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Review

Metabolic reprogramming and its role in plant cold acclimation

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Abstract: Plants have evolved tightly regulated strategies to adapt and acclimate to a changing environment to ensure their survival. Various environmental factors affect plant distribution, growth and yield. Low temperature belongs to those abiotic factors which significantly constrain range boundaries of plant species. Exposing plants to low but non-freezing temperature induces a multigenic processes termed cold acclimation, which finally results in an increased freezing tolerance. Cold acclimation comprises reprogramming of the transcriptome, proteome and metabolome and affects communication and signaling between subcellular organelles. Reprogramming of the central carbohydrate metabolism plays a key role in cold acclimation. This review summarizes current knowledge about the role of carbohydrate metabolism in plant cold acclimation. A focus is laid on subcellular metabolic reprogramming, its thermodynamic constraints under low temperature and mathematical modelling of metabolism.

Keywords: cold acclimation, metabolic reprogramming, carbohydrates, subcellular metabolism, sucrose cycling, enzyme activity, Arrhenius equation, kinetic modelling, *Arabidopsis thaliana*

1. Introduction

Due to their sessile lifestyle, plants have to cope with fluctuating environmental conditions. Fast acclimation to unfavourable surroundings is necessary to ensure survival. The responses of plants to abiotic stress, e.g. cold, heat or drought, are diverse and highly dynamic. Exposing plants to abiotic stress induces an immediate response before plants acclimate to establish a new metabolic homeostasis [1-4]. Range boundaries of many temperate herbaceous plants, and also of *Arabidopsis thaliana*, are defined by low temperature [5]. It is estimated that only around 5% of the earth's surface is frost free which immediately implies that frost has a significant impact on agricultural production [6]. In many temperate plant species, the exposure to low, non-freezing temperatures results in an increase of freezing tolerance through a multigenic process termed cold acclimation. After immediate stress response which is characterized by transient physiological, biochemical and molecular reprogramming, a stable long-term adjustment of metabolism is gained [7]. Plant acclimation to low temperature is of interest since a long time [8] and continues to be of relevance with respect to modern changes in climatic conditions, leading to local stronger temperature extremes and locally increased risk of frost damages in spring [9-13]. Environmental factors differ across a species range. For example, comparison of Swedish *Arabidopsis thaliana* accessions showed that the most tolerant ones have historically experienced more precipitation combined with low temperature, and this combination of factors seems to play a crucial role for increasing freezing tolerance [14].

Across the plant kingdom, diverse strategies are employed to react to decreasing temperature. Therefore, trying to define a universally valid response model has been difficult, although there are

certain general responses which are common between different species, e.g. from grasses to trees [15]. Even within the well-studied model plant *Arabidopsis thaliana* there is a strong variation in cold response, cold hardiness and freezing tolerance between different natural accessions [16, 17], because genetic architecture varies across the species range [14]. Nevertheless, finding common mechanisms, as well as understanding specialized responses, will help breeders to find new strategies to stabilize crop yields with respect to potential frost risks. This review provides an overview of the role of carbohydrates and the regulation of their metabolism during plant cold acclimation. A focus is laid on metabolic reprogramming, subcellular metabolism and mathematical modelling to study biochemical regulation under low temperature.

Perception of low temperature

Cellular membrane systems are primary targets for freezing injury due to extracellular ice formation and cellular desiccation [18, 19]. Beyond, a change of membrane fluidity represents a signal which is involved in cold perception [20-22], e.g. leading to the opening of Ca^{2+} channels [23, 24]. Plasma membrane located kinases, e.g. Cold Responsive Protein Kinase 1 (CRPK1), were found to transduce signals into the nucleus [25]. Modified Ca^{2+} concentrations result in signaling cascades through a multitude of MITOGEN ACTIVATED PROTEIN KINASES (MAPKs), eventually triggering comprehensive transcriptome changes (for a summary see [23] and [26]). Furthermore, decreased temperature and thereby decreased membrane fluidity activates a diacylglycerol kinase (DAGK) pathway, consequently products might prevent membrane damage in freezing conditions [22, 27].

