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Research review paper

Fructans of the saline world

Onur Kırtel[a], Maxime Versluys[b], Wim Van den Ende[b,*], Ebru Toksoy Öner[a]

[a] Industrial Biotechnology and Systems Biology Research Group, Marmara University, Bioengineering Department, 34722 Istanbul, Turkey
[b] Laboratory of Molecular Plant Biology, KU Leuven, 3001 Leuven, Belgium

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ABSTRACT

Saline and hypersaline environments make up the largest ecosystem on earth and the organisms living in such water-restricted environments have developed unique ways to cope with high salinity. As such these organisms not only carry significant industrial potential in a world where freshwater supplies are rapidly diminishing, but they also shed light upon the origins and extremes of life. One largely overlooked and potentially important feature of many salt-loving organisms is their ability to produce fructans, fructose polymers widely found in various mesophilic Eubacteria and plants, with potential functions as storage carbohydrates, aiding stress tolerance, and acting as virulence factors or signaling molecules. Intriguingly, within the whole archaeal domain of life, Archaea possessing putative fructan biosynthetic enzymes were found to belong to the extremely halophilic class of Halobacteria only, indicating a strong, yet unexplored link between the fructan syndrome and salinity. In fact, this link may indeed lead to novel strategies in fighting the global salinization problem. Hence this review explores the unknown world of fructanogenic salt-loving organisms, where water scarcity is the main stress factor for life. Within this scope, prokaryotes and plants of the saline world are discussed in detail, with special emphasis on their salt adaptation mechanisms, the potential roles of fructans and fructosyltransferase enzymes in adaptation and survival as well as future aspects for all fructanogenic salt-loving domains of life.

1. Introduction

Arguably the most fundamental question that has been preoccupying humankind's mind since time immemorial still remains the same: how and where did life start? The beginning of life marks the foundation of biology. We are still not able to give a definite answer to how life began; however, theories about the location of its beginning are far more well-supported. Although there have been recent reports indicating the possibility of the emergence of first living organisms in terrestrial hydrothermal pools (Damer and Deamer, 2015; Mulkidjanian et al., 2012), it is still widely accepted that life began in the oceans. Oldest known putative fossils predate back to 3,770 – 4,280 million years ago and were found in hydrothermal-vent related ferruginous sedimentary rocks on the seafloor of Nuvvuagittuq belt in Quebec, Canada (Dodd et al., 2017). The authors suggested that the shapes and mineral compositions of haematite tubes and filaments found on these rocks resemble the morphology of modern filamentous microorganisms which reside on hydrothermal deep sea vents. Longo and Blaber (2014) investigated the presence of prebiotic α-amino acids that were present before the beginning of life in modern day proteins and found out that modern halophilic proteins harbor significantly higher numbers of these amino acids compared to mesophilic ones. The authors concluded that the key biomolecule foldable peptide that emerged during abiogenesis may be of halophilic origin. If life indeed began in the ocean, this means that salinity has been, and still is, a fundamental driving force in evolution.

Saline (seas and oceans) and hypersaline (deep sea brines, salt lakes, salterns, salt mines, some rock sediments) environments make up the largest ecosystem on Earth, and they are rich with a plethora of organisms from all three domains of life (Oren, 2016). While Eubacteria and various Eukarya are dominant in saline environments, inhabitants of hypersaline environments are predominantly Archaea, followed by Eubacteria (Loukas et al., 2018; Ventosa et al., 2012) and a small number of microbial Eukarya such as some *Dunaliella* species (Polle et al., 2017; Wang et al., 2016a) and ancient fungi (Gunde-Cimerman and Zalar, 2014; Plemenitaš et al., 2014). Moreover, many viruses also exist in these environments and make up a significant part of the total biomass (Roux et al., 2016; Sabet, 2012). According to their requirement of/tolerance to salt, these salt-loving organisms can be classified as halophiles (organisms that require certain amounts of salt to survive) and halotolerants (organisms that can withstand varying concentrations of salt). The term halophytes, on the other hand, is a specific term used for those plants that are specialized to live in high-salt environments.

With the combined effects of climate change, deforestation, and

* Corresponding author.
E-mail address: wim.vandenende@kuleuven.be (W. Van den Ende).

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other natural and human-related causes, the saline world is expanding at an uncontrollable rate and salinization has become a global issue with large economic, social and ecological impacts resulting in huge losses of money, arable land and biodiversity. Salinization reduces the trophic diversity in freshwater environments, thus significantly altering the structure of these ecosystems (Castillo et al., 2017). Salinized soils become dense upon drying, hindering the rooting and emergence of plants, and also show reduced water holding capacity (Daliakopoulos et al., 2016). Additionally, increasing salt content in soil reduces the overall microbial activity, resulting in detrimental effects on N cycling (Singh, 2016). 25% of the agricultural lands in the Mediterranean region of Europe is affected by soil salinization (Mateo-Sagasta and Burke, 2017). The global annual cost of the salinization of the agricultural lands is estimated to be around 27.3 billion US $ due to reduced crop production (Qadir et al., 2014).

The inefficiency of currently applied strategies against salinization warrants to adopt novel perspectives where salt-loving organisms, with their unique ways of coping with high salinity may serve as valuable resources. Especially, the capacity to produce and accumulate fructans, the so called “fructan syndrome” (Versluys et al., 2018), is a largely overlooked area that deserves to be put into the spotlight. Not only are fructans (like inulin and levan) some of the most widespread biomolecules in nature with established commercial significance due to their health promoting effects (Toksoy Öner et al., 2016), but there is also increasing evidence on their multi-functional roles in (a)biotic stress resistance mechanisms and signaling which are believed to give an adaptive advantage for plants and microbes to survive under water-limiting conditions (Versluys et al., 2018). Moreover, many Archaea also harbor putative proteins that are required for fructan biosynthesis, namely the J clan of glycoside hydrolases (GH-J). However, any experimental evidence for the presence of fructans themselves in Archaea is still missing. Intriguingly, Archaea that harbor putative GH-J clan enzymes are all halophiles found under the Halobacteria class. This raises the question whether fructan biosynthesis and salinity are linked, especially considering the hypothesis that fructan biosynthesis is related to water scarcity in many species (Versluys et al., 2018). With the motivation to find possible links between fructans and salinity, this review aims to summarize the most recent findings on fructan biosynthesis in life forms of the saline world and discuss the possible functional roles and evolutionary origins of the fructan syndrome.

2. The saline world

Over 97% of Earth’s water is saline, mainly consisting of oceans and seas. The remaining 2.5% is freshwater, including ice mass, ground water and surface water bodies. The main saline water source is ocean water, forming 96.5% of total water and estimated at a volume of 1.338 billion km^3. Other sources of saline water are saline ground water (0.93% of total water, 12.87 million km^3) and saline lakes (0.006% of total water, 85400 km^3; Erakins and Sharman, 2010; Shiklomanov, 1993). Salinity is also a major determining factor in other aquatic zones and coastal regions, such as coastlines, mangroves and deltas. Salt marshes are located in the coastal zones where there is a frequent flooding with saline water due to tides (Tempest et al., 2015). Deep sea brines are areas of hypersaline conditions near the ocean floor, containing much higher salt concentrations than the surrounding water (Eder et al., 2001). In many of these environments, salinity stress is accompanied by other types of abiotic stress such as drought or flooding (Souid et al., 2018).

The salinity of 50% of all ocean water ranges from 54 to 54.3 dS/m, with an annual variation of about 0.78 dS/m. Sea water with a salinity of 54.7 dS/m contains mostly Cl^- and Na^+ (55% and 31% of total salinity, respectively), but also SO_4^{2-}, Mg^{2+}, Ca^{2+}, K^+, HCO_3^- in proportions ranging from 0.5% to 7.5% of total salinity composition (Castro and Huber, 2016).

Water salinity and temperature both influence water density, thus influencing vertical mixing processes in the ocean. Evaporation and freezing can cause an increase in surface salinity, while rain, runoff and melting of ice will cause a decrease. For example, salinity levels are lower near the equator, due to greater precipitation. In regions with high evaporation rates, higher salinity values are observed, such as in the Mediterranean region and the Red Sea (ca. 60 dS/m). Salt concentration of the Dead Sea is as high as 550 dS/m, with Mg^{2+} and Ca^{2+} concentrations around 2 M and 0.5 M, respectively. It should be noted that salinities of hypersaline environments may change with seasonal rain floods, tidals or evaporation, triggering the bloom of different species and forcing the microbial communities to adapt to ever-changing osmotic stress values (Oren, 2015). Besides the Dead Sea, extreme values of over 400 dS/m can also be reached in closed lagoons in arid regions (Millero, 2005; Tsontos and Vazquez, 2016). Water salinity functions in ocean circulation dynamics and large-scale ocean climate signals. Sea surface salinity is an important parameter to study these processes using satellite-based techniques (Reul et al., 2014).

Recently, NASA’s Aquarius instrument (Lagerloef et al., 2008) has mapped the first global salinity distribution of the water surface. Many of the observed trends were already previously characterized in the literature. Salinity is higher in the Atlantic Ocean as compared to the other oceans, but with a low salinity region near the equator. This zone of low salinity is also found in the other oceans and caused by the heavy rainfall (Tsontos and Vazquez, 2016). European Space Agency (ESA) has also made efforts in global salinity monitoring. In November 2009, they launched the SMOS (Soil Moisture and Ocean Salinity) mission (Mecklenburg et al., 2012). This satellite provides important information for use in climate and weather predictions and apart from soil moisture and salinity levels, it can also measure additional factors, such as sea ice levels (Mecklenburg et al., 2016).

Apart from saline water bodies, a large part of our planet is covered with saline soils. Over 9 Mha of soil is prone to soil salinization (Aladin et al., 2009). This resembles about 6% of all the land area being affected by salinity. According to Food and Agriculture Organization of United Nations (FAO), soils with an electrical conductivity (EC) of 4 dS/m or more and a sodium adsorption rate (SAR) of less than 13% are considered saline. While several cations (Na^+, Ca^{2+}, Mg^{2+}) and anions (Cl^-, SO_4^{2-}, HCO_3^-) are associated with this phenomenon, Na^+ and Cl^- are considered the most important (Butcher et al., 2016; Yadav et al., 2011). While salinization is most common in arid and semi-arid regions, all regions are to a certain extent prone to salinization (Rengasamy, 2006).

The two main causes for soil salinity are either nature- or human-related, termed primary and secondary salinization, respectively. Natural processes include weathering and transport of parent rock material. Deposition of oceanic salts by wind and precipitation is another important factor. Rainwater can contain an EC of up to 0.1 dS/m, mainly NaCl. Irrigation and deforestation are examples of secondary salinization. Land clearing and irrigation change the hydrological balance of the soil, which is based on water application and water usage by plants (crops). Excess water applications can cause rises in soil water table, thereby mobilizing stored salts upwards. Moreover, irrigation water already contains low amounts of salt (Aladin et al., 2009; Bui, 2013).

