A Story of Carbohydrates in Plants: From a Basic Concept to Advanced Technology.

By Syahnada Jaya Syaifullah

Bogor, 15th June 2026

APPROVAL PAGE

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Carbohydrates first step: photosynthesis

Carbohydrates are crucial to natural macromolecules in plants. Carbohydrates can be a substrate to produce energy and gene expression regulator for plant growth in entire plant life cycle (Hill, 1998; KE Koch, 1996; Smeekens, 2000; Smeekens et al., 2010). The carbohydrate function is strongly related to its capability to be transformed from one substance into others (MacNeill et al., 2017; Ruhland & Wolf, 1936) (Fig. 1) especially being able to provide building blocks for most of essestial constituents in plant such as amino acid, fatty acid and some other compounds (Smeekens, 2000). Carbohydrate is primary final product of photosynthesis in green plants (Blechschmidt-Schneider et al., 1989). Plants produce carbohydrates from free C02 in the air and H20 taken by roots which is for each carbohydrate product, 02 will be released (Baslam et al., 2021). Long time ago, photosynthesis was broadly thought in the school consisted of two major reactions: light and dark reaction and it was inspired by a historical report in photosynthesis by Blackman in 1905 (Blackman, 1905). Later, the carbon reaction replaced dark reaction when some important fact was revealed about photosynthesis reaction light dependency.

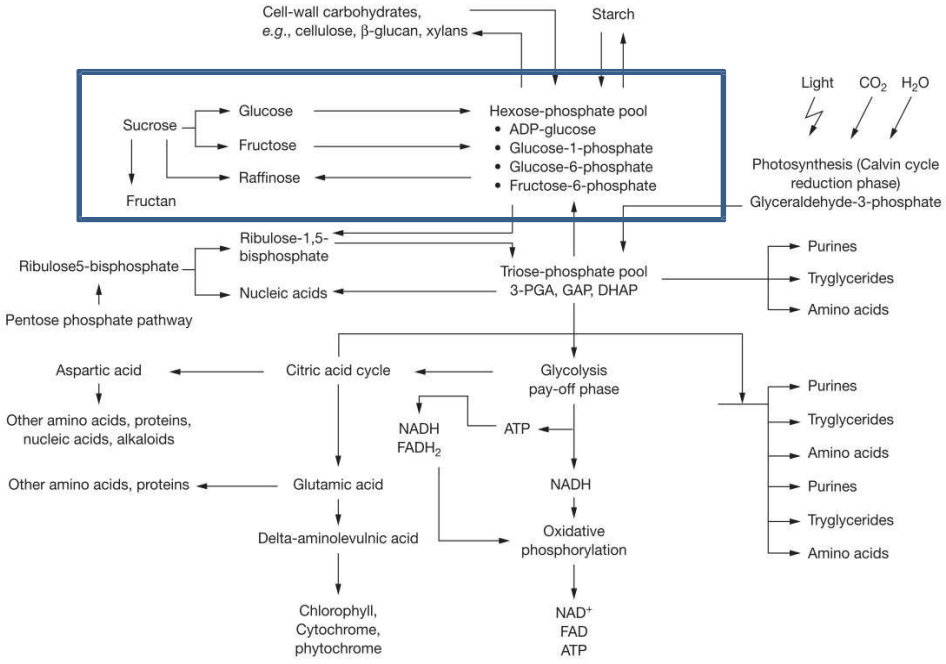


Figure 1. An overview of carbohydrate roles on other biosynthesis pathways in plants (MacNeill et al., 2017).

In the late 1960 and 1970, the central enzymes of Calvin cycle requires light to regulate the reaction and it made the dark reaction no longer relevant for photosynthesis whole process (Buchanan, 1980). The light reaction was undergoing in thylakoid membrane of chloroplast. It happened when chlorophyll molecules absorb energy from the light. Hereafter, the photosystems I and II convert the electron

excitation energy into chemical energy such as ATP and NADPH (Baslam et al., 2021). The light involvement in the first process of photosynthesis was the inspiration for the reaction name. Furthermore, the chemical energy that is produced in the light reaction is critical for the downstream reaction in photosynthesis where C02 fixation is conducted. The reaction is taking place in the photosynthesis organelle: chloroplast and the first reaction of fixation is catalyzed by Rubisco: Ribulose-1,5-bisphosphate carboxylase/oxygenase. The major activity in this Calvin-Benson cycle (carbon fixation) can be the reason why this reaction was named carbon reaction (Baslam et al., 2021) (Fig. 2).

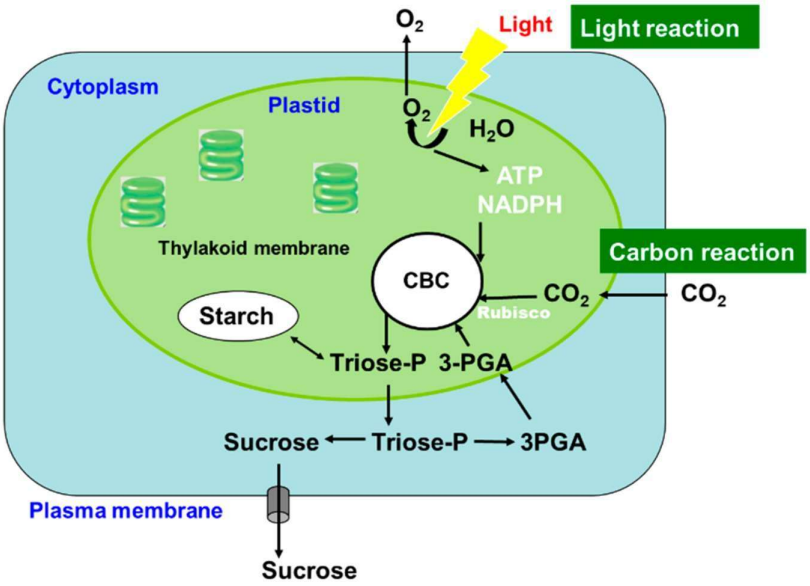


Figure 2. Two reactions of photosynthesis in the leaves of plants: Light reaction and carbon reaction (Baslam et al., 2021).

In general, carbohydrates can be divided into two big groups based on their specific function on building up plants' structure: non-structural carbohydrates (starch and sugars) and structural carbohydrates (cellulose, lignin, dextrans, mannans, inulin, pentosans, and pectic acids). Furthermore, non-structural carbohydrates or commonly known in brief as NSC will be a main actor on central metabolism in plant consist of primary metabolism (growth, development and reproduction) and secondary metabolism (defense, protection and attraction and stimulation) (Hartmann & Trumbore, 2016).

The importance of sucrose metabolism in plants.

The major form of transported carbohydrates in plant is sucrose (α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside) as it is actively used as substrate, signal and for starch or sucrose when it is projected to be reserves (Blechschmidt-Schneider et al., 1989; Lunn, 2016; Peat, 1946; Ruan, 2014). The sucrose is translocated from mature leaves (source tissue) to the growing sink tissues such as buds, flowers, and fruits (Baslam et al., 2021; Lunn, 2016). Therefore, carbohydrates are a versatile material by function and existence. In addition, Lunn (2016) summarized that sucrose was made from fructose-6-phosphate (Fru6P) throughout a set sequential step by sucrose-phosphate synthase (SPS) and sucrose phosphatase (SPP) in the cytosolic compartment

of plant cells. However, for the purpose of carbon allocation and diverse uses, this disaccharide is further processed to become its derivatives. The central enzymes to conduct this sucrose cleavage are sucrose synthase (reversible) and invertase (irreversible).

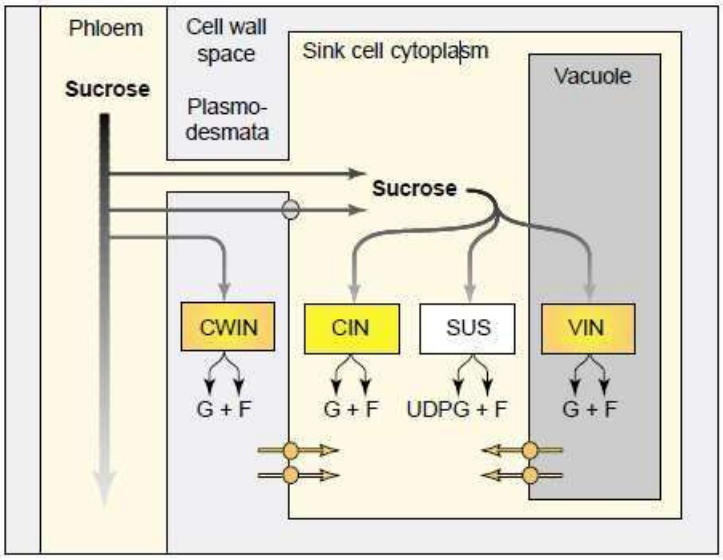


Figure 3. Sucrose mobility and cleavage contributing on hexose-based signal in plant cell (Karen Koch, 2004).

