates (total soluble, glucose, fructose and sucrose, starch) and proline; phenomena that have been strongly correlated with an increased cold resistance in plants. Furthermore, the earlier reduction of lipid peroxidation markers and hydrogen peroxide contents after 1 week in bacterized compared to non-bacterized plantlets, suggested a significant activation of cold acclimation mechanisms in the former resulting to better adaptation to cold (Fig. 1).

iii) Transcript analysis by cDNA-AFLP technique to further investigate the primed- physiological state of *V. vinifera* L. induced by *Burkholderia phytofirmans* strain PsJN

cDNA-AFLP analysis, is a RNA fingerprinting technique that has been used extensively in recent years to display differentially expressed genes in plants (Yang *et*

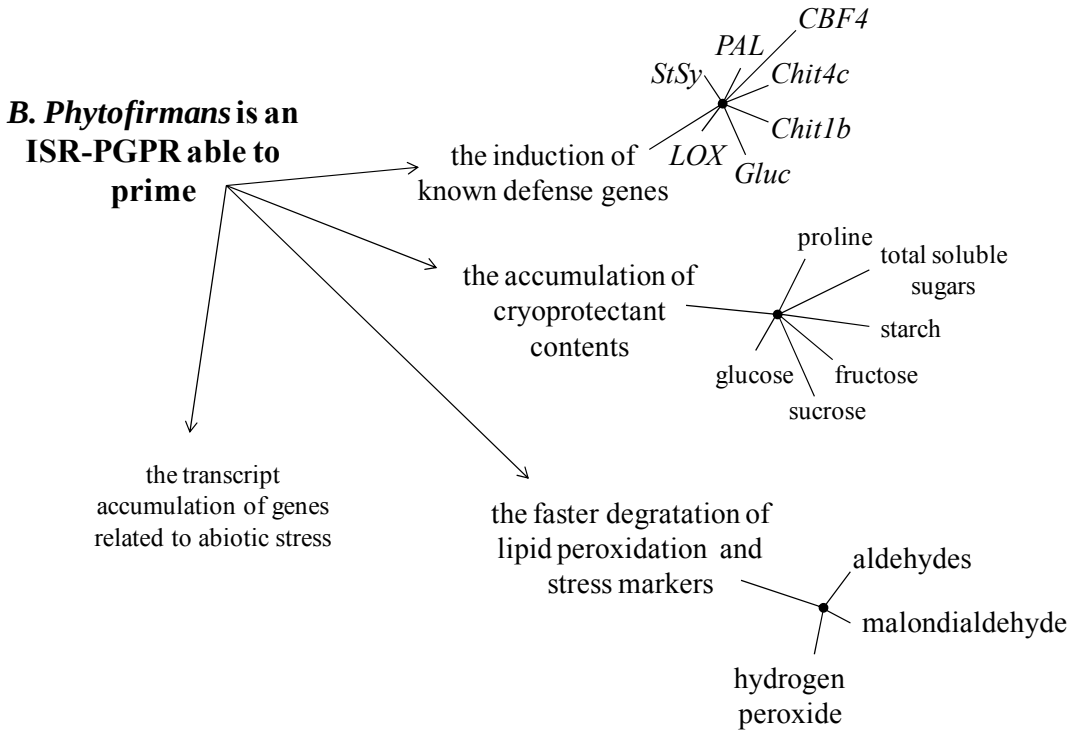


Fig. 1: Study of grapevine primed physiological responses with *Burkholderia phytofirmans* strain PsJN upon cold stress.

al., 2003; Mao *et al*., 2004; Burger & Botha, 2004; Ongena *et al*. 2005). The investigation of expressed bands in non-bacterized and bacterized grapevine plantlets by cDNA-AFLP technique has revealed that strain PsJN prime the physiological state in grapevine plantlets by stimulating the expression of about 50% of detected genes, while the identification of three isolated bands showed that bacterized plantlets can react to cold by over-expressing genes involved in response to for abiotic stress. Although the analysis of isolated genes needs further investigation, an overview of gene expression in priming phenomenon induced by strain PsJN upon low non-freezing temperature was successfully detected.