Transcriptional regulation plays an essential role in cold acclimation. The analysis of freezing tolerance across a diverse set of natural accessions of *Arabidopsis* thaliana revealed a strong correlation between freezing tolerance and MYB transcription factors PRODUCTION OF ANTHOCYANIN PIGMENT 1 and 2 (PAP1 and 2) which are involved in regulation of flavonoid metabolism [16]. Supportingly, a more recent study provided further evidence for a function role of PAP1 and 2 in *Arabidopsis* cold acclimation and freezing tolerance [28]. Yet, in addition to PAP1 hundreds and up to thousands of transcripts are reprogrammed during plant cold acclimation. The C-repeat Binding Factor (CBF) pathway in *Arabidopsis* is a central player in freezing tolerance illustrates the complexity of cold acclimation [29]. The CBF locus and its three genes CBF1-3 encode transcription factors (TFs) which are induced within minutes of exposure to low non-freezing temperatures [30]. CBF TFs alter the expression of about hundred cold-regulated (COR) genes, also known as the CBF regulon [31, 32]. Finally, the coordinated response of COR genes contributes to higher survival rates of plants under freezing stress [33]. Also natural accessions of *Arabidopsis* thaliana show differential regulation of the CBF pathway, which was discussed to be correlated with variation of freezing tolerance and which emphasizes the role of the CBF pathway as an evolutionary factor [34-36]. The CBF pathway itself is tightly regulated by other transcription factors, phytohormones, Ca^{2+} binding receptor kinases and key components of the circadian clock, light intensity and light quality via the phytochrome system, and post-translational modifications (PTMs) [37-39]. These reaction cascades highlight the interconnection of regulation mechanisms taking place as soon as a cold signal is perceived. Carbohydrates are involved in regulation of the CBF pathway as it was indicated that sucrose enhances transcription of COR78 especially in epidermal cells [40]. Adaptation to cold is not only apparent by changed expression of genes, but also in the levels of proteins. Focusing solely on changes of transcript levels is often not enough to reveal the actual state of metabolism. Post-transcriptional mechanisms, post-translational modifications and differential regulation of protein isoforms are crucially involved in cold stress response [2, 3, 41]. For complementation, intensive proteome and enzyme activity analysis together with metabolome analysis are necessary as vice versa changes in protein concentration affect transcriptional and translational processes. Moreover, modification of gene expression at the transcript level frequently does not correlate with protein level [42] and similarly, at low temperatures enzyme activities often do not correlate with protein level changes. Low temperature stress affects groups of proteins involved in photosynthesis, carbohydrate metabolism, polyamine synthesis, ROS scavenging, protein folding, stabilizing cell structure and cell membrane integrity [2, 43] which are frequently affected by the CBF regulon [44]. For example, abundance and

phosphorylation of COR 78 protein was described to be positively correlated with acclimation state of natural *Arabidopsis* accessions and their carbohydrate accumulation capacity [45]. In addition, increased protein abundance of COR78 and COR15B during low temperatures seems to be a common feature, also for mutants deficient in starch and sucrose biosynthesis [46]. These observations might allow to link cold responsive gene regulation and sugar signaling networks [45].

A tight regulation of photosynthesis and carbohydrate metabolism is essential under low temperature

