What kinds of osmotic materials induce stomatal opening

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September 17, 2020

Abstract

In the mechanisms of stomatal opening, the transports of osmotic materials between the guard cell cytoplasm and vacuole have not been studied much. There were also important lacks of understanding about tonoplast transport proteins and channels. Tonoplast has been found to have many types of channels related to K+ transport, among which are inward-K+ channels/FV, outward-K+ channels/FV, outward-TPK/VK channels and TPC1/SV channels. The two H+ transport enzymes in tonoplast, H+-ATPase and H+-PPase, transport H+ from the cytoplasm to vacuole very actively. They serve to create an ideal pH condition between vacuole and cytoplasm to facilitate the many metabolisms in the cell. The cytosolic K+ cannot easily enter the vacuole to fill the charge balances, because vacuole is too full of positive charges. Therefore, in order to increase the osmotic pressure of the guard cell vacuole, it is necessary to transport solute that can replace K+. Tonoplast contains sucrose-H+ antiports, an active transport protein that can transport cytoplasmic sucrose to vacuole. Although various solutes including K+ are required for stomata to open, sucrose is believed to be the most important substance that can increase the vacuole's osmotic pressure.

Introduction

Starch-sugar hypothesis was the basic concept of stomatal physiology in the early 20th century. This theory was brought up by Kohl in 1895. When the plant receives light, photosynthesis occurs, the amount of CO_2 in the cell decreases, the pH of the guard cell increases. At high pH, starch phosphorylase, which decomposes starch into sucrose, is activated, increasing the osmotic pressure of the guard cell. On the contrary, it was considered that the photosynthesis did not occur in the dark-treated leaves, resulting in an increase of CO_2 concentration. As a result, at low pH, the starch does not decompose into sucrose and the stomata close. It is now known that carbon assimilated by photosynthesis during the day is used for starch synthesis of chloroplasts or transported to the cytoplasm for sucrose synthesis. Therefore, the initial starch-sugar theory is not perfect, but it is still a partially accepted theory that it was understood as a sucrose as the main osmotic material that opens stomata. In 1943, Imamura isolated epidermis from the mesophyll cells and cultured epidermal strips in a high concentration of KCl solution. And then, he observed an increase of K^+ concentration in the guard cell. Experimented in the same way as Imamura, in 1976, Hsiao announced that the accumulation of K^+ occurs when stomata open. From this point on, many stomatal researchers began to recognize K^+ as the main osmotic material for stomata opening.

At this time, most stomatal researchers, including me, knew that stomata's main osmotic material was K^+. In this atmosphere, a paper has been reported that the accumulation of K^+ concentration beyond the imagination occurs in the guard cell when stomata are opened. In fact, this high concentration of K^+ was never observed in plant cells, but it was a time when stomatal opening was believed to be opened by K^+. When stomata were opened, a paper was published stating that up to 800mM of K^+ was accumulated in the guard cell (Talbott & Zeiger 1996). Even today, many scientists understand that stomatal opening is caused by K^+. Some of the stomatal researchers actually measured the K^+concentration of the guard cell to see if it needed so much potassium for the stomatal opening (Travis & Mansfield 1977, Bowling 1987, DeSilva *et al.* 1996). When the K^+ concentration of the guard cell was measured, the total concentration of K^+ ions

presents in the cytoplasm, apoplast, and vacuole was 100˜150 mM, and most $K^+$was known to exist in the apoplast (50˜75 mM).

The above results showed that the concentration of $K^+$for stomatal opening was not higher than expected. Even in this situation, many stomatal researchers recognized K^+ as the main osmotic material for stomatal openings, but papers that sucrose was actually the main osmotic material for stomatal openings were constantly published (Outlaw 1989, Reckmann *et al.* 1990, Gautier*et al.* 1991, Poffenroth *et al.* 1992, Outlaw 1996, Lu*et al.* 1997, Asai *et al.* 2000, Outlaw & De Vleighere 2001, Lawson *et al.* 2002, 2003, von Caemmerer *et al.* 2004, Outlaw 2003, Kang *et al.* 2007).

