CHAPTER-2

REVIEW OF LITERATURE
Potato (Solanum tuberosum L.) is one of the unique and most potential crops having high productivity, supplementing major food requirement in the world. It is rich in carbohydrates, proteins, phosphorus, calcium, vitamin C, and β-carotene and has high protein calorie ratio. Amongst the world's important food crops, potato is the world’s third most important food crop after wheat and rice (Sundaram, 2011). India ranks second in global potato production after China (FAOSTAT data, 2008) and produces 8% of total global potato, which is pegged at about 320 million tonnes for the year 2010.

2.1 Potato crop

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The major potato species grown worldwide is Solanum tuberosum (a tetraploid with 48 chromosomes). There are about five thousand varieties of potato present all over the world. Three thousand of them are found in the Andes alone, mainly in Peru, Bolivia, Ecuador, Chile, and Colombia. Solanum tuberosum is a hybrid between the diploid species S. stenotomum and the diploid weed S. sparsipilum with subsequent chromosome doubling. The ploidy levels vary in potato with different series, based on a haploid number of 12, ranging from diploid (2n=24) to hexaploid (6n=72) including triploids, tetraploids and pentaploids (Dodds, 1962). The cultivated potatoes are autotetraploid (4n=48) and many wild species are diploid.

Potato plants are herbaceous perennials that grow about 60 cm high, depending on variety. They bear white, pink, red, blue, or purple flowers with yellow stamens. Potatoes are cross-pollinated mostly by insects, including bumblebees, which carry pollen from other potato plants, but a substantial amount of self-fertilization occurs as well. After potato plants flower, some varieties produce small green fruits that resemble green cherry tomatoes, each containing up to 300 true seeds. Potato fruit contains large amounts of the toxic
alkaloid solanine and is therefore unsuitable for consumption. All new potato varieties are grown from seeds, also called "true seed" or "botanical seed" to distinguish it from seed tubers.

2.2 Potato as a future crop

Potato is viewed to have the potential for alleviating hunger and malnutrition of the developing world especially in view of the shrinking arable land. When compared to other grain crops, Potato tubers are exceptionally high yielding per acre of land (Burton, 1969) and find use in variety of ways like, for table purpose, processing, livestock feed and industrial uses (Feustel, 1987; Talburt, 1987).

Potato is a labour and capital-intensive crop and is cultivated by farmers with holdings of all size-classes, i.e., marginal, small medium and large farmers. Potato is an ideal food crop. It is highly efficient in converting sunlight into nutritious food. It produces the highest amount of food, dry matter, protein and other nutrient per unit time, among all the major food crops. It is also nutritionally superior to the traditional food crops. In addition, it has wide adaptability and has great potential for yield increase by genetic manipulation. It is a short duration crop and can be harvested as early as 60 days after planting. Therefore, it is an ideal crop to fit in intensive cropping system. In fact potato is a low calorie food containing protein with biological value of 75% of egg and milk protein and is also rich in minerals, vitamins B and C. With the shrinking arable land resources and increasing population, potato has turned to be a food crop having great potential in supplementing and to partially replacing, the traditional cereal foods.

2.3 Potato processing

Processing is a fast growing sector within the world potato economy. Due to the increased demand for convenience food and the expanding institutional market, the consumption of processed potato products is of increasing importance. The most popular processed products of potato are chips, french fries, powder, cubes, slices and starch. Processing is mainly confined to developed countries, and it is under-exploited in most of the developing countries with the exception of China (12%), Korea (6%) and Mexico (8%). Currently, about half of the annual crop in the USA is processed. In India processing has
flourished in the past decade and there are considerable scopes for expansion of this industry (Marwaha, 1999). Total raw material requirement of potato processing sector in India was 0.9678 MT in 2005-06, which was 4.1% of the total potato production during 2004-05. In 2007-08 total raw material requirement was about 1.68 MT (7.12% of total production during 2004-05). Out of total 32.55 MT potatoes produced in India during 2009-2010 about 2.67 MT was utilized as raw material by the processing industry including the processing at the household, cottage and unorganized sector level, which resulting into 0.42 MT of the final processed product. The raw material requirement is expected to be about 11.5% (by the year 2010-11) of the average potato production in India during 2006-07 (Rana, 2011). The demand for processed potato products is growing by 15% per annum, and it is estimated that after five years total consumption will be 5.2 MT.

As India is the second largest producer of potato in the world, multinationals like M/s Frito-Lay and M/s McCain Food Ltd. and other foreign multinationals are directly or indirectly tying up with their Indian counterparts on different collaborative ventures to supplement and diversify this emerging sector.

There are two major cropping seasons in India, kharif, during the south-west monsoon (June/July through September/October), when agricultural production takes place both in rain fed areas and irrigated conditions and rabi, during the winter, when agricultural activities take place only in the irrigated areas. ~80% of the crop is raised during winter season. So, tuber has to be stored for round the year availability for processing industry. But, none of the so far bred Indian potato cultivars is good cold-chippers and this poses a serious impediment to otherwise fast emerging potato processing industry in India. It is the one of the reasons of the unavailability of quality raw material for the processing industry throughout the year. If processing industries could utilize potatoes stored in the cold stores located in their vicinity from early April onwards, they could save a lot of expenses incurred on transportation of processing potatoes from far off places like Hasan in Karnataka and lower hills. For solving this problem Central Potato Research Institute has developed specialized Indian processing varieties, namely Kufri Chipsona-1 and Kufri Chipsona-3, but they were also unfit for chipping after merely 10 days of storage at low temperature (Kumar, 2011).
To be competitive in global potato trade the most important challenge for India is how best to stabilize the potato production for processing purposes round the year to meet industry's demand.