If temperature drops, photosynthetic light reactions and the central carbohydrate metabolism need to be immediately reprogrammed to prevent any imbalances which would cause production of reactive oxygen species (ROS), cell damage or even cell death [7, 47]. Photosynthetic rate and the expression of genes related to photosynthesis are closely connected to temperature [48-51] and decreasing temperature causes strong inhibition of photosynthesis [52, 53]. Further, low temperature might induce reduction of size of PSII associated antenna [7]. Within the first minutes of cold exposure, plants compensate for the high PSII excitation pressure either by state transition from PSII to PSI or dissipation of heat via non-photochemical quenching [7]. Earlier it was shown that under such conditions expression of genes coding for photosynthetic proteins was downregulated [54]. The photosynthetic rate is not only controlled by the amount of associated proteins, but also fine-tuned by the specific production of isoforms with optimized performance or adaptions in the activation state according to the prevalent temperature [55, 56]. An initial decrease in photosynthetic rate is not only due to direct effects in the photosynthetic apparatus, as systemic thermodynamic effects influence enzyme activities of whole metabolism. For example, the electron transport chain in chloroplasts relies on a continuous supply of $NADP^+$ as electron acceptor, which is mainly provided by usage of NADPH in carbon fixation reactions. A decreased activity of Calvin cycle enzymes has recently been found to possibly contribute to overreduction-associated damage to the photosystem and inhibition of the photosynthetic rate [57]. The continuous function of photosynthesis and the Calvin cycle relies on the exchange of triose phosphate (TP) from the chloroplast with orthophosphate (Pi) from the cytosol via the triose phosphate/phosphate translocator (TPT), which directly links photosynthetic processes to the energy balance in the cytosol [58, 59]. Enzymatic sucrose biosynthesis via sucrose phosphate synthase (SPS) significantly affects Pi concentration in the cytosol and it has been indicated that limitation in SPS capacity disturbs the triose phosphate export from the chloroplast [58]. If triose phosphate cannot be exported in sufficient amounts and starch metabolism might not be able to compensate the excess triose phosphates, photosynthesis may run into disequilibrium resulting in ROS production [60]. In this context, a higher capacity of sucrose biosynthesis was linked to increased cold tolerance in *Arabidopsis* by avoidance of a bottleneck in metabolism under cold exposure [61, 62]. The accumulation of TP and resulting Pi limitation in the chloroplast might lead to a strong inhibition of photosynthesis by damaging the photosystem through overreduction of the electron transport chain [63].This could be relieved by direct supplementation with Pi [64]. Decreased enzyme activity of carbon fixation and sucrose biosynthesis might be intensified by product inhibition due to affected metabolic sink activity and decreased assimilate export [65, 66]. It is discussed that regulation of the photosynthetic apparatus via redox regulatory networks is crucial for chilling stress acclimation [67]. A possible link between chloroplast antioxidant abilities and carbohydrate availability was shown in *Arabidopsis*. Transcripts of genes encoding for photosynthetic electron transport chain and chloroplastidic antioxidant enzymes were decreased upon external sucrose feeding [68]. Nevertheless, through acclimation the optimum temperature for photosynthesis can be shifted and similar rates of photosynthesis as under ambient temperatures can be reached [51, 69].

Metabolic reprogramming during cold acclimation

The process of cold acclimation is induced after a short time period of exposure to low but non-freezing temperatures [70]. It plays an important role for plant evolution and ecology, indicated by the