Currently, according to stomatal researchers, K^+ or sucrose is believed to be the main osmotic material, so two types of theories are compatible. When this atmosphere was created, a paper was reported that sucrose and K^+ have similar importance and influence stomatal opening (Tallman & Zeiger 1988). They reported that stomata were opened by k^+ in the early morning and sucrose acts as an osmotic material in the afternoon. Of course, for stomatal opening, most stomatal researchers recognize that Cl^- and $malte^{2-}$ are necessary in addition to K^+ and sucrose.

Recently, many papers have been published that the stomatal mechanism is regulated by the sugar-sensing enzyme Hexokinase (HXK), and the function by HXK promotes the decomposition of sucrose, resulting in stomatal closing (Kelly *et al.* 2013, Li *et al.* 2016, Hei *et al.* 2017, Kottapalli *et al.* 2018, Lugassi *et al.* 2019, 2020). HXK is an enzyme that catalyzes to fructose-6-phosphate and glucose-6-phosphate from promoting the phosphorylation of fructose and glucose in the glycolysis process. It regulates the concentration of sucrose in the guard cell vacuole. Enzymes that control the concentration of sucrose include sucrose synthase, sucrose phosphate synthase and sucrose phosphate phosphatase. Therefore, enzymes that may be related to stomatal opening may include sucrose phosphate synthase and sucrose phosphate phosphatase, which synthesize sucrose.

Zeaxanthin and phototropins (*pho1* and *pho2*), blue light photoreceptors for stomatal openings, have been identified. Blue light has been shown to promote regulatory 14-3-3 protein as the activity of PM (plasma membrane) H^+-ATPase by IAA is mediated by regulatory 14-3-3 protein (Eigo & Kinoshita 2018). However, despite the discovery of a mechanism for stomatal opening by blue light, stomata are also opened by red and white light. The size of the stomatal apertures caused by white light was about 18μm in *Commelina communis* , but increased by about 6μm stomatal aperture by single blue light and stomatal aperture of about 7.3 μm by red light (Schwarz & Zeiger 1984, Lee & Bowling 1992). The stomatal aperture by blue light was estimated to be the sum of the stomatal opening by chlorophyll and carotenoid and the stomatal opening mediated by blue light photoreceptors. After that, the first and last paper to measure stomatal opening using blue light photoreceptors-deficient mutant plants was published (Talbott *et al.* 2003). After the blue light receptors-deficient mutant plants were made with *Arabidopsis thaliana* , the stomatal opening by blue light was observed. In wild type, stomatal opening increased by 0.7 μm when treated with blue light, but stomatal opening of the *npq1*mutant was suppressed by 0.3 μm. The *photo1 /photo2* mutant had a rather increased stomatal opening of about 0.3 μm. In the experiment using the blue photoreceptor mutation, the wild type increased about 0.4 μm compared to the *photo1 /photo2*mutant. SEM (The standard errors of the mean) of about 20 stomatal apertures repeated twice in the *Commelina communis* was ± 0.89 μm (Lee & Bowling 1992). Therefore, it is difficult to see that the effect of the distinct blue light receptor appeared in Talbott *et al.*(2003)'s experiment.

Recently, stomatal researchers who studied stomata in relation to blue light photo-receptors were difficult to find, but review papers were available (Inoue & Kinoshita 2017, Matthews *et al.* 2020). In addition, photosynthetic activity occurs even with red light alone, but when blue light is added, photosynthetic activity and plant growth are greatly increased. Recently, photosynthesis activity has been known to occur with blue light alone. Therefore, when studying stomata, if red light is continuously added to the background while adding blue light on it, the effect of photosynthesis activity by blue light cannot be blocked. Therefore, the stomatal opening by blue light can partially add photosynthetic effect. In this paper, the environmental characteristics of ion and sucrose transport between the guard cell cytoplasm and vacuole are examined, and

attempts are made to clarify the opinions on stomatal opening by blue light.