The breakdown of sucrose *in vivo* will affect the sugar signal in the plants that further regulates the carbon portioning, sink-source mechanism, growth and development in plants (KE Koch, 1996; Lalonde et al., 1999; Tang et al., 1999; Wobus & Weber, 1999). In detail, Karen Koch (2004) narrated the physical path of sucrose and how it regulates: the abundant sucrose in source tissue moves through phloem tissue to sink tissue's cytoplasm and vacuole. The sucrose gets into cytoplasm of the sink tissue via two ways: plasmodesmata or across the cell wall space. In the sink tissue's cytoplasm, the sucrose entering via plasmodesmata, is cleaved by cytoplasmic invertase and sucrose synthase. Uniquely, sucrose synthase produces uridine di-phosphate glucose (UDPGlucose) instead of glucose. Moreover, cytoplasmic invertase in general has low activity. These two conditions made the cytoplasmic sucrose cleavage produce limited hexose-based sugar signal. Hereafter, the sucrose across the cell wall space will be cleaved by an active cell wall invertase and it will produce significant amounts of glucose and other hexoses. Later, it affects hexose-based signals both in the membrane of plant cell and in the cytoplasm for following metabolism. The same mechanism is also observed in the vacuole membrane with vacuole invertase as an enzyme that also gives an additional hexose-based signal in the cell (Fig. 3).

The dynamic source-sink status

Source-sink relationship is commonly agreed by scientists to have direct and vital roles in plant growth and performance (White et al., 2016). Moreover, prior to understand the source-sink concept, there are two phrases that should be defined clearly beforehand: source strength is photosynthesis rate and sink strength refers to a

competition among the organs to attract assimilates and it can be gauged by potential growth rate (Marcelis & Baan Hofman-Eijer, 1995). By that definition, plant growth is strongly connected to source and sink strength and their balance relationship (Gifford, 1981; A. M. Smith & Stitt, 2007; Wardlaw, 1990). Furthermore, in the last report by Osorio et al., (2014), the interaction among source and sink will modulate soluble carbohydrate levels in plants.

Moreover, T. Li et al., (2015) showed that the sink-source balance was dynamically changing by the age of the plant to respond to the light stimulation in tomato and will affect tomato fruit size in the end. The finding was in agreement with a previous study presenting that source-sink status in wheat leaves can be interrupted by fungal infection. The sucrose, glucose, fructose and hexose-phosphate were increasing into huge amount in leaves after 5 days of powdery mildew inoculation. The increment of soluble carbohydrates continues until 7 days after inoculation both in leaves with and without fungicide application. Signal from invading fungi was concluded to cause the carbohydrate soluble accumulation in the leaves which was stimulated by acid invertase enzyme to work double than in fungi free leaves (wright et al., 1995). To sum up, the sink-source balance in plants is actively changed until the sink-source relationship reaches the equilibrium condition. In addition, the balance is prone to be interfered with by the stimulus from the growth environment (abiotic and biotic) due to change that might be taking place in the photosynthesis process. In context of sink-source relationship, carbohydrates play roles as both substrate and signal at the same time in this mechanism (Bihmidine et al., 2013).

Carbohydrate enzymes and profiling.

Carbohydrates are the most abundant and important biological molecules in plants. This compound varies on function and structure. Jammer et al., (2015) analyzed 13 highlighted major enzymes activity patterns in carbohydrates metabolism (13 c-enzymes) and uncovered that they have a strong association to plant growth, crop yield and quality and stress responses behavior.

The 13 c-enzymes in this context are three invertase isomers; cell wall invertase (cwInv), vacuolar invertase (vacInv), cytoplasmic invertase (cytInv), and the following 10 carbohydrate enzymes; sucrose synthase (Susy), fructokinase (FK), hexokinase (HXK), phosphoglucoisomerase (PGI), phosphofructokinase (PFK), aldolase (Ald), ADP-glucose pyrophosphorylase (AGPase) and phosphoglucomutase (PGM), UDP-glucose pyrophosphorylase (UGPase) and glucose-6-phosphate dehydrogenase (G6PDH) (Fig. 4, table 1) (Jammer, Gasperl, Luschin-Ebengreuth, et al., 2015).

Table 1. List of the 13 primary carbohydrate enzyme and function (adapted from Jammer et al., (2015))

No	Selected Carbohydrate Enzymes	Biochemical reaction	Specific Key-roles
1	cell wall invertase (cwInv)	sucrose cleavage (sucrolytic)	plant biomass, fertility, fruit quality, and senescence
2	vacuolar invertase (vacInv)		
3	cytoplasmic invertase (cytInv)		
4	sucrose synthase (Susy: EC 2.4.1.13)		
5	fructokinase (FK: EC 2.7.1.4)	sucrose biosynthesis	plant growth and development
6	hexokinase (HXK: EC 2.7.1.1)	glycolysis	plant growth and development
7	phosphoglucoisomerase (PGI: EC 5.3.1.9)		plant growth and development
8	phosphofructokinase (PFK: EC 2.7.1.11)		plant growth and development (less clear)
9	aldolase (Ald: EC 4.1.2.13)		plant growth and development
10	ADP-glucose pyrophosphorylase (AGPase: EC 2.7.7.27)	starch biosynthesis	plant growth and development
11	phosphoglucomutase (PGM: EC 5.4.2.2)		plant growth and development
12	UDP-glucose pyrophosphorylase (UGPase: EC 2.7.7.9)	cell wall biosynthesis	plant growth and development
13	glucose-6-phosphate dehydrogenase (G6PDH: EC1.1.1.49)	oxidative pentose phosphate pathway	plant growth and development

The Susy enzyme plays a key role in sucrose metabolism mostly in sink tissues beside invertase. Susy is prominent because it breaks sucrose down to fructose and either uridine diphosphate glucose (UDP-G) or adenosine diphosphate glucose (ADP-G) via reversible cleavage. The products of this reaction catalyzed by Susy enzyme are vital for many metabolism pathways in plants including complex carbohydrates synthesis (Stein & Granot, 2019). Likewise, Stein & Granot (2018) summarized that fructose metabolism or fructose phosphorylation happened after sucrose cleavage yields glucose and fructose. FK and HXK are two decisive enzymes in fructose metabolism. FK both cytosolic and in plastid phosphorylate fructose to further go into cytosolic glycolysis or plastidic metabolic pathways, respectively. In addition, HXK phosphorylates glucose or fructose in mitochondria prior to glycolysis and respiration or in plastids to feed the plastidic metabolic pathways. HXK is the only carbohydrate enzyme that is capable to phosphorylate glucose (Paulina Aguilera-Alvarado & Sanchez-Nieto, 2017). FK and HXK are initial essential enzymes for most organic metabolism in plants (Granot et al., 2013). Furthermore, the second phosphorylation stage in glycolysis after HXK activity on fructose is conducted by PFK (Mustroph et al., 2013). PGI is taking part in glycolysis and the regeneration of glucose-6-P molecules in the oxidative pentose phosphate pathway (OPPP) (Bahaji et al., 2015). Aldose is one of nonregulated enzyme in Calvin-cycle and has potential to control the photosynthetic carbon flux (Uematsu et al., 2012).