2.1. Salinity as a global stress

Salinity poses a worldwide problem, especially for plants as it greatly impedes agricultural yield. On average, over 80 million ha or 20% of global irrigated land is affected by salinity. On top of that, agricultural practices and irrigation increase soil salt content even more (Shrivastava and Kumar, 2015). In Europe for example, about one tenth of the saline or sodic soils are affected by human-related processes (Aladin et al., 2009; Bui, 2013) and this has a large economic impact (Qadir et al., 2014). Several strategies have been proposed to reduce salinization, such as drainage of underground saline water, flooding of

heavily salinized lands or more efficient use of irrigation water (for example drip irrigation). Nevertheless, these strategies are not efficient enough or create problems under specific circumstances (Pannell, 2001; Pannell and Ewing, 2006).

Overall, climate change will heterogeneously affect the world. In those areas with increased evaporation combined with less precipitation, salinization will increase even faster, especially in semi-arid regions. Additionally, coastal areas with very low altitude will be flooded due to sea level rise, leading to an increase in salinity stress in those regions. Several studies indicate that a rise in sea level may be the main causal agent for an increase in soil salinity (Durack and Wijffels, 2010; Kapur et al., 2017; Teh and Koh, 2016). A clear example on the influence of climate change is the shrinking of the Aral Sea in central Asia, which was the fourth largest lake in the world in 1960. The area of this water body has decreased by more than 50%, accompanied by high salinity levels and a break-up into four separate bodies (Aladin et al., 2009). These changes dramatically alter the biodiversity composition, leading to diversity loss (Jeppesen et al., 2015).

The harmful effects of salinity on plants are widely studied (Butcher et al., 2016). Soil salinity is known to reduce crop growth and yield in many salt-sensitive crops (Munns and Tester, 2008; Tilbrook et al., 2017). The sensitivity to salt stress, however, strongly depends on the crop species. Because of high ion concentrations, osmotic stress will influence water potential and thus water availability. In the roots, an increase in ROS is measured within minutes after salt stress exposure, activating cation-permeable ion channels which leads to disturbances in ionic homeostasis, generating detrimental effects such as for instance impeding the uptake of necessary ions such as K^+ (Demidchik et al., 2003; Luna et al., 2000; Miller et al., 2008; Pottosin et al., 2014; Zepeda-Jazo et al., 2011). As a consequence, caspase-like proteases are activated, followed by programmed cell death mechanisms in the roots (Shabala, 2009). These responses are very specific for salt (NaCl) and are not observed in roots treated by isotonic organic osmolytes (Affenzeller et al., 2009). In the roots of salt tolerant species however, temporal hydrogen peroxide increases may act as positive signals (Formentin et al., 2018) that induce an array of salt-responsive genes with involvement of ABA (abscisic acid), SnRK2 kinase and salt overly sensitive (SOS) signaling (Liang et al., 2018; Yang and Guo, 2017). Even though such direct effects are found in the roots, there is an expected delay in salt-mediated effects in above ground plant parts, potentially compromising for instance photosynthesis and generative development. As an overall defense strategy in all plant parts that are affected, plant cells produce several osmolytes, such as proline, betaine and soluble sugars, for osmotic adjustment. Additionally, transporter systems are activated to exclude excess toxic Na^+ from the cytosol, including vacuolar and plasma membrane Na^+/H^+ antiporters. An increase in antioxidative enzymes is another common response under salinity stress, to prevent prolonged periods of excessive ROS levels, causing cellular damage (Parihar et al., 2015 and references therein).

Salt stress is often combined with flooding (coastal regions, salt marshes, …). The problems for the plants are different from normal salt stress in the sense that there is no acute problem of water shortage. However, flooding causes soil hypoxia and due to O_2 consumption under very low O_2 flux the soil will become increasingly anoxic. In addition, plant growth is influenced through the accumulation of other compounds and ions as a consequence of flooding (Colmer and Flowers, 2008). Salinity stress also has an influence on disease resistance in plants, but the outcome of such stress combinations depends heavily on the severity of the salt stress and on which pathogen is involved. On the one hand, studies show an increase in susceptibility to *Phytophthora* spp. in tomato plants exposed to higher salt (Dileo et al., 2010). On the other hand, several studies present a positive effect on disease resistance in tomato against different pathogens through the induction of salt-pathogen combinatorial stress-specific gene expression (Achuo et al., 2006; Bai et al., 2017).

Both terrestrial and aquatic communities of invertebrates suffer from the increase in salts as well, leading to a dominant position for more salt-tolerant species (Khlebovich and Aladin, 2010). Loss of soil fauna can greatly impede important processes such as decomposition and nutrient cycling (Pereira et al., 2015). Min et al. (2016) studied the effect of salinity on soil microbes. Under high saline water use for irrigation, the microbial community was characterized by a decrease in biomass (both C and N), basal respiration and total phospholipid fatty acid. Such reduction in microbial biomass and diversity has a large impact on soil quality (Min et al., 2016). However, it has also been illustrated that soil communities can adapt to natural soil salinity increases, showing a different scenario from the secondary salinization effect measured by Min et al. (2016) (Bencherif et al., 2015). Generally, many reports on how increasing salinity can seriously alter the microbial activity, diversity and composition in soil can be found in the literature (Chen et al., 2017a,b; Morrissey et al., 2014; Van Horn et al., 2014). Nevertheless, several organisms can survive and even thrive under high salinity conditions.

3. Life forms of the saline world

Salt-loving and salt-tolerant organisms can be classified into three categories, namely halophiles, halotolerants and halophytes, although it should be noted that this is not a strictly taxonomical classification (Fig. 1; Colmer and Flowers, 2008; Larsen, 1962).

Halophiles are microorganisms that require low (0.34 M) to extreme (saturation point) concentrations of salt to survive and they are comprised of various organisms from all three domains of life. Salinity ranges that cover the degree of halophilicity are defined differently by various researchers. Schneegurt (2012) defines a halophile as a microorganism which requires higher salinity values than seawater for survival. Larsen's (1962) distinction between slight (0.34–0.85 M salt), moderate (0.85–3.4 M salt) and extreme (3.4–5.1 M salt) halophilicity is widely accepted among halophile researchers. Halotolerants, on the other hand, are organisms that can withstand substantial amounts of salt, but thrive better in the absence of it.

Halophilic and halotolerant microorganisms show a wide variety and distribution in nature. Halophilic Archaea are included within the classes *Halobacteria*, *Methanomicrobia* and 'Methanonatronarchaeia' which are under the phylum *Euryarchaeota* (Sorokin et al., 2017; Ventosa et al., 2012). Except for strictly anaerobic methanogenic Archaea or facultative anaerobic species like *Halorhabdus tiamatea*, most halophilic Archaea grow aerobically. Apart from some members of the *Natrialba* genus, most of them form colonies with red to pink pigmentation, which protect them from the detrimental effects of intense UV radiation (Jones and Baxter, 2017). Among extreme halophiles, Archaea are the most intensely studied ones, due to their dominant presence in habitats with extremely high salt concentrations, such as the Dead Sea.

Unlike halophilic Archaea, halophilic bacteria are found under a very wide range of different phyla, which makes them a very

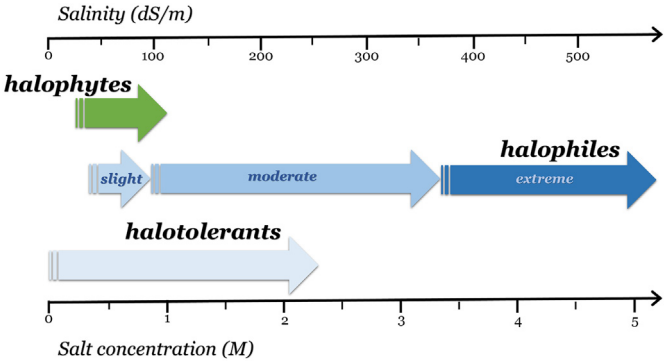

Fig. 1. Salinity ranges (M) at which different life forms of the saline world thrive.

heterogeneous group. According to both Ventosa et al. (2012) and Loukas et al. (2018), they are mainly found under at least eight different phyla, though it should be kept in mind that with the recent developments in –omic technologies, it is now better understood that there may be many more unculturable halophilic species in nature, due to their unknown growth requirements (Oren, 2015). In general, halophilic bacteria thrive under more moderate salt concentrations as compared to halophilic Archaea (Ventosa et al., 2012). Nevertheless, recent studies indicate the significant presence of some halophilic Archaea in many low-salt or fluctuating-salinity environments, such as *Haladaptatus paucihalophilus* (Youssef et al., 2014), *Halomarina oriensis* (Inoue et al., 2011) and *Halococcus hameliensis* (Gudhka et al., 2015).

Halophyte is the term used specifically for plants that can tolerate significant concentrations of salt and even benefit from the presence of salt. Most recently, plant halophytes have been defined as species that complete their lifecycle in at least 200 mM NaCl (Colmer and Flowers, 2008). The most pertinent halophytic terrestrial plants can withstand NaCl concentrations up to 0.5–1.0 M (Flowers et al., 2010; Khan et al., 2005). Of course, these salt levels are remarkably low when compared to the microbial halophiles (Edbeib et al., 2016). The exceptional unicellular green alga *Dunaliella salina* can thrive at NaCl concentrations between 0.05 – 5.5 M and shows a great tolerance to sudden changes in salinity (Wang et al., 2016b). Being a unicellular organism, however, it is rather debatable whether *D. salina* is either a halophile or a halophyte.

Similar to the distribution of halophilic bacteria, halophytes can be found under various orders of plants. Although there are no strictly halophytic families, some families like *Amaranthaceae* have significantly higher numbers of halophytic species. Genera of *Aster, Glycine, Plantago* and *Solanum* are well-known to involve both halophytic and non-halophytic species (Cheeseman, 2015).

Although mostly overlooked, halophilic Eukarya are also present in nature. For instance, the ancient fungus *Wallemia ichthyophaga* requires at least 1.7 M NaCl to grow, with an optimum range of 2.6 - 3.4 M (Zajc et al., 2014). Harding et al. (2017) reported the presence of the halophilic protozoa *Halocafeteria seosinensis* and proposed that it acquired the genes required for salt tolerance via duplication, lateral gene transfers and transcriptional modifications. Species of the halophilic brine shrimp *Artemia* show a wide distribution over hypersaline lakes of the world and survive even complete dry-out events in those lakes by forming highly resistant cysts that contain their genetic info (Gajardo and Beardmore, 2012). Other halophilic Eukarya commonly found in hypersaline lakes include insects such as *Ephydra* sp. and *Trichocorixa verticalis* (Barnes and Wurtsbaugh, 2015).