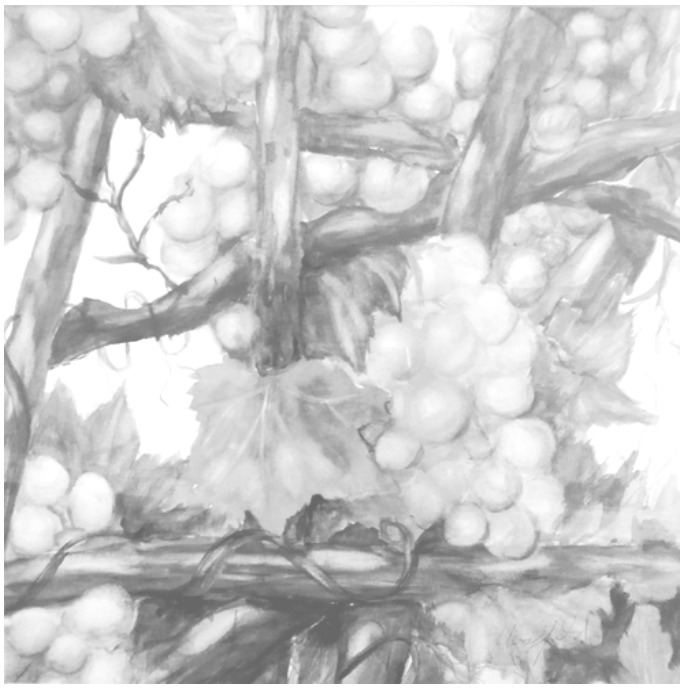
Cost and benefits of defense induced by *Burkholderia phytofirmans* strain PsJN

Induced resistance can protect plants against a wide spectrum of abiotic and biotic stresses. However, it can be costly due to allocation of resources or toxicity of defensive products. Most theories of plant defense assume that there will be a cost to fitness resulting from the production of defense chemicals or structures, which at least must be balanced by the resulting benefit (Simms & Rausher, 1987; Simms & Triplet, 1994). These costs should ideally be measured as reduction in fitness or they may be estimated by measuring reduction in growth rates (Simms & Rausher, 1987; Sagers & Coley, 1995). Most studies on costs and benefits of induced resistance have focused on situations in which the defense is activated directly by the inducing agents. Determining the cost and benefits of induced direct defense and comparing those to priming, van Hulten *et al*. (2006) demonstrated that fitness costs of priming are substantially lower than those of the directly induced defense against pathogens, while the benefits of priming outweigh its cost when disease occurs. From ecological point of view, priming is the plant's solution to the trade off dilemma between environmental stress protection and costs involved in defense activation, representing an important adaptive defense strategy in plants. Apparently, induced resistance uses priming as a common mechanism by which plants acquire sustainable protection against environmental stress (Conrath *et al*., 2006).

B. phytofirmans strain PsJN is a well characterized PGPR able to protect plants against abiotic and biotic stresses. In this study further analysis of this symbiotic relation with grapevine, showed that strain PsJN induces an ISR-type response, but without any significant cost to grapevine fitness, in view of the fact that several physiological parameters, like growth rates and photosynthesis in grapevine plantlets are improved after plant inoculation by strain PsJN (Ait Barka *et al*., 2000;

2002). Additionally, *B. phytofirmans* strain PsJN induces a save-energy defense mechanism in grapevine plantlets to low non-freezing temperatures, according to phenomenon of priming. Therefore, *B. phytofirmans* protects plantlets by priming grapevine defense mechanism when stress occurs, offering a low-cost adaptive defense strategy against cold stress.

Consequently, grapevine plantlet responses to PsJN inoculation are consistent with an induced systemic response and priming phenomena as documented by activation of the expression of the defense-related genes and change in several biochemical markers upon low non freezing temperature, addressing for the first time that the phenomenon of priming in grapevine could be induced by interaction with a microorganism offering a sustainable protection of vineyard in “cool” climates.



