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Influence of carbon starvation and of TOR inhibition on *Arabidopsis thaliana* cell cultures

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Abbreviation table:

ASN1/DIN6: Asparagine Synthase1/ Dark Induced6
ATG: AuToPhagy related
(At)TOR: (Arabidopsis thalina) Target Of Rapamycin
AZD 8055: 5-[2,4-bis[(3S)-3-methyl-4-morpholinyl]pyrido[2,3-d]pyrimidin-7-yl]-2-methoxy-benzenemethanol
bZiP: basic leucine ZIPper
CESA1: Cellulose Synthase1
DEG: Differentially Expressed Genes
DMSO: DiMethyl SulfOxyde
E2F: E2 factor
FKBP12: FK506 Binding Protein12
FAT(C): FRAP-ATM-TRRAP (C-terminal)
FRB: FKBP12-Rapamycin Binding domain
F6P: Fructose-6-Phosphate
FW: Fresh weight
GC-MS: Gas Chromatography – Mass Spectrometry
GFP: Green Fluorescent Protein
GLN1;5 : Glutamine Synthase 1;5
HEAT: Huntingtin, Elongation factor 3, A subunit of PP2A, and TOR1
HK: Hexokinase
LB: Luria-Bertani
LES: Low Energy Syndrome
LST8: Lethal with SEC13 protein 8
MIOX2: Myo-inositol oxygenase2
MS: Murashige and Skoog
mTOR: mammalian Target Of Rapamycin
OFAT: One Factor At a Time
PCA: Principal Component Analysis
ProDH2: Proline Dehydrogenase2
RAPTOR: Regulatory-Associated Protein of mammalian Target Of Rapamycin
RICTOR: Rapamycin-Insensitive Companion of mammalian Target Of Rapamycin
ROS: Reactive oxygen species
RPS6: Ribosomal Protein S6
SAG12: Senescence Associated Gene12
SnRK1: SNF1-Related Kinase 1
SPP: Sucrose-Phosphatase
SPS: Sucrose-Phosphate Synthase
SUSY: Sucrose Synthase
S6K: S6 Kinase
TCA: Tri Carboxylic Acid
TORC: Target Of Rapamycin Complex
TRE1: Trehalase1
T6P: Trehalose-6-Phosphate
UPSC: Umeå Plant Science Center

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I- Introduction:

A- Description of host organism organization

UPSC is a public center of experimentation which is located in Umeå (Sweden). It has been created in 1999 by the fusion of the department of Plant Physiology at Umeå University (UmU) and the department of Forest Genetics and Plant Physiology at the Swedish University of Agricultural Sciences (SLU). One of the key work of the center is the optimization of nanocellulose production by using the CRISPR-Cas9 technology. It contains 37 groups of variable size. There are around 200 staff members of over 35 different countries. The metabolic reprogramming group (directed by Johannes Hanson) especially contains 3 employed members and looks for the plant responses to stress. The group is particularly interested in the LES (Low Energy Syndrome) and by two master kinases: TOR and SnRK1.

B- Presentation of *Arabidopsis thaliana*

Arabidopsis thaliana is one of the first model organisms for plant biology. It has been the first plant genome to be fully sequenced in 2000 and contains around 25 500 genes coding for proteins (Kaul et al., 2000). Its numerous advantages (small size, short generation time, small genome, large number of seeds) made it quickly almost unavoidable in plant sciences.

C- TOR description and importance

C.1 – Rapamycin inhibitor

Rapamycin ($C_{51}H_{19}NO_{13}$) is an immunosuppressive compound produced by *Streptomyces hygroscopicus* which is a bacteria found in Easter Island. It can stop yeast division and mimic an N starvation phenotype by binding to a specific protein called FKBP12 and to a specific fixation site of the TOR protein called FRB (Dobrenel et al., 2016). However some mutants were identified in yeast exhibiting a resistance to this drug due to a mutation in the FKBP12 protein or in the FRB domain (Heitman et al., 1991).

C.2 – Presentation of TOR complex, a major sensor

TOR is a very well conserved protein kinase in the eukaryotic kingdom (between 40 and 60% of common identity between mTOR and AtTOR). It belongs to the PIKK family, which is characterized by its carboxy-terminal serine/threonine protein kinase domain (Wullschleger et al., 2006). It is a large protein (280-300 kDa) and contains 5 different domains: from N terminal to C terminal domain, there are the HEAT repeat domain (containing around 20 repetition of the HEAT motifs), the FAT domain (supposed to have a scaffolding function), the FRB domain (which is targeted by rapamycin), a kinase domain and finally the FATC domain which seems to have a function similar to the FAT one (Richardson et al., 2004; Wullschleger et al., 2006). The inactivation of TOR by rapamycin is effective when the three-membered complex FKBP12-Rapamycin-FRB is formed **(Figure 1).** In mammals TOR is present under the form of two complexes: TORC1 and TORC2. TORC1 is composed of three different proteins: mTOR , RAPTOR and LST8. TORC2 is composed of mTOR, RICTOR and LST8. TORC1 is the only complex which is rapamycin sensitive (Wullschleger et al., 2006; Yip et al., 2010). Nevertheless, no trace of RICTOR or other TORC2 specific compounds has been found leading to the conclusion that this complex could be absent in plants (Dobrenel et al., 2013).

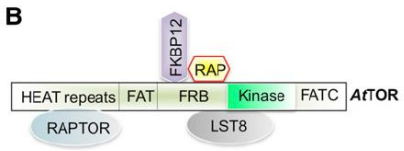

Figure 1: Theoretical interaction between rapamycin and TORC1 (Xiong and Sheen, 2014)

However, higher plants do not seem to be rapamycin sensitive, at least at physiological concentrations (Menand et al., 2002; Sormani et al., 2007) although this point is subject to discussion, some studies show a small effect of a large amount of rapamycin on plant growth and development. Interestingly FKBP12 expression in higher plants seems to be very low which could explain their resistance to rapamycin (Xiong and Sheen, 2012). But this is not the only hypothesis, this could be due to FKBP12 which fails to bind with AtTOR or to the incapacity of the triplex to inhibit AtTOR (Menand et al., 2002). AtTOR is a kinase which is regulated by both endogenous signals (as auxin, energy levels, nutrient availability (internal sugars and nitrogen) (Bögre et al., 2013; Xiong and Sheen, 2014; Dobrenel et al., 2016)) and exogenous signals as light or stress (Laurie and Halford, 2001; Robaglia et al., 2012). TOR activity is upregulated by favorable conditions and down regulated by unfavorable conditions (e.g. pathogen attack, nutrient starvation, drought): it acts like an integrator of the plant environment and a coordinator of cell response.

C.3 – TOR is a key actor in cell adaptation

TOR has several substrates which are phosphorylated by its protein kinase activity: TOR phosphorylates TAP46 which stimulates the nitrogen uptake (Nunes-Nesi et al., 2010). It also phosphorylates E2F which upregulates cell division (Henriques et al., 2010) (notably by stimulating cell wall synthesis). Finally RAPTOR interacts with S6K (Mahfouz et al., 2006) which phosphorylate the RPS6 proteins and promotes translation (Dobrenel et al., 2016): it stimulates the transcription of genes related to those activities and represses the ones which are involved in stress response or anabolism process. This shift is responsible for the enhancement of other biological process generally related to growth or energy production (like glycolysis) and the production of some signaling molecules (raffinose, myo-inositol, ROS) (Leiber et al., 2010; Moreau et al., 2012). TOR stimulates translation activity by drastically increasing the number of polysomes (Deprost et al., 2007; Sormani et al., 2007). Finally TOR inhibits autophagy by phosphorylating ATG1 and ATG13 preventing the formation of the phagophore (Liu and Bassham, 2010; Avila-Ospina et al., 2014). **Figure 2** displays the key role of TOR in metabolic adaptation**.** TOR is vital in plant development and adaptation to varying conditions due to its central position in the metabolism. However it is important to notice that TOR is not the only protein kinase to regulate metabolism and works in balance with an antagonist kinase called SnRK1 generating a crosstalk between them. SnRK1 is activated by unfavorable conditions (starvation, hypoxia) or stress-related markers (abscisic acid) and allows metabolism reprogramming in response to LES (Lastdrager et al., 2014; Tomé et al., 2014) by increasing soluble sugar and amino acid contents (Mair et al., 2015), increasing starch content (Dobrenel et al., 2016) and repressing energy demanding processes like growth, amino acid anabolism (Hanson et al., 2008) or triglyceride anabolism (Ghillebert et al., 2011).

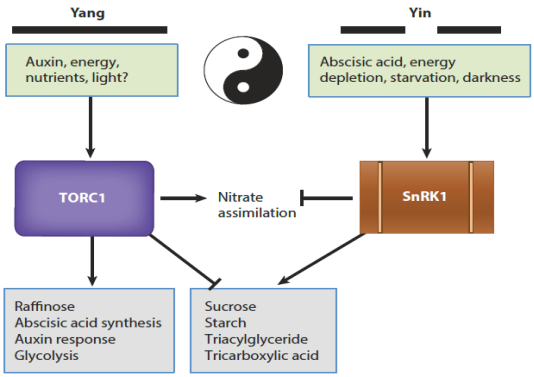

Figure 2: Metabolic reprogramming of the cell driven by the TOR-SnRK1 crosstalk (Dobrenel et al., 2016)

Its direct targets are part of the bZiP family (e.g. bZiP63) (Lastdrager et al., 2014). When TOR is upregulated, SnRK1 is strongly inhibited and vice versa. However this direct TOR-SnRK1 crosstalk is nowadays based on an hypothetical phosphorylation of RAPTOR by SnRK1 causing its dissociation from TORC1 (Oshiro et al., 2004). Nevertheless, many antagonisms have been found in their respective pathway (Robaglia et al., 2012). **Figure 3** summarizes the importance of the two actors in the cell adaptation to its environment.