2.4 Cold-storage induced sweetening

Low temperature (8-12°C) storage of potato is an integral post-harvest handling activity of this semi-perishable crop to prevent sprouting, reduce respiration and minimize disease losses (Rausch and Greiner, 2004). However, 8-12°C is not an ideal temperature for storage, and storage at 2-4°C is more preferable because of lesser need to use fungicides and bactericides, lesser loss of solids through respiration and the lesser need for chemical sprout suppressants (Sowokinos, 2007). During cold storage dormant potato tubers degrade starch and synthesize sucrose with energy provided by glycolysis and respiration (Isherwood, 1973). This accumulated sucrose is then split into reducing hexoses, glucose and fructose, catalyzed by vacuolar acid invertase. This phenomenon of conversion of starch to reducing sugars at low temperature is known as cold-induced sweetening (CIS) (Mullar-Thurgau, 1882). The level of sugars in potato tubers is an important factor affecting quality of potatoes, especially colour of processed products.

2.4.1 Sucrose and reducing sugars

Sucrose is a 12-carbon, non-reducing sugar that occupies a critical position in potato tuber development and has been found to be the most abundant sugar in potato (Burton, 1965). It is the major source of carbon and energy for starch synthesis in potato and is important for plant growth. The sucrose concentration is high in immature potatoes because in tubers the rate of sucrose translocation is higher than the rate of its metabolism. Potato cultivars differ in the amount of sucrose they accumulate during the growing season. Although it does not participate directly in the Maillard reaction which is responsible for darkened chips but it does serve as an intermediate product in the formation of reducing sugars from starch. Invertases break down the sucrose into the reducing sugars and reaction becomes active during storage (Isherwood, 1973). Sucrose and reducing sugar content vary under different storage conditions. Samotus et al. in 1974 reported that the predominant sugar in tubers
following short term storage at low temperature was sucrose. However, upon prolonged storage reducing sugars content increased and the sucrose content decreased. This shift of sucrose to reducing sugar level is due to increase in invertase activity during cold storage.

Sowokinos in 1973 suggested that the amount of sucrose at harvest may be used to predict the initial rate of reducing sugar formation. As higher sucrose concentrations ultimately lead to increased reducing sugar concentration which has direct correlation with the browning of chips. He also noticed higher concentration of sucrose in non processing varieties and found a direct correlation between the sucrose content in tubers at the time of harvest and chip colour.

2.4.2 Maillard reaction

Maillard reaction was named after the name of French chemist, Louis Camille Maillard, who first described it in 1912 (Maillard, 1912). It comprises a series of non-enzymatic reactions between reducing sugars and amino groups of amino acids (Fig. 1). Maillard reaction occurs at high temperature and low moisture conditions and, therefore, frying, baking and roasting provide the perfect environment for the reaction. The reaction requires a reducing sugar such as glucose, fructose or maltose. Sucrose does not participate unless it is hydrolysed through enzymatic, thermal or acid-catalysed reaction (De Vleeschouwer et al., 2009).

On potato processing at high temperature, carbonyl groups of reducing sugars (glucose and fructose) react with the amino group of free amino acids through Maillard reaction; which causes an unwanted brown coloration in potato chips and crisps, thereby negatively affecting the processing quality of potato tuber (Shallenberger et al., 1959; Sowokinos, 2001a; Kumar et al., 2004). The melanoidin pigments are one of the products of Maillard reaction responsible for brown colour in fried, backed and roasted foods (Fig. 1). These 'melanoidins' have variable structures, molecular weights and nitrogen content. The other products of Maillard reaction includes volatile compounds that contribute to the aroma associated with many cooked foods; flavored compounds, often bitter substances; reducing compounds that may help prevent oxidative deterioration and increase the stability (shelf life) of the food and mutagenic compounds. In
addition, this reaction also produces acrylamide, a toxin and potential carcinogen (Keijbets, 2008). Though amino acids are important substrate in the Maillard reaction, but only the amount of reducing sugars are rate limiting factor (Marquez and Anon, 1986).