3.1. Survival strategies at high salinity

Throughout their evolution, salt-loving microorganisms developed two main strategies to cope with high salt concentrations: i) the salt-in strategy, where high concentrations of ions are accumulated in the cytoplasm (Oren, 2013), ii) accumulation of compatible solutes in the cytoplasm (Hagemann, 2016; Yin et al., 2015). In the salt-in strategy, the halophile accumulates high amounts of KCl in its cytoplasm while excluding Na^+ ions as much as possible. Historically, it was thought that the salt-in strategy was limited to *Halobacteriaceae* (Archaea) and *Halanaerobiales* (Eubacteria) (Gunde-Cimerman et al., 2018). However, it was found later that some extremely halophilic Eubacteria such as *Salinibacter ruber* and *Salisaeta longa* also accumulate molar concentrations of salt in their cytoplasm with no significant organic compatible solutes detected (Oren, 2013), while *Halobacillus halophilus* seems to be able to combine both osmoadaptation strategies (Saum et al., 2013). Additionally, Vavourakis et al. (2016) reported the presence of putative sodium-pumping rhodopsins in draft genomes of *Flavobacteriaceae, Chitinophagaceae* and *Rhodothermaceae* in uncultured samples from the soda lakes of Kulunda Steppe (Altai, Russia), suggesting the presence of the salt-in strategy in these Eubacterial families. Similarly, recent studies indicate that halophilic Archaea are not limited to the salt-in strategy for osmoadaptation. Youssef et al. (2014) analyzed 83 genomes belonging to *Halobacteriales* order and found genes related to trehalose and glycine betaine synthesis in 38 and 60 of these genomes, respectively. Synthesis of trehalose or sulfotrehalose was also experimentally shown in 17 of these species. It was observed that in *Haladaptatus paucihalophilus,* a low-salt adapted Archaea, trehalose synthesis decreased with increasing salinities, which may indicate its importance in adaptation to low-salt conditions. Compatible solutes such as ectoine, glycine betaine, trehalose, sucrose and glycerol are produced and stored intracellularly by a wide range of halophilic Eubacteria and Eukarya (Waditee-Sirisattha et al., 2016; Zajc et al., 2014), which provide a more “flexible” protection against fluctuating environmental salt concentrations. This strategy is sometimes also referred to as “salt-out strategy”, since these hydrophilic organic solutes increase the cytoplasmic osmotic pressure while keeping salt out via the active transport system, thus ensuring water uptake (Hagemann, 2016).

Other high-salinity adaptations such as evolving higher numbers of acidic residues on protein surfaces to prevent their aggregation (DasSarma and DasSarma, 2015; Oren, 2013; Versluys et al., 2018), or increasing their membrane fluidity (Bergmann et al., 2013; Harding et al., 2017) emerge as consequences of above-mentioned main adaptation strategies. For instance, generally acidic proteomes are observed in halophiles which use the salt-in strategy (Oren, 2013). However, cytoplasmic proteins of halophiles which accumulate compatible solutes do not need to be acidic. Since the salinity of the cytoplasm is not as high as the surrounding environment, those proteins do not require excessive negative surface charges to stay active.

Halophytes use two main strategies as an adaptation to salt tolerance. Some halophytes exclude the salt, which can involve the shedding of leaves when toxic concentrations are reached, or the exclusion at the root level (Himabindu et al., 2016; Flowers and Colmer, 2015). Alternatively, some halophytes accumulate the salt inside the cell, for example in specialized salt glands (Shabala et al., 2014; Yuan et al., 2016). Many species transport the ions into their vacuolar compartment to avoid toxic effects in the cytosol, where central metabolism occurs. However, this requires accumulation of compatible solutes in the cytosol, to balance the osmotic equilibrium between the vacuole and the cytosol. Both ion exclusion and salt compartmentalization strategies are also found in non-halophyte plant species. In addition, some plant species maintain growth independent of Na^+ accumulation in the shoot (Negrão et al., 2017; Shrivastava and Kumar, 2015).

Generally, presence of salts restricts the availability of water for organisms, thus forcing them to develop various survival strategies. Across all domains of life, there is a common water availability limit, due to physicochemical constraints, under which vitality and functionality of the organism is lost (Stevenson et al., 2015). The above paragraphs show that across the domains of life, two general strategies emerge in salinity tolerance, namely ion transport and the biosynthesis of compatible solutes.

4. Fructan syndrome in the saline world

Fructans are fructose-based oligo- and polysaccharides synthesized using sucrose by fructosyltransferases (FTs). These carbohydrates are mainly present in both Gram-positive and Gram-negative bacteria, as well as in certain plant families. They are built up out of β-2,1- (inulins), β-2,6- (levans) glycosidic bonds or a combination of both (branched fructans). Important differences between plant and microbial fructans include the degree of polymerization (much higher in microbes) and the localization of biosynthesis (extracellular in microbes, vacuolar in plants; Toksoy Öner et al., 2016; Van den Ende, 2013). While historically these carbohydrates are known as storage compound for energy, more recently, a multifunctional role has become evident. Fructans are involved in (a)biotic stress resistance mechanisms and signaling. Especially due to water retracting properties, producing

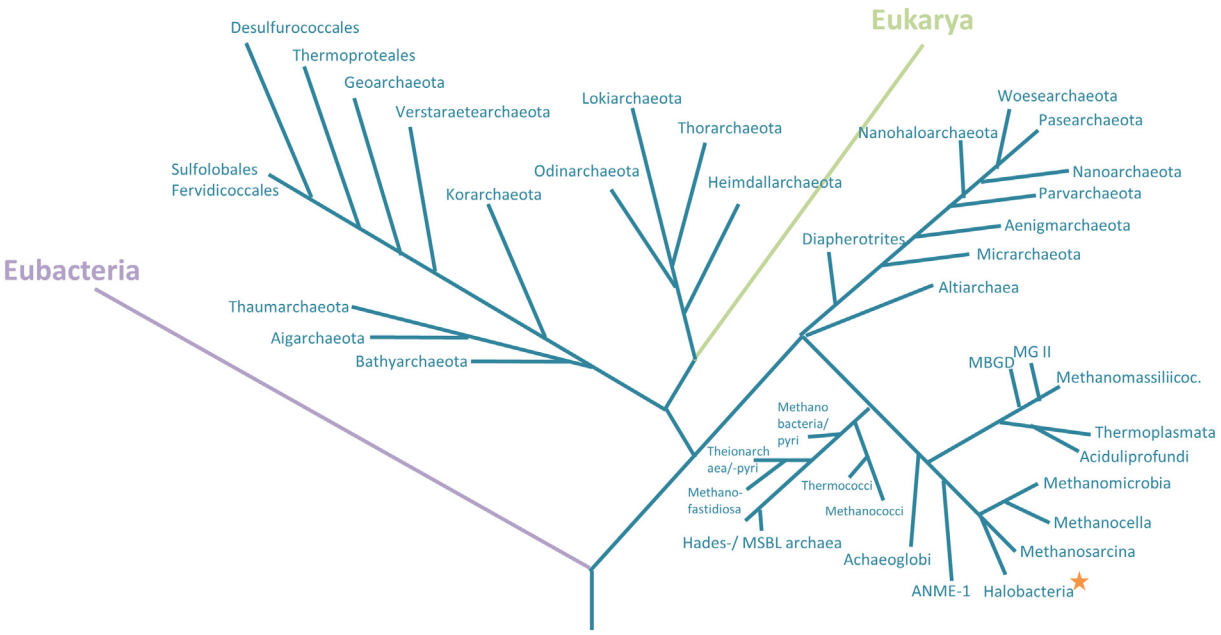

Fig. 2. The modern tree of life. Denoted with a star, Halobacteria is the only Archaeal class harboring GH-J clan enzymes. Adapted from Spang et al. (2017).

fructans may give an adaptive advantage for plants and microbes to survive under water-limiting conditions (Versluys et al., 2018).

Fructan metabolic enzymes belong to the GH32 (glycoside hydrolase 32) and GH68 family, both making up the GH-J clan enzymes. Generally, the members of these families catalyze the hydrolysis of sucrose, however, some enzymes show strong transfructosylating activity, leading to the production of fructooligosaccharides and fructans, hence named FTs (Nagaya et al., 2017). Apart from these FTs, invertases and fructan hydrolases are also part of these enzyme families. Fundamentally, these enzymes all carry out the same type of reaction, characterized by the transfer of a fructosyl residue (or chain) from a donor (sucrose, fructan) to an acceptor (sucrose, fructan, water) substrate (Versluys et al., 2018). In fructan synthesis, the first acceptor is a sucrose molecule, while the elongating fructan serves as a substrate in subsequent reactions (Edelman and Jefford, 1968).

Within microbial FTs, levansucrases, enzymes producing levan-type fructans with mainly β-2,6-linkages, are characterized in many species of both Gram-positive and Gram-negative bacteria. Inulin-type fructans are produced by inulosucrases, enzymes that are only identified in a relatively low number of Gram-positive bacteria (Kralj et al., 2017). Both levansucrases and inulosucrases belong to the GH68 family and preferentially use sucrose as a donor substrate (Pijning et al., 2011; Rapoport et al., 1966). Microbial FTs can also use other acceptor substrates such as various mono- and disaccharides, or even organic solvents for their transfructosylation reactions (Li et al., 2015), and all known levansucrases also exhibit levanase activity (Méndez-Lorenzo et al., 2015; Toksoy Öner et al., 2016; Yanase et al., 1992).

The GH32 family harbours all plant FTs as well as plant and microbial invertases and fructan hydrolase enzymes. Plant FTs and invertases also use sucrose as donor substrate, while the acceptor varies depending on the enzyme. The plant kingdom uses several different FTs to produce structurally different types of fructans. Inulin-type fructans are produced by subsequent action of sucrose: sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT), while levan production involves 6-SST and 6-SFT activities. A concerted activity of both 1-FFT and 6-SFT is necessary for the production of branched graminan-type fructans, containing both β-2,1- and β-2,6-linkages. Additionally, some plants have a fructan:fructan 6^G-fructosyltransferase to produce neoseries of fructans, containing an internal glucose moiety (Van den Ende, 2013; Versluys et al., 2018).

All members of GH32 and GH68 families, thus all FTs, have classical Koshland retaining properties, so the configuration of the substrate during hydrolysis is retained (Koshland and Stein, 1954). FTs contain a 5-bladed β-propeller fold around a central negatively charged cavity (Kralj et al., 2017). In addition, FTs from the GH32 family encompass a β-sandwich structure of six β-strands (Lammens et al., 2009). Both plant and microbial FTs use the same catalytic mode of action involving an aspartate residue near the N-terminal part as a nucleophile and a glutamate as general acid/base (Meng and Fütterer, 2003; Yanase et al., 2002).

Presence of GH-J clan enzymes in the saline world is a largely overlooked area, thus their investigation may present novel and unique aspects regarding their enzymes, halozymes. Within the scope of this review, increased acidity in halophilic proteins is of special interest, since higher numbers of negatively-charged residues in putative halophilic Archaeal FTs compared to halophilic Eubacterial FTs have been observed recently (Versluys et al., 2018), indicating these proteins' adaptation to changing salinities. It was suggested that the conservation of acidic FTs may indicate that they are essential for adaptation to high salinity, with uncertain functions. Also, simplicity of fructan synthesis via single extracellular enzymes compared to complex synthesis of heteropolysaccharides may have been favored throughout evolution. Increasing numbers of acidic residues on a protein is thought to increase surface hydration via carboxylate binding of solvated cations under water-limited environments, thus providing solubility to the protein (Deole et al., 2013; Longo and Blaber, 2014; Ueno et al., 2016).