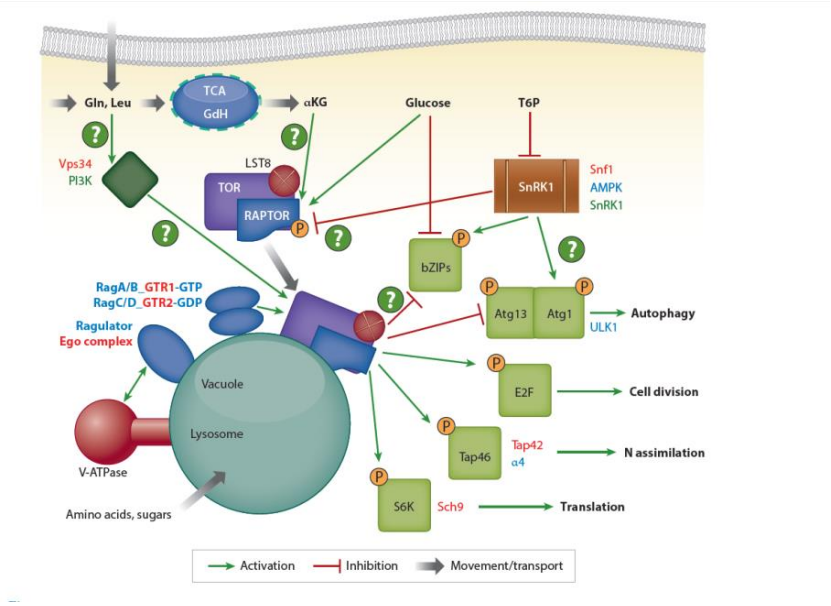

Figure 3: TOR pathway regulation by nutrients in animals (blue), yeast (red) and plants (green) (Dobrenel et al., 2016)

C.4 –Very different ways to study TOR

Studying TOR is complicated for three reasons: first its central position in the metabolism (and its probable interaction with SnRK1) can make difficult to discriminate between the direct and the potentially numerous indirect targets. Then TOR expression changes a lot between the tissues considered: it seems not to be expressed in differentiated plant cells (Menand et al., 2002) but it is crucial in cells still undergoing divisions (Menand et al., 2002) as it is in other eukaryotic organisms (Schmelzle and Hall, 2000). In addition a TOR disruption is embryo lethal: a classical approach by knocking it out by inserting a T-DNA seems not to be the best way to study the effect of TOR inactivation (Menand et al., 2002). Several types of approaches have

been developed, which can be separated in two groups: genetic approaches and chemical approaches. For the genetic ones, one of the idea was the use of hypomorphic TOR alleles (Wu et al., 2013) which led to an increased life span due to a slow life cycle. Another one was the use of RNAi with a VIGS approach (Ahn et al., 2011) or induced by either ethanol (Deprost et al., 2007) or estradiol (Xiong et al., 2013). An inducible micro RNA approach was used too and allowed a good description of metabolic and transcriptomic changes (Caldana et al., 2013). Finally mutations in one of the interactors of TOR (RAPTOR and LST8) have been tested (Anderson et al., 2005; Deprost et al., 2005; Moreau et al., 2012; Kravchenko et al., 2015). For chemical approaches, rapamycin itself had been used but as said above, plants are relatively insensitive to rapamycin and the rapamycin-dependent inhibition of TOR seems to be only partial (Ren et al., 2012) or linked to very specific conditions (Xiong and Sheen, 2012). A hybrid approach between genetic and chemical has been used by expressing yeast FKBP12 in plants before treating them with rapamycin and showed a restored sensibility to rapamycin (Sormani et al., 2007). Recently some specific inhibitors of TOR (e.g AZD 8055) have been described and showed the most promising results in terms of transcriptional changes. However it is important to notice that the overlap in the DEG (Differentially Expressed Genes) is very small probably because of a change in the level of inhibition intensity and in the indirect effects caused by a long-term treatment (Dong et al., 2015).

D- Sugar metabolism, a central point in cells' life

D.1 – Sugars as energetic and structural molecules

Sugars which have a general formula of $C_nH_{2n}O_n$ (simple sugars) can have many different forms and roles in the cell from a structural role (cell wall formation) to signaling molecules and obviously energy rich molecules. In all cases sugars are very important because they contribute to many essential processes in the cells. Soluble sugars which are used in those biological processes are a good indicator of the energetic state of the cell: in the contrary to animals, plants don't have the ability to move to avoid environmental pressure. Photosynthesis which consists in sugar synthesis by CO_2 fixation is one of the major anabolic process which helps the plant to survive in carbon depleted environment. Those sugars constitute a negative feedback loop which inhibits this process when they are accumulated (Goldschmidt and Huber, 1992). On the other hand they are consumed by catabolic processes such as glycolysis, respiration, fermentation to produce energy and by some anabolism to produce amino acids (photorespiration), cell wall compounds (cellulose, hemicellulose), fatty acids (β oxidation) or secondary metabolism compounds. Carbon is vital for plant and a starvation induces an arrest of the growth, a rapid consumption of the soluble sugars and a decrease in the gave off amount of CO_2 that could be linked to a reduction of the respiration rate, a degradation of lipids and proteins and a decline of glycolytic enzyme activities resulting in the death when the starvation is prolonged for a longer period (Yu, 1999). Among these soluble sugars, three are of major interest for the primary metabolism: sucrose, glucose and fructose. Sucrose is the major form of transported sugar in the plant, it is synthetized by a SPS and a SPP from an UDP-glucose and a fructose (Wind et al., 2010) and degraded by either a SUSY or an invertase. It can be hydrolyzed in almost all cell compartments including cytosol (Barratt et al., 2009) mitochondria (Szarka et al., 2008) or chloroplast (Gerrits et al., 2001). Glucose is the most active sugar in primary metabolism and derives either from sucrose or

from starch degradation. It takes an essential place in energy producing pathways like glycolysis or fermentation. It can be regenerated in some conditions by gluconeogenesis (Sung et al., 1988). It has an important structural role too, by contributing as monomer to synthesize cellulose. Fructose, even if less active than glucose in metabolism, is important too, especially in glycolysis but more often under a phosphorylated form in another pathway (e.g.: the pentose phosphate pathway which uses fructose as carbon source). In addition to this soluble sugars, starch, the most common polysaccharide in plants, is an important storage molecule which is quickly available for the plant. Two forms of starch exist: transient starch which is located in photosynthetic cells and used in darkness period and reserve starch which is located in dedicated organs (seeds, bulbs, tubers) and used for germination or growth after the seasonal unfavorable period. Finally some sugars (trehalose, sorbitol) have a minor role in energy production (but aren't negligible in some specific conditions (Berüter et al., 1997; Iturriaga et al., 2009) and can regulate the quantity of starch as signaling molecule by decreasing it (Cheng et al., 2004). In supplement of this energetic role, sugars can have a very important role by contributing to the cell wall synthesis (Delmer, 1999; Ridley et al., 2001).

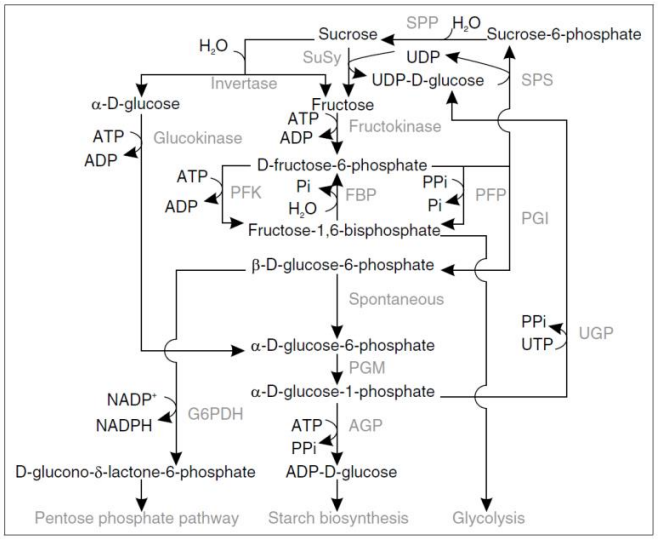

Figure 4: Metabolic relations between the different carbohydrates (Keurentjes et al., 2008)