Maillard Reaction

\[
\text{Reducing Sugars} + \text{Amino Group} \xrightarrow{\text{Amadori Rearrangements}} \text{N-Substituted Glycosylamine} + \text{Water} \\
\text{1-amino-1-deoxy-2-Ketos (1,2-Enol Form)} \xrightarrow{\text{High Temperature}} \text{Aldols and N-free Polymers} + \text{Ketamines} + \text{Aldamines} \\
\rightarrow \text{Melanoidin Pigments (Cause of brown coloured chips)}. 
\]

**Fig. 2.1:** Formation of melanoidin pigments in Maillard Reaction.

2.5 Factors affecting sugar accumulation

Plant cells subjected to cold temperatures accumulates soluble sugars consisting mainly of sucrose, glucose and fructose. Low temperature-induced sugar accumulation has also been verified with a number of other plant species (ap Rees et al., 1981) including *Vitis vinifera* (Berbezy et al., 1997) and tulips (Komiyama et al., 1997). Sugar accumulation in response to low temperature is a useful process for survival of the plant species as sugars act as osmoprotectant and cryoprotectant during cold. But this process is of negative importance in case of potato because it affects the quality of the processed potato product. The sugar content of potato is determined by genotype, several post and pre-harvest factors, including temperature during growth, storage temperature, and storage atmosphere. There are some other factors like soil moisture, mineral nutrition, tuber maturity and mechanical stress, but there is divergent opinion about their contribution for sugar accumulation.
2.5.1 Genotype - The genetic component has a strong influence upon initial reducing sugar level in a mature tuber as well as during storage (Kumar et al., 2004). The tendency to contain a high or low content of total sugar or of a particular sugar is a heritable trait (Cunningham and Stevenson, 1963). In a study based on several good or poor processing cultivars, Sowokinos (1973) related the processing quality to sucrose synthase activity. Sowokinos (2001b) reported that alleles A and B for enzyme UGPase were present in different ratio in cold resistant and cold susceptible cultivars. Cold resistant cultivars exhibited predominance of $ugpA$ allele, while cold susceptible cultivars showed predominance of $ugpB$ allele.

2.5.2 Temperature during growth - The temperature and the length of growing season are second to cultivar selection, as transpiration (water movement), translocation (carbohydrate movement), photosynthesis (carbohydrate production) and respiration (carbohydrate breakdown) are temperature dependent (Pavlista and Ojala, 1997).

An increase was recorded in sugar content of tuber exposed to temperature below 8-12°C (Arreguin-Luano and bonner, 1949) and above 29-30°C (Kumar et al., 2004).

Optimum temperature for tuber formation and growth for most varieties ranges between 15°C and 20°C with few exceptions (Burton, 1966) and all studies until now on temperature during growth seem to validate this temperature range.

2.5.3 Storage temperature - Reducing sugar content of potato increased during storage at low temperature (Sowokinos, 1990b), and even incidence of increase in sugar content was noticed during high temperature (Nielson and Todd, 1946).

Tubers that have undergone low temperature sweetening can be reconditioned by increasing the storage temperature (Pritchard and Adam, 1994). The degree to which quality can be restored with reconditioning varies with cultivar. Edwards and his co-workers in 2002 also found decrease in sugar content of potato upon reconditioning.
Pritchard and Adam (1992) have demonstrated that even preconditioning of tubers at 15°C can limit the increase in reducing sugars during subsequent storage. Potato seems to have an ideal temperature range, where equilibrium is achieved and net production of free sugar is at its minimum. Any kind of temperature stress above and below that ideal temperature range results in excessive accumulation of sugars.

2.5.4 Storage atmosphere - Potato, being a living entity, respires, exchanging CO₂ and O₂. Low oxygen level has been shown to suppress the sugar accumulation in potato tubers stored at low temperature (Harkett, 1971). Zhou and Solomos (1998) reported that low oxygen concentration inhibited the cold induced enzymes, such as invertases, alternative oxidase and one form of amylase. In a study conducted in commercial cold store, Mazza and Siemens (1990) found that increased CO₂ concentration led to rise in reducing sugar and sucrose values.

2.5.5 Reconditioning - Reconditioning of tubers at higher temperatures after cold storage is a common practice followed in order to reverse the physiological reaction thereby decreasing the reducing sugar content of stored tubers. Reconditioning of stored potatoes increases the reverse reaction i.e. conversion of sugar to starch and is a common practice prior taking the produce into processing (Pritchard and Adam, 1994). A demonstration by Pritchard and Adam has clearly shown to decrease or limit the reducing sugar content of potato tubers by preconditioning them at 15°C before storage at low temperatures. Recondition is a cultivar dependent phenomenon and degree of reconditioning also varies from cultivar to cultivar. Kyriacou et al. (2009) have clearly mentioned that poor-chipping cultivars exhibit inadequate response to reconditioning. Edwards et al. (2002) has also accounted for decrease in sugar content of potato upon reconditioning. In some recent studies, reconditioning period up to three weeks (Knowles et al., 2009) or 30 days (Kyriacou et al., 2009) have been found to be useful in reducing the sugar content after cold storage. During reconditioning the sugars are incorporated back into starch or are used for growth in reconditioning. Therefore, identification of a genotype for a particular geographical location or climate and then standardization of crop and storage management factors to get minimum amount of sugars are very important.
2.6 Potato carbohydrate metabolism

Carbohydrate metabolism is a complex process in potato (Sowokinos, 2007) and is thought to be a quantitative genetic trait (Menendez et al., 2002). The actual concentration of free sugars in potato involves the interaction of several pathways of carbohydrate metabolism, including starch synthesis/degradation, glycolysis, respiration and sweetening. These pathways are controlled at many levels, including hormonal, membrane structure and function, compartmentalization and concentration of enzymes, key ions, and substrate; and of course, enzyme expression levels and activity (Sowokinos, 2007).