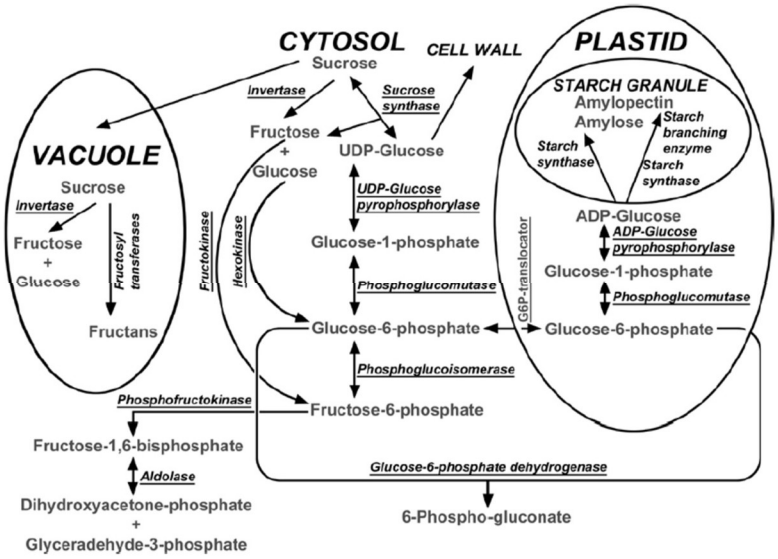


Figure 4. A map of the 13 c-enzyme (including three invertase isomers) distribution regarding their function in the carbohydrate metabolism in plants (Jammer, Gasperl, Luschin-Ebengreuth, et al., 2015)

AGPase and PGM are involved in starch biosynthesis. AGPase catalyzes the synthesis of ADP-glucose which is a glucosyl donor for α-1,4-glucosidic chains elongation (Ballicora et al., 2004). PGM has two isoforms, they are respectively cytosolic and plastids-localized PGM (cPGM and pPGM). The pPGM in chloroplast plastids is taking in

transitory starch biosynthesis, whereas cPGM is necessary for sucrose and cell wall synthesis (X. Y. Li et al., 2015; Malinova et al., 2014). UDP-glucose is the forerunner material to build up the carbohydrate cell wall in plants, and the reaction is driven by UGPase (Park et al., 2010). G6PDH is the central actor of the Oxidative Pentose Phosphate Pathway (OPPP) (Esposito et al., 2005; Landi et al., 2016). The summary of these enzymes above is shown in table 1.

Furthermore, as described before, sucrose is the basis for the formation of all carbohydrates in plants as it is the product of photosynthesis. Sucrose metabolism is taking part on modulating stress responses, for example against low temperature and is playing strategic roles in the end stage of plant development, senescence (Ruan, 2014).

Besides Susy, Invertase is also a key enzyme in sucrose utilization as a carbon source and energy production (Albacete et al., 2011). Invertase (EC 3.2.1.26) is a hydrolase and cleaves sucrose into the two monosaccharides glucose and fructose through irreversible hydrolyzation (Karen Koch, 2004; Stein & Granot, 2019; Sturm, 1999; Sturm & Tang, 1999). Invertase plays an essential role on sugar translocation and metabolism relating to plant growth, plant development, natural responses to surrounding stimuli, abiotic stress tolerance ability such as low temperature and reproduction (Fotopoulos, 2005; Oliver et al., 2005; Thomas Roitsch & González, 2004; Sturm, 1999; Wan et al., 2018). There are three invertase isomer (cell-wall, vacuole and cytosolic invertase) according to the biochemical properties, pH optima, solubility, isoelectric points, primary function and subcellular location (Thomas Roitsch & González, 2004; Sturm & Tang, 1999; Wan et al., 2018). Invertases are regulated by several stimuli: sugar level (T Roitsch et al., 1995; Tymowska-Lalanne & Kreis, 1998), gibberellic acid, auxin, light and some plant protein inhibitors (e.g. cell wall/vacuolar inhibitor of beta-fructosidase) (Kaufman et al., 1973; Tymowska-Lalanne & Kreis, 1998) pathogen attack and wounding (Chrispeels, 1990; Tauzin & Giardina, 2014) and low temperature as seen in potato storage (Zhou et al., 2016). Furthermore, regarding low temperature hardiness, one study showed that invertase activity in potato leaves increased when the environment was under 5oC. These results directed to a conclusion and hypothesis that the pattern of the invertase activity in potato leaves during hypothermia (2oC) corresponds to the role of invertases on cold adaptation (Sin et al., 2008).

Based on the fact how important carbohydrates and the metabolism of these are in plants, Jammer et al 2015 developed a simple and robust assay to measure carbohydrate enzymes to generate an overview of the physiological state of model plants and crops under certain environmental treatment such as elevated CO^2 and heat stress. The method had been proven as an effective approach to determine enzymes signature on woody gravepines after bacteria infection (Prezelj et al., 2016), on ryegrass to study fructant metabolism and its regulation link to seven carbohydrate enzymes (Gasperl et al., 2015, 2016). This enzyme measurement was also utilized to investigate certain enzyme activity profiling change on tobacco, potato, barley and chrimast tree after particular treatment to the plants (Andersson et al., 2018; Cuesta-Seijo et al., 2019; Garcia-Lemos et al., 2019; Marc Goetz et al., 2016; Kuska et al., 2018).

Arabidopsis is a Drosophila of plant science (Leonelli, 2007)

Krämer (2015) summarized that *Arabidopsis thaliana* is an annual, small plant with a rosette. Moreover, Arabidopsis is a higher flowering plant with short generation times (4-5 weeks), less work and less field space for mutant screening, high seed yield, diploid and has high frequency of self-fertilization (E. M. Meyerowitz & Pruitt, 1985). This plant looks (Fig. 5) in general appearance physically like the oilseed crops, vegetables, and spice plants such as rapeseed, Brussel sprouts, various cabbages, cauliflower, garden radish and mustard. Those facts are in agreement with its taxonomy data as follow:

Plantae (kingdom)

Tracheophyta (phylum)

Magnoliopsida (class) Brassicales (order)

Brassicaceae (family)

Arabidopsis (genus)

Arabidopsis thaliana (species)

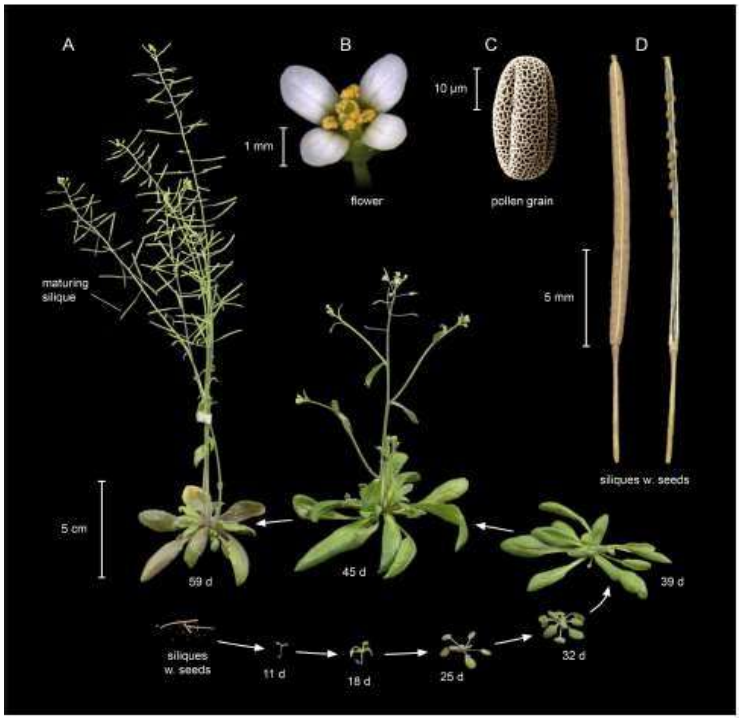


Figure 5. Arabidopsis morphology and life cycles: Mature plant (A), whole flower (B), pollen grain (C), siliques (D) (Krämer, 2015)