4.1. Fructanogenic halophiles

Up until a decade ago, Archaea were thought to be divided into two phyla, namely Euryarchaeota and Crenarchaeota. Thanks to developments in cultivation-independent genomics, it is now known that Archaea are divided into four different phyla/groups: Euryarchaeota, Proteoarchaeota (or TACK group), Asgard group and DPANN (Diapherotrites, Parvarchaeota, Aenigmaarchaeota, Nanohaloarchaeota, Nanoarchaeote) group, in which more than 40 taxonomical classes are identified (Spang et al., 2017). Halophilic Archaea are found under three taxonomical classes of Euryarchaeota, namely Halobacteria, Methanomicrobia and 'Methanonatronarchaeia' (Sorokin et al., 2017). According to NCBI, putative GH-J clan enzymes are found under all three orders of Halobacteria (Halobacteriales,

Table 1
Archaea that harbour putative GH-J clan enzymes, which are all halophiles.

Halobacteriales	Predicted GH-J	Accession number (GI)	Subcellular localization
Haladaptatus litoreus	GH32	1131913889	Cytoplasmic
Haladaptatus paucihalophilus DX253	GH32	320548467	Cytoplasmic
Halalkalicoccus jeotgali B3	GH32	299123994	Cytoplasmic
Haloarcula amylolytica JCM 13557	GH32	445766972	Cytoplasmic
Haloarcula argentinensis DSM 12282	GH32	445767431	Cytoplasmic
Haloarcula californiae ATCC 33799	GH32	445758388	Cytoplasmic
Haloarcula hispanica ATCC 33960	GH32	343783670	Cytoplasmic
Haloarcula hispanica N601	GH32	564122585	Cytoplasmic
Haloarcula japonica DSM 6131	GH32	445781969	Cytoplasmic
Haloarcula marismortui ATCC 43049	GH32	55230985	Cytoplasmic
Haloarcula rubripromontorii	GH32	926546216	Cytoplasmic
Haloarcula sinaiiensis ATCC 33800	GH32	445765186	Cytoplasmic
Haloarcula sp. CBA1127	GH32	972338916	Cytoplasmic
Haloarcula vallismortis ATCC 29715	GH32	445749982	Cytoplasmic
Halomicrobium katesii	GH32	517069893	Cytoplasmic
Halomicrobium mukohataei DSM 12286	GH32	257170293	Cytoplasmic
Halorhabdus tiamatea SARL4B	GH32	528525781	Cytoplasmic
Natronoarchaeum philippinense	GH32	1247601902	Cytoplasmic
Haladaptatus litoreus	GH68	1131913890	Extracellular
Haladaptatus paucihalophilus DX253	GH68	320549682	Extracellular
Halalkalicoccus jeotgali B3	GH68	299123995	Extracellular
Haloarcula amylolytica JCM 13557	GH68	445766973	Extracellular
Haloarcula argentinensis DSM 12282	GH68	445767432	Extracellular
Haloarcula californiae ATCC 33799	GH68	445758389	Extracellular
Haloarcula hispanica ATCC 33960	GH68	343783669	Extracellular
Haloarcula japonica DSM 6131	GH68	445781970	Extracellular
Haloarcula marismortui ATCC 43049	GH68	55230984	Extracellular
Haloarcula rubripromontorii	GH68	926546217	Extracellular
Haloarcula sinaiiensis ATCC 33800	GH68	445765185	Extracellular
Haloarcula vallismortis ATCC 29715	GH68	445749983	Extracellular
Halomicrobium katesii	GH68	517069892	Extracellular
Halomicrobium mukohataei DSM 12286	GH68	257170292	Extracellular
Haloferacales			
Haloferax alexandrinus	GH32	910012694	Cytoplasmic
Haloferax elongans ATCC BAA-1513	GH32	445730353	Cytoplasmic
Haloferax gibbonsii	GH32	909825081	Cytoplasmic
Haloferax prahovense DSM 18310	GH32	445713481	Cytoplasmic
Halogeometricum limi	GH32	1097353645	Cytoplasmic
Halogeometricum pallidum JCM 14848	GH32	445681596	Cytoplasmic
Halogeometricum rufum	GH32	1097625477	Cytoplasmic
Halogranum salarium B-1	GH32	399237541	Cytoplasmic
Halopelagius inordinatus	GH32	1097333809	Cytoplasmic
Halopelagius longus	GH32	1086430161	Cytoplasmic
Haloprofundus marisrubri	GH32	966675555	Cytoplasmic
Halorubrum aidingense JCM 13560	GH32	445816820	Cytoplasmic
Halorubrum kocurii JCM 14978	GH32	445817181	Cytoplasmic
Halorubrum lacusprofundi ATCC 49239	GH32	222453801	Cytoplasmic
Halorubrum lipolyticum DSM 21995	GH32	445807818	Cytoplasmic
Halorubrum saccharovorum DSM 1137	GH32	445688897	Cytoplasmic
Halorubrum tropicale	GH32	926550336	Cytoplasmic
Halorubrum vacuolatum	GH32	1215971838	Cytoplasmic
Halobaculum gomorrense	GH68	1109802745	Extracellular
Haloferax alexandrinus	GH68	910012695	Extracellular
Haloferax elongans ATCC BAA-1513	GH68	445730352	Extracellular
Haloferax gibbonsii	GH68	909825082	Extracellular
Haloferax prahovense DSM 18310	GH68	445713480	Extracellular
Haloferax sp. SB3	GH68	966682190	Extracellular
Halogeometricum limi	GH68	1097354285	Extracellular
Halogeometricum pallidum JCM 14848	GH68	445681597	Extracellular
Halogeometricum rufum	GH68	1097625476	Extracellular
Halogranum amylolyticum	GH68	1103313741	Extracellular
Halogranum gelatinilyticum	GH68	1086401046	Extracellular
Halogranum rubrum	GH68	1097855435	Extracellular
Halogranum salarium	GH68	496827144	Extracellular
Halohasta litchfieldiae	GH68	1094368324	Extracellular
Haloparvum sedimenti	GH68	961364407	Extracellular
Halopelagius inordinatus	GH68	1097333808	Extracellular
Halopelagius longus	GH68	1086430162	Extracellular
Haloprofundus marisrubri	GH68	966675554	Extracellular
Halorubrum aidingense JCM 13560	GH68	445816819	Extracellular
Halorubrum ezzemoulense	GH68	1231488362	Extracellular
Halorubrum halodurans	GH68	1231479639	Extracellular
Halorubrum kocurii JCM 14978	GH68	445817182	Extracellular
Halorubrum lacusprofundi ATCC 49239	GH68	222453796	Extracellular

(*continued on next page*)

Table 1 (*continued*)

Halobacteriales	Predicted GH-J	Accession number (GI)	Subcellular localization
Halorubrum lipolyticum DSM 21995	GH68	445806941	Extracellular
Halorubrum saccharovorum DSM 1137	GH68	445688893	Extracellular
Halorubrum sodomense	GH68	1097141538	Extracellular
Halorubrum tropicale	GH68	926550334	Extracellular
Halorubrum vacuolatum	GH68	1215971277	Extracellular
Halorubrum sp. J07HR59	GH68	541196243	Extracellular
Natrialbales			
Haloterrigena turkmenica DSM 5511	GH32	284016978	Cytoplasmic
Natronococcus jeotgali	GH32	495695260	Cytoplasmic
Natronococcus amylolyticus	GH32	491715294	Cytoplasmic
Natrialba taiwanensis	GH32	909671641	Cell wall
Natrialba aegyptia DSM 13077	GH32	445650759	Cytoplasmic
Natronococcus occultus SP4	GH68	433675125	Extracellular
Natronococcus amylolyticus DSM 10524	GH68	445600129	Extracellular
Natronococcus jeotgali DSM 18795	GH68	445612496	Extracellular
Halostagnicola kamekurae	GH68	1097596491	Extracellular
Natrialba taiwanensis	GH68	493879455	Extracellular
Haloterrigena turkmenica	GH68	502710025	Extracellular
Natrialba aegyptia	GH68	909660753	Extracellular
Haloterrigena salina	GH68	909671317	Extracellular
Halostagnicola larsenii	GH68	909711239	Extracellular

Haloferacales and Natrialbales), but not in Methanomicrobia or 'Methanonatronarchaeia'. Fig. 2 shows the location of fructanogenic Archaea on the tree of life. Recently, Asgard group of Archaea has been suggested to be very closely related to Eukarya since they harbor many proteins that were thought to be specific to Eukarya (Zaremba-Niedzwiedzka et al., 2017). Absence of GH-J clan enzymes in Asgard may suggest that these enzymes have evolved separately in Eukarya, or the production of fructans might have been lost in some Archaea at some point in the evolutionary timeline.

A query for Archaeal GH-J clan enzymes (GH32 and GH68 families) on NCBI database gave 32 results for Halobacteriales, 47 results for Haloferacales, and 14 results for Natrialbales (Table 1). Identical proteins and halotolerant species were excluded from the lists for both Archaea and Eubacteria.

As a result of our analyses, it was intriguing to observe that the presence of archaeal GH-J clan enzymes is limited to halophilic Archaea, which suggests that these enzymes may contribute to survival in hypersaline environments. However, it should be remembered that there are also halophilic Archaea which do not seem to harbor any GH-J clan enzymes, even within the same genus (i.e. *Haloferax gibbonsii* and *Haloferax lucentense;* while the first one has GH68- and GH32- like proteins, the latter does not). The number of species that harbor GH-J clan enzymes make up 21%, 31% and 16% of all known species of Halobacteriales, Haloferacales and Natrialbales orders, respectively.

For the halophilic Eubacteria, GH-J clan enzymes were mostly found under the γ-proteobacteria class and the phylum of Firmicutes, with total numbers of 22 and 52 proteins, respectively (Table 2). Number of Eubacterial species that carry GH-J clan enzymes make up 13% and 22% of all known halophilic γ-proteobacteria and Firmicutes, respectively. Apart from these, several GH32-like enzymes were identified in *Actinopolyspora alba, Actinopolyspora mortivallis, Cellulosimicrobium cellulans, Cyclobacterium halophilum, Fabibacter pacificus, Gramella echinicola, Haloactinobacterium album, Longimonas halophila, Nocardiopsis salina, Rhodotermus marinus, Salinivenus iranica, Salinivenus lutea* and *Spirochaeta africana* DSM 8902. GH32-like enzymes seem to be more prevalent in halophilic Eubacteria: in halophilic γ-proteobacteria, 82% of all GH-J are GH32-like enzymes, while in Firmicutes this proportion is 71%. On the other hand, halophilic Archaea seem to be harboring higher proportions of GH68-like enzymes out of all their GH-J: 44% in Halobacteriales, 62% in Haloferacales, and 64% in Natrialbales.