It is thought that cold induced hexose accumulation is caused by an imbalance between starch breakdown and glycolytic activity. The amount of starch degrading enzymes (amylases and phosphorylases) increases (Cottrell et al., 1993) during cold storage, while the cold lability of several enzymes is believed to restrict glycolysis (Pollock and après, 1975a; Dixon et al., 1981).

During cold storage of potato, starch breakdown in amyloplast is triggered through induction of amylolytic enzymes or by phosphorolytic enzymes and products are exported from amyloplast to cytosol either as hexose-phosphates (hexose-P) via the glucose- glucose translocator or as free sugar via the glucose and /or maltose transporters (Nielsen et al., 1997; Weber, 2004; Smith et al., 2005). Sucrose is formed in cytosol via UDP-glucose pyrophosphorylase, sucrose phosphate synthase and sucrose phosphatase (Isherwood, 1973; Krause et al., 1998) and is subsequently hydrolyzed in the vacuole by acid invertase yielding the reducing sugars, glucose and fructose (Pressey and Shaw, 1966; Richardson et al., 1990). Process of hexogenesis (reducing sugar formation) in the plant cell is given in the Fig. 2.
Even though glucose and fructose are produced in equimolar amounts, glucose has been shown to be present at higher concentration in potato cells during growth and in storage at 4°C (Merlo et al., 1993). This may be due, in part, to high concentration of fructokinase found in potato extracts that could cycle fructose back into pool of hexose phosphates.

Several explanations have been put forward to explain cold induced accumulation of sugars, including: 1. an increase in activity of starch degrading enzyme (Sowokinos et al., 1985; Cochrane et al., 1991; Claassen et al., 1993; Cottrell et al., 1993); 2. an increased concentration of hexose phosphates as a result of cold-liability of phosphofructokinase and other enzymes in glycolysis (Pollock and ap Rees, 1975b; Dixon and ap Rees, 1980; Trevanion and Kruger, 1991); 3. increased activity of enzyme involved in sucrose synthesis (Sowokinos, 1990a); and 4. increased activity of vacuolar acid invertase (Pressey and Shaw, 1966; Richardson et al., 1990). It has been concluded that sweetening results from altered gene expression and/or modulation of post translational activity of key enzymes influencing the flux of carbon towards sugar formation (Sowokinos, 1990a).
2.7 Direct approach for reduction of cold induced sweetening in potato

In direct approach, efforts have been made to modulate/suppress expression of genes involved in hexogenesis (i.e. genes for UDP glucose pyrophosphorylase (UGPase), Sucrose phosphate synthase (SPS), Sucrose phosphatase (SPP), and acid invertase (valINV)) using different strategies.

2.7.1 UDP glucose pyrophosphorylase

UGPase (UTPα-D-glucose-1-phosphate uridylyl transferase) is one of the important enzymes of carbohydrate metabolic pathway. It catalyzes the reversible conversion of Glc-1-P and UTP into UDP-Glc and PPI. UDP-Glc is the key metabolite that contribute glucose residue to Fru-6-P towards formation of Suc-6-P, which is immediately dephosphorylated to sucrose. Activity of this enzyme is strongly correlated with amounts of sugars accumulated during cold storage (Sowokinos, 1990b). UGPase expression in potato was analyzed with respect to sink source interactions and during cold storage. Storage of mature tubers at low temperature led to an increase of steady state level of UGPase mRNA (Zrenner et al., 1993; Bagnaresi et al., 2008). High level of UGPase mRNA was observed in sink tubers, which decreased dramatically after harvesting and storage at room temperature. Expression of UGPase was found to be significantly higher in cold stored tubers as compared to tubers stored at room temperature, implicating a role of this enzyme in cold sweetening.

It has been suggested that availability of UDP-Glc limits the flow of carbon through SPS (sucrose phosphate synthase) for the formation of sucrose (Sowokinos et al., 2000). UGPase in potato is encoded by single gene (Borovkov et al., 1996) and in the most tested potato cultivars UGPase was represented by at least two alleles (UgpA and UgpB), differed by presence or absence of BamHI recognition sequence. It was observed that the allele without BamHI site (UgpA allele) was usually associated with cultivar having higher resistance to cold sweetening (Sowokinos, 2001b). Antisense and ribozyme mediated repression of UGPase resulted in partial decrease of sucrose and reducing sugars in cold stored tubers (Spychalla et al., 1994; Borovkov et al., 1996).
2.7.2 Sucrose phosphate synthase

Sucrose phosphate synthase (SPS) has been widely studied in the context of sweetening. SPS catalyzes the penultimate and rate limiting step of sucrose synthesis; it is a highly regulated enzyme, undergoing modulation through phosphorylation and dephosphorylation by allosteric effectors (Glc-6-P and Pi) (Huber and Huber, 1996; Pattanayak, 1999). Sucrose synthesis forms the interphase between starch degradation and hexogenesis in potato during exposure to cold. The formation of sucrose involves two enzymatic steps: sucrose phosphate synthase that catalyzes the synthesis of sucrose-6-phosphate, which is further, hydrolyzed by sucrose phosphatase to yield sucrose. Reaction catalyzed by SPS is freely reversible, and rapid hydrolysis of sucrose-6-P displaces the equilibrium into the direction of net sucrose synthesis (Stitt et al., 1987). SPS is generally assumed to be a key enzyme controlling flux of carbon into sucrose (Huber and Huber, 1996). Studies with transgenic plants have shown that SPS activity is the major detriment of partitioning of photosynthate between sucrose and starch. Transgenic tomatoes over-expressing maize SPS had higher concentration of sucrose and lower concentration of starch in leaves (Worrell et al., 1991). The onset of sugar accumulation in cold stored potato tubers coincides with an activation of SPS (Hill et al., 1996; Nielsen et al., 1997). Plants contain several isoforms of SPS which appear to be, at least in part, functionally distinct (Langenkamper et al., 2002; Chen et al., 2005).