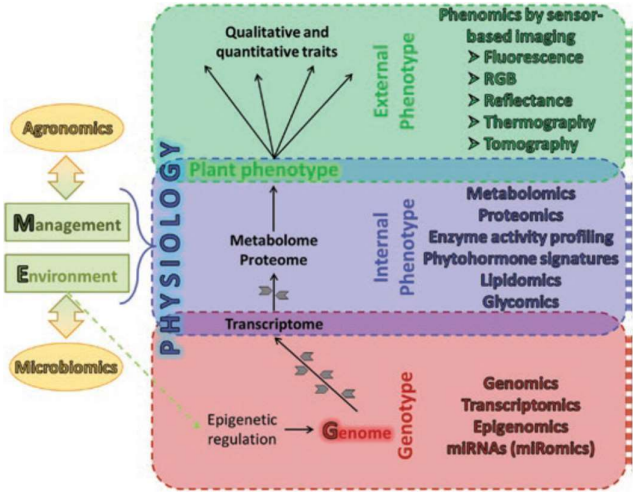
This plant was first described by the physician Johanes Thal in the Harz Mountains of Northern Germany in 1577, published by Linnaeus in 1753 in *Species Plantarum* II and got its first name officially by Gustav Heynhold in 1842: *Arabidopsis thaliana* (L.) Heynh. In addition, Koornneef & Meinke (2010) and Somerville & Koornneef (2002) restated that undoubtedly Friedrich Laibach was the first scientist on using Arabidopsis in experimental research (cytogenetic study) for his PhD research and the result was published in 1907 in his native language, Germany. Laibach's publication fostered the further use of Arabidopsis in genetics research in 1940s and stimulated an initiative movement to collect the wild types to study the intraspecific phenotypic variation in 1950s before this plant entered scepticism in the mid of 1970s (Koornneef & Meinke, 2010). After about 10 years, some groups of biologists begun to take Arabidopsis as their research object especially in genetics, provocating by Meyerowitz & Pruitt (1985) who reminded some fundamental reason why Arabidopsis is better than permitted plants at that time. In the molecular wise, Arabidopsis has some advantages. Its genomic size is only 70.000 kilobase pairs (kb) (a haploid nuclear genome size) and it is the smallest genome size among higher flowering plants (E. Meyerowitz, 1987; E. M. Meyerowitz & Pruitt, 1985). This fact made genetics work easier especially on doing positional cloning and its function subsequently analyzed (Somerville & Koornneef, 2002). Therefore, *Arabidopsis thaliana* became a model plant in plant biology and genetic system in general (Provart et al., 2016; Takou et al., 2019), similar to the use of Drosophila in animal science (Leonelli, 2007).

Figure 6 . The Arabidopsis distribution map based on the map by Mathias Hoffmann and additonal the map to the southern hemiphere by Bresinsky (Krämer, 2015).

Many theories has tried to explain the origin of Arabidopsis, one report said that Arabidopsis was spread from Western Eurasia (Krämer, 2015), another argued that this weed is coming from two *refugia*: the Iberian Peninsula and central Asia, later the population was mixed in central and eastern Europe (Sharbel et al., 2000). Those theories have some discrepancy on tracing the plant distribution pattern, but they share the same argument that Arabidopsis covers vast climate types, from cold to warm growing areas (Fig. 6).

Carbohydrates and senescence

As mentioned before, regarding primary metabolism, carbohydrates play a pivotal role in plant growth and developmental processs. Plant coordinates growth exerting carbohydrate availiability (Eveland & Jackson, 2012) either in the range of plant cell complexity: cell level, tissue level and whole plant level (Araya et al., 2006) or in many stages of plant life cycle (Smeekens, 2000) such as seedling (Yuan & Wysocka-Diller, 2006), flowering (Bernier et al., 1993) and senescence (Doorn, 2008; Wingler & Roitsch, 2008). Moreover, the responsiveness of sucrose synthase and the invertase genes in particular maize organs to carbohydrate concentration were clearly summarized by KE Koch et al., (1996). Ainsworth & Bush (2011) added some interesting explanations that carbohydrate concentration in the range of the leaves age is perfectly controlled to ensure that plants will have optimal primary and secondary metabolism. Even in the ultimate stage of plant life cycle: senescence, carbohydrate had been reported contributing a key role on stimulating the senescence phenomena in



plant in combination with the concentration of nitrogen (SI Gibson, 2005).

Figure 7. The available- omics to study plants senescence at different levels. (Großkinsky et al., 2015)

Senescence is a complex cell death program in plants. However, senescence is not always relating to the age of the plant as an individual (Caswell & Salguero-Gómez, 2013; Lanner & Connor, 2001). Senescence is from Greek word: *senēscere*, which mean to grow weak, become exhausted and to be in a decline (Miryeganeh, 2021). Therefore, senescence is more about an indication on how the plant is managing sink-source and further, how the process will affect the secondary metabolism (production) of plants (Bertheloot et al., 2008; Lv et al., 2020). The most well studied senescence is leaves

senescence as leaves is a factory for plants to manage photosynthesis, a fundamental process to guarantee plant growth and survival. The senescence process in leaves is distinctive and observable (Großkinsky et al., 2018). There is a global regulator, SnRK1 (Snf1-Related Kinase1) which has the central senescence program and actively interact with the sugar-signal pathway (Baena-González et al., 2007). This regulator is activated by several internal and external factors. Jongebloed et al., (2004) observed that the concentration of cellular sucrose and glucose is one of the external factors that controls senescence as a sequential process in castor bean leaf. Eventually, senescence is necessary to be studied in more detail, precisely how this phenomenon coordinates with other metabolism pathways in plants. My first review presented in this thesis suggest that we need to study senescence comprehensively using an integration of multi-omics techniques and physiological phenotyping (holistic phenomic approaches) (Fig. 7) (Großkinsky et al., 2018).

Carbohydrates and cold temperature adaptation

Lately, due to global warming temperature has become one of the fastest changing abiotic factors in the world (Hurry, 2017). In addition, cold temperature is one of abiotic limitions for plants to distribute geographically and reach properly agricultural yield (Korn et al., 2010). Plants are sessile creatures that need a particular strategy to acclimate to any abiotic change in the environment to survive. Fürtauer et al., (2019) concluded that plants responded to environmental changing, especially to environment temperature decreasing by doing metabolite re-programing. Moreover, in that re-programing process, carbohydrate play an essential role (Fürtauer et al., 2019a; Ristic & Ashworth, 1993). This is in agreement with the research using the starchless mutant of plastidial phospho-gluco mutase (pgm) Arabidopsis. The results showed that the mutant had lower freezing tolerance than the wild type and the metabolite profiling indicated redox imbalance because it the lack of carbohydrate content (Hoermiller et al., 2017). On the other hand, soluble sugar accumulation was one of the factors for cold tolerance enhancement in Arabidopsis observed 2 hours after shifting to low temperature (Wanner & Junttila, 1999).

The temperate plants were recorded developing a cold tolerance mechanism when exposed to low, but non-freezing temperature for a short time (Ristic & Ashworth, 1993). The whole process from cold temperature exposure to improving the freezing tolerance trait in plant is the definition of cold acclimation in plant science (Fürtauer et al., 2019a; Korn et al., 2010; Ristic & Ashworth, 1993; Scarth, G W & Levitt, 1937). Moreover, a recent study showed that two garden rose cultivars (more cold-hardy and less cold-hardy) under low temperature treatment reached the same level of cold hardiness but the carbohydrate metabolism profiling was significantly different (Ouyang et al., 2020). In addition, the worldwide plant model, Arabidopsis is growing well at numerous altitude and longitude positions, and it is reflecting that Arabidopsis accessions have evolved for a broad range of temperature living condition (François et al., 2008; Krämer, 2015). Therefore, Arabidopsis is a good plant to study cold acclimation. Furthermore, in some articles *Arabidopsis thaliana* is mentioned in general as one example of cold or chilling tolerant species. Cold temperature is one of the major restrictions for plants to grow, be productive and be able to be broadly distributed (Miura & Furumoto, 2013; Rihan et al., 2017; J. Xu et al., 2020). Arabidopsis may show a mechanism how they can be a worldwide weed regardless of their domain temperature (Fig. 6 & Fig. 8).

Eventually, based on all these impressive facts about low temperature in controlling plants distribution; a strong relationship between climate change and plant production; plus carbohydrate is central in plant strategy to survive in adverse temperature, inspired us to investigate how low temperature adaptation can be described by primary carbohydrate enzymes profiling (Jammer, Gasperl, Luschin-ebengreuth, et al., 2015) in the model plant and in well-adapted flower species to early spring temperature (Fig. 9). This stimulating study can be read in the second manuscript: "Carbohydrate Metabolic Enzymes Activities Signatures of Plant Adapted to Different Temperatures Climate"

Figure 8. Map of origin sites of 41 *Arabidopsis* accession (AT) and three Early Spring Flower (ESF) plants group. group high (AT), group medium (AT), group low (AT), Group low (ESF).

Figure 9. Early Spring Flowers: 1) Chionodoxa luciliae, 2) Eranthis hyeminalis, 3) Galanthus sp**.** These well adapted to low temperature flowers species were investigating for c-enzymes profiling. 1. Pistil, 2. Pollen, 3. Modified filament, 4. Petal, 5. Stem.

Carbohydrates as a reserve in plant: starch and potato story.