high mortality at -8°C of non-acclimated plants compared to acclimated plants which has been described for more than 70 natural *Arabidopsis* accessions [71]. Adverse effects of low temperature are counteracted during the phase of cold acclimation by extensive reprogramming of transcriptome, proteome and metabolome. Not a unique metabolite, transcript or pathway can be assigned to be responsible for cold tolerance but rather the whole sum of multifaceted reorganisation of metabolic homeostasis is necessary [72]. Carbohydrates are the primary photosynthetic products, thus playing a central role in energy metabolism, developmental processes and stress signaling. Reprogramming of primary metabolism during cold acclimation typically results in the accumulation of soluble sugars, sugar alcohols, organic acids, amino acids, and polyamines [72-75] and central building blocks for secondary metabolites [76]. This accumulation finally allows plants to withstand lower freezing temperatures when compared to non-acclimated plants [17]. Reactions in primary metabolism are tightly regulated and closely linked to the circadian clock to ensure a continuous carbohydrate availability [77, 78]. Clock components are significantly influenced by low temperature [41] and sugars are also known to be important for the entrainment of the clock [79]. Besides of the clock components, light itself is deemed to be essential for cold acclimation. As it was shown that enhanced freezing tolerance by cold acclimation can only be reached if light is available [73, 80]. Starch is a direct product of photosynthesis and a storage compound for carbon, and its biosynthesis and breakdown are tightly regulated during abiotic stress. Many enzymes involved in starch metabolism are redox regulated [81, 82]. During cold stress starch metabolism in *Arabidopsis* harbours great plasticity to react to differences in growth conditions and is discussed to be a determinant of plant fitness under abiotic stress [83]. Starch degradation is an initial response [84] as starch metabolism has the potential to relieve product inhibition effects on Calvin cycle associated enzymes and might allow the release of Pi in the chloroplasts under cold stress. Increased activity of beta-amylases supplements maltose accumulation during cold exposure [85, 86]. Further, mobilisation of starch seems to differ between different natural accessions of *Arabidopsis* and might influence their cold acclimation capacities [45]. Interestingly, impairment of plastidial α-glucan phosphorylase resulted in no significant changes in the starch content of *Arabidopsis* leaves, but reduced survival under stress [87]. This highlights that not the absolute amount of starch but rather the dynamics of synthesis and breakdown pathways might be responsible for metabolic reprogramming and survival under abiotic stress. Starch has several roles in both source and sink tissues and starch degradation into sugars has a pivotal role for plant cold stress response via offering osmoprotective sugars and rapid energy supplies [88]. Membrane damage by ice crystal formation poses a large threat to plant cells, and sugars can directly influence cell membrane stability by interacting with the membrane interface and therefore support to maintain membrane integrity under freezing conditions [89, 90]. Various sugars are proposed to directly stabilize biological membranes [91], e.g. sucrose can interact with the phosphate in lipid headgroups, thereby decreasing membrane permeability [90]. Carbohydrates have been found to stabilize *in vitro* liposomes against leakage of aqueous content which suggests a cryoprotective role *in vivo* [19]. Fructans might move via vesicle transport from vacuoles to the apoplast where they could assist in stabilizing the plasma membrane [92]. Further, sugar transport proteins might play a role of vacuolar fructan export [78]. Raffinose Family Oligosaccharides (RFO) are known to protect membranes under cold stress, contributing to higher freezing resistance [93]. Raffinose is synthesised in the cytosol and transported into plastids to protect thylakoid membranes, ensure PSII integrity and act as ROS scavenger [94-96].