Various experiments conducted on alteration of SPS gene expression showed that its activity is not rate limiting for cold induced sweetening but its regulatory kinetics had acceptable effect on the process (Hill et al., 1996; Krause et al., 1998). In antisense SPS transformants where SPS activity is less than 30% activity compared to that of wild type, sucrose synthesis and accumulation were found to be strongly inhibited (Deiting et al., 1998). Experiments on antisense SPS transformants provide evidence that low temperature induce alterations in kinetic property of SPS (Deiting et al., 1998). It is concluded that alteration in kinetic properties in response to cold stress can be used as an effective way to suppress the sugar accumulation than by changes in SPS gene expression because on response to the cold incubation SPS does not alter its amount of protein. Hence, SPS activity is substrate limited and it is expressed over the cell...
demand (Krause et al., 1998). However, it is likely that SPS is not the only step at which sugar accumulation at low temperature is regulated.

### 2.7.3 Sucrose phosphatase

Sucrose phosphatase (SPP) catalyses the final step in the pathway of sucrose biosynthesis and higher plants contain multiple isoforms of enzyme encoded by different genes. Chen et al. (2008) performed RNAi experiment to investigate the role of SPP in potato carbohydrate metabolism at low temperature (4°C). From the molecular and biochemical characterization of cold-stored, SPP silenced tubers, they concluded that reduced SPP activity can block the flux through sucrose synthesis in cold. However, SPP does not seem to operate with maximum catalytic activity *in-vivo*, as the massive accumulation of Suc-6-P in transgenic tubers compensated for reduced SPP activity eventually leaving the long term sucrose production largely unchanged in cold stored SPP silenced tubers.

Moreover, Suc-6-P accumulation seemed to prevent cold induction of vacuolar acid invertase. Reduced vacuolar invertase activity in cold stored SPP silenced tubers dramatically lowered the hexose to sucrose ratio as compared to control tubers.

The role of suc-6-P as a signaling molecule is currently unknown. Interestingly, trehalose-6-P, an intermediate of trehalose biosynthesis and similar molecule as suc-6-P, is an important regulator of carbohydrate utilization in plants (Paul, 2007). It needs to be established whether suc-6-P plays a similar role.

### 2.7.4 Acid invertase

In plants, acid invertases are known to be the key enzymes cleaving sucrose into reducing sugars (glucose and fructose). The enzyme activity increases several fold during storage of potato at low temperature (Pressey, 1969). qPCR studies on acid invertase in cold stored tubers showed drastic increase in mRNA transcript level from 400 fold expression on day 3 of cold storage to 1100 fold on day 25 (Bagnaresi et al., 2008).

Zrenner et al. (1996) used antisense approach to inhibit particular cold inducible vacuolar invertase (valNV) activity and consequently formation of sucrose derived hexoses. No effect was found on hexose concentration and
hexose sucrose ratio in fresh harvest transgenic tubers. However, hexose to sucrose ratio reduced significantly in transgenic tubers after storage at 4°C. An absolute reduction in cold induced hexose accumulation (~34%) was observed only in the strongest antisense line, where residual vacuolar invertase activity was <18% as compared to wild type plants. Based on these results, it was concluded that other invertase isoforms might contribute to cold induced hexose accumulation. Greiner et al. in 1999 over-expressed tobacco vacuolar invertase inhibitor (Nt-lnhh) in potato to inhibit the activity of cold induced vacuolar acid invertase in tubers and circumvent the possible limitations of isoforms specific repression of the enzyme. Nt-lnhh lines showed reduction of reducing sugars in tubers, stored at 4°C for six weeks. Chips prepared from cold-stored transgenic tuber showed desirable chip colour with reduction in browning during frying at higher temperature as compared to wild type plants. No adverse effect was found on tuber growth, yield and starch content in Nt-lnhh transgenic lines (Greiner et al., 1999).

Liu et al. (2010) performed transcript analysis of potato invertase inhibitors and invertases among six potato genotypes with distinct cold induced sweetening activity. Four genes with homology to tobacco invertase inhibitor were identified, and their contribution to cold induced sweetening was investigated. It was observed that St-lnvlnh2 (invertase inhibitor) played critical role in post-transcriptional regulation of St-lnv1 in potato and suggested that it might play critical role in controlling cold induced sweetening.

2.8 Indirect approach for reduction of cold induced sweetening

It involves the engineering of genes involved in starch synthesis, starch breakdown and glycolysis.