D.2 – Sugars as sensing molecules

In addition to those roles, sugars are very important sensing molecules. They indeed reflect the energetic status and nutrient availability of the cell to allow metabolic reprogramming when needed. The three major sugars involved in energy production are essential for this signaling function. This sensing is often realized by a kinase which is activated by the presence of those sugars. As examples hexokinase, the sensor of glucose, which is one of the enzymes taking part of the glycolysis (pathway which stimulates growth and life cycle) (Jang and Sheen, 1994; Smeekens et al., 2010) are regulated by sugars too. Hexokinase has strong effects on transcription by repressing some compounds of SnRK1 (Kunz et al., 2015). Glucose can possibly have indirect effects on TOR too independently of HK (Xiong et al., 2013). Fructose has also an important role and seems to act in an hexokinase independent way (Li et al., 2011). In addition the fructose moiety is essential to repress α-amylase by non-metabolized disaccharide signaling. It indicates a high level of soluble sugars (Loreti et al., 2000). Sucrose could have the most essential role in sensing, first because glucose and fructose come from

its hydrolysis, their pattern are at least indirectly sucrose dependent. Then sucrose beneficiates from receptors which are not related to either glucose, or fructose, showing a specific role in sugar signaling. In addition sucrose accumulation is negatively correlated with growth (Sulpice et al., 2009) and photosynthetic activity although this mechanism seems to be indirect (Goldschmidt and Huber, 1992). Although the whole sucrose signaling pathway has not been identified (Wind et al., 2010), some compounds have been discovered, like members of the bZiP family (especially bZiP11, transcription factor targeted by SnRK1) which are repressed by sucrose. They contribute to an amino acid and trehalose metabolism reprogramming (Hanson et al., 2008; Ma et al., 2011). Finally, sucrose controls organ formation by inhibiting root elongation and lateral root formation when supplied in the medium, almost in the same way as nitrate does (Wind et al., 2010). Even sugar derivative molecules, like polyols (sorbitol, mannitol) or sugars with a smallest importance in the energy production (like trehalose, raffinose), can be very important in their signaling function. To begin all of this three sugars have an osmoprotection function. Trehalose has a bounding energy 27 times less than the one of sucrose which makes it very stable in comparison and allows it to fulfill this role. In addition T6P seems to activate plant development and to inhibit glycolysis by reducing the number of phosphorylated sugars (Iturriaga et al., 2009). In case of starvation its level decrease suddenly to activate this catabolic way (Lunn et al., 2006). Even insoluble sugars have a role to play in signaling : as an example starch reserve abundancy is strongly negatively correlated with plant growth, so do pigment involved in photosynthesis (chlorophyll) (Sulpice et al., 2009).

D.3- Carbon metabolism link with other metabolisms

Carbon is however far to be the only required nutrient for life. Nitrogen is a very important element which is among other implicated in chlorophyll synthesis and amino acid metabolism. It is precisely on that points that it interacts with carbon metabolism. A nitrogen starvation induces a chlorosis (Sauer et al., 2001) which strongly affects carbon fixation. Nevertheless it doesn't cause chloroplast autophagy, which is a much more drastic answer on the contrary of carbon starvation (Izumi et al., 2010). Nitrogen starvation has a very strong impact on amino acid metabolism by increasing catabolism to facilitate nitrogen remobilization (Veith and Komor, 1993; Avila-Ospina et al., 2014). However, the level itself of nutrients is not the only important point but the balance between them (especially carbon and nitrogen) seems to be considered too, a very imbalanced ratio seems to be more prejudicial than two low levels (Gao et al., 2008). Finally other metabolisms, like phosphorus metabolism, are important by shifting the nature of amino acids. It is to notice that phosphorus and sulfur are negatively correlated, the lack of one of them leads to the accumulation of the other (Veith and Komor, 1993).

D.4 – A strong interaction between TOR-SnRK1 pathway and sugar metabolism

AtTOR is a very important plant kinase, which makes the link between plants and their environment. It allows plant to adapt themselves to their changing environment by reprogramming both transcriptome and metabolome. Beyond this reprogramming, carbon metabolism is one of the most important by its central position in the cell life cycle (production of energy, sensing, growth, cell wall synthesis…). A plant which is not able to adapt itself to varying condition will suffer from damages, possibly even death (Moreau et al., 2012), showing the crucial role of such kinases. As an example, a carbon starvation inhibits TOR

(Dobrenel et al., 2016) and causes the responses described above (reserve mobilization, growth inhibition) to help the plant to survive. TOR seems to control partially this carbon metabolism reprogramming but some response to a starvation seems to be at least partially independent from TOR pathway (and linked to another plant kinase like HK). Nevertheless, sugars have been shown to have a great influence on TOR activity (Schmelzle and Hall, 2000; Xiong et al., 2013; Dobrenel et al., 2016) and to regulate it by their availability. However TOR integrates other signals independent from carbon starvation. It is now important to understand how this feedback loop is working to be able to determinate the acclimation processes *in planta*.

E – Aim of the project

This project handles with the importance of carbon in plant as autotrophic and mixotrophic organism. Carbon metabolism is indeed crucial for plant development and survival. It is strongly correlated with TOR kinase activity which is vital for plant development and environmental adaptation. Based on the transcriptional and metabolic reprogramming, these pathways are partially overlapping, mainly because of the importance of the TOR-SnRK1 axis in the starvation signal transduction as well as in the remobilization of the sugar resources. In addition plants are complex organisms composed by many different types of cells which have very different behaviors (sink or source behavior, photosynthetic or not, differentiated or dividing cells) and this natural variation could have make difficult the precise deciphering of the different pathways. That is the reason why two *Arabidopsis thaliana* cell cultures have been chosen for this study : a non-photosynthetic one (Pesquet et al., 2010) and a photoautotrophic one (Hampp et al., 2012). In addition, cell cultures grow much faster than whole plants, allowing more experiments. A protocol already exists to transform white cells to make them overexpress TOR with a high yield. White cells ability to grow without hormone (which is a main signal integrated in TOR pathway) and their lack of photosynthetic machinery doesn't make them ideal to study carbon metabolism. The green ones, on the contrary are able to perform photosynthesis but they have never been transformed until now. The first part of this project consists in transforming the green cells to make them overexpress TOR. Then the study of the response of those cells to a carbon starvation will be mandatory: some are linked to the TOR inhibition (caused by the starvation) but some are totally independent: TOR is indeed far to be the only kinase used to adapt metabolism. A specific inhibition of TOR by using AZD 8055 will be performed to be able to discriminate which transcriptomic and metabolic response are TOR dependent and which are just linked to carbon starvation.

II- Materials and Methods:

Cell cultures : Two types of *Arabidopsis thaliana* Col-0 cell cultures have been used in this study: a photoautotrophic cell line CH (Hampp et al., 2012) and a white (non-photosynthetic) one (Pesquet et al., 2010). The CH line is growing on a Gamborg 1X liquid medium with 2% sucrose and 1 mg/L 2,4D. They grow at 23,5˚C with a relative humidity of 45 to 55%. Cells are subcultured every second week and the culture is set in continuous day. The white line grows on Murashige and Skoog medium with 3% sucrose, is subcultured every week and grows in constant darkness. They grow at 24˚C with a relative humidity of 25 to 30%. Both lines are cultivated at 150 rpm on an orbitary shaker. Sugar-free and hormone-free autoclaved media

were supplemented with filter-sterilized (pore size of 20 µm) sucrose and 2,4-D at the above-cited final concentrations. Prior the autoclave, both media are adjusted to a pH of 5,7-5,8. The subculture is performed by diluting the cells in fresh medium with a ratio of 1/11.

Agrobacterium culture: The transformation protocol used the *Agrobacterium tumefaciens* GV3101 strain with two helper plasmids: pMP90 and pSOUP. They were pre cultivated on a Luria-Bertani solid medium with 50 mg/L rifampicin and kanamycin, 25 mg/L gentamicin and 5 mg/L tetracyclin for four days before the transformation. 24 hours before transformation, a colony was transferred into a liquid medium of the same composition. Cultures were growing at 28˚C.

Cell harvesting: The volume of the medium was measured and the cells filtered and rinsed extensively using ice-cold sterile distilled water. The fresh weight was immediately determined and cells were snap frozen in liquid nitrogen.

RNA and real-time PCR analysis: Frozen cell samples were manually grinded with a mortar and a pestle. RNA extraction was performed with E.Z.N.A. RNA Plant Kit (Omega) according to the Standard Protocol in manufacturer's instruction with minor modifications. RNA integrity was confirmed by electrophoresis and total RNA was quantified by using NanoDrop 2000 (Thermo Scientific). To perform cDNA synthesis, 1,5 µg total RNA was digested by using 1,5 U DNAse (Thermo Scientific). cDNA was synthetized with anchored oligo-T primers (Thermo Scientific). cDNA synthesis was performed by using 200 U Reverse Transcriptase H Minus (Thermo Scientific) according to the manufacturer's instruction. QPCR was performed using Thermal Cycler C1000 (BioRad) and Cybergreen (Roche). Relative expression was calculated in comparison to ACBP5 (At5g27630) or to AP2M (At5g46630) according to the methods described (Muller et al., 2002). Reference genes were chosen according to the literature and a Genevestigator analysis (see the Results) (Czechowski et al., 2005)(Graaff et al., 2006). The full list of primers (Eurofins MWG) and the qPCR program are available in supplementary data **(Table SS1 and Figure SS1)**.

Standard transformation and selection: Transformation was done by co-culture according to Pesquet et al. (2010) with minor modification. Concisely, cells were co-cultivated with overnight grown *Agrobacterium tumefaciens* for two days. Then the co cultivated cells were rinsed three times with 30 mL of fresh medium by centrifugation (200×g, 20°C, 1 minute, acceleration 2 AU): supernatant was eliminated and the pelleted cells were plated on selection medium. The selection medium contained 30 mL of fresh medium (Gamborg 1X, 2% sucrose, 0.8% agar, 1 mg/L of 2,4-D, 50 mg/L of kanamycin and 500 mg/L of carbenicillin if not precised otherwise.