Plant growth and photosynthesis could occur under single blue light

In the 1960s, mechanisms for photosynthetic electron transport have been revealed, which is the fact that electron transport is centered around photosystem II (P680) and photosystem I (P700). Since then, it was recognized that photosynthesis was caused mainly by red light. In 1882, Engelmann used a prism to disperse sunlight into a rainbow, then illuminated aquatic filaments, *Spirogyra* sp. and observed the migration of the O_2-seeking bacteria population. As a result, O_2-seeking bacteria gathered at the sites that generate the most oxygen, where the similar numbers of bacteria gathered at blue and red light regions. This experiment was surprisingly the first to show that photosystem II works perfectly by single blue light only. Recently, many papers were reported that photosynthetic electron transport by blue light occurred perfectly (Evans *et al.* 2017, Gruszecki *et al.* 1997, Miao *et al.* 2016). The absorption spectrum regions of chlorophyll a and b are much larger and have a wider range than red light region. The absorption spectrum of chlorophyll b showed the peak between 400nm and 500nm and the absorption spectrum of the red light showed peak between 600nm and 700nm. The sizes of blue light absorption spectrum region are twice higher than that of red light in chlorophyll b. The highest absorption spectrum of chlorophyll a showed a larger and broader absorption spectrum from 300 to 450 nm than the highest red light absorption spectrum in 670nm (Comar & Zscheile 1942). β-carotene absorbs only the regions of blue light band. These facts imply that the main absorption spectrum of chlorophyll is blue wavelength, which is very important for photosynthetic activities. It was reported that blue light increased net leaf photosynthetic rates when those are compared to red light alone in wheat (Goins *et al.*1997). Only 50 % blue light doubled the photosynthetic activities over red light alone (Hogewoning *et al.* 2010). The most surprising facts are that plants could grow only with single red or blue light. The seedlings of *Salvia splendens* F. Sello ex Roem & Schult. were treated with single blue light only for 4 weeks and then growths were observed for the first time (Runcle 2017). In the absence of blue radiation, the plants had purplish leaves. However, in outdoors, the plants had green leaves. Seedlings grown indoors with blue light were often shorter and had smaller leaves than grown under the red light only. They also observed the induction of flowers from *Salvia splendens* which was grown under the blue light alone. Two rose cultivars, Rosa hybrid 'Radrazz' and *Rosa chinensis* 'Old blush', were cultivated under blue or white light. While plant development was totally inhibited in darkness, blue light could sustain full development from bud burst until flowering (Abidi *et al.* 2013). These mean that the plants operate photosynthetic electron transports and the Calvin cycles even though they were only treated by blue light. It was reported that blue light was more essential than red light for the activities of photosystem II and I in cucumber leaves (Miao *et al.* 2016). The similar effects of blue light on biochemical composition and photosynthetic rate of *Isochrysis* sp. were reported by Marcetti *et al.* (2013). The evidences for the complete operation of the photosynthetic electron transport by blue light and the complete operation of the Calvin-Benson cycle has been published, and now the operation of the CO_2 assimilations function by single blue light has gained confidence. The most representative paper is Abidi*et al.* (2013). They reported that blue light perfectly performs CO_2 assimilation as well as photosynthetic electron transport. *Mesembryanthemum crystallinum* was cultured aeroponically for a 16-h photoperiod at an equal photosynthetic photon flux density of 350 μmol m^{-2} s^{-1}under red and blue light. Compared to plants grown under red light condition, blue light treated plants had similar but higher total chlorophyll contents, carotenoid contents and higher Chl.a /b ratios (He *et al.*2017).

The study of stomatal opening by blue light began with the idea that photosynthetic activities were induced only by red light. In the studies of the stomatal opening mechanism, those were recognized that the treatment of red light as a background could eliminate all photosynthetic effects and observed the stomatal opening reactions by blue light. Surprisingly, the studies of the stomatal opening mechanisms caused by the blue light originated from this experimental prerequisite. Nevertheless, some stomatal researchers did not think that blue light was essential for the activities of photosynthesis.

Fig. 1. The possible mechanisms of stomatal opening. The explanations of the figure are below.

Is IAA the first essential signal hormone for the stomatal opening?