The first two studies emphasized carbohydrates as a signal in plant metabolism pathways. Carbohydrates also function as a critical source for energy production either for the plant itself or for human consumption. Therefore, in the third part of this dissertation, carbohydrate as a reserve will be the research with the title: "CRISPR-Cas9 mediated of loss *Glucan Water Dikinase* gene function in 2 Potato Cultivars: Royal (Denmark) and PAU-1 (Indonesia)".

A brief story about Potato

Potato is originally from South America (new world). Farmland around Lake Titicaca (Andean region) is mentioned as the origin of cultivated potatoes (Brown & Henfling 2014). There were two important periods in potato history regarding its adventure to the Europe continent (old world) (Fig. 10). Those years were 1570's and late 1800's. The first sub species, which arrived at the first period of the introduction of potato to Europe, was *Andigena* in 1570 and it was no longer cultivated after *Phytophthora infestants* attacked potato farming severely in 1840. After the potato famine, *Solanum tuberosum* ssp took Adigena's place for the dominating potato farming in Europe (some decade after the famine) (Birch *et al.* 2012).

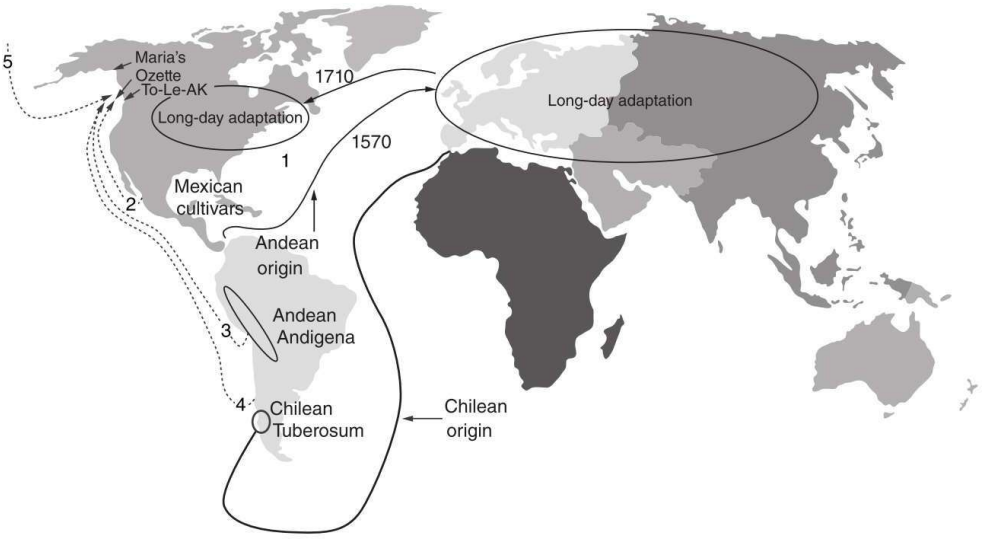


Figure 10. The potato's journey from the New World (South America) to the Old World (Europe) and back to the North America (Brown & Henfling 2014)

Moreover, potato was reaching Asia in the last period through the European colonization expeditions. Particularly, in Indonesia there are two different stories on how potato was introduced: the first that the potato was brought by Dutch people in 1794 (related to their occupation for about 350 years in Indonesia) and the second explained that Indonesia had potato already after Spanish conquest in the Philippines early of 16th century (Forshee, 2006). Eventually, potatoes became a global cultivated crop. It can grow on virtually all continents, except Antarctica. Nowadays potato is cultivated in 149 countries lying down at latitudes 65°N to 50°S and broad range altitudes sea level to 4,000 m, from southern Chile to Greenland (Birch *et al.* 2012, CIP 2018). This fact indicates how good potato adapts to various environmental factors and is versatile in existence and uses. Machida-hirano (2015) wrote a clear description about *Solanaceae* family diversity and how potato, which is one member of this family, genetically can survive to broad cultivation abiotic parameters since its first domestication in upland Latin America.

Regarding productivity, potato shows various data on harvest yield. The lowest potato production recorded is in Africa more precise at the sub-Sahara region and the highest yield is found in seven north European countries (including Denmark), which are the 10 highest potato yielding zones together with USA, New Zealand and Australia. Birch *et al* 2012 categorized potato as one of the top three crops in the world with an impressive increasing production rate in the last two decades especially in developing countries. Furthermore, in Indonesia, which is just gaining its status as a new emerging economic.

Potato is not really popular as in European countries. In the past time, potato in Indonesia was mostly used in meat and vegetable dishes as addition garnish and flavoring instead of a staple food (Woolfe 1987) and it appeared only at special occasions (Poats 1981). Recently, Indonesia's potato market started to change with the increased demand for French fries.

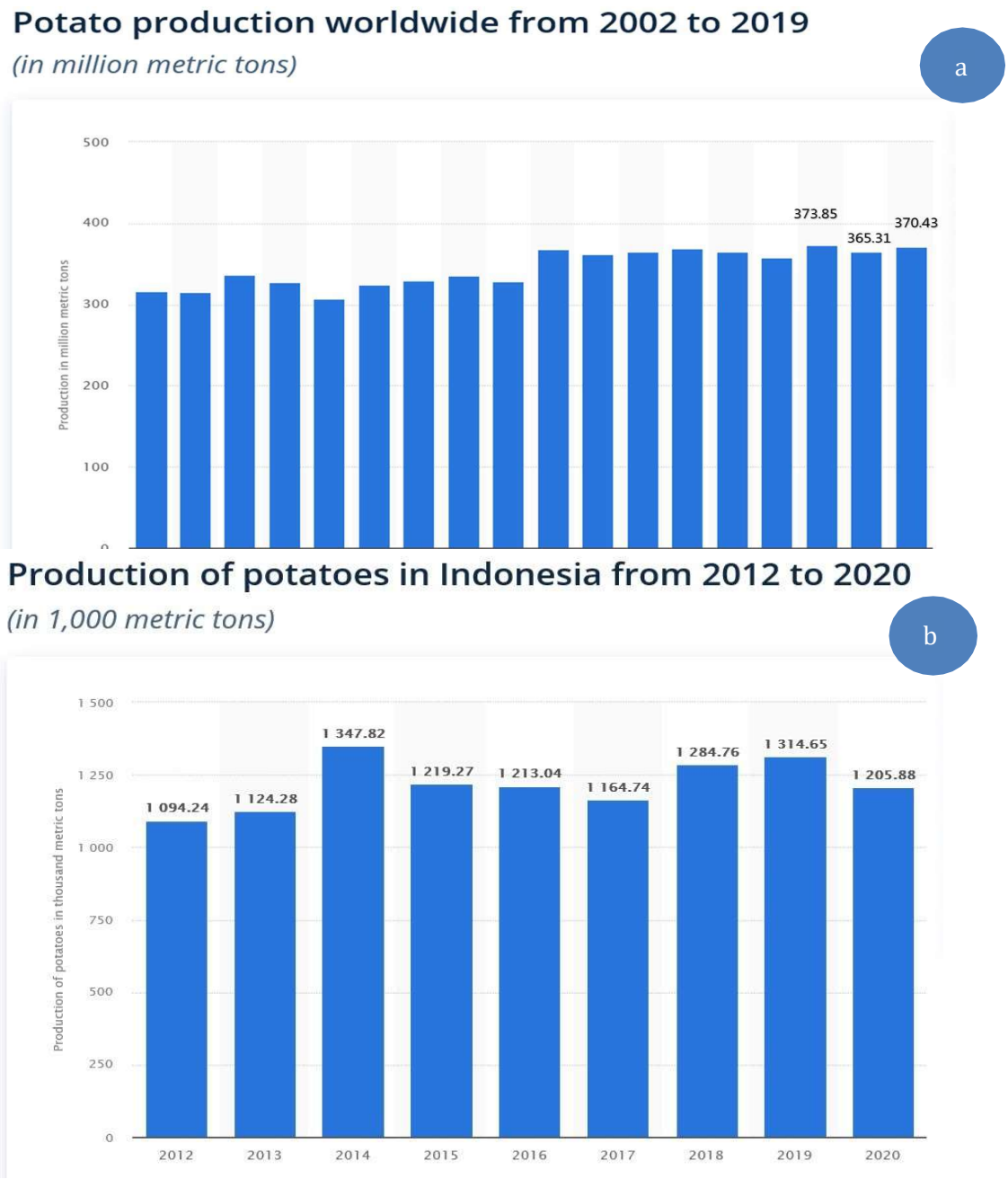


Figure 11. World potato production 2002-2019 (a), potato production in Indonesia 2012-2020 (b) Shahbandeh M (statista 2021) & Statista Research Department (statista 2021).