The occurrence of GH-J clan enzymes in a limited number of halophilic Archaea and Eubacteria, sometimes even within the same genera, strongly suggests that although they may be functional for their survival in hypersaline environments, they may not be absolutely crucial for these microorganisms, with the possibility of their replacement by other polysaccharides. Then again, it is possible that fructans may be central players in symbiotic relationships between these microorganisms and sucrose-producers in hypersaline habitats (see the last paragraph of this section). Unfortunately, any experimental study that investigates such relationships is still missing.

Annotating functions to GH-J clan enzymes *in silico* is rather impractical. Although GH68 and GH32 families are mainly differentiated by the presence of a β-sandwich domain in GH32 in addition to the 5-bladed β-propeller domain found in both families, functionalities may differ drastically in unexpected ways. A good example is the β-fructofuranosidase from *Microbacterium saccharophilum* K-1 (PDB ID: 3WPU). Structurally, this enzyme lacks a β-sandwich domain and shows highest similarity to *Gluconacetobacter diazotrophicus* levansucrase (PDB ID: 1W18). However, it is classified as a β-fructofuranosidase since its main activity is hydrolysis in a wide range of sucrose concentrations, and it does not produce fructose polymers (Tonozuka et al., 2012). Thus, during our *in silico* analyses, functional annotations by NCBI were ignored and GH-J clan enzymes were classified as "GH32-like" or "GH68-like", according to the presence or absence of the β-sandwich domain. NCBI BLAST, NCBI COBALT and SWISS-MODEL (Arnold et al., 2006) were used for alignment of sequences and detecting the presence of above-mentioned domains.

Subcellular localizations for GH-J clan enzymes were also predicted via PSORTb 3.0.2 tool (Yu et al., 2010), and it was revealed that all archaeal GH32-like enzymes were cytoplasmic while all GH68-like enzymes were extracellular. The only exception belongs to *Natrialba taiwanensis,* which seems to have a cell wall-associated GH32-like enzyme. As seen in Table 2, Eubacterial GH32-like enzymes can be cytoplasmic, cytoplasmic membrane-associated, periplasmic or cell wall-associated. Similar to Archaea, Eubacterial GH68-like enzymes were all predicted to be extracellular, with one exception, which is a cytoplasmic membrane-associated enzyme from *Pontibacillus halophilus.*

Cytoplasmic GH32-like enzymes may be acting mainly as sucrose hydrolases, thus doubling the osmolarity of sucrose upon cleaving it into glucose and fructose. Extracellular GH68-like enzymes may be responsible for the formation of fructans, whose occurrence and putative functions in Archaea are unknown for the moment. However, it is possible that extracellular fructans might be crucial components of biofilms, increasing the availability of water in water-restricted environments such as hypersaline habitats (Versluys et al., 2018). Cell wall-anchored GH32 enzymes were described (Margetić and Vujčić,

Table 2
Halophilic Eubacteria that harbour putative GH-J clan enzymes.

γ-Proteobacteria	Predicted GH-J	Accession number (GI)	Subcellular localization
Halomonas campaniensis	GH32	641742445	Cytoplasmic
Halomonas hydrothermalis	GH32	730431293	Cytoplasmic
Halomonas meridiana	GH32	764089132	Cytoplasmic
Halomonas taeanensis	GH32	1086450357	Cytoplasmic
Halomonas aquamarina	GH32	1094483515	Cytoplasmic
Kushneria avicenniae	GH32	1097389246	Cytoplasmic
Carnimonas nigrificans	GH32	647288935	Cytoplasmic
Halomonas axialensis	GH32	1011463898	Cytoplasmic
Halomonas lionensis	GH32	1178502808	Cytoplasmic
Halomonas alkaliantarctica	GH32	1180695171	Cytoplasmic
Marinobacterium rhizophilum	GH32	916400201	Periplasmic/ Cytoplasmic membrane
Pseudoalteromonas atlantica T6c	GH32	109702403	Periplasmic/ Cytoplasmic membrane
Pseudoalteromonas haloplanktis ANT/505	GH32	332035858	Cytoplasmic membrane
Pseudoalteromonas fuliginea	GH32	633467542	Cytoplasmic
Pseudoalteromonas distincta	GH32	743327974	Periplasmic/ Cytoplasmic membrane
Pseudoalteromonas arctica	GH32	498239960	Cytoplasmic
Psychromonas aquimarina	GH32	655484285	Cytoplasmic
Psychromonas ingrahamii 37	GH32	119863337	Cytoplasmic
Halomonas smyrnensis AAD6	GH68	452755863	Extracellular
Marinobacterium rhizophilum	GH68	648600102	Extracellular
Pseudoalteromonas haloplanktis ANT/505	GH68	332035857	Extracellular
Pseudoalteromonas telluritireducens	GH68	1009073709	Extracellular
Firmicutes			
Alteribacillus bidgolensis	GH32	1086730358	Cell wall
Alteribacillus bidgolensis	GH32	1086729967	Cytoplasmic
Bacillus aurantiacus	GH32	916707854	Cell wall
Bacillus aurantiacus	GH32	651976535	Cytoplasmic
Bacillus campisalis	GH32	816385092	Cytoplasmic
Bacillus salsus	GH32	1086768167	Cytoplasmic
Bacillus shacheensis	GH32	1177501344	Cytoplasmic
Gracilibacillus massiliensis	GH32	960414052	Cytoplasmic
Gracilibacillus massiliensis	GH32	1177189985	Cytoplasmic
Gracilibacillus orientalis	GH32	1097917763	Cytoplasmic
Gracilibacillus halophilus YIM-C55.5	GH32	477569196	Cytoplasmic
Halanaerobium kushneri	GH32	1131796866	Cytoplasmic
Halanaerobium kushneri	GH32	1131796873	Cytoplasmic
Halanaerobium salsuginis	GH32	1097245539	Cytoplasmic
Halobacillus aidingensis	GH32	1086630634	Cytoplasmic
Halobacillus dabanensis	GH32	635348945	Cytoplasmic
Halobacillus dabanensis	GH32	635346008	Cell wall
Halobacillus mangrovi	GH32	1181755621	Cell wall
Halobacillus massiliensis	GH32	1176393270	Cell wall
Halobacteroides halobius DSM 5150	GH32	433669988	Cytoplasmic
Halonatronum saccharophilum	GH32	653090487	Cytoplasmic
Jeotgalibacillus malaysiensis	GH32	747141697	Cytoplasmic
Lentibacillus jeotgali	GH32	498217618	Cytoplasmic
Oceanobacillus jeddahense	GH32	751268659	Cell wall
Paludifilum halophilum	GH32	1230800952	Cell wall
Paraliobacillus ryukyuensis	GH32	1160865958	Cytoplasmic
Pontibacillus yanchengensis Y32	GH32	701590568	Cytoplasmic
Pontibacillus halophilus JSM 076056	GH32	717933775	Cell wall
Salinicoccus halodurans	GH32	820757724	Cytoplasmic
Salinicoccus roseus	GH32	748402524	Cytoplasmic
Salipaludibacillus agaradhaerens	GH32	1154205045	Cytoplasmic
Salsuginibacillus kocurii	GH32	517752615	Cell wall
Sediminibacillus massiliensis	GH32	1148933178	Cytoplasmic
Terribacillus aidingensis	GH32	664799307	Cell wall
Thalassobacillus devorans	GH32	1188385981	Cell wall
Thalassobacillus devorans	GH32	1188385981	Cell wall
Thalassobacillus sp. TM-1	GH32	1011552066	Cell wall
Virgibacillus halodenitrificans	GH32	983529701	Cytoplasmic
Alteribacillus bidgolensis	GH68	1086730359	Extracellular
Bacillus salsus	GH68	1086768998	Extracellular
Halobacillus aidingensis	GH68	1086631779	Extracellular
Halobacillus alkaliphilus	GH68	1097757826	Extracellular
Halobacillus kuroshimensis	GH68	654486067	Extracellular
Halobacillus massiliensis	GH68	1176393269	Extracellular
Halobacillus sp. BAB-2008	GH68	432189158	Extracellular
Halobacillus sp. BBL2006	GH68	725818662	Extracellular
Halobacillus sp. KGW1	GH68	1011666739	Extracellular
Halobacillus mangrovi	GH68	1181752450	Extracellular
Halobacillus mangrovi	GH68	1181755734	Extracellular
Pontibacillus chungwhensis BH030062	GH68	701631088	Extracellular
Pontibacillus halophilus	GH68	1175003164	Cytoplasmic membrane
Pontibacillus halophilus JSM 076056	GH68	717929998	Cytoplasmic membrane
Terribacillus aidingensis	GH68	664799306	Extracellular
Other species			
Actinopolyspora alba	GH32	1098127403	Cell wall
Actinopolyspora mortivallis	GH32	518692128	Cell wall
Cellulosimicrobium cellulans	GH32	922583922	Cell wall
Cyclobacterium halophilum	GH32	1094940803	Cytoplasmic
Fabibacter pacificus	GH32	1094716305	Cytoplasmic
Gramella echinicola	GH32	652540875	Periplasmic/ Cytoplasmic membrane
Haloactinobacterium album	GH32	1089028138	Cytoplasmic
Longimonas halophila	GH32	1267200448	Cytoplasmic
Nocardiopsis salina	GH32	516208643	Extracellular/Cell wall
Rhodotermus marinus	GH32	262333784	Cytoplasmic
Salinivenus iranica	GH32	1333939480	Cytoplasmic
Salinivenus lutea	GH32	1333903702	Cytoplasmic
Spirochaeta africana DSM 8902	GH32	383107025	Cytoplasmic

2017; Rouwenhorst et al., 1990; Velikova et al., 2017) and their presence in Archaea may indicate their sucrose-/fructan-degrading functions. Nevertheless, one cannot dismiss the possibility of pseudogenes, which are inactive genes that are not expressed, and usually take longer time to disappear in Archaea compared to Eubacteria (van Passel et al., 2007). All these hypotheses set new questions regarding halophilic GH-J enzymes, and require extensive experimental validation at the DNA, RNA, enzyme and product levels.