The hypothesis which is still widely accepted to explain stomatal activities involves fluxes of inorganic cations and anions across the plasma membrane and tonoplast of guard cells associated with the synthesis and degradations of photosynthetic products. It was reported that previously darkened leaves expose to light showed quenching of fluorescence in the apoplast surrounding the guard cells up to 20 min. before stomatal opening (Edwards *et al.* 1988). They showed that proton efflux originating at the guard cells preceded stomatal opening, confirming earlier works which suggested that proton efflux was a necessary precursor of stomatal opening (Raschke & Humble 1973). Therefore, when stomata open, protons are first pumped out from the guard cell, resulting in hyperpolarization of plasma membrane potential difference. Consequently, K^+ may then passively enter guard cell cytoplasm from the guard cell wall or subsidiary cells. Therefore, the first reaction that occurs when stomata are opened, is the activity of PM (plasma membrane) H^+-ATPase present in the guard cell. How does PM H^+-ATPase activate? It can be assumed that PM H^+-ATPase can be activated by light simply because stomata open when plants receive light and stomata close in the dark. Very important points here are understandings of the structures and activities of PM H^+-ATPase. PM H^+-ATPase can be activated by phosphorylation of their penultimate residue (a Thr) and the subsequent binding of regulatory 14-3-3 proteins. The confusions are that PM H^+-ATPase is activated by regulatory 14-3-3 proteins by IAA, fusicoccin and light (Olsson *et al.* 1998, Svennelid *et al.* 1999, Takahashi*et al.* 2012, Eigo & Kinoshita 2018). These results suggest that the first signal material required for stomata to open can be IAA or light. Therefore, it means that the initial signal transduction pathway of stomatal opening can be induced by light regardless of IAA. However, delicate reactions such as stomatal opening require light, but it must be speculated that IAA, which acts as a signal for cell wall growth, phototropism, thigmotropism, and many metabolisms of plants as a signal material, is highly likely to be the first signal material for stomatal opening (Fig. 1). Plant V (vacuolar)-H^+-ATPase and H^+-ppase maintain the vacuole acidity, so their activities are very high. IAA transports to guard cells through PGP/ABCB using ATP in the plasma membrane of the mesophyll cells (Fig. 1). The main synthesis sites for IAA are the cytoplasms of young leaf mesophyll cells, which are also synthesized in mature leaves (Fig. 1). IAA promotes the activity of PM H^+-ATPase to acidify the cell wall. Plant V-H^+-ATPase could be also activated by IAA. This is because PM H^+-ATPase and V-H^+-ATPase are monophyly, so they have high phylogenetic relationships, similar structures and functions. The high activities of many V-H^+-ATPase and H^+-ppase promote H^+ transport to vacuole and increases sucrose transport to vacuole by H^+-sucrose antiporters to balance H^+charges (Fig. 1). It was also reported that the transcription of H^+-PPase gene *IbVP1* in sweet potato plants was strongly induced by auxin in hydroponics (Fan *et al.* 2017).

Sucrose is the main osmotic material for the stomatal opening and most of sucrose comes from the mesophyll cells

Sucrose is much more efficient osmotic material than K^+ ions. The sucrose in increasing the spacing of the water solution was mainly responsible for osmotic potential; this contrasted with K^+ and Cl^- ions where their spacing effects were only a little higher to that of water held to those ions (Cochrane & Cochrane 2007).

During the day, sucrose synthesized in the cytoplasm of mesophyll cells is actively transported to the guard cell by the H^+-sucrose symport through the plasma membrane (Fig. 1). All plant cells usually have plasmodesmata. However, guard cells initially are coupled symplastically to adjoining epidermal cells. With time, however, their plasmodesmata become truncated and eventually nonfunctional, eliminating intercellular communication between mature guard cells and surrounding epidermal cells (Roberts & Oparka 2003). It has now become a general theory that light-induced stomatal openings are made from metabolites or signals synthesized from mesophyll cells (Mott*et al.* 2008, Mott 2009, McAdam & Brodribb 2012, Fujita *et al.* 2013, Sibbernsen & Mott 2010, Fujita *et al.* 2013, Lawson*et al.* 2014). In recent years, it was reported that the sucrose produced in the mesophyll cells can be transported to the vicinity of the guard cells via the transpiration stream (Lima *et al.* 2018). It has been reported that starch decomposition in guard cells can be decomposed in a very short time, and some of sucrose may be sourced from starch decomposition (Sabrina *et al.* 2020).