Middle class economic society has developed rapidly by number, and this community typically adopts western lifestyle with no exception on food consumption preferences. They begin to eat other carbohydrate source dishes than the traditional. Corresponding to this topic, instant Potato is one western lifestyle product. According to Biro Pusat Statistik Indonesia (Indonesian Statistic Board) on food consumption report 2011-2015 (2015), Indonesian potato consumption is dominated by fresh potato instead of manufactured potato or potato-based products. There are some issues related to this fact, the limitation of the local cultivar trait, which cannot be fried for French fries could be one factor of many other reasons.

As mentioned before, potato production trend is increasing year by year to fulfill consumer demand, especially in Asia and Africa. Potato world production recorded a huge number of 316.44 million tons produced in 2002 and significantly increasing in 2016 to 370.43 million tons. The production pattern is going on the same direction in Indonesia. The Indonesian potato production was reported to be about 1094.24 thousand metric tons in 2002 and 1205.88 thousand metric tons in 2021 (Fig. 11) (statista.com 2021). Utilizing potatoes has evolved significantly and globally. 7000 years ago, Latin America people used potatoes to survive. When it started to grow in Europe, potato was only a second-class crop. It was planted to secure an adverse situation when major crops were facing harvesting (McNeill, 1999). Potato was called food for poor people. By the time, back in 1811 Kirchoff hydrolyzed potato starch with acid to get sugar. It was the first report regarding starch industry processing. Thereafter, potato was widely used in Europe's starch industry in the 18^{th} century after the earlier phase of starch industry where wheat was dominating as raw material (starch.eu). In addition, now people admit that this starchy tuber plant is sophisticated by content and function for starch industry uses (Table 2).

Table 2. Potato tuber dry matter composition

Constituent	% in DM	
	Normal value (approx.)	Range (approx.)
Starch	70	60-80
Sucrose	0-5-1	0.25-1.5
Reducing sugars	0.5-2	0.25-3
Citric acid	2	0.5-7
Total N	1-2	1-2
Protein N	0.5-1	0.5-1
Fat	0.3-0.5	0.1-1
Fibre (dietary	6-8	3-8
Ash	4-6	4-6

(Burton 1989 and Woolfe 1987)

Gibson & Kurilich (2013) showed that potato contributed significant nutrition values: 7% of total daily energy intake, potassium and vitamin B6 (15%), vitamin C (14%), fiber (13%), folate (10%) and magnesium (9%) and 4% of saturated fatty acids. Therefore, it is not surprising in 2008 on a program named The International Year of the Potato, FAO declared that potato is an essential crop for producing more quickly and quite healthy diet (Freedman & Keast 2011) and require less land; therefore, potato is a suitable crop to feed society. Furthermore, potato has potential as a future staple food in a consumption niche and production on global level (Lutaladio N et al. (FAO). 2009). About 9 years ago, a report made by FAO Rome mentioned that potato production rate in developing countries even take about 50% of the global potato share market. This number is believed to grow in the years to come.

International Potato Center (cipotato.org) (2021) recorded that potato uses are not limited on food and feed anymore. For instance, in New Brunswick, Canada remnant of potato industry such as potato peel and 'zero value' waste, which are rich in starch was transformed to fuel grade ethanol. Moreover, potato has also been popular as raw material for pharmaceutical, textile, wood, and paper industries. In context of food, some various functions and trends of potato are lately presented. World potato consumption is still predominated for fresh potato, yet value added potato products apparently have become a new life preference all over the world including the developing countries. Increasing potato consumption has taken place in several places, especially in Asia and Africa (Camire 2009).

Starch industry is one of most popular commercial use of potato. Batchelor et al. (1996) presented that the market for starch was increasing. Potato starch is unique compared to

other starch plants sources in term of yield, properties and characteristics. Bertoft & Blennow (2009) explained the scientific reasons why potato starch is better than others regarding several starch industry applications. Potato starch has huge and smooth granules, quite high on phosphate content, long chains of amylopectin and high-molecular weight of amylose. In Denmark, more than 60% of the potato production is utilized in the starch industry. This percentage is higher than other EU countries potato allocation for starch industry (18%) (Birch et al. 2012).

In addition, Potato breeding has their own explanation regarding the benefits of genetic engineering application on potato. Not only because potato is one of the most valuable crops in the world, documenting a specific gene corresponding to the biological process in potato is necessary in terms of its uniqueness on a breeding perspective view. The breeding of potatoes is challenging because of the trait's complexity. Muthoni (2015) and his colleagues elucidated that most cultivated potatoes are tetraploid with tetrasomic inheritance, this genetic characteristic makes potato conventional breeding complicated due to high heterozygosity, polyploidy with broad range, and tetrasomic inheritance (Batchelor et al., 1996). Another possibility, desired characters of cultivated potato could be gained from its wild relatives via traditional breeding, but the process will take about 15-20 years and there is high risk of failing. Fortunately, the last genetic engineering technology in biotechnology let researchers deal with an opportunity on understanding wanted traits genes in depth and performing direct and precise gene editing afterward without involvement of any foreign gene (Halterman et al., 2016). Hameed et al (2018) reported some gene engineering projects on potato, which used new breeding technology such as TALEN (transcription activator-like effector nucleases) and CRISPR-Cas9 (clustered regularly interspaced palindromic repeats-CRISPR-associated 9). Table 3 encompasses a list of genes that had been successfully altered by CRISPR-Cas9 technology in potato within the last 14 years.

Table 3. Potato genes studied utilising the CRISPR system (modified from Das Dangol, Barakate, Stephens, Çalıskan, & Bakhsh, 2019; Hameed et al., 2018)

No	Genes targets	Genes functions	Editing targets & results	Cultivar/ Type	References
1	Exon 2 of *Aux/IAA* family (*StIAA2*)	Involved in Auxin/indole-3-acetid acid proteins synthesis (petiole hyponasty & shoot morphogenesis)	Altered Aux/IAA protein expression	Bintje/ Double haploid DM	Kloosterman, Visser, & Bachem, 2006; Wang et al., 2015
2	*Acetolactate synthase1* (*StALS1&2*)	Biosynthesis of branched-chain amino acids	Targeted mutation	Desiree	Butler et al., 2015; Butler, Baltes, Voytas, & Douches, 2016
3	Exon 8,9 of *Granule-bound starch synthase* (*GBSS*)	Involved in starch synthesis pathway	Altered starch quality	Kuras	Andersson et al., 2017, Andersson et al., 2018
4	*St16DOX*	Production of steroidal glycoalkaloids (SGAs) α-solanine and α-chaconine. Bitter taste and toxic compound in tuber of potato hairy roots.	Complete knockdown of steroidal glycoalkaloids (SGAs) production	Mayqueen	Nakayasu et al., 2018
5	*potato coilin*[2)] and *phytoene desaturase*[2)]	[1)] Nuclear protein for resistance mechanism [2)] Involved in carotenoid biosynthesis	[1&2)] Analyze the functional feature of gRNA after DSB [1)] Study virus and salt stress tolerance system	Chicago	Khromov et al., 2018; Makhotenko et al., 2019
6	*Granule-bound starch synthaseI (GBSSI)*	Starch synthesis (amylose)	Low amylose starch in potato tubers	Sayaka	Kusano et al., 2018
7	*Granule-bound starch synthase* (*GBSS*)	Starch synthesis (amylose)	Modified *GBSS of* 4 allel	Desiree, Wotan	Johansen et al., 2019
8	*Polyphenol oxidase (ppo)*	Catalyze enzymatic browning	Lower *ppo* activity	Desiree	González et al., 2020