The starch degradation product maltose might serve as a direct osmoprotectant in chloroplasts [85] and is as substrate for the production of other carbohydrates like hexoses, raffinose or proline [84, 86] to fuel and maintain carbon metabolism. A direct correlation of freezing tolerance with *Arabidopsis* accessions and the degree of accumulation of raffinose, proline and glutamine has been observed earlier [97]. Further, ROS as well as redox regulatory networks play a central role during acclimation to stress [67]. This specialized role of protecting the photosynthetic apparatus under cold stress potentially explains the widespread accumulation of this metabolite in plants, but also why the artificial increase of raffinose accumulation was not enough to increase freezing tolerance, measured by leakage assays [93]. Additionally, sugars act as signaling molecules which influence expression and

activity of transporters, subcellular redox homeostasis, scavenging mechanisms, secondary metabolism and hormone metabolism [75, 90, 98]. Diverse signal transduction networks are connected to sugar metabolism and need to be reprogrammed during cold exposure, e.g. comprising sucrose non-fermenting kinases (SnRKs), target of rapamycin (TOR) kinases (high carbon availability), hexokinase 1 (HXK1) and abscisic acid (ABA) networks [99, 100]. Proline is well-known to accumulate during stress response affecting signaling events, cryoprotection and redox balance in several plant species [101]. In a protein-protein interaction network delta 1-pyrroline-5-carboxylate synthase 2 (P5CS2), which is a central enzyme in proline biosynthesis, indicated a linkage to heat-shock proteins and to the interface of primary and secondary metabolism which shapes stress response across a wide range of metabolic states during cold stress [46]. In general, gene expression related to secondary metabolism is well correlated with freezing tolerance [16, 102]. In *Arabidopsis*, biosynthesis of flavonoids, anthocyanins, glucosinolates and phenylpropanoids is induced during cold exposure [103, 104]. Several flavonoid biosynthesis mutants with reduced flavonoid content showed impaired freezing tolerance, and contribution of flavonoids to freezing tolerance was shown to be genotype-dependent [28]. Flavonoid metabolism seems to be regulated via post-transcriptional mechanisms as the corresponding transcripts and metabolites correlated poorly in response to cold [105]. During cold acclimation, the amount, composition and structure of cell wall and extracellular components are massively changed [106, 107] and have been suggested to be related to accumulation of cell wall modifying enzymes [107]. In field studies it was shown, that accessions with the lowest cold acclimation potential benefited most from cold pre-treatment regarding seed yield [108]. Moreover, it was shown that a memory of cold acclimation, i.e. cold priming, exists which suggests *Arabidopsis* can memorize cold stress for several days over a stress free period [109, 110]. After a lag phase of seven days following a cold pulse, the priming effect could be described by changed gene expression of lipid, secondary and stress metabolism. This cold memory effect improved plant freezing tolerance and seems to differ between natural accessions [109]. Additionally, it was suggested that the plastid antioxidant systems transmit information of a previous cold stress over time [110]. As fast as freezing tolerance is increased, it can decrease again [73] but the time frame of de-acclimation differs between natural accessions of *Arabidopsis* [111]. Likewise, as the acclimation process, de-acclimation induces various changes in the transcriptome and metabolome of plants. Therefore, a tight regulation of freezing tolerance loss, re-activation of the circadian clock and activation of growth is necessary [112]. Plasma membrane located proteins were identified to play a major role during de-acclimation, as some of them stayed highly abundant, possibly to protect the membrane against a threat of sudden temperature drop [113]. With respect to the challenge induced by climate change, investigation of the molecular basis of de-acclimation in herbaceous plants and trees will be necessary to allow predictions about survival rates during spring freezing events. This knowledge might further support breeding of new varieties of e.g. crop plants which can better adapt to challenges of a rapidly changing climate [114].