When the pH of the vacuole is acidic, Cl^- and malate^{2-} may be transported to the vacuole for the charge

balances (Fig. 1). Humble and Raschke (1971) reported that only 5% of K^+ was balanced by Cl^- in *Commelina communis* . In the same species, the accumulation of malate^{2-} could account for half of the K^+ uptake (Allaway 1973). At that time, the stomatal researchers believed that the stomatal opening was caused by K^+. However, if the main osmotic material was supposed to be sucrose, the importance of Cl^- and malte^{2-} as the osmotic materials needed for stomata to open will be lower.

Assuming that sucrose is the main osmotic material of the stomatal opening, sucrose has to be transported from mesophyll cells. It is generally accepted that all Calvin-Benson cycle enzymes are present and functional in guard cells, but their activities of the chloroplasts definitely low, and they cannot supply all the osmotic materials to guard cells (Lawson *et al.* 2002, 2003). If the guard cell itself could not supply all the requirements of the energy, then imports from the mesophyll cells must occur (Outlaw 1989, Reckman *et al.*1990). It has been observed that pulse-labelling solutes are actively transferred from labelled mesophyll cells to the epidermis (Outlaw &d Fisher 1975, Outlaw *et al.* 1975). There were rapid exchanges of photosynthetic products between the mesophyll and epidermis. These metabolites include glucose, sucrose, sugar phosphates, malate, glycine and serine (Thorpe & Milthorph 1984). Guard cells usually contain from 10 to 15 chloroplasts. In case of *Selaginella* , the number of guard cell chloroplast were 3 6. *Erigeron annuus* (L.) PERS. had 9 chloroplasts per guard cell: *Sedum sarmentosum* , 7;*Chamaesyce supina* MOLD, 8; *Trifolium repens* , 7;*Persicaria tinctoria* , 9; *Portulaca oleracea* L., 8) (Lee & Park 2016). In the early days, plant without chloroplasts in the guard cells was firstly known in *Paphiopedilum insigna* var. (Nelson & Mayo 1975). The succulent plant, *Pelagonium zonale* cv.*Chelsia gem* . have no chloroplasts in guard cells (Avrill & Willmer 1984). Slow photosynthetic induction and low photosynthesis in*Paphiopedilum armeniacum* are related to its lack of guard cell chloroplast and peculiar stomatal anatomy (Zhang *et al.* 2011). Although these plants do not have chloroplasts in the guard cell, stomata work normally, meaning that these guard cells are sink cells that must receive photosynthetic products from mesophyll cells.

The thickness of the guard cell wall can be reach about 5μm and the width of mesophyll cell wall is only under 100nm (depicted in Fig. 1). It is estimated that about 90% sucrose of the total amount needed by the guard cell comes from mesophyll cells. 80% of which may be transported to vacuole, and 10% of which can be used for maintenances and repairs of guard cells including the wall structures (Fig. 1). Sucrose synthesized by guard cell chloroplasts may be under 10%. It could be estimated that about 5% sucrose may be transported to vacuole, and the rest can be used for maintaining the structure of the guard cell wall and metabolism in the guard cell (Fig. 1).

What is the role of K^+ for the stomatal opening?