Starch Structure

Starch granules comprise of two polyglucans with different structure. The most dominant component is named Amylopectin. Vamadevan & Bertoft, 2015 delineated this part as a branched structure, influencing on the semi-crystalline level and lamellar construction of starch (Fig. 12). In addition, the author also introduced the terminology of building blocks and treelike branches pattern on determination how Amylopectin is organized in the plants (Bertoft & Blennow, 2009; Vamadevan & Bertoft, 2015; (Bertoft, 2017). The second glucan is Amylose. Hanashiro (2015) described Amylose in plants in very detail. The recent concept on Amylose structure showed that this polysaccharide has linear and branched chains. These chains could be classified by its position whether they are the main chain (linear one or C-chain) or short chains (side chains or A-, B-chains) this classification method also applies for Amylopectin (Bertoft, 2015). The Amylose percentage has a strong association with starch functional properties such as gelatinization, retrogradation and waxy texture of starch (Tester and Morrison 1990, Fredriksson et al. 1998, Jane et al. 1999, Sasaki et al. 2000). The wild type of starchy plant has about 20-30% Amylose in its granules, this value indicates that the texture of extracted starch will not have a high rate of viscosity, otherwise starch from a waxy maize reported by Cheetham and Tao (1998) and Shi et al (1998) has high viscosity as Amylose content in this modified maize was about 80-90%. Eventually, not only for Amylopectin but also Amylose, the certain starch characteristics which are mentioned previously will vary depending on the plant's botanical profile (Bertoft, 2015, 2017; Hanashiro, 2015)

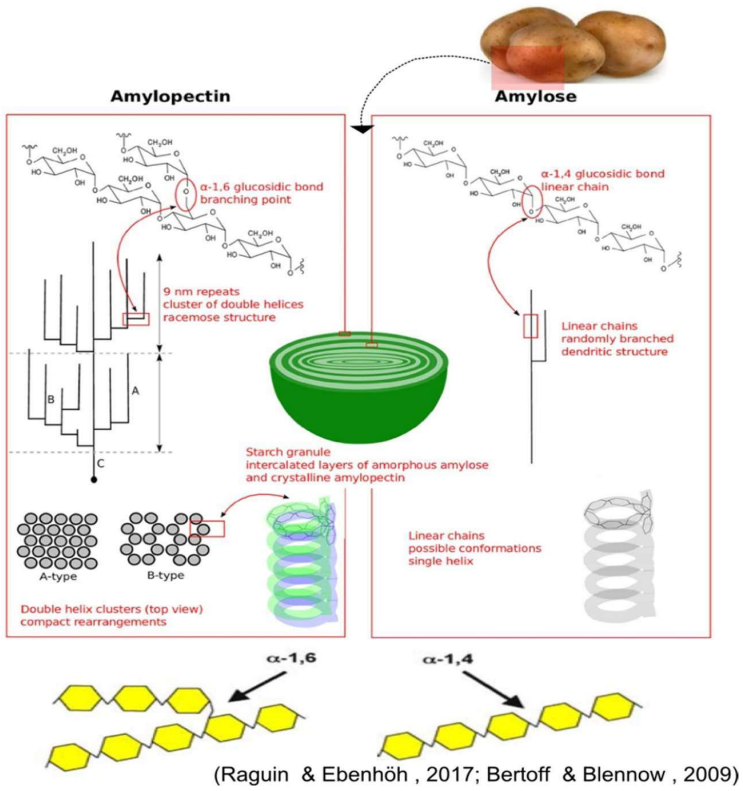


Figure 12. Primary construction of Starch: Structure of Amylopectin and Amylose

Starch synthesis

In higher plants there are two starch biosynthesis pathways defined by the biological function 1) starch production for reservoir in organs and 2) starch metabolism in the cells of the leaves (transitory) (Fig. 13a and 13b). Moreover, starch metabolism and photosynthesis are two natural processes, which are linked. In case of transitory starch, it is synthesized right after a continued active photosynthesis phase during the presence of light. Furthermore, the starch in the leaves will be degraded and used for energy and running all biosynthesis in a plant especially during darkness (Graf A et al. 2010). Cases, starch catabolism can take place when photosynthesis is inactive. Therefore, starch will not be stored in non-photosynthetic organs if the plant basic need to live has not been fulfilled yet (Pfister & Zeeman, 2016). Fettke et al., (2010), summarized that starch formation requires several primarily chain reactions: a carbohydrate-like glucosyl acceptor construction, α-1, 4 interglucose bonds formation, branching assembling (α-1, 6 bonds formation) and α-glucan arrangement. However, there are major differences between starch synthesis in leaves (transitionary starch) and storage organs.

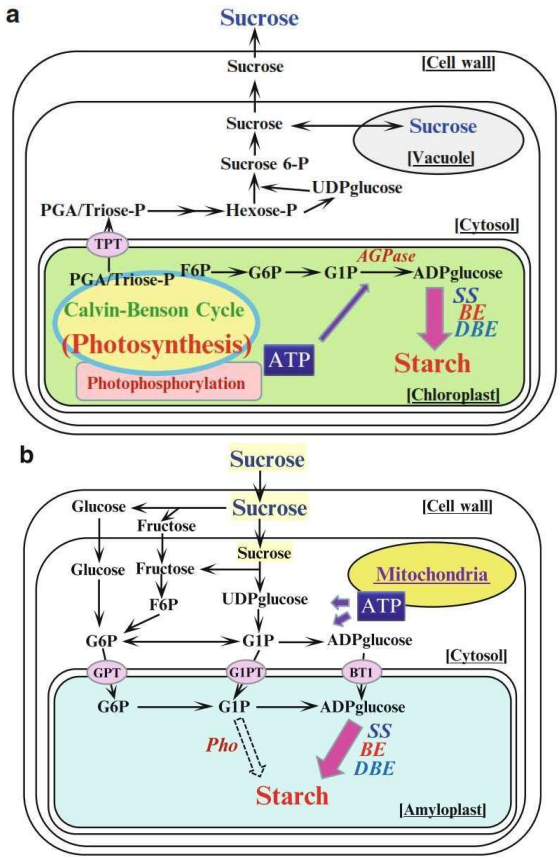


Figure 13. Starch biosynthetic in leaf a) and storage organ or non photosynthetic tissue b) (Nakamura, 2015)

The ADP-glucose source will also affect all the polymerizing enzymes involved. In leaves ADP-glucose is produced from fructose-6-P (Smith, 2012; Streb et al., 2009; Bahaji et al., 2011). In tubers sucrose from leaves is transported and changed to become ADP-glucose (Zrenner et al., 1995; Kühn et al., 1999). In addition, regarding

starch biosynthesis, the phosphorylation is a crucial and versatile in-built process that supports formation of well-functional starch granules (Blennow, 2015) and starch degradation (Ritte et al., 2004). Phosphorylation is broadly found at some starch biosynthesis processes when many enzymes are depending this biochemical reaction.

In a certain case, phosphorylase was proven to run the extending of the starch chain distribution, and the reaction is reversible (Blennow et al., 1998; Zeeman et al., 2010). Glucan Water Dikinase (GWD) and Phosphoglucan Water Dikinase (PWD) are responsible for phosphorylation in starch, transferring ɣ-phosphate to water and β-phosphate group to the C6 or C3 position (Mahlow et al., 2016a). Phosphate content relates to several starch properties: gel starch stability during freezing and thawing, granule morphology, amylose content, gelatinization characteristics, starch fine structure, physicochemical properties (Carpenter et al., 2015; Xuan Xu et al., 2017), which are functional traits useful for starch industry implementation. Moreover, in starch industry, potatoes are one of the dominant raw materials in the world and number one in Europe (Jobling, 2004). Many scientific investigations reported that potato tuber starch have the highest phosphate content compared to other starchy plants organs (Blennow et al., 1998, 2000, 2002). This fact let potato starch have a broad application not only for food but also non-food (Jobling, 2004). Therefore, a reliable and well-established knowledge corresponding to phosphorylation on potato would be necessary. Last chapter of this PhD thesis describes the design of experiments to confirm GWD role on starch phosphorylation of two different potato cultivars. Royal is a Danish potato cultivar for use in starch industry and PAUS cultivar, an Indonesian potato cultivar, which is claimed as a prospective candidate for potato chip industry especially for Indonesia market.

CRISPR-Cas9 technology

CRISPR-Cas9 stands for Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9 of *Streptococus pyogenes* (later the cas9 will be known solely as protein cas9), had it first debut in 2012 as a new generation of precise genome editing (PGE) tool (Knott & Doudna, 2018; Sukegawa et al., 2021). This technology originally existed in bacteria and archaea as an adaptive immune system (Knott & Doudna, 2018). There are three original stages of adaptive immune system development in bacteria and archaea. 1) Foreigner genetic material from particular virus that invades bacteria is acquired by Cas1-Cas2 protein. The introduced genetic element was integrated into CRISPR array, and it works alike memory system to remember and give the appropriate response to the future attack from the same type of virus (adaptation). 2) Cas proteins and CRISPR array are expressed to produce surveillance complex which consists of Cas effector nucleases and a crRNA (expression). 3) When the virus with the same genetic material in first phase attacks the bacteria by injecting their genetic elements, Cas effectors will welcome the element (this process guided by crRNA) and digestion will be happened in context of immune system in bacteria and archaea (Knott & Doudna, 2018) (Fig. 14).