2.8.1 Engineering of genes involved in starch synthesis

Starch synthesis occurs in amyloplasts of potato cells. Inside the amyloplast, ADP-glucose is synthesized via ADP-glucose phosphorylase (AGPase). ADP-glucose is the substrate for the synthesis of starch polymers, amylase and amylopectin and is converted to starch polymer via action of starch synthase and starch branching enzyme. Although the metabolic pathways and enzymes involved in starch-hexose interconversion are well characterized, little is
known about their contribution to cold sweetening and \textit{in-vivo} regulation (Greiner \textit{et al.}, 1999).

It is widely accepted that most of the control of carbon flux through the pathway of starch synthesis in storage organs rests with AGPase (Stark \textit{et al.}, 1992). Alteration of the activity of AGPase in storage organs changes the amount of starch that they accumulate. Large reduction in enzyme activity and low starch content were observed in transgenic potato harbouring antisense construct for the large subunit of the enzyme, (Muller-Rober \textit{et al.}, 1992; Okita, 1992). Overexpression of a mutated AGPase gene (\textit{glgC16}) from \textit{Escherichia coli} led to an increased rate of starch synthesis in transgenic potato (Stark \textit{et al.}, 1992), and less accumulation of reducing sugars during cold storage. Sweetlove \textit{et al.} (1996) reported four to five fold increase in AGPase activity in \textit{glgC16} over expressed potato and found no detectable effect on starch content in mature tuber during development. They also observed that transgenic tubers demonstrated greater ability to degrade starch.

Lorberth \textit{et al.} in 1998 tried to silence protein involved in starch phosphorylation and created antisense R1 transgenic plants. So its antisense inhibition led to reduced phosphate content of potato starch resulting in less digestible starch compared to that of wild type, and consequently inhibition of cold induced hexose accumulation.

Mckibbin \textit{et al.} in 2006, modified carbohydrate content of potato by manipulating the metabolic regulator sucrose non fermenting-1- related protein kinase -1(SnRK1). Antisense suppression of \textit{SnRK1} had been shown to reduce gene expression in potato (Pruell \textit{et al.}, 1998) and also prevent sucrose dependent redox reaction of AGPase (Tiessen \textit{et al.}, 2003). Over expression of \textit{SnRK1} in potato resulted increase in starch content up to 30% and decrease in glucose level up to 83%, due to increase in level of expression and activity of two key enzymes involved in starch biosynthetic pathway, sucrose synthase and AGPase.

Although these approaches were successful for reducing hexose accumulation during cold storage but are of limited value, as these approaches compromised with quality and quantity of starch.
2.8.2 Engineering of genes involved in starch degradation

It is generally accepted that cold sweetening is fuelled by starch derived hexoses due to increase in activity of starch degrading enzymes, i.e. amylases and phosphorylases (Cottrell et al., 1993). Starch exists as semi-crystalline granules composed of two glucose polymers: amylopectin (highly branched structure) made of short chain of α-1,4-linked glucose residues joined by α-1,6-linkage, and amylase, a much smaller and relatively unbranched structure, made of α-1,4-linked glucose residues. Various studies have been carried to investigate the role of starch degrading enzymes. These enzymes (glucan pyrophosphorylase, α and β amylase and α-glycosidase) decrease the degree of polymerization of starch which contains predominantly α-1, 4-linked glucose unit (Steup, 1990).

Glucan phosphorylases has long been argued as being responsible for cold triggered starch degradation (Sowokinos, 2001a), and transgenic antisense approaches succeeded to some extent in lowering glucose accumulation during cold storage for over three months (Rommens et al., 2006). However, several studies do not support the prominent role of phosphorylytic starch degradation in early stages of cold storage. Cochrane et al. (1991) found higher activity of α-amylase, β-amylase and α-glucosidase in tubers stored at 4°C as compared to tubers stored at 10°C. Hill et al. in 1996 reported the activity of cold triggered amylase by zymograms. These amylolytic activities were identified as β-amylase based on substrate specificity (Nielsen et al., 1997) but still remained uncharacterized at molecular level.

Glucan-water dikinase (GWD) is the another key enzyme controlling degree of starch phosphorylation and its susceptibility to degradation, possibly as a consequence of relaxed steric hindrance and increased accessibility of amylolytic enzymes (Mikkelsen et al., 2005). Antisense suppression of GWD gene expression resulted reduction in the level of sugars in tubers, stored at 4°C for two months.

2.8.3 Genetic engineering of genes involved in glycolysis

Repression of glycolysis is known to be involved in cold induced sweetening since the activity of enzymes such as phosphofructokinase and pyruvate kinase are reduced during low temperature storage (ap Rees, 1988; ap
Rees and Morrell, 1990). Hajirezaei et al. (2006) investigated the first non-equilibrium step in glycolysis, i.e. the phosphorylation of fructose-6-phosphate to fructose-1, 6-biphosphate by PFK (ATP: fructose-6-phosphate-1-phosphotransferase). Decrease in Q10 (temperature coefficient) at low temperature (Hanlmond et al., 1990) and cold induced dissociation of PFK (Dixon et al., 1981) could be responsible for reduction in glycolysis rate more than other pathways that consume hexose-phosphates (ap Rees et al., 1981). However, it is hard to reconcile that respiration of potato tuber increases concomitant with initial increase in sugar concentration (Barker et al., 1968; Cseke et al., 1982). Furthermore the conversion of fru-6-P to fru-1, 6-bisphosphate is also catalyzed by a completely different PFP, utilizing PPI instead of ATP as phosphate donor (Carnal and Black, 1983; Kruger and Hammond, 1988). This reaction is readily reversible in vivo and is regarded as an adaptive step due to its dependence on variation in biological and physical environment. Claassen et al. (1991) has postulated that PFP activity contributes to cold induced accumulation of sugars by controlling PPI concentration and thus facilitates UDP-glucose and subsequent sucrose synthesis.