Another interesting property of haloarchaeal GH32-like enzymes is the presence of *ca.* 250-260 amino acid long sequences at their N-terminal, which are absent in Eubacterial, fungal or plant GH32 enzymes. Local alignment (NCBI BLAST) of these enzymes reveals only their homologues among Archaea, and multiple alignment (NCBI COBALT) shows that although there are conserved regions among all domains of life, these N-terminal sequences are specific to Archaea. Homology modelling (SWISS-MODEL) could not be carried out due to lack of any suitable template sequence. Secondary structure prediction via PSIPRED (Jones, 1999) revealed that these archaeal sequences are comprised of various beta-strands and alpha-helixes (Fig. 3). For now, the function of these N-terminal sequences is completely unknown and

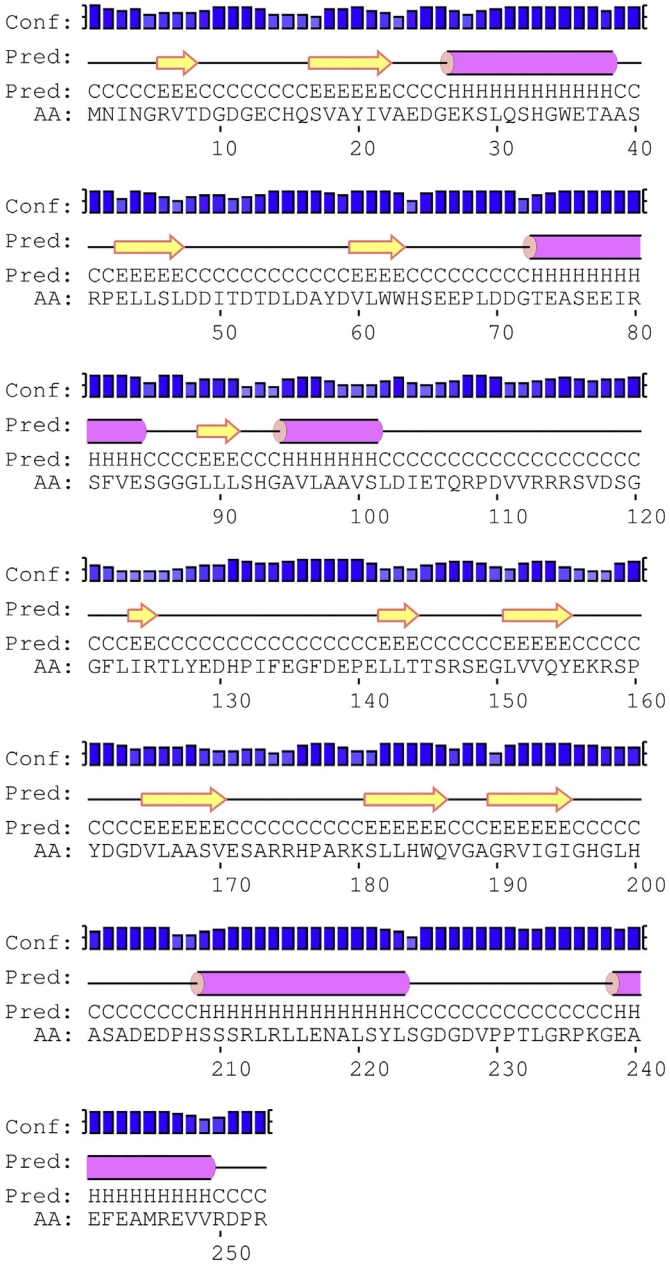

Fig. 3. Secondary structure prediction for *Haladaptatus paucihalophilus* DX253 GH32-like enzyme's 253 amino acid long N-terminal sequence, obtained with PSIPRED. Arrows: β-strands, cylinders: α-helixes, straight lines: coils, vertical bars: confidence of prediction.

they present a unique and novel challenge for archaeal protein research.

According to homology modeling (SWISS-MODEL) using PDB: 3WPV as the template, active site residues D48, R200, D201, E264 and E266 in *H. litoreus*' GH68-like protein are all located and exposed in suitable positions for enzyme activity. Upon further inspection of halophilic GH68-like proteins in Tables 1 and 2, Archaeal GH68-like proteins were shown to carry many strictly conserved amino acid residues that are absent in Eubacteria. Homologues of R53, R55, P79, D124, Y129, Q147, H172, W203, F204, P222, N233, Y277, Y331, W333, H330, F347 and F373 in *Haladaptatus litoreus*' GH68-like protein (accession number: 1131913889) are all strictly conserved among all investigated Archaea, and not conserved in crystal structures of *Erwinia amylovora, Bacillus subtilis,* or *Gluconacetobacter diazotrophicus* levansucrases. Interestingly, W203 homologues are also strictly conserved in halophilic Eubacteria genera of *Halobacillus* and *Pontibacillus*, but not in halophilic γ-proteobacteria or any mesophilic Eubacteria. Furthermore, all Archaeal GH68-like proteins show a conserved TFAGPL sequence, occurring only in one Eubacterial GH68-like protein (accession number: 701631088), and further research into its functional significance is warranted.

With sucrose being the main fructosyl donor for the actions of GH-J clan enzymes, the question of its origin in hypersaline environments arises. All halophilic Archaea and Eubacteria in Tables 1 and 2 are heterotrophic organisms, which means that they rely on external nutrients for energy generation. According to NCBI, neither sucrose-phosphate synthase (EC 2.3.1.14) nor sucrose phosphate phosphatase (EC 3.1.3.24) are present in any Archaea. However, there are known Eubacterial and algal sucrose producers in hypersaline environments, such as two haloalkaliphilic *Methylobacter* strains from moderately saline lakes of Tuva, Central Asia (Khmelenina et al., 1997), several cyanobacterial species such as *Chlorogloea fritschii, Coleofasciculus chthonoplastes, Dactylococcopsis salina, Halothece* sp. and *Nodularia spumigena* (Oren, 2012; Loukas et al., 2018), and the green alga *Dunaliella tertiolecta* (MacRae and Lunn, 2012; Müller and Wegmann, 1978). If GH-J clan enzymes are functional in halophilic Archaea and Eubacteria, their actions would require sucrose synthesized by other organisms in their environment. However, due to the lack of any study that elucidates such relationships to the best of our knowledge, these hypotheses remain speculative, but they present an exciting subject of further investigation.

5. Fructans and FTs in plant adaptation to salt

Moderately salt-tolerant crop species include mostly cereals such as barley, oat, wheat and rye (Shahbaz and Ashraf, 2013). Nevertheless, many important crops and fruits are salt-sensitive, such as bean (Hoffman and Rawlins, 1970), lettuce (Ayers et al., 1951), banana (Israeli et al., 1986), tomato (Shalhevet and Yaron, 1973) and rice. Especially in the latter, the combination with flooding, so called salt flooding, is an important constraint. Ethylene signaling and its effects on central metabolism, including sugar dynamics, are involved in such processes (Locke et al., 2018; Xu et al., 2006) with dedicated roles for starch degrading enzymes (amylases; Ismael et al., 2009) and sucrose splitting enzymes (Susy's, invertases). Vacuolar and cell wall invertases may in fact be considered as hydrolytic type of GH32 FTs (Versluys et al., 2018). While Susy dominates carbohydrate metabolism in the deeper submerged parts suffering from hypoxia (Guglielminetti et al., 1997), vacuolar invertases are involved in the cellular elongation processes of the uppermost stem internode in an attempt to grow above the water surface level (Hirano et al., 1996). Ram (2000) found an increase in vacuolar invertase activity during flooding of two *Brachiaria* species, together with an increase in the hexose reducing sugars, which was higher in the flooding tolerant *Brachiaria mutica*.

The role of vacuolar invertase under osmotic stress, including salinity, has been studied in several plant species. In *Schenkia spicata* root cultures, although activity levels of vacuolar invertase were generally very low, there was a significant rise in protein abundance in the salt-tolerant species under high salt conditions (100-200 mM NaCl). Cell wall invertase levels, however, decreased in both the salt-sensitive and salt-tolerant genotype under such saline conditions (Misic et al., 2012). An increase in vacuolar invertase expression and activity was also observed in the salt sensitive species Arabidopsis after salt stress. However, the activity of the enzyme was much higher under drought or ABA treatment than under salt stress. Cell wall invertase showed no increase in activity to any of the treatments (Yamada et al., 2010). In maize (*Zea mays*), another salt-sensitive species, the induction of vacuolar invertase IVR2 was shown in different tissues under water stress (drought), while no significant increase was apparent for cell wall invertase or neutral invertase. Concomitant with an increased activity, hexose levels were higher under stressed conditions. The upregulation of vacuolar invertases has thus been observed under different stresses, indicating a possible strategy for salt sensitive or moderately salt tolerant species to increase hexose levels during osmotic stress (Kim et al., 2000).

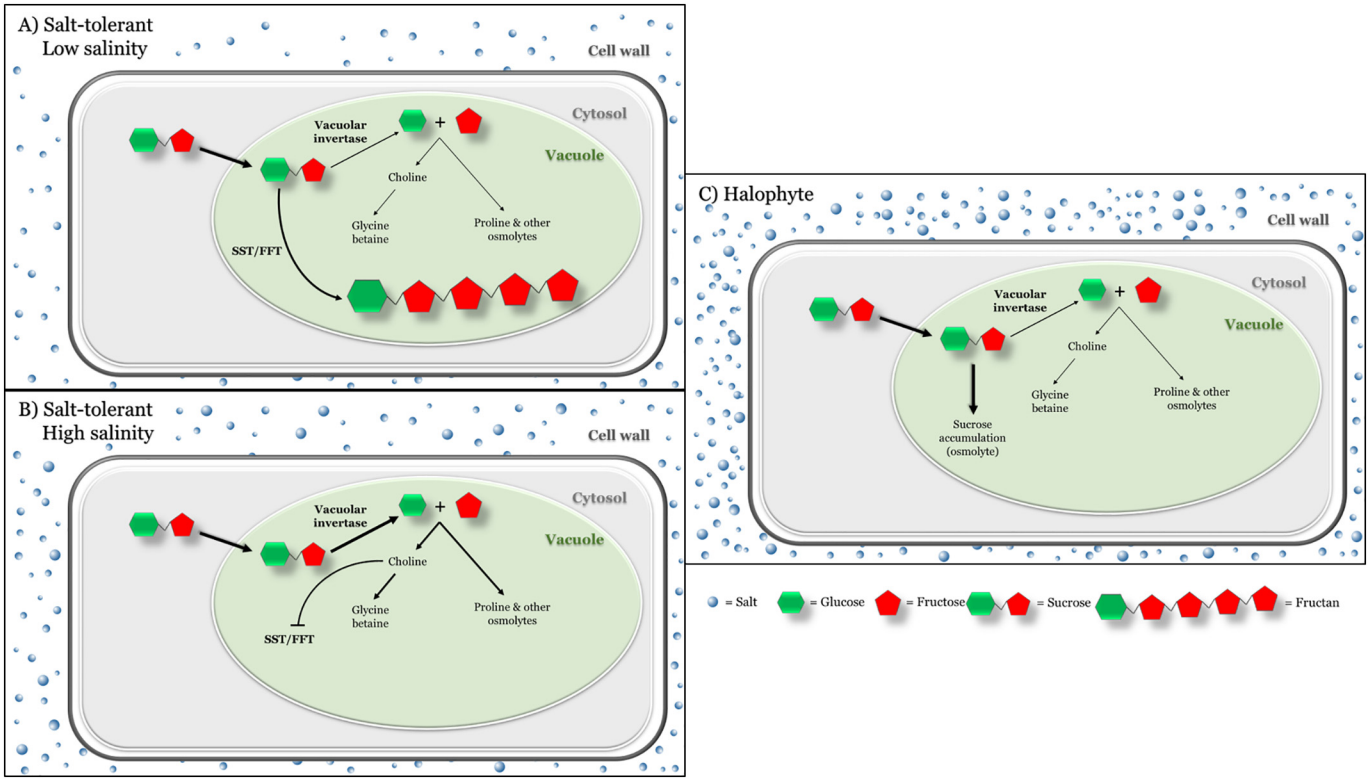

Fig. 4. Salt tolerance mechanisms in salt-tolerant plants and halophytes. A) Under low to moderate salinity, salt-tolerant species such as barley can use sucrose for fructan synthesis or degradation to the hexoses glucose and fructose for osmolyte production. B) Fructan synthesis is suppressed under (discontinuous) higher salinity conditions as it provides no advantage in salt tolerance mechanisms. All sucrose is diverted to vacuolar invertase-based hydrolysis to increase osmotic potential or fuel osmolyte production. C) To date, no real halophytes are known to produce fructans. Sucrose is often accumulated as an osmolyte itself, and/or converted to glucose and fructose by vacuolar invertase, which is readily used to fuel osmolyte production. NaCl concentrations for salt-tolerant species can be similar or even higher than for halophytes, but since these peaks in salinity are temporary, on average the salinity stress is lower than for halophytes, usually prone to continuous levels of salts.