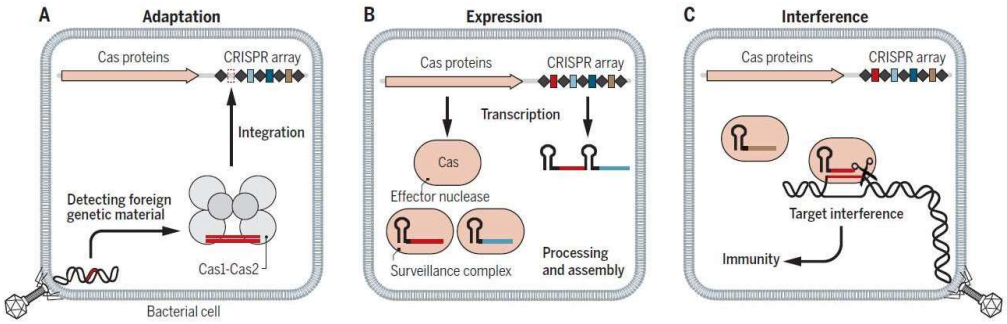


Figure 14. The stage of an adaptive immune system in bacteria and archaea which is the original concept of CRISPR-Cas9 technology. a) Adaptation: lead by Cas1-Cas2 protein, the injected virus genetic element is acquired to CRISPR array. b) Expression: the complex of surveillance is form and ready to block c) an interference: the genetic element that is complementary to crRNA will be digested and the immunity in bacteria and archaea is successfully modulated. (Knott & Doudna, 2018).

Within 3 years, 1000 publications were reported using CRISPR technology in the method and it was undebatable proof that this new PGE technology suddenly becomes center of researchers' interest around the globe (Quétier, 2016; Sternberg & Doudna, 2015). Up to date, it had been about 20 years for the CRISPR-Cas to be used for massive applications including gene therapy uses since it had been been reported for the first time at 1987 (Barrangou & Doudna, 2016; Lander, 2016; Quétier, 2016). This is promising technology because it has some essential benefits compared to the previous PGE technology: it is simpler by utilizing reprogrammable short guide RNA and is inexpensive. Briefly, the intended genome could be opened at certain sites appointed by designed guide-RNA (20 nucleotides) (Fig. 15). A denaturation process conducted by a DNA endonuclease (Cas9): double-strand break (DSB). Ensuing cellular response after DSB takes place is a natural repairing process by endogenous cell machinery which opens the possibility for mutagenesis occurs at the site within the genome around DSB (J. Li et al., 2015). Schaeffer & Nakata, (2015) reported some of high value crops that have been engineered by CRISPR-Cas9 such as soybean, tobacco, rice, tomato, corn, chines white poplar, wheat and potato.

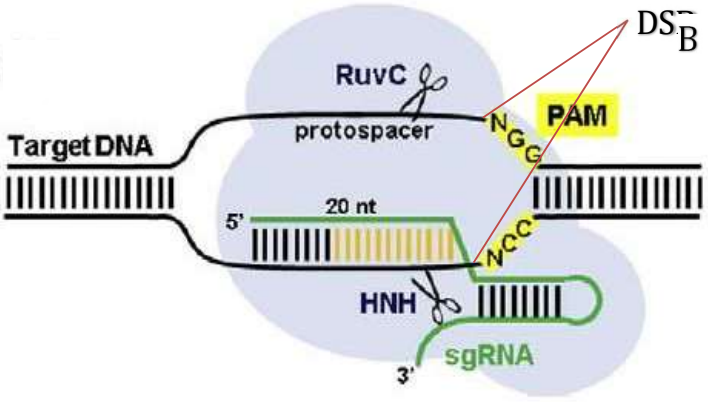


Figure 15. CRISPR-Cas9 components: Light blue in the back is Cas protein with two catalytic sites where the scissors signs indicate cleave of the DNA target (black) and generation of a double strand break (DSB). The PAM (Protospacer Adjacent Motif), which is a short and unique sequence (yellow) is adjacent to the single guide RNA (sgRNA) (green), for Cas9 binding and enables the Cas9 protein to bind and cut the target gene. (Bortesi & Fischer, 2015).

IDAA method for editing confirmation

When precise genome editing technology had been established and improved in term of site target precision, cost issues and easy for application then a further analysis should be required. The confirmation tool should be rapid, reproducible, and inexpensive. A rapid and high accuracy tool of gene alteration detection will make genetic engineering work more amenable and reliable at the same time not only in the stage of how researchers make a gene modification but also on controlling them whether editing as expected or not. Bennet and his group at University of Copenhagen, Panum, Denmark developed a promising method on how they proof gene editing even if the modification is ± 1bp (Yang et al., 2015). The promoted technology is named Insertion Deletion Amplicon Analysis (IDAA). Referring to the name this method is useful for detection of insertion and deletion utilizes three labeling primers (tri-primers) and capillary electrophoresis (Fig. 16) (Lonowski et al., 2017; Yang et al., 2015). There are two steps for the IDAA method for indels confirmation. At the first step, the nuclease site target is amplified by genomic PCR using three primers: first primer is a locus-specific pair forward (Fwd) and second primer is the reverse type of primers (Rev) and the third is an additional primer which is named as universal primer (FamFwd) and this last type of primer has a direction of amplification same as Fwd primer type. Furthermore, this FamFwd primer is labeled with 6-Fam (Fig. 15). This label will generate a fluorescent of every amplicon. Related to this method function, amplicon of alleles with deletion will be shortest and the alleles with insertions are the longest compared to the wild type allele amplicon. The wild type is commonly designed to have an amplicon of 200-450 bp. The second phase will be the analysis steps. The amplicons are then analyzed employing a fragment analyzer (common Sanger sequencer for instance ABI3010 sequenator). The output will be like sequence chromatograms justifying amplicons size by the Peak Scanner software. It will be references to identify indels mutation of the alleles. (Lonowski et al., 2017) (Fig. 16).

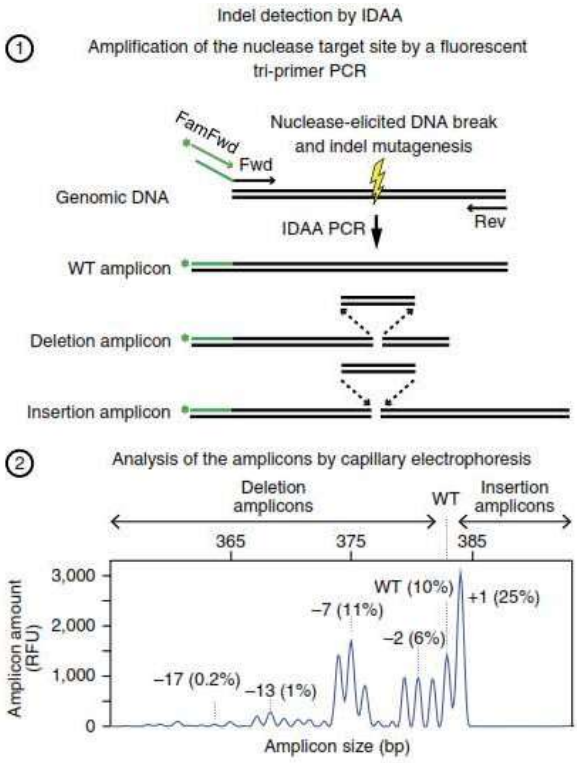


Figure 16. IDAA protocol consists of 2 stages: 1) First stage is fragment amplification. The nuclease site target (yellow arrow) is amplified by genomic PCR. Three primers are involved: first primer forward (Fwd) and second primer is the reverse (Rev) and the third is a universal primer (FamFwd). The FamFwd primer is labeled with 6-Fam (green asterisk in the figure). This label will render fluorescent on amplicon. The product of PCR will be categorized into three different groups of amplicons regarding size discrepancy (normal: wild type (200-450 bp), the longest: insertion, the shortest deletion. 2) The amplicons then are analyzed by a common Sanger sequencer to identify indels mutation of alleles. (Lonowski et al., 2017)

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