To find out whether the metabolic shift imposed by the inhibition of phosphorylating GAPC (cytosolic phosphorylating glyceraldehyde3-phosphate dehydrogenase) would further stimulate the accumulation of reducing sugars in potato tubers at low temperature, transgenic tubers with reduced GAPDH activity (up to 97% compared with wild type controls) were stored at 4°C and sugar contents were monitored after 42 days and 85 days. Very little differences were found in sugar accumulation between transgenic and control tubers, suggesting that phosphorylating GAPC also does not play an important role in accumulation of soluble sugars during cold (Hajirezaei et al., 2006).

Claassen et al. (1993) studied accumulation of sucrose, hexose phosphates and reducing sugars, during storage at 2, 4 and 8°C in potato tubers. They found no involvement of glycolytic enzymes except phosphorylases, in sugar accumulation.

So, the hypothesis that cold liability of glycolytic enzymes is the cause of cold induced sweetening is not tenable at this point.
2.9 RNA silencing

First hints of RNAi emerged in 1990, when Napoli and colleagues generated transgenic petunia flowers by introducing an additional gene copy of enzyme chalcone synthase for pigment synthesis. Unexpectedly transgenic flowers produced with mosaic pigmentation patterns or even completely white petals instead of deep purple flowers. This phenomenon was termed as co-suppression or post-transcriptional gene silencing (PTGS), as the introduced gene copy did not enhance, but blocked the expression of the endogenous gene (Napoli et al., 1990). However, insights into the mechanism did not arouse until 1998, when Craig Mello and Andrew Fire identified double-stranded RNA (dsRNA) to be the PTGS-inducing agent (Fire et al., 1998). They demonstrated that introduction of unc-22 dsRNA into C. elegans resulted in a more effective down regulation of unc-22 mRNA than injecting only sense or antisense strands of the same RNA. Based on this and other observations, Fire and Mello could rule out the formerly proposed antisense mechanism, which would suggest the formation of a hybrid-RNA duplex between the introduced strand and the endogenous mRNA, leading to a block in translation. Shortly thereafter, a correlation was found between PTGS and the presence of ~25-nucleotide (nt) antisense RNAs (Hamilton and Baulcombe, 1999). These small interfering RNAs (siRNAs) were complementary to target mRNAs and were never detected in the absence of PTGS, indicating a direct role in this mechanism. In the following years more and more studies in different organisms showed that the introduction of DNA or RNA sequences of any internal gene in organisms resulted in down-regulation of corresponding mRNAs (Zamore and Haley, 2005).

2.9.1 Mechanism of RNAi

RNAi is triggered by an RNase III-like enzyme, termed dicer, which cleaves long dsRNA into ~21-nt short interfering RNAs (siRNAs) with 2-nt 3’ overhanging ends and phosphorylated 5’ ends (Matranga and Zamore, 2007). dsRNAs derive from exogenous sources like viruses or internal aberrant transcription of repetitive sequences. The siRNA duplexes are incorporated into a protein complex, comprising argonaute (Ago) proteins where one of the strands is removed (“passenger” strand). The Argonaute effector complex is guided by its remaining siRNA strand (“guide”) to the target mRNA, where a single
phosphodiester bond is cleaved between nucleotide position 10 and 11 counting from the 5' end of the siRNA guide (Matranga and Zamore, 2007; Grosshans and Filipowicz, 2008). **A simplistic diagram of siRNA-mediated gene silencing pathway is depicted in Fig. 3.**

Fig. 2.3: RNA silencing pathways. Left: siRNA pathway, Right: miRNA pathway. (Figure adapted from Grosshans, 2008).
2.9.2 microRNAs - A new class of small RNAs

A small RNA, lin-4 was identified in 1993 as a development regulator in the worm, Caenorhabditis elegans (Lee et al., 1993; Wightman et al., 1993), followed by let-7 in 2000 (Reinhart et al., 2000). Later, in the same year let-7 was found to be conserved in a wide range of animal species. These development regulating RNAs formed a new class of non-coding RNAs known as "small temporal RNAs" (stRNA) (Pasquinelli et al., 2000). The existence of additional stRNAs involved in developmental control was proposed at the same time (Pasquinelli et al., 2000). Only one year later, several groups announced the discovery of many more stRNAs in various organisms, which were termed microRNAs (Quintana et al., 2001; Lee and Ambros, 2001). The term microRNA (miRNA) describes a class of small non-coding, RNA molecules that are encoded by specific genes and possess a crucial regulatory function in diverse biological processes, development and stress responses (Kim, 2005; Kim and Nam, 2006; Grosshans and Filipowiez, 2008; Wu and Belasco, 2008). After transcription and several processing steps a 19-24-nt single-stranded RNA stretch is formed which is incorporated into an effector complex. Nowadays, hundreds of miRNAs in plants, worms, flies, mammals and even viruses in various tissues and organs are known. Their function is not restricted to development, but they are involved in many regulatory pathways. In humans, more than 600 miRNAs have been sequenced according to the miRBase Sequence Database (http://miRNA.sanger.ac.uk), predicted to regulate the expression of more than 30% of the genes in the human genome (Lewis et al., 2005).