Carbon starvation: 5 day-old green cells were rinsed with fresh medium or medium containing 2% mannitol (instead of sucrose) and transferred to the dark for 11 days. They were harvested every second day (see Cell harvesting for more details) and the death rate was estimated at day 0, 2, 5 and 11 by using Evan's Blue. As mock treatment, cells were rinsed with a fresh medium containing 2% sucrose.

TOR inhibition: AZD 8055 was solubilized in DMSO. 5 day-old green cells were treated with 500 nM AZD 8055 for three days. Cells were harvested every day and death rate was estimated every day by using Evan's Blue. DMSO was used as mock treatment.

GC-MS analysis: Metabolites were extracted from plant tissues by using a 20/60/20 chloroform/methanol/water mix supplemented with stable isotope reference compounds ([1,2,3-13C3]-myristic acid, [1,2,3,4-13C4]-hexadecanoic acid, [2,2,3,3-2H4]-succinic acid, [13C5, 15N]-glutamic acid, [25,26,26,26,27,27,27-2H7]-cholesterol, [13C5]-proline, [1,2, 3,4-13C4]-disodium 2-oxoglutarate, [13C12]-sucrose, [2,2,3, 3-2H4]-putrescine, [2H6]-salicylic acid, and [13C6]-glucose) for normalization between the samples. The extraction was performed by using tungsten bids and vibration mill (30 Hz). The supernatant was kept after centrifugation (10 min, 16 100xg, 4˚C). Derivatization was processed in two steps: first the methoxiamination was realized by adding 0,045 mg of methoxyamine in dry pyridine to each sample for 16 hours incubation at room temperature in the darkness and then derivatization itself by adding 30 µL of MSTFA +1% TMS for one hour incubation. GC-MS was run by loading methylstearate every 10th sample to check the sensitivity of the run. N-alkanes series (C12-C40) were used to calculate the retention indices. 1µL of each sample was injected into an Agilent 7890 gas chromatograph (Agilent) which contained a 10m x 0,18 mm column coupled with 0,25 µm DB 5-MS stationary phase (Agilent). The injection temperature was set to 260ºC with a flow rate of 20 mL/min. The purge was turned on after 75s. The gas flow rate was set to 1 mL/min. The temperature of the column was set at 70ºC for 2 minutes and then increased of 40ºC/min up to 320ºC and held there for 2 minutes. Pegasus HT TOFMS (Leco Corp) at 200 ºC was used as ion source for the column effluent with a transfer line of 250ºC. The ions were generated with a -70eV beam which displayed a current of 2mA and 20 spectra/s which was recorded in the mass range 50-800 m/z. The acceleration voltage started after the solvent underwent a 290 s delay. 1600V was used as detection voltage.

Chlorophyll content: Chlorophyll determination was performed according to Porra et al. (1989) with minor modifications. CH cell powder was solubilized in 80/20 acetone- Tris/HCl 1M at pH 7.0. Samples were centrifuged at 14 000xg, 4˚C, 10 minutes and the absorbance of the supernatant was measured at 647nm and 664 nm by using Lambda 18 Spectrophotometer (Perkin Elmer).

Sugar content: CH cell powder was suspended in 80% ethanol for soluble sugar extraction. After heating at 50°C, samples were centrifuged 5 minutes at 13 000 rpm. The supernatant was collected without disturbing the pellet. After resuspending the pellet once again in 80% ethanol and another time in 50% ethanol, the pellet was stored at -20°C for further starch analyzes. Soluble sugar analyses was performed according to Stitt et al.(1989) on the pooled ethanol extracts. Concisely, HEPES/KOH buffer (100mM) at pH 7.0 with 3mM $MgCl_2$ was added to the supernatant with 100 mM ATP and 45 mM NADP and 0,55U G6PDH (Roche). Just after 0,9U HK (Roche) was added to the supernatant and optical density (340nm) was measured every minute until stabilization of the signal by using Epoch Spectrophotometer (Biotek). When the OD reaches a plateau, 0,6U PGI (Roche) is added to the mix. Finally, 20U Invertase (Roche) are added with the same protocol.

Starch analysis: Starch was gelatinized according to Smith and Zeeman (2006) with minor modifications. Concisely starch was gelatinized by using NaOH and heating at 95˚C. The pellet was then resuspended in 0,1M sodium acetate/NaOH + 0,5M HCl. The aliquot was then treated with starch degradation mix (50mM acetate buffer, 0,45U α-amyloglucosidase (Roche), 0,5U α-amylase (Roche)) and incubated over night at 37˚C on an orbitary shaker. The supernatant was collected every day and the starch degradation mix was refreshed. Finally, glucose derived from hydrolized starch was quantified(see above).

Consumables: Gamborg, MS (both with vitamins), Agar and antibiotics were provided by Duchefa. Sugars came from Fischer. Medium composition is available in Supplementary Data **(Table SS2).**

III- Results

A- Study of cell behavior and green cell transformation

1- The different cell lines have different growth characteristics

Both green and white cell lines have been cultivated in their optimal conditions (see material and methods) to determine their growth characteristics. White cells have an exponential growth during 7 days with a doubling time of 1,35 days. They reached a final density close to 400 mg cells/mL medium. Green cells have an exponential growth until the 12th day before reaching a plateau at around 200 mg cells /mL medium. In addition, their doubling time is much longer: 3,11 days **(Figure 4)**.

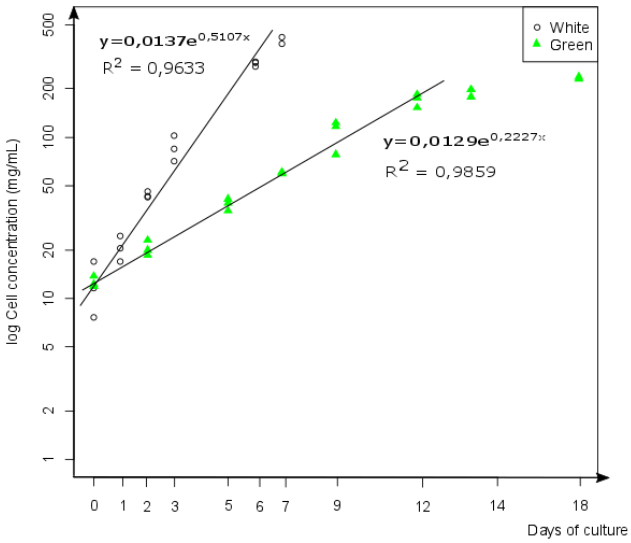

Figure 5: Growth kinetic of the two lines in their optimized conditions

2- Design of a protocol to transform green cells

Green cell transformation was one of the major axis of this project in the reason of their better link with the whole plant model. The new transformation protocol was based on the already existing for white cells (Pesquet et al., 2010). This protocol has been tested and produced some kanamycin resistant callus with white cells and modifying it with an OFAT method: one factor was changed between this protocol and the ones which were tested. Several experiments were set to determine the effect of: antibiotics on both *Agrobacterium tumefaciens* and *Arabidopsis thaliana* cells, nature and concentration of sugar, lighting

duration, Gamborg salt concentration, transformation duration and proportion of fresh medium, *Arabidopsis* green cells and *Agrobacterium tumefaciens.* Antibiotic effect was determined only during selection, the influence of the ratio of different transformation actors only during transformation but all other parameters were tested on both transformation and selection steps in duplicates for a total of 70 levels tested, all in duplicates. The whole experiment procedure is presented in **Table 1.** A picture of each experiment was taken every second week.

Parameters tested	Description	Number of parameters
Antibiotic	Effect of carbenicilin on both Arabidopsis thaliana and Agrobacterium tumefaciens	28
Sugar type	Sucrose / Glucose / Fructose as carbon source. The same sugar was kept for both coculture and selection	6
Sugar concentration	4 concentrations were tested: 0 g/L, 0.5 g/L, 1g/L and 2g/L. The sugar concentration have been crossed between coculture and selection	32
Lighting duration	Darkness, long days and continuous days were tested for transformation and the two last ones were tested for selection. The duration have been crossed between the coculture and selection	12
Gamborg salt concentration	3 concentrations were tested 0,5X, 1X, 2X. The same concentration was kept for the coculture and selection	6
Transformation duration	Three different transformation have been tested : 1 day, 2 days, 3days	6
Proportion of different compounds	5 different Medium / *Arabidopsis* cells ratio have been tested (0.2, 0.5, 1, 2, 5) with five different *Agrobacterium* quantities (50, 100, 200, 400, 800 µL)	50

Table 1: Experiment procedure used to transform green cells

No data was available about the sensitivity of *Arabidopsis thaliana* green line and *Agrobacterium tumefaciens* to carbenicilin which should inhibit *Agrobacterium* growth without killing *Arabidopsis thaliana* **(Figure 5).**