The role of subcellular metabolic regulation in plant cold acclimation

Cell organelles and compartments are interconnected by various transport and shuttle systems which enable a regulated exchange of metabolites across biological membrane systems [115, 116]. Analysis of crude whole cell extracts of metabolites and proteins are suitable to record the overall stress response of metabolism. Nevertheless, information content about organelle specific subcellular alteration of biosynthetic pathways is strongly limited by analysis on the whole cell level. As a result, through analysis on a whole cell level, the role of specific metabolites and proteins might be hidden and overseen which can lead to misinterpretation of results. Specific changes in subcellular concentrations of potential stress protectants can have a massive influence on successful stress responses. Connection of subcellular metabolite information with subcellular transport activity and/or expression is crucial to unravel regulatory responses to cold. For example, induction or level changes of soluble sugar transporters like Tonoplast Sugar Transporters (TSTs) [117, 118], Sugars Will Eventually be Exported Transporters (SWEETs) [119], plastidic Sugar Transporter (pSUT) [120] and their phosphorylation [121] might play a crucial role [90]. Applying the method of nonaqueous fractionation (NAF), it is possible to resolve metabolites from one sample on a subcellular level

revealing chloroplastic, cytosolic, vacuolar and mitochondrial information [122-124]. NAF was applied in several studies to investigate subcellular metabolism under cold exposure. Knaupp and colleagues indicated stabilization of photosystem II by accumulation of plastidial raffinose [96]. Leaves developed in cold showed lowered cytosolic pyruvate and 3-phosphoglycerate levels but increased dark respiration compared to cold shifted leaves. It was discussed that either there are higher maintenance costs of the reprogrammed metabolism or it might be a precautionary effect due to new environmental changes which require rapid reorganization of metabolism [125]. Analysis of natural accessions of *Arabidopsis thaliana* indicated distinct mechanisms of carbohydrate reallocation between cold-tolerant accessions [126]. Further analysis indicated that a freezing sensitive accession enhanced its subcellular re-allocation of metabolites during cold acclimation while a freezing tolerant accession was found to invest in a stronger accumulation of sugars and amino acids [124]. Plastidial metabolism was indicated to be important to prepare for continuation of growth [127], and the hexokinase 1 deficient *Arabidopsis* mutant *gin2-1* showed a delayed accumulation of protective plastidial metabolites like proline in response to cold treatment [128]. Especially subcellular sucrose cycling was shown to be significantly influenced during lowered temperatures, and is thought to play an essential role in stabilizing photosynthesis during stress [129, 130]. In leaf mesophyll cells, sucrose is synthesized in the cytosol by a sequential reaction of sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP). UDP-glucose and fructose-6-phosphate are substrates for SPS to synthesize sucrose-6-phosphate, whereas SPP releases orthophosphate (Pi) yielding sucrose [131]. Sucrose biosynthesis is a central part of energy metabolism and was shown to be a limiting factor in cold acclimation [61, 62, 132]. Overexpression lines of SPS showed an improved photosynthetic performance and increased freezing tolerance after cold acclimation [61]. Regulation of SPS activity is multi-layered, and also comprises protein phosphorylation which inactivates SPS [133]. Additionally, its activity is stimulated by glc-6-phosphate and inhibited by UDP and Pi [134, 135]. Sucrose is constantly cleaved and resynthesized in a cyclic reaction. Invertases (Inv) catalyse the hydrolytic cleavage of sucrose to glucose and fructose. Invertases are located in several compartments comprising cytosol, vacuole, mitochondria, chloroplast, cell wall and extracellular space (see e.g. [131, 136, 137]). From an evolutionary perspective, it is hypothesized that different invertases have evolved for different functions, e.g. coevolution of cell wall invertases and vascular tissue [137]. Particularly in sink tissues, sucrose can also be cleaved by sucrose synthase (SuSy) to form fructose and UDP-glucose or ADP-glucose (for a summary about SuSy see e.g. [138]). Glucose and fructose are re-phosphorylated by hexokinases to yield hexose phosphates, which are again substrate for sucrose biosynthesis. Continuous sucrose breakdown and re-synthesis appears to be energetically wasteful, but is handled to allow precise control over carbohydrate partitioning [131]. For cotyledons and leaves of various species and experimental setups, a sucrose recycling flux of 10-30% is estimated [139-141]. Stability analysis of kinetic parameters pointed towards hexokinase to be the important regulator of the cycle, while the step of sucrose degradation by invertases appeared to be secondary [139, 142]. Confirming limitation of sucrose cycling via hexokinase, it was shown that deficiency of glucokinase activity resulted in sucrose accumulation and enhanced root respiration [143]. Supplementary, impairment of hexokinase1 in the *gin2-1* mutant might indicate problems of assimilate transport and shoot growth [144]. *Vice versa*, in detached cotyledons in *Ricinus communis* a strong stimulation of sucrose cycling was observed [140]. In addition to cytosolic sucrose cycling and export to phloem, another cyclic reaction across the tonoplast was suggested to play a role in stabilizing metabolism due to environmental cues. Sucrose can be cleaved by vacuolar invertases, yielding hexoses, which can be transported into the cytosol to fuel the cytosolic hexose pool. Simulation of fluctuating environmental conditions of primary carbohydrate metabolism under consideration of millions of possible enzyme kinetic parameters were analysed regarding the stability behaviour of the system [129]. Analysis revealed a non-intuitive link of vacuolar and plastidial metabolism, as a perturbation of vacuolar sucrose and hexose metabolism caused an interference with the regulation and stabilization of plastidial and cytosolic carbohydrate metabolism and/or photosynthetic performance [129]. Supporting this theory, kinetic modelling of carbon metabolism under cold stress revealed different strategies of partitioning sucrose cleavage between cytosolic/ neutral and vacuolar/acidic invertase comparing a cold tolerant and a cold sensitive natural accession of *Arabidopsis thaliana*. The cold tolerant accession shifted

sucrose cleavage capacity from the cytosol into the vacuole, whereas the cold sensitive accession maintained a high rate of cytosolic sucrose cleavage [130]. Deficiency in vacuolar sucrose cleavage capacity lead to a disturbed cytosolic hexose metabolism, an affected ADP/ATP ratio and finally lead to decreased photosynthetic CO_2 uptake under cold and high light stress conditions [130]. The central role of invertases during early stress conditions, e.g. drought and water stress, was further shown to play a crucial role in maize leaves [145-147]. In conclusion, those examples show that sucrose cycling not only allows a precise control over carbohydrate partitioning, it also serves as energy balancing mechanism to efficiently react towards sudden environmental changes. Subcellular information is not only necessary to unravel metabolic pathway regulation but also for biotechnological applications, e.g. metabolic engineering [148] and to feed mathematical models of plant metabolism to quantify non intuitive dynamics of metabolic systems [149].