Stomata open slowly without lag time under the sun light (Lee & Bowling 1992). Lu *et al.* (1997) have pulse-labelled the leaflets of broad bean with $^{14}CO_2$, then harvested whole leaf pieces and rinsed epidermal peels for subsequent processing in a histochemical analysis. There, sucrose–specific radioactivity shows a peak (111GBq mol^{-1}) in the palisade cells at 20 min. Therefore, when the plant receives light, sucrose uptake into the guard cell vacuole could occur firstly. According to the increases of photosynthetic activities in mesophyll cells over time, stomatal apertures increase as the synthesized sucrose accumulations into vacuole. The number of chloroplasts in mesophyll cells is known to be unexpectedly high. It is expected that the number of chloroplasts in mesophyll cells may vary depending on the plant species. For the first time, the number of chloroplasts in mesophyll cells was measured using an optical microscope and reported to reach up to 70 (Humble & Rashke 1971). Zuzana *et al.* (2014) applied, for the first time, the stereological method of an optical dissector based on counting chloroplasts in stacks of spruce needle optical cross-sections acquired by confocal laser-scanning microscopy. They reported that, unlike what was measured with a two-dimensional optical microscope, when measured with a three-dimensional microscope, the number of chloroplasts increased by about ten times. Therefore, they estimated that chloroplasts in mesophyll cells reached hundreds. According to a recent report, the number of chloroplasts in mesophyll cells was generally estimated to 100 (Lee 2019). The number of chloroplasts in Fig. 1 was estimated to be 60 to 100 based on the published data. Except for apoplast, the remaining K^+ concentration of 50 75 mM will be divided into cytoplasm or vacuole. According to the activity of PM H^+-ATPase, K^+ concentration of the cytoplasm

will increase as K^+ is transported to the cytoplasm. Some of the increased K^+ in the cytoplasm will be transported to the vacuole (Fig. 1). Tonoplast has with very selective K^+/FV-inward and outward channels (Lebaudy *et al* . 2007). Since these two-channels are shaker channels that are activated equally by voltage, it is presumed that the difference in slope between K^+ concentrations of vacuole and cytoplasm will not be large. TPK/VK channels present in the tonoplast, which are very selective to K^+ ions and release K^+ into the cytoplasm. Tonoplast also have TPC1/SV channels that transports two ions of K^+ and Ca^{2+} to the cytoplasm non-selectively (Fig. 1). Therefore, it is presumed that the transport of K^+ to vacuole from cytoplasm will be limited (Fig. 1). V-H^+-ATPase and H^+-ppase are present in tonoplast, and they transport cytoplasmic H^+ into vacuole. Therefore, vacuolar pH maintains generally around 5˜5.5 (Fig. 1). It was also reported that vacuolar pH was below 3 in 9 plant species. The fruit vacuolar pHs in *Citrus aurantifolia* and *Prunus cerasus* were 1.7 and 2.5, respectively. The leaf vacuolar pH of the very common *Rumex* sp. was 2.6, and the vacuolar pH of the lowest plant, *Perpetual begonia* , was 0.9˜1.4 (Small 1946). This means that tonoplast has a strong ability to transport cytosolic H^+ to vacuole. In this state, K^+ transport by tonoplast-inward K^+ channels must be limited (Fig. 1).

Conclusion

Plant photosynthesis is the primary producer on earth, making it an essential energy source for the survival of all living things, including humans. In addition, many metabolisms of plants are associated with photosynthesis, including plant growth and morphogenesis, stomatal opening, photorespiration, energy production, enzyme activity, ions transport and cell wall synthesis. It is assumed that the photosynthesis products, inorganic ions and hormones, are very likely to be transported between the guard cells and the mesophyll cells. Chloroplasts in mesophyll cells have evolved optimally for photosynthesis for hundreds of millions of years. However, chloroplasts in guard cells have evolved to be less optimized for photosynthesis. This may be seen as a reduction in the role of chloroplasts in the guard cell or a decrease in photosynthetic activity. There were much lacks of understanding of guard cell apoplast when explaining the stomatal opening mechanism. The guard cell vacuole is normally maintained at pH 5˜5.5. It is regulated by the high activities of V-H^+-ATPase and H^+-PPase in apoplast. Due to the activity of V-H^+-ATPase and H^+-PPase in the guard cell vacuole, vacuole has a high positive charges by H^+, so the transport of K^+ into vacuole through the inward vacuolar K^+ channels can be limited. Tonoplast has outward-K^+-channels/FV, outward-TPK/VK-channels and TPC1/SV channels that release vacuole K^+ into the cytoplasm. These channels are responsible for balancing the positive charges as well as the K^+concentration in the vacuole. Sucrose-H^+ antiporters transport sucrose to vacuole relatively easily. The above results support that sucrose is clearly the main osmotic material for stomatal opening.

Conflict of Interest

The author declare that I have no conflict of interest.

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