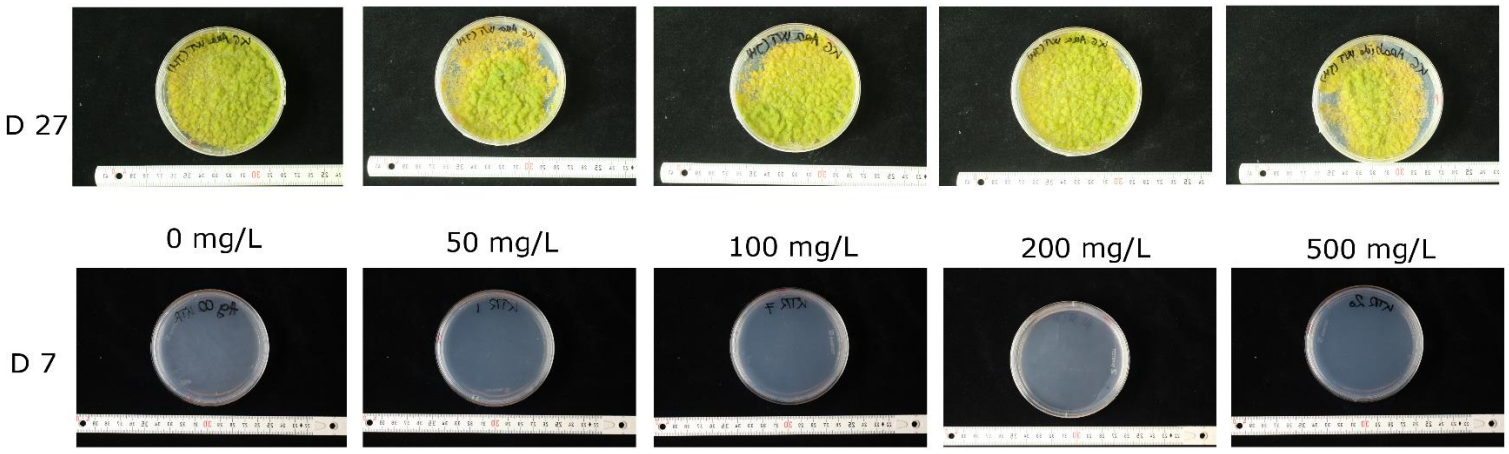

Figure 6: Effect of carbenicilin on *Arabidopsis thaliana* CH line (D27) and Agrobaterium tumefaciens (D7) development. Carbenilin concentration is written between the pictures, duration in day is on the left

There was no effect of carbenicilin on *Arabidopsis thaliana* green cells even at concentrations as high as 500 mg/L, the differences in the pictures are mostly due to a difference in the cell spreading on the plates. As an example there is no difference between the mock treatment

and the 200 mg/L treatment. *Agrobacterium tumefaciens* didn't really manage to grow on Gamborg medium even without carbenicilin. However, the strain was able to grow on LB medium **(Figure SS2).** 500 mg/L was chosen to minimize the risk of bacteria development. However, not a single trace of callus has been obtained in the 112 transformation experiments, meaning that it is impossible to transform CH line with such a protocol at least with a reasonable success rate. **Figure SS3** shows a summary of this project.

B- Influence of a carbon starvation on *Arabidopsis thaliana* CH line

1 – Soluble sugar content decrease quickly but not starch content

Arabidopsis thaliana 5 day old green cells were rinsed with a sugar free fresh medium and transferred to extended darkness for 11 days. As mock treatment cells were rinsed with a fresh medium containing 2% sucrose. Cells were harvested every second day and an enzymatic quantification of soluble sugars was performed **(Figure 6).**

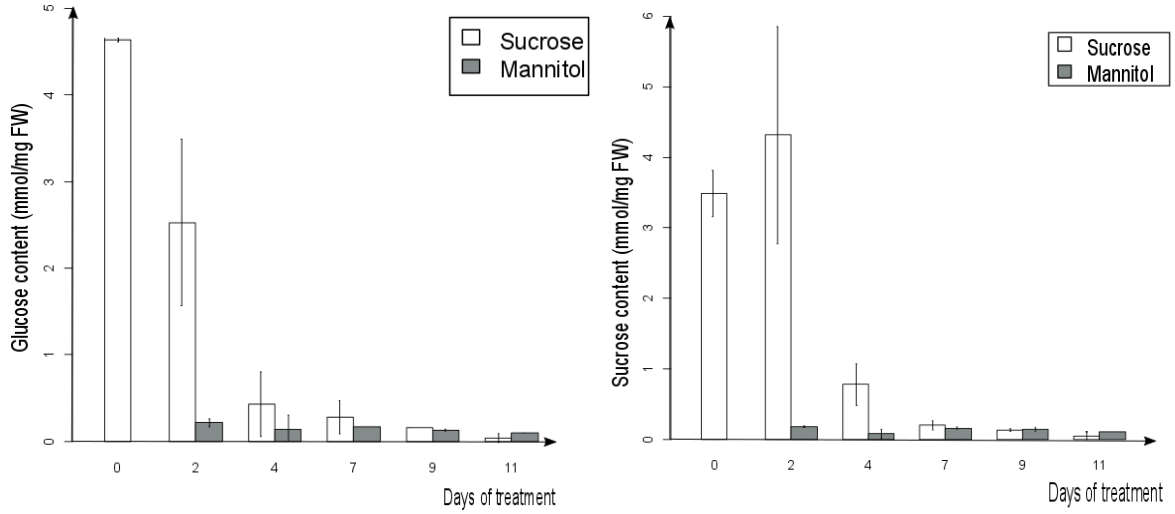

Figure 7: Glucose and sucrose content of green cells during carbon starvation. Mean value ±SD are shown for 2 independent cultures for each condition.

Glucose is relatively quickly consumed in cells with the new medium (50% every two days) to be almost depleted in only four days. In cells growing in the medium without sugar around 95% of glucose has been consumed in two days and this level doesn't change anymore after that. Concerning sucrose, the level first increases in cells growing on sugar and then quickly decreases to be almost depleted at day 7. When there is no sugar in the medium, sucrose is directly consumed and almost depleted (95%) in two days. This intensive use of sucrose can be explained by the lack of sugar in the medium and the impossibility to perform photosynthesis which means that the cell has to use its reserve to produce glucose. Glucose content doesn't increase probably because its consumption is quicker than its production. Surprisingly starch content doesn't decrease in any of the treatments **(Figure SS4).**

2- Carbon starvation stops growth

Carbon starvation seems to quickly stop growth. This could be explained by the fact that cell division and cell wall synthesis are very energy and carbon consuming processes and are down-regulated in case of stress **(Figure 7)**. Cells treated with the new medium seem to have a normal growth rate.

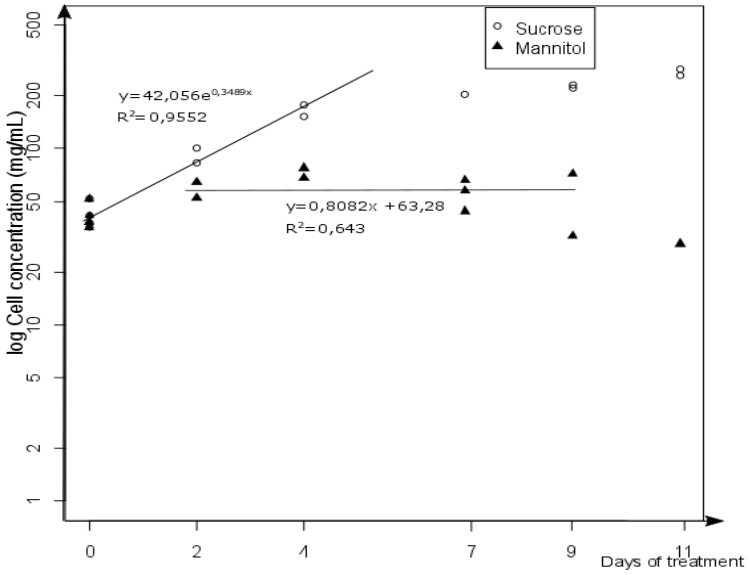

Figure 8: Growth kinetic of green cells under carbon starvation

3- Carbon starvation strongly induces cell death

To explain this sudden arrest of growth, death rate of the different cultures was investigated with Evan's Blue staining. If the death rate of the mock treatment never overcomes a physiological rate of 4%, the one of starving cells reaches around 10% after 7 days and more than 90% after 11 days **(Figure 9)**. In addition, the absence of reaction of the mock treatment allows to brush aside the influence of rinsing. This strong increase in death rate seems to be explained only by an energy depletion.

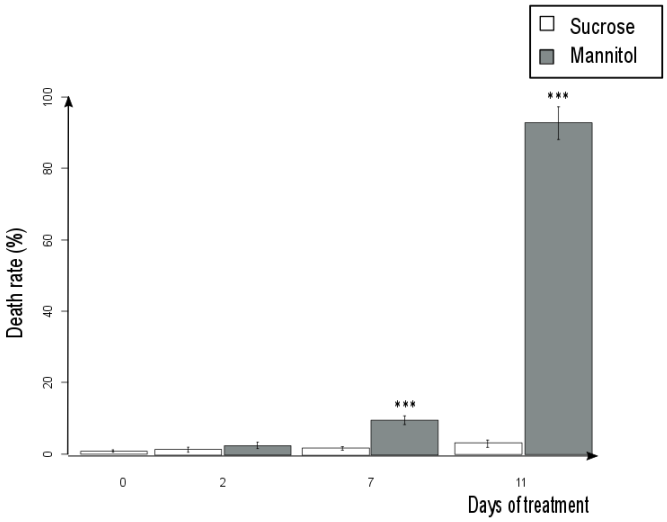

Figure 9: Death rate of green cells under carbon starvation. Mean value ± SD are shown for 4 independent cultures. Student test, significant differences between treatment are shown by asterisk (* P<0.05, **P<0.01, ***P<0.001)