Mathematical modelling at low temperature: kinetics and thermodynamic constraints

Cold acclimation is a multigenic process. Thus, it seems very likely that a synergy of various cellular events triggers and integrates this complex physiological process rather than a single integrator of environmental conditions [4]. Evaluation of plant responses via comprehensive and multidimensional data sets created by *omics* techniques has significantly enhanced the knowledge about synergetic effects of cellular events. Recorded transcript abundance, protein levels, metabolite concentrations and enzyme activities enable the simultaneous elucidation of pathway regulation. For the analysis of such data sets regression and correlation analysis are widely used to characterize system dynamics. Yet, analysis of plant temperature response also needs to consider non-linear system dynamics due to thermodynamic constraints [150]. Numerous, often unknown, regulatory effectors like feedback/feedforward loops significantly affect metabolic reprogramming. A combination of multivariate statistics, mathematical modelling, and pattern recognition seems promising to yield predictive information about biochemical regulation of plant metabolism [151, 152,153]. The availability of genome-scale metabolic reconstructions of plant metabolism has supported functional integration of experimental high-throughput data, which played a crucial role in predicting observed phenotypes [154, 155]. Further, the need of a combined sink-source model of plant metabolism is essential for crop engineering and emphasizes the essential role of mathematical modelling [148]. Thus, it can be expected that future *in silico* concepts for analyzing plant metabolism will crucially support functional data integration from genome to ecosystem scale [156].

Many mathematical models of plant metabolism consist of ODEs (ordinary differential equations) describing time dependent changes, e.g. of metabolite concentrations, by the sum of synthesizing and degrading reaction rates. Various parameters like enzyme abundance, posttranslational modification, thermodynamic constraints as well as inhibitor and activator concentrations define these reaction rates [149, 151]. To study plant metabolism, the most commonly used modelling approaches are constraint-based modelling (CBM) and kinetic modelling. CBM is applied to large networks and compares the steady-state behavior of different conditions, while kinetic models are the method of choice to elucidate dynamic system behavior [4, 157]. In general, kinetic models tend to consider only a relatively low number of reactions because of experimental limitations in recording enzyme kinetics and activities. Consequently, kinetic model construction comprises critical steps of metabolic network simplification and various assumptions about the comparability of *in vivo* and *in vitro* measurements [158]. Mathematical modeling has been proven to efficiently support analysis of plant-environment interactions. For example, Calvin-Benson cycle enzyme activity was shown to be affected by metabolite concentrations outside of the chloroplast [159]. Further, ODE model simulation and mathematical analysis revealed diurnal pathway regulation of plant metabolism [160], diurnal and circadian sensors [161] and critical temperatures for sucrose biosynthesis [62]. Mathematical modelling supported the analysis of subcellular sugar metabolism during cold exposure [126, 130], and sink-source dynamics [162]. Conclusively, although kinetic modeling frequently comprises strong network simplification, it represents a powerful strategy to reveal and predict temperature-induced metabolic reprogramming.

Changes in the environmental temperature regime immediately affect enzymatic activities and reaction rates following thermodynamic laws. According to the van't Hoff rule, the velocity of enzymatically catalysed reactions decreases by a factor 2-3 per each 10°C reduction [163]. This theory was developed further to the so called Arrhenius equation [164, 165]. The Arrhenius equation (Equation 1) is a simple, yet precise way to investigate temperature dependent changes of enzyme reaction velocities in biological systems [166], nevertheless there are limits due to thermal stability of proteins [167]. It describes the rate constant k of a chemical reaction as the product of a constant C and an exponential term (Eq. 1). The constant C comprises information about collision frequency and geometric molecule positions. The unit of C varies depending on the reaction order, and for a first order reaction is denoted by [s^{-1}]. The exponential term comprises the activation energy E_A [J mol^{-1}], temperature T [K] and the universal gas constant R [J K^{-1} mol^{-1}].

$$k = C * \exp(-E_A R^{-1} T^{-1}) \quad (1)$$

In biochemical reactions, E_A frequently ranges between 40 and 50 kJ mol^{-1}, and comprises various steps of catalytic mechanism [168]. To yield an approximate value of *in vivo* enzyme activity and flux estimation under low temperature, maximal enzyme activity might be experimentally determined applying the plant growth temperature, e.g. 5°C, for enzyme activity measurements. Yet, frequently this results in a complicated experimental setup which might also affect statistical robustness of experimental output. Alternatively, maximal enzyme activities recorded under optimum temperature might be adjusted to growth temperature by applying the Arrhenius equation [62].