4. FUTURE PROSPECTS

*B. phytofirma*ns strain PsJN has been well characterized as a PGPR that triggers induced resistance in grapevine against fungal pathogens. Recently, it has been demonstrated that PGPR may also enhance resistance to stress in low non-freezing temperatures. In this study, the induction of ISR-type resistance by PsJN strain in grapevine plantlets has been proposed while the analysis of bacterized grapevine reaction to low non-freezing temperatures revealed that strain PsJN can prime the physiological response of grapevine defense mechanisms. Therefore, it could be suggested that strain PsJN acts similarly to other ISR-inducing PGPRs, able to prime the defense mechanism of grapevine tolerance against stresses. Through the further analysis of ISR-type resistance and the further study of primed physiological responses upon cold, the future prospects of this project may include investigation of several physiological, biochemical and molecular aspects of this symbiotic relation.

i) Further analysis of ISR response in grapevine plantlets after root inoculation

The study of grapevine reactions to inoculation by strain PsJN showed an ISR-type response after root inoculation with bacteria and further investigation is needed by studying grapevine responses of root-colonized plantlets by strain PsJN, upon pathogen contamination. Although, the beneficial effects of strain PsJN against *Botrytis cinerea* have already been revealed (Ait Barka *et al.*, 2000; 2002), the specific role of the systemic signal induced after root inoculation in defense against pathogen, need to be clarified. For that reason, the next step of this study is the analysis of differential expressed genes induced by strain PsJN in correlation with the induced resistance of grapevine plantlets to pathogen.

Previous study also showed that strain PsJN can also enhance the rate of photosynthesis (Ait Barka *et al*., 2006). The further analysis of stimulation of

photosynthetic activity induced by strain PsJN is an interesting field to further investigate. The photosynthetic status in grapevine after root inoculation could be analysed through the ratio of chlorophyll fluorescence variable to maximal chlorophyll fluorescence (Fv/Fm) and any changes in expression levels of several genes correlated with changes in photosynthesis.

ii) Investigation of carbohydrate accumulation mechanisms in grapevine plantlets upon low non-freezing temperature, and analysis of the potentiated role of strain PsJN

By several studies, the exposure of plants to low temperatures has been connected with an increase in total carbohydrate concentration. Even if low temperature leads to the inhibition of photosynthesis, a possible explanation about the relation between cold and accumulation of sugars has been provided by activation of specific enzyme activities of the Calvin cycle. Therefore, the study of changes in gene expression levels of several genes, including Rubisco and hexokinase, correlated with changes in photosynthesis and tissue sugar concentrations, and the analysis of post-translational activation of enzymes such as Ribulose bisphosphate carboxylase oxygenase (Rubisco) and Fructose-1,6-bisphosphatase in sucrose synthesis pathways, in bacterized and non-bacterized grapevine plantlets, is a key point of this future work.

iii) Isolation of antifreeze proteins and analysis of beneficial effects of strain PsJN in grapevine plantlets

As it was shown, grapevine plantlets under low non-freezing temperature increased the accumulation of transcripts encoding PR proteins which are homologous

to antifreeze proteins (AFPs). AFPs accumulated in cold-acclimated plants exhibit both antifreeze and antifungal enzymatic activity (Yeh *et al*., 2000; Huang & Duman 2001). Although AFPs have been reported in apoplast of many acclimated plants since 1992, their enzymatic activity has not been reported in grapevine yet. Therefore, the first goal of this prospect will be to isolate the antifreeze proteins from the young acclimated grapevine leaves which are frequently exposed to low non-freezing temperature during spring. After isolation, further investigation of their thermal hysteresis activity (the difference between the melting point and freezing point) in grapevine could be analysed in relation with the possible beneficial effect of PsJN strain.

iv) Further investigation of primed- physiological state of *V. vinifera* L. induced by *Burkholderia phytofirmans* strain PsJN by transcription analysis

By cDNA-AFLP analysis, it was revealed that strain PsJN induce the physiological state of fully colonized grapevine plantlets by stimulating the expression of about 50% of detected genes in comparison to non-bacterized, at normal growth condition. Although, previous studies showed that bacteria can be involved in several physiological parameters (e.g. growth rates and photosynthesis activity, sugar and proline accumulation), there is a lack of knowledge regarding the molecular status in bacterized grapevine plantlets, and especially the identity of expressed genes and their relation with the phenomenon of priming. Therefore, the isolation and identification of these expressed bands may support our knowledge about the primed physiological state in grapevine plantlets induced by *B. phytofirmans* strain PsJN.

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