4- Carbon starvation preserves chlorophyll content

Chlorophyll content was measured by spectrophotometry every second day. In presence of sugars, chlorophyll content first increases (respectively 10% and 20% more of chlorophyll a and chlorophyll b compared to the D0) but then quickly decreases. Only 30% of initial chlorophyll content is still present at day 7 and no chlorophyll b anymore. After 9 days cells

doesn't have any chlorophyll. This could be explained by the growth in extended darkness which decreases the chlorophyll content. In starving cells chlorophyll content doesn't decrease at all and is even slightly increasing after 9 days. The ratio between chlorophyll a and b is constant, in the contrary to the mock treatment. The final decrease which is observed is probably caused by the cell death **(Figure 9).**

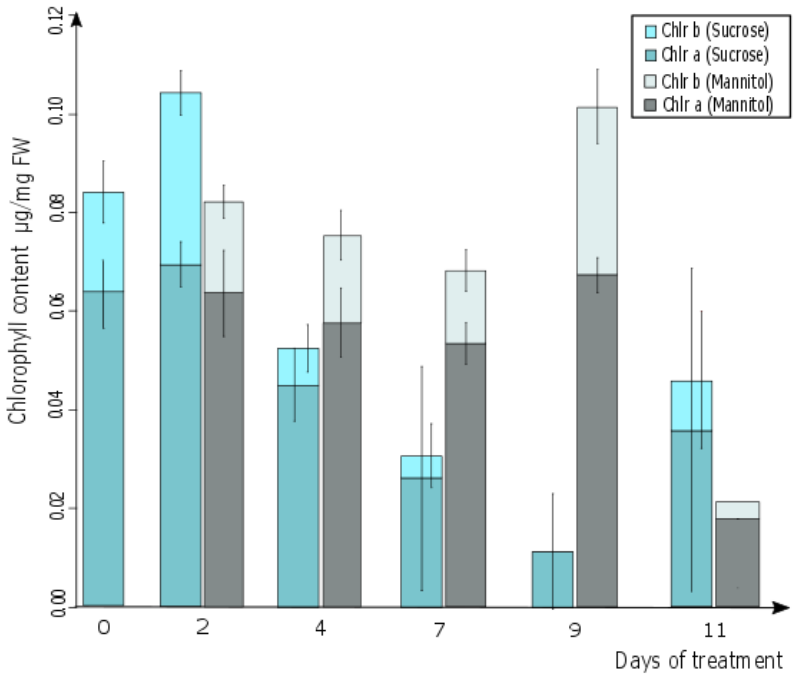

Figure 10: Chlorophyll a and b content of green cells under starvation. Mean value ±SD are shown for 2 independent cultures for each condition.

5- Carbon starvation strongly reprograms metabolism

GC-MS analyzes were performed to study the role played by carbon starvation in metabolism reprogramming **(Figure 10).** A PCA analysis was performed and clearly showed a treatment effect and a time effect in mock treatment which seems to be absent in starved cells **(Figure SS5).** However, this effect is present when starving cells are plotted alone. A first response is observed after two days of starvation **(Figure SS6)** but its intensity increases until day 9. After 11 days all the metabolism is strongly inhibited **(Figure SS7)** which is concomitant with a massive cell death. TCA cycle intermediates and amino acids synthesis derivate from Calvin cycle are both strongly depleted in starved cells. This can be explained by the carbon starvation: no soluble sugars are available for those processes. On the contrary, metabolites linked to signaling (trehalose, polyols, inositol, phosphorylated sugars), redox potential (ascorbate) are strongly enhanced. Interestingly, most of the amino acid contents increased strongly which could be explained by a protein hydrolysis. More information about the importance of the changes (fold ratio) are available in **Table SS3.**

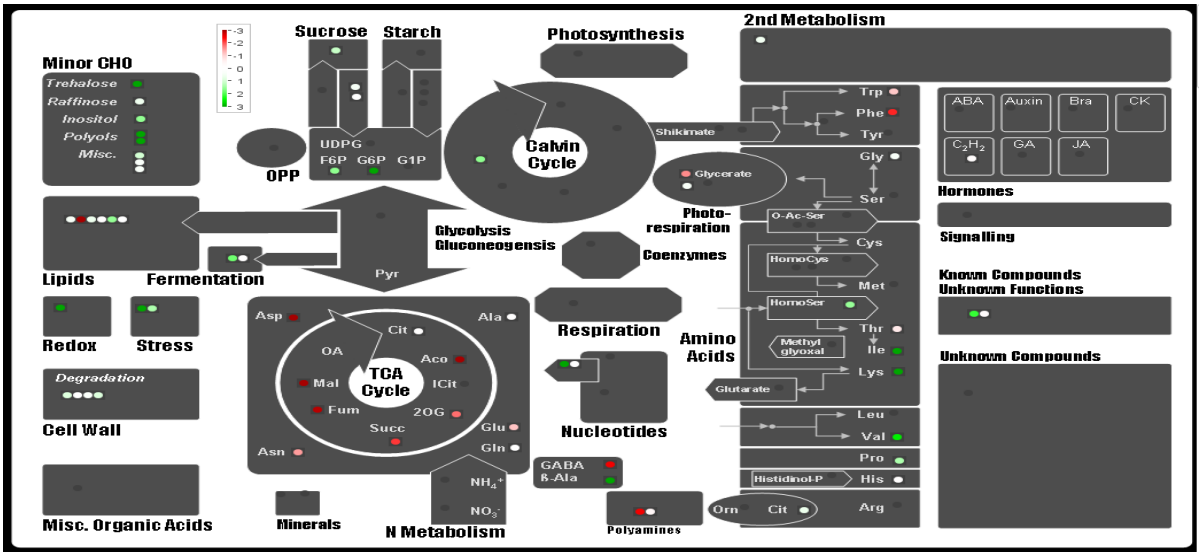

Figure 11: Metabolic modification in response to a carbon starvation (D9). Mean value ±SD are shown for 2 independent cultures for each condition. Data are shown in log (2) fold change, red color corresponds to a decrease, green color to an increase

C- Influence of TOR inhibition on *Arabidopsis thaliana* CH line

1- TOR inhibition changes sugar content

AZD 8055 is a specific inhibitor of TOR and needs to be dissolved in DMSO. For the DMSO treated cells, 50% of the glucose is consumed the two first days but its content increases from 30% the last day. In the meantime sucrose level doesn't change the two first days and increase from 70% the last day, meaning that the increase in glucose content doesn't come only from a sucrose hydrolysis. When TOR is inhibited, glucose content doubles in one day and even triples the last day. In the meantime sucrose content is 40% lower than in the cell with active TOR the two first days and increases the third day but it kept the difference with the non-treated cells. It seems possible to consider that TOR inhibition causes a partial sucrose hydrolysis which led to a glucose content increase **(Figure 11)**.

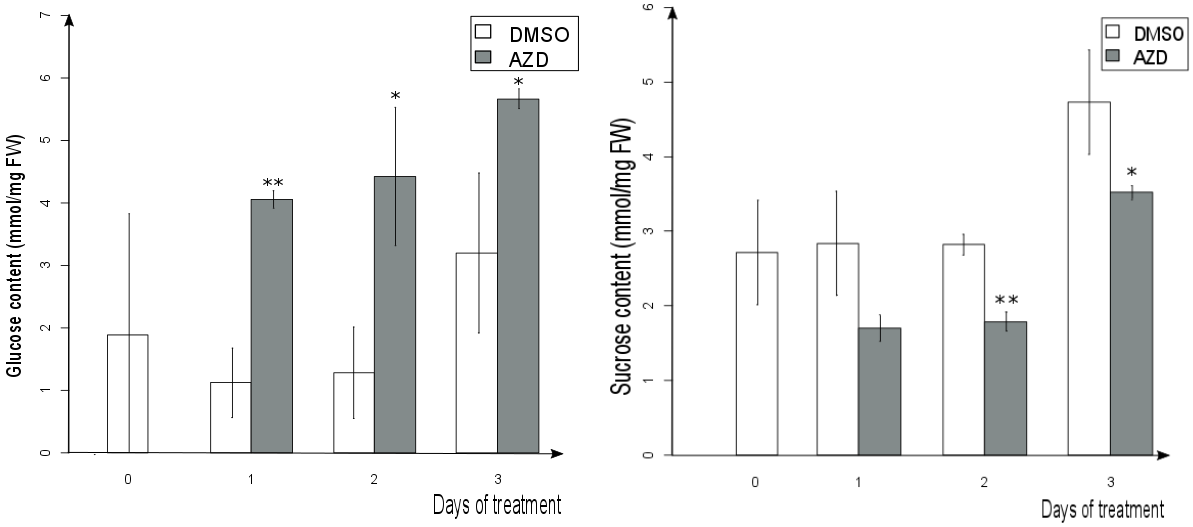

Figure 12: Glucose and sucrose content of green cells during TOR inhibition Mean value ± SD are shown for 6 independent cultures at D0 and 3 otherwise. Student test, significant differences between treatment are shown by asterisk (* P<0.05, **P<0.01, ***P<0.001)

2- TOR inhibition decreases chlorophyll content

For the mock treated cells, chlorophyll a content almost doubles the first day but doesn't change after that. Chlorophyll b content strongly increases the two first days (twelve times more the second day) but then degraded the third day (around half of the second day content is degraded). When TOR is inactivated, there is a small synthesis of both chlorophyll a and b the first day but then both rates return to their initial rate and don't change anymore **(Figure 12)**.