Irrespective of the method applied, thermodynamic adjustments need to be considered when enzyme activity and protein amounts are discussed under changing temperature regimes. For example, plants exposed to 5°C (278.15K) might contain a doubled amount of an enzyme compared to plants exposed to 22°C (295.15K). Consequently, doubled enzyme amount results in a doubled maximal reaction rate measured under optimal laboratory conditions (Fig. 1).

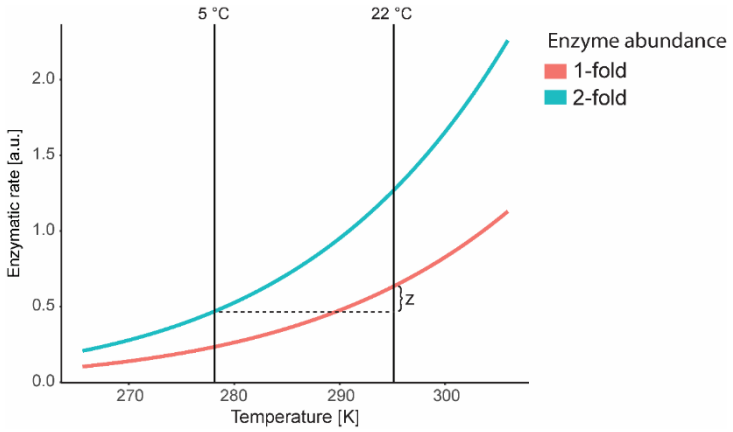

Figure 1. Enzymatic rates with single (1-fold, red, 22°C) and doubled (2-fold, blue, 5°C) enzyme abundance. The adjusted v_{max} enzyme activity with doubled abundance at 5°C is lower than the adjusted activity at 22°C (factor: z). Enzymatic rates were calculated using the Arrhenius equation (Eq. 1).

Nevertheless, the actual maximal enzymatic rate in plants at 5°C would be lower than in plants exposed to 22°C by the value of z (difference of enzymatic rate), even though a doubled amount of the investigated enzyme is available (Fig. 1). Application of the Arrhenius equation allows the statement of the actual v_{max} prevalent in the plant under the applied cold condition. Conclusively, cold-induced protein accumulation does not necessarily result in a higher reaction rate *in vivo*.

Conclusions

Carbohydrates are central players in plant cold acclimation and future work on involved signaling and metabolic regulation will extensively broaden our knowledge about cold-induced metabolic reprogramming. Combination of findings about subcellular carbohydrate, amino- and organic acid metabolism with dynamics of protein amount and enzyme activities will support the understanding of initial and long-term stress responses as well as acclimation processes. Thermodynamics need to be considered for physiologically meaningful interpretation of enzyme kinetics and pathway regulation. To be able to transfer knowledge about abiotic stress factors to natural habitats, it will be important to investigate the combination of stressors. For example, cold and high light occur simultaneously in the field and extrapolation from single stress responses to a combined one is complicated as there might be antagonistic or synergistic effects [169, 170]. Considering climate change, insights into molecular mechanisms controlling plant growth and productivity versus protection during abiotic stress will be critical for the improvement of crop plants [171]. Mathematical modelling approaches promise to support the analysis of nonlinear, non-intuitive compartment specific responses of metabolism [172]. In addition, the field of artificial intelligence and its application in plant science is rapidly growing. How data from multiple sources are incorporated into a learning system is a crucial step for successful analysis. Finally, machine learning algorithms have provided useful classification of multivariate data and helped to uncover characteristic metabolic patterns and flux predictions in plants [46, 173-175]. In conclusion, interconnected reprogramming of plant metabolism affects diverse molecular levels and a combination of experimental and mathematical strategies promises to reveal central regulatory processes of plant-environment interactions.

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