2.9.3 microRNA biogenesis in plants

Like any other family of cellular RNAs, the origin of miRNAs resides in the genome. They originate from intergenic region, introns as well as from coding region, by action of RNA polymerase II. Transcription of miRNA encoding genes, MIR, produces long stem loop primary transcript, primary-microRNA (pri-miRNA). Primary transcript, pri-miRNA, has been processed in plant nucleus by dicer, DCL1, into duplex miRNA through an intermediate hairpin-stem structure called pre-miRNA. DCL1 interacts with HYL1 to make both cuts within the miRNA precursor to liberate the miRNA/miRNA* duplex. The mature miRNA is methylated by HEN1, which also methylates siRNA. Either the duplex miRNA or
mature single-stranded miRNA is exported into cytoplasm mediated by plant protein HASTY. In the cytoplasm mature miRNA gets incorporated into ribonucleoprotein complex, miRNP, which is similar to RISC. miRNP-miRNA assembly prevent target gene expression either by cleavage of homologous mRNA similar to PTGS or translational repression after binding to homologous mRNA at 3' untranslated region (Pattanayak et al., 2005; Chen, 2005; Vaucheret, 2006). Plant miRNA have very few (zero-five) mismatches to their targets (Schwab et al., 2005).

2.9.4 amiRNA mediated gene silencing in plants

Recent studies have shown that in both plants and animals miRNA precursor can be modified to express a small RNA with a sequence that is unrelated to the miRNA normally produced by the precursors (Zeng et al., 2002; Alvarez et al., 2006; Niu et al., 2006; Schwab et al., 2006). Alteration of several nucleotides within a miRNA sequence does not affect its biogenesis, as long as the initial base-pairing in the stemloop structure of the precursor remain unaffected (Herve et al., 2004). This makes it possible to modify natural miRNA sequences and to generate artificial microRNA (amiRNA) to target any gene of interest (Parizotto et al., 2004; Niu et al., 2006). The amiRNA technology was first used for silencing genes in human cell lines, and recently it was successfully employed to down-regulate individual genes or groups of endogenous genes in transgenic plants (Schwab et al., 2006; Warthmann et al., 2008; Fahim et al., 2012). These plant amiRNAs are expressed from vectors derived from precursors of Arabidopsis thaliana, ath-miR159a, ath-miR164b, ath-miR164a, ath-miR172a and ath-miR319a. Genome-wide expression analyses in transgenic Arabidopsis thaliana show that plant amiRNAs exhibit high sequence specificity similar to natural miRNAs (Schwab et al., 2005), so the amiRNA sequence can be easily optimized to knock down the expression of a single gene or several highly conserved genes without affecting the expression of other unrelated genes.

Based on in-vitro analysis and study of plant miRNAs and their targets, specific sequence parameters important for target selection by plant miRNA have been proposed (Schwab et al., 2005). Using these determinants artificial miRNA (amiRNAs) were designed to target individual genes and groups of endogenous genes in Arabidopsis. These miRNA were found to be highly specific in targeted
genes without any off-target effects (Schwab et al., 2006). Arabidopsis based synthetic pre-miRNAs were found to be processed effectively in tomato and tobacco (Alvarez et al., 2006). Real breakthrough in amiRNA mediated gene silencing was achieved through development of turnip yellow mosaic virus (TYMV) and turnip mosaic virus (TuMV) resistant transgenic Arabidopsis by overexpression of amiRNA targeted against the viral genes (Niu et al., 2006). In another study Qu et al. (2007) also got resistance to cucumber mosaic virus (CMV), using expression of amiRNA against viral suppressor protein in transgenic tobacco plants. Ai et al. in 2011, using Arabidopsis thaliana miR159a, miR167b and miR171a precursors as backbones, designed two types of amiRNA targeting sequence that encode the silencing suppressor HC-Pro of Potato virus Y (PVY) and the TGBp1/p25 (p25) of Potato virus X (PVX). They detected amiRNAs efficiently inhibited HC-Pro and p25 gene expression and conferred highly specific resistance against PVY or PVX infection in transgenic Nicotiana tabacum. The resistance was also maintained under conditions of increased viral pressure. Moreover, they found that resistance was strongly influenced by the complementarily between the target sequence and amiRNA, and it was well correlated to amiRNA expression level. They also found that the expression level of amiRNAs was also related to the precursor backbones. During the study they encountered a new fact that transgenic plants with multiple virus-specific resistance can be obtained through co-expression of several amiRNAs targeting multiple viruses. Recently, Fahim et al. (2012) used polycistronic amiRNA to develop transgenic wheat resistant to wheat streak mosaic virus.