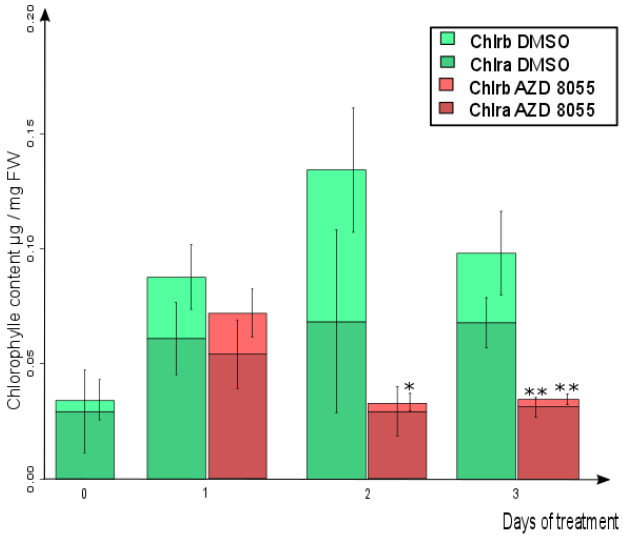

Figure 13: Chlorophyll a and b contents of green cells under TOR inhibition. Mean value ± SD are shown for 3 independent cultures. Student test, significant differences between treatment are shown by asterisk (* P<0.05, **P<0.01, ***P<0.001)

3- TOR inhibition strongly increases amino acid contents

GC-MS analyzes were performed to study the role played by carbon starvation in metabolism reprogramming **(Figure 13).**

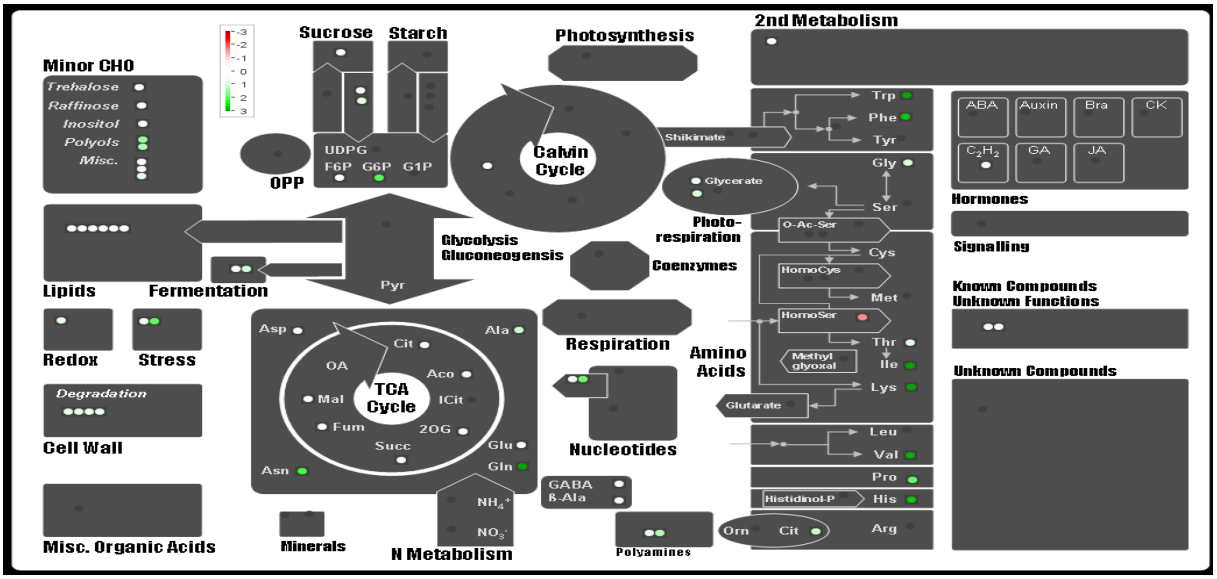

Figure 14: Metabolic modification in response to TOR inhibition (12 hours). Mean value ± SD are shown for 3 independent cultures. Data are shown in log (2) fold change, red color corresponds to a decrease, green color to an increase

A PCA analysis was performed and clearly showed a treatment effect and a time effect in TOR inhibition treatment which is absent in mock treatment **(Figure SS8)** both quite well supported. Interestingly the inhibition of TOR seems to cause a very quick response (around 12 hours) (**Figure 13)** which quickly decreases after that (**Figure SS9)** and mostly consist in an increase of almost all amino acids and in a moderate increase of signaling molecules content **(Table SS4 and SS5).** Surprisingly almost no evolution in TCA cycle compounds has been detected.

4- TOR inhibition modulates gene expression

qPCR analyses were performed to determine changes in gene expression. Six genes linked to carbon metabolism or response to stress were tested: CESA1 (cellulose synthase1), ProDH2 (Proline Deshydrogenase2), GLN1;5 (Glutamine sythase1; 5), TRE1 (trehalase1), SAG12 (senescence induced gene 12) and MIOX2 (myo-inositol dehydrogenase2). Reference genes were first investigated by using Genevestigator and geNorm **(Figure SS10).** SAG12 and GLN1;5 have never been found expressed in our conditions (data not shown). MIOX2 expression is between 10 and 20% lower without TOR activity which could be connected to a higher myo inositol content. CESA 1 activity doesn't change before the third day where it drops from 25% **(Figure 14)**.

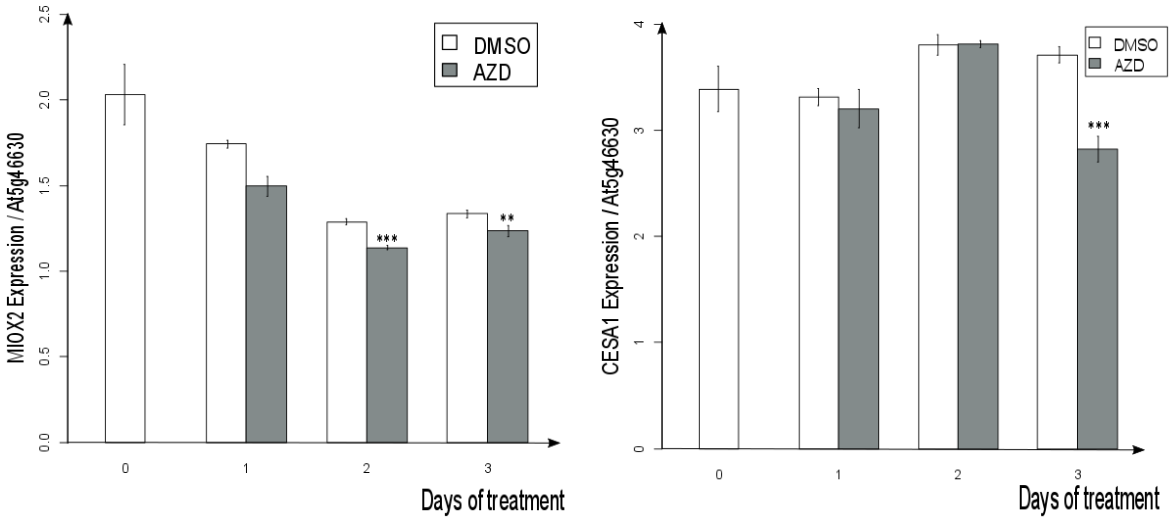

Figure 15: MIOX2 and CESA1 expression in comparison to At5g46630. Mean value ± SD are shown for 3 independent cultures. Student test, significant differences between treatment are shown by asterisk (* P<0.05, **P<0.01, ***P<0.001)

IV- Discussion

Green cell transformation: In none of the 112 different tested conditions did transgenic callus develop. This means that it is not possible, at least with a realistic success rate, to transform green cells with the tested protocols. We confirmed that the construction has not been transferred because the kanamycin resistance gene has not be expressed. This could be due to the big size of TOR gene (17,5 kb) causing a huge difficulty to transfer it with a plasmid construction. However, this hypothesis can be rejected because the same construction was used to transform white cells with a high success rate, which was totally comparable to the one obtained during their transformation with GFP which is only 717 bp long. A more realistic hypothesis is that the co culture didn't last enough time: the doubling time of CH line is indeed

3,11 days and the coculture never last more than three days which can explain this result. One of the other possibility which could be tested is the use of more virulent *Agrobacterium tumefaciens* or the treatment of green cells with acetosyringone to facilitate the transfer. Possibly the age of the cells have importance for transformation efficiency. This was not tested in this set of experiments due to the slow growth of the cells. Transient transformation (by using protoplast for example) could have been a good alternative but the natural variation in results and the necessity to perform it at every study are two real inconvenient to its use.