Few studies have already indicated a role for sugars in salt tolerance mechanisms in halophytic species. Hartzendorf and Rolletschek (2001) showed changes in small sugars glucose, fructose, and sucrose under different salinity levels in *Phragmites australis*, a species occurring in both fresh- and saline waters. These changes in hexose to sucrose levels can indicate a role for invertase enzymes during salinity stress. In 2007, Gagneul et al. measured sugars as an important class of active osmolytes in the halophyte *Limonium latifolium*. Nevertheless, a different study in *Tecticornia pergranulata* showed a decrease in sugars when salinity was combined with flooding stress, indicating the higher complexity in sugar response for halophytes prone to submergence (Colmer et al., 2008). A comparison between drought and salt stress in the xero-halophyte *Atriplex halimus* shows a potential difference in invertase regulation. While an increase in glucose and fructose was seen under drought, salinity caused an increase mainly in sucrose levels, although hexose levels were somewhat higher compared to the control treatment (Fig. 4C; Ben Hassine and Lutts, 2010). In *Thellungiella halophila*, sugars and proline are the most important osmolytes under salt stress. Using comparative proteomics, many proteins involved in carbohydrate metabolism were upregulated under higher salinity. The pathways that were most affected include starch production and sucrose metabolism. Concerning invertases, no up- or downregulation was found in this study (Wang et al., 2013).

While data on the presence of fructans and genuine fructan biosynthetic enzymes (stricto senso FTs) in pertinent halophytes are completely lacking, the literature contains some data on FTs during salinity in non-halophyte species that can tolerate relatively high amounts of salt (moderate salt tolerant species). Among these, *Lolium perenne* is a neofructan producing grass species widely used as turf or forage grass (Lasseur et al., 2006; Pavis et al., 2000). In a study on ten cultivars, fructan content increased when salinity went up to 300 mM NaCl (Jiang et al., 2013). Another study by Hu et al. (2013) further investigated changes in carbohydrate metabolism under salinity stress. Expression levels of 6-SFT from the *L. perenne* cv. Overdrive increased under increasing NaCl levels up to 400 mM in all organs measured. Compared to cv. Overdrive, cv. PI 538976, a salt-sensitive cultivar, showed only a small increase in the roots. Since cv. Overdrive survives under 400 mM salt, it may be considered to survive in similar conditions as halophytes. In *Helianthus tuberosus*, another fructan accumulator, salt treatments of up to 250 mM led to a decrease in sprouting and a reduction in fructan levels in the tubers, although fructans with a low degree of polymerization originally increased. The proportions of fructans with high and low degree of polymerization changed significantly at different time points as well as under different salt concentrations. These experiments indicate that these oligosaccharides may aid sprouting and salt stress resistance by changing the degree of polymerization, while the total fructan content remains unchanged (Bhagia et al., 2018; Luo et al., 2018; Zhang et al., 2018).

Among the cereal crops, wheat and barley are important fructan accumulators. Bread wheat (*Triticum aestivum*) is one of the most important cereals worldwide with a production of over 700 million tonnes (Kumar et al., 2017). It produces branched type of fructans, graminans, containing both β-2,1- and β-2,6-linkages, which function as reserve carbohydrate and specifically during kernel development (Pollock and Cairns, 1991; Verspreet et al., 2013). Wheat is known as a relatively salt-tolerant crop compared to many crop species (Munns et al., 2006; Tanji and Kielen, 2002), although salt tolerance differs between cultivars (Kafi et al., 2003). Barley (*Hordeum vulgare*), as one of the most salt-tolerant crops, has a higher salt tolerance than bread wheat (Henry, 1988). It produces several types of fructans with differing functions during grain development. During the pre-storage phase it accumulates levans and graminans, while inulin-type fructans are produced during

the storage phase. While the former supports cell division by maintaining low sucrose levels, the latter may be involved in ROS detoxification and possibly water retention (Peukert et al., 2014; Versluys et al., 2018). Like in wheat, barley cultivars have different levels of salt tolerance.

Salinity stress influences wheat at different levels in development by delaying germination and subsequent seedling growth (Guo et al., 2015), affecting photosynthesis and causing a decrease in relative water content, which reduces yields significantly (Hasanuzzaman et al., 2017). On the other hand, barley can maintain growth and photosynthetic capacity through Na^+ sequestration in the vacuole and osmolyte production in the cytosol (Witzel et al., 2009). Widodo et al. (2009) showed a significant difference in salt tolerance between barley cultivars Clipper and Sahara. The salt tolerant cv. Sahara showed increased sugar concentrations after 3 weeks of salt exposure, a response that has also been observed in certain halophyte species (Gagneul et al., 2007; Wang et al., 2013). In a recent manuscript, the importance of different salt tolerance mechanisms was also investigated in wheat cultivars with differing salt tolerance. The most tolerant cultivar, cv. Kharchia, showed increased membrane stability, antioxidant potential and a higher K^+/Na^+ ratio when exposed to 200 mM NaCl. Additionally, a higher accumulation of osmoprotectants was observed. Cultivars more sensitive to salt stress were characterized by low content of proline and soluble sugars (Kumar et al., 2017).

Carbohydrates are known to often be accumulated under salt stress in function of osmotic adjustment. In wheat, there is a trend towards increased small sugar levels under increasing salinity (Weimberg, 1987). By comparing salt-tolerant and salt-sensitive cultivars, Kafi et al. (2003) measured a more significant increase in the tolerant species up to 300 mM NaCl. Generally, an increase in sucrose levels is also observed in barley as salinity increases. Wild barley is better adapted to high salt conditions than the cultivated barley and showed a higher increase in several compatible solutes (Ashraf and Foolad, 2007; Chen and Murata, 2011; Ueda et al., 2002; Wu et al., 2013). In wheat, Kafi et al. (2003) also showed a significant increase of proline, which is a common osmotic response of wheat during salinity (Poustini et al., 2007).

Besides small soluble sugars and non-carbohydrate osmoprotectants, fructans may be involved in salt tolerance mechanisms in these salt-tolerant species, similar as observed in *L. perenne*. Fructans indeed have special physicochemical properties when compared to other polysaccharides: longer linkages between fructosyl units give them a more flexible structure, the ability to sequester water and stabilize membranes. This positions them as potential membrane receptor cross linkers or hydroxyl radical scavengers in the vicinity of membranes (Keunen et al., 2013; Matros et al., 2015; Peukert et al., 2014; Valluru et al., 2008). In plants, however, fructans are localized in the vacuole, which limits their functioning as osmolyte and ROS scavenger during salinity stress to the tonoplast (Peshev et al., 2013). Nevertheless, if plants can bring fructans to the outer side of the plasma membrane through a mechanism of exocytosis, as proposed by Valluru et al. (2008), their role in salinity tolerance could increase vastly. Indeed, under stress, the presence of fructans in the apoplast of fructan accumulators has been shown (Livingston and Henson, 1998; Valluru and Van den Ende, 2008 and references therein). In other cellular organelles such as chloroplasts and mitochondria, where ROS homeostasis is also of critical importance, plants will likely rely on other players, such as glucose, sucrose and raffinose-family oligosaccharides (Nägele and Heyer, 2013; Xiang et al., 2011).

In general, no simple correlations could be found between overall fructan contents and reported salt tolerance thresholds focusing on yield in a large array of crops (http://www.fao.org/docrep/005/y4263e/y4263e0e.htm). Besides the involvement of additional factors determining salt tolerance, this discorrelation may be explained by differential fructan quantification methodologies and not properly taking into account the plant physiological context and the species-specific spatio-temporal dynamics of fructans during sampling. In the study by Kafi et al. (2003) the salt-tolerant wheat cultivar showed the highest fructan content at high salinity. Kerepesi et al. (2002) exposed four cultivars with differing tolerance to drought and/or salinity to water stress (PEG), followed by salt treatment (200 mM NaCl). While an overall increase in fructans was measured after drought exposure, fructan content only increased further in the salt-tolerant cultivars. In a more recent study, upregulation of gene expression of two fructan enzymes, 1-SST and 6-SFT, was apparent in the salt-tolerant cultivar, but not in the salt-sensitive one (Fig. 4A). Higher expression of fructan exohydrolase and vacuolar invertase was also observed in the more resistant cultivar. The authors proposed that the salt-tolerant cultivar shows less yield loss during salt stress due to an increased fructan production and more efficient fructan degradation and sucrose export from the stems (Sharbatkhari et al., 2014, 2016), followed by sucrose import in the kernels and temporal fructan accumulation, prior to starch synthesis and accumulation (Verspreet et al., 2013; Zhang et al., 2015). This suggests that a close interplay between the different fructan pools and a good connection between fructan and sucrose metabolisms are critical, especially under stress. In barley, Bagheri and Sadeghipour (2009) showed an increase in fructan content in four cultivars after exposure to 100 mM NaCl. When exposed to higher salinity, fructan levels dropped, while sucrose increased, thus showing a potential mechanistic difference in fructan accumulation between wheat and barley under saline conditions. Nevertheless, the barley cultivars used in this study may all be salt-sensitive ones, making a comparison to the mechanisms in wheat difficult.

Recently, salt stress responses have been compared between wheat and barley in one study, using wheat cv. Asakaze and barley cv. Manas. The authors showed higher proline and glycine betaine content in the roots of the barley cultivar, together with a decrease in sugar content. In wheat roots, both proline and glycine betaine accumulated to lesser extent (Darko et al., 2017). There may be a direct link between the accumulation rates of glycine betaine and fructan in barley and wheat, as suggested in Chevalier and Rupp (1993). The authors showed an inhibitory effect of choline chloride, choline being a precursor in glycine betaine biosynthesis (Sakamoto and Murata, 2002), on a 1-SST enzyme from wheat. Thus, in fructan accumulators, accumulating glucose (because of growth cessation due to increasing salt) may induce choline synthesis, which inhibits 1-SST to arrest further fructan synthesis in favor of glycine betaine synthesis (Fig. 4B). This elegant feedback mechanism may explain why the fructan content in barley cultivars in the study by Bagheri and Sadeghipour (2009) decreased at higher salt concentration, as Darko et al. (2017) showed an increased accumulation of glycine betaine in the tested barley cultivar in comparison to wheat. In the latter study, the leaves of both wheat and barley showed a significant increase in sugars. Since an increase in fructose and glucose may be caused by increased invertase activity, the authors also measured vacuolar invertase activities and showed higher activity for barley cv. Manas as compared to the wheat cultivar. However, invertase upregulation is only partially responsible for the increased osmotic potential in the leaves (Darko et al., 2017).

Nevertheless, when we would consider the most 'pertinent halophytes' as those species that grow and reproduce at continuous salt levels of > 500 mM, a role for GH32 stricto sensu FTs in salt stress seems to be missing in this category. Indeed, after extensive searching efforts within such species, we could not find any notions on the presence of fructans or active GH32 FTs. It can thus be hypothesized that under conditions of continuous very high salt, evolution did not recruit the fructan syndrome.

While a positive correlation between fructans and halophiles seems to exist in microbes, in plants the accumulation of fructans seems to exist only in non- or moderate halophytic species, but not in the most pertinent halophytes. The biggest difference with the microbial world is most likely the localization of fructans and fructan synthesis. While microbes produce their fructans extracellularly, in plants this process takes place within the cell inside the vacuole. This creates a very different situation under high salt stress and we hypothesize that fructan

synthesis under such conditions can have detrimental consequences for the plant. Firstly, carbohydrate solubility decreases with degree of polymerization (Mensink et al., 2014). Under such high salt concentrations, the total amount of solutes, including the fructan polymers, would just become too high and leading to overall precipitation and vacuolar instability, on its turn leading to cell death (Hara-Nishimura and Hatsugai, 2011). Secondly, the production of fructans from sucrose does not lead to an increase in osmotic potential. In addition, there would be a release of glucose molecules which may, depending on the growth dynamics, lead to glucose accumulation. Above a certain temperature dependent threshold level, this may lead to unwanted Maillard reactions with the free amino acid pool disturbing biosynthesis and energy production pathways (Businge and Egertsdotter, 2014; Wettlaufer and Leopold, 1991). Since fructose is much more reactive than glucose in Maillard reactions (Businge and Egertsdotter, 2014), this is expected to play an even more extended role when sucrose is hydrolysed to fructose and glucose by invertase, at least in the case where growth and synthetic abilities are restricted, allowing hexose accumulation. Although this sucrose splitting process could increase osmotic potential, several studies show that pertinent halophytes rather accumulate sucrose to high concentrations as compatible solute. Indeed, in several monocot halophyte species such as *Juncus maritimus*, sucrose constitutes over 50% of total soluble sugar content (Gil et al., 2013 and references therein). In the study by Ben Hassine and Lutts (2010), the authors also found an increase in sucrose content in *Atriplex halimus* under salt stress in both roots and leaves.

6. Halofructans and halophy(t/l)es for the future

In light of increasing soil salinization worldwide, aided by global climate change, creating more stress-tolerant crops will be a necessary goal. One of the most important targets in this aspect is rice as it is very important in global food production and very salt sensitive. Increases in soil salinity as well as sea-level rises are already affecting rice yields (Hoang et al., 2016). Nevertheless, the effects are cultivar-specific since some cultivars are less salt-sensitive than others (Rahman et al., 2017). Very recently, Chinese researchers reported the development of a rice cultivar that is able to grow in seawater, tolerating salt flooding (http://independent.co.uk/news/rice-seawater-chinese-scientists-food-200-million-a8017971.html). This clearly indicates the efforts that are being made in salt stress research in crops. Nevertheless, rice is not a typical fructan accumulating plant, and a transcriptome analysis of the salt-resistant rice cultivar SR86 (Sea Rice 86) by Chen et al. (2017) revealed no role for FTs or fructans in salt tolerance, although the occurrence of fructan at very low concentrations and the involvement of fructan signaling had not been investigated.

Spraying with different osmoprotectants has already shown some promising results (Abdel Latef and Tran, 2016; Hasanuzzaman et al., 2014; Zhang and Rue, 2012). Several osmoprotectants, including glycine betaine and proline, have already been tested on different wheat cultivars, showing an increase in salt tolerance (Hasanuzzaman et al., 2017 and references therein). Within the category of sugars, trehalose priming has been investigated at the seed stage (Yan and Zheng, 2016). Since an upregulation of FTs/acid invertases is observed on multiple occasions in more salt-tolerant plants, boosting invertase expression in salt-sensitive crops (or FTs in fructan crops) may increase salt tolerance. The expression of an apoplastic yeast invertase in transgenic tobacco shows a significant increase in salt tolerance due to the accumulation of sucrose in source organs and hydrolysis to glucose and fructose, thus creating a change in osmotic pressure (Fukushima et al., 2001).

Furthermore, the introduction of fructan producing enzymes in non-fructan crops may enhance salinity tolerance as well. Bie et al. (2012) already showed fructan production in tobacco plants transgenically expressing wheat FTs (both 1-SST and 6-SFT), resulting in increased tolerance to different abiotic stresses, including salinity. A study by Li et al. (2007) evidenced a strong increase in cold tolerance by transgenic expression of a lettuce 1-SST in tobacco. Since overall levels of 1-kestotriose produced were extremely low, this points towards signaling effects. Several studies have tested the transgenic production of fructans in crop species with promising results. In species such as potato (van der Meer et al., 1994), maize (Caimi et al., 1996), sugar beet (Sévenier et al., 1998; Smeekens, 1998), and rice (Kawakami et al., 2008) transgenic expression of FT genes, either from plant or microbial origin, led to significant fructan production. These plants not only show increased abiotic stress tolerance, but also their fructans have health-stimulating effects for the consumer (Peshev and Van den Ende, 2014). The behaviour of these transgenics under salt stress, however, has never been cleary investigated and it would be interesting to put more focus on salinity in this context. Alternatively, non-transgenic approaches may also be used to produce crops with higher fructan contents. For example, Jin et al. (2017) recently obtained barley with higher fructan content through crossbreeding, which can be used to evaluate salinity stress under increased fructan content. Nevertheless, it is unclear whether a further increase in fructan content will be reflected in higher salinity tolerance. In this regard, introducing fructans in plants with no fructan background may probably lead to more pronounced improvements in stress tolerance.

In agriculture, halophytes have since long been investigated because they allow agricultural practice on dry and saline soils. Halophyte species such as *Salicornia* and *Atriplex* have been tested as potential fodder plants for the feeding of livestock. In combination with fodder from non-halophyte species, they can provide sufficient nutritional value. In the context of human food consumption, some halophyte species are already sold on the market as sea vegetables and salad crops. A well-known example is *Chenopodium quinoa*, or just quinoa, a seed crop with high tolerance to salinity (Adolf et al., 2013). Halophyte production for ornamental use has also been investigated as many halophyte species produce attractive flowers (Ventura et al., 2015 and references therein). The use of halophytes as bioenergy crops in biofuel production is advantageous as it doesn't compete with conventional agriculture when grown on saline soils (Sharma et al., 2016). Besides halophytes, halophiles are also considered valuable sources for industrial applications thanks to their robust nature and ease of non-sterile production conditions made possible by high salinity. Halophiles have been utilized in the production of bio-plastics, enzymes, ectoines and bio-surfactants (Yin et al., 2015).

Apart from their industrial importance, halophiles are also of special interest to astrobiology field. After the discovery of perchlorate salts on Mars soil, Oren et al. (2014) investigated the growth of several halophilic Archaea and the bacterium *Halomonas elongata* in the presence of perchlorate and they observed that all strains grew well in the presence of 0.4 M perchlorate concentration. The authors concluded that if brines containing perchlorate are present in Mars as suspected, perchlorate may act as an electron acceptor for halophiles under anaerobic conditions on Mars.

Fructans constitute one of the most widespread functional biomolecules in nature and escalating number of evidences on their health promoting effects made these polymers an important class of platform chemicals. In fact, they have the largest market share among the natural functional additives in food sector and their recognition as multi-purpose adjuvants in drug delivery and health sector is expected to further boost up their uses in high value biotechnological applications. Whereas plants are the main resources of inulin, graminan and agavin type fructans, levan type fructans are commercially produced by microorganisms. Challenges associated with resource availability due to seasonal climate changes as well as the higher titers reached by optimized and fully controlled bioprocesses make microbial systems the preferred choice for fructan production. However, there are also major limitations of microbial fermentations like the risk of contamination, which requires the use of expensive infrastructure to maintain sterility. Especially for mesophilic industrial producer strains demanding mild conditions, this issue becomes more cost intensive since they call for

additional strain improvement investments for obtaining robust, stress and contamination resistant mutants via expensive and lengthy strategies. On the other hand, with their metabolic abilities to survive under challenging conditions, extremophiles are recognized as valuable sources for next generation processes of industrial biotechnology (Chen and Jiang, 2018), especially since we will be forced to use sea water as the cheapest water source due to increasing fresh water scarcity. The risk of contamination will be greatly reduced in the case of halophiles, due to high salt concentrations which can be used.

7. Conclusions

Microbes typically produce their fructans in the extracellular environment, while in plants this takes place in the intracellular environment, more precisely the vacuole. This may be linked to plant/microbe differential physiological reactions, including an inhibition of fructan syndrome persistence (plants) and a boost for fructan syndrome development (microbes) under strongly increasing (and continuous) salt stresses.

A clear fructan/salt tolerance relationship is still missing for fructan accumulating crops. Although a further increase in fructan levels may contribute to increased salt tolerance in fructan and non-fructan accumulating crops, likely this approach has its limitations (fructan solubility/oversaturation issues). However, it would be interesting to investigate salt tolerance in the formerly developed transgenic crops (potato, maize, sugar beet, rice, listed above) carrying FT genes of plant or microbial origin.

Most genuine halophytes opt to accumulate sucrose as such, and choose not to polymerize it into fructan. Sucrose splitting for hexose synthesis is only occurring when these hexoses can be used for the synthesis of other osmolytes (glycine betaine, proline), avoiding excessive hexose accumulation above certain, temperature dependent threshold levels that would potentially lead to unwanted Maillard reactions *in planta*. For now, apart from the development of rice cultivars that can grow in sea water, applications with genuine halophytes are more limited compared to halophiles, which are much more straightforward and diverse.

Fructan syndrome among extremophiles is an underexplored area with very few literature reports mostly limited to *Bacillus* and *Halomonas* species (Versluys et al., 2018). Halophiles are of particular interest since they enable non-sterile and continuous production in saline (even sea) water and they serve as feasible genetic sources for industrially important compounds like osmolytes and extremozymes (Chen and Jiang, 2018). As such, their use for microbial levan production has also been studied in depth (Sarılmışer Kazak et al., 2015; Toksoy Öner et al., 2016) and on-going studies are focusing on the development of low-cost non-sterile production processes. With the emergence of new studies and reports on fructanogenic halophiles, they are expected to become a serious alternative to the current production processes relying on mesophiles.

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