Carbon starvation: Several response to a carbon starvation were expected: a reduction of growth rate (or even an arrest), a strong decrease in soluble sugars and starch content, an increase in cell death caused by energy depletion, and a decrease in chlorophyll content (at least caused by the extended darkness) (Veith and Komor, 1993; Izumi et al., 2010). Concerning other metabolites, a decrease in TCA cycle intermediates and an enhancement in stress signaling molecules were expected coupled with an increase in amino acid content (Contento et al., 2004). All these responses previously detected in whole plants were detected in our cell culture system. Transcriptomic responses were expected too, especially an increase of stress response expression and a decrease of anabolism gene expression. An immediate and total arrest of growth has been shown and corresponds to the expectation as does the strong increase in death rate, almost all the cells being dead after 11 days. Soluble sugars were quickly depleted (around 90% after 11 days) but starch content didn't change. Both chlorophyll a and b were higher under starvation which was unexpected too. TCA cycle intermediates were harshly depleted (between 5 and 10 times less) during starvation but both amino acids and signaling molecules content increased (both more than 3 log (2) fold changes). Finally no really striking changes in the six genes (CESA1, ProDH2, MIOX2, TRE1, SAG12, GLN1;5) tested have been observed except for MIOX2 which was slightly repressed after 7 days. The impossibility for the cell to perform an uptake of sugar in the medium as well as the impossibility to perform photosynthesis because of the extended darkness logically led to the use of the soluble sugars which were present in the cell as only source of carbon available. Sucrose was probably converted to glucose which is mandatory for vital biological process like respiration which explains their quick depletion. Once carbon sources were exhausted, those biological process were turned off, degrading the energetic state of the cell and explaining the strong decrease in TCA intermediates content. Without those processes the cells will lack of energy, slow their metabolism (notably the most energy demanding process) and finally die as observed. This increase in death rate is concomitant with the arrest of the growth which is anyway a very both energy and carbon (for cell wall synthesis) demanding process. CESA1 is known to be implicated in this synthesis so a strong reduction of its activity was expected but it was not the case although it is with CESA3 one of the major protein to synthesize cellulose. However CESA1 mutants have shown a reduced cell length rather than cell number (Burn et al., 2002) and this work deals with cell culture which does not elongate so much but rather divide explaining this result. In addition, MIOX2 expression which is required for cell wall synthesis decreases after 7 days from 20 to 30% which corresponds to this turn off of cell wall synthesis.

Carbon starvation causes nutrient recycling and turns off anabolism so it was possible to think that chlorophyll content should decrease with this condition either because of a slower

synthesis or because of a stronger degradation. However, carbon starvation seems to prevent chlorophyll degradation. In addition, there is no nitrogen starvation which could cause chlorosis before 7 days (Thomas Dobrenel, unpublished observation). The first hypothesis is that starving cells slowed their metabolism, especially anabolism to survive and by doing that consumed less nitrogen as the cells growing with sugars. That means that they could have only reached the nitrogen starvation after 11 days and not after 7 days. This drop in chlorophyll content would have been caused by a nitrogen starvation and not by a carbon starvation and would consist in a higher degradation rate. Goldschmidt and Huber (1992) showed that photosynthesis activity was down regulated by end product especially sucrose. Even if they don't look for chlorophyll content (rather for stomatal resistance and O_2 content) it could be possible that a depletion in this sugar as the one which was observed here causes an enhancement in the chlorophyll content to stimulate photosynthetic activity. This hypothesis is partially confirmed by the fact that chlorophyll content and growth are negatively correlated (Sulpice et al., 2009) but cells undergoing a carbon starvation hold their growth which could increase their chlorophyll content too.

It was expected that starch rate decrease to produce some soluble sugars but it was not the case. However, in the meantime amino acid contents which are not related to TCA cycle strongly increase, meaning that either amino acid synthesis increases (but it is unlikely because amino acid biosynthesis is a very energy demanding process: 4,7 to 4,9 ATP per amino acid which would be contradictory with the low energetic state of the cell) or protein degradation increases. Beyond this amino acids, glutamine has several roles (energy production, carbon and nitrogen donation) so a change in GLN1;5 was expected but it was even not found express (data not shown). There are 5 cytosolic GLN and one chloroplastic one. GLN1;4 and 1;5 are not expressed in vegetative tissues (Dragićević et al., 2014). For those reason, although CH line is not a fully vegetative line, it may explain why no expression of GLN1;5 has been found. Kölling et al.(2015) suggest an explanation for both of those results: a carbon starvation seems to increase carbon allocation to starch and protein degradation which produces more energy than starch degradation.

In addition, carbon starvation causes a strong accumulation of signaling molecules like sugars (trehalose), sugar derivate (sorbitol, mannitol), stress amino acids (proline) or myo-inositol (which is concomitant with the drop in TRE1 and MIOX2 expression obtained (data not shown), although this changes in expression fold are not so important. Sorbitol and mannitol have the role of osmoprotection, although sorbitol seems to be slightly use in metabolism (Berüter et al., 1997) and mannitol is proposed to have a role against pathogen in whole plant (Juchaux-Cachau et al., 2007) especially to protect against ROS during infection. In addition, some evidences (Szarka et al., 2008) showed that mannitol could be transported in the cell and could play its osmoprotection role in response to sugar depletion which makes the osmotic potential decrease.

As this carbon starvation treatment induced cell death, a senescence was expected but ProDH2 expression which is strongly induced in case of senescence or in abscission zone (Funck et al., 2010) did not change (data not shown). This is to be correlated with SAG12 expression that was not detected in CH line during the whole experiment, including in the

dying cells. It could be possible to think that SAG12 is not expressed in cell cultures but it is not the case (De Michele et al., 2009) and ProDH2 is induced by a very broad spectrum of stimuli. There are also three main hypotheses to explain this result, either this senescence step is independent of those two genes which seems unlikely or there is a lack of hormone signaling (especially abscisic acid) in these cells which is mandatory to express those genes or even those cells doesn't undergo a senescence step.

TOR inhibition: As for carbon starvation many responses were expected to a TOR inhibition: an arrest of growth with an increase in the death rate, an increase in both soluble sugar and starch contents, and a decrease in chlorophyll content. An increase in TCA cycle intermediates, amino acid contents and stress response metabolites were expected too (Caldana et al., 2013). Finally, an increase in stress response and senescence genes expression coupled with a decrease in anabolism genes expression were predicted. Surprisingly neither growth arrest nor increase in the death rate were observed. If glucose content strongly increased, that is not the case for fructose content which was depleted after 3 days (data not shown) or sucrose content which underwent a slight diminution after 24 hours. Chlorophyll a and b contents were lower for the cells without TOR activity and more precisely were exactly at the same level as before the treatment whereas chlorophyll content increased in the cells which exhibited TOR activity. The cells without TOR activity showed a much higher content in amino acids (around three times for most of them) but the effect seemed to decrease after twelve hours. A slight increase in signaling molecule content was observed but no effect has been detected on TCA cycle intermediates. No real striking effect was observed on the expression of the tested transcripts except maybe for CESA1 and MIOX2 both repressed of 20% after 3 days and between 10 and 15% the two first days respectively. First it is important to notice that the concentration of AZD 8055 used in this study (0,5µM) is very low (only 25%) in comparison to other studies (Dong et al., 2015). In addition, the inhibition duration is much shorter (3 to 4 times) than the one of other studies (Menand et al., 2002; Dong et al., 2015) which can explain the difference in the magnitude of the answers. This was done to determinate very accurately which answers are only due to TOR inhibition and to remove as many side effects as possible. Surprisingly cells do not hold their growth with an AZD 8055 treatment and more, display a 10 to 15% biomass augmentation (data not shown). It is to remain that growth is normally composed from two different processes: cell division and cell elongation. This augmentation could not be attributed to a faster division, AZD 8055 has indeed a cytotoxic effect (Shao et al., 2012). Further investigations were done on death rate and cellular water content. If the death rate doesn't change at all (data not shown), water content increase from 5 to 10% (data not shown) in AZD-treated cells, probably because AZD 8055 interferes with cell wall integrity by inhibiting TOR (Henriques et al., 2014) causing a water entry and increasing their weight. Those data correspond with the drop observed in CESA1 expression. Nevertheless many authors showed that TOR is essential for cell division (Menand et al., 2002; Henriques et al., 2014), it seems possible to expect an arrest of the division with a longer or stronger inhibition.

TOR inhibition causes a quick increase in glucose content which could be either related to a decrease in its consumption which seems unlikely because there is not any change in TCA cycle intermediates content. This increase could explain the restoration of cell state after one day

because glucose is supposed to activate TOR (Xiong and Sheen, 2012), this strong increase could have helped to counteract the inhibition caused by AZD 8055. This increase could come from sucrose hydrolysis or from starch hydrolysis. Sucrose content effectively decreased even the first day but then the difference in its content in cells with or without TOR activity remains constant. But the decrease in sucrose content was not strong enough to explain the augmentation of glucose content alone so starch content was investigated (data not shown). No real significant change has been obtained although TOR inhibition or SnRK1 is known to increase it (Dobrenel et al., 2013) which could mean that in the other hand its hydrolysis increased in the meantime. As explained above CESA1 activity dropped and cell wall integrity was probably affected. Cell wall synthesis is a high carbon demanding process and could explain the augmentation in glucose content by its reduction if we consider the non-evolution of TCA cycle.

The other idea which could have explained the increase in glucose content would have been an increase in photosynthetic activity. However, it seems really unlikely because both chlorophyll a and b contents decrease after a TOR inhibition. The first hypothesis could be the autophagy of chloroplasts caused by TOR inhibition as suggested by Avila-Ospina et al. (2014). Another hypothesis is that TOR inhibition mimics a nitrogen starvation (as observed in yeast) (Dobrenel et al., 2016) and this could decrease chlorophyll content a lot by degrading it. Both hypothesis are supported by Li et al. (2015) who showed that an AZD treatment rather increases chlorophyll degradation than decreases the biosynthesis rate.

Chlorophyll degradation could be one of the first sign of senescence procedure which could occur during TOR inhibition as part of autophagic procedure (Liu and Bassham, 2012). However no increase in death rate was found and no expression of SAG12 neither. In addition, as for carbon starvation no real increase in ProDH2 expression has been found (data not shown) with the same hypothesis as before. Nevertheless this time, both weak duration and intensity of inhibition could explain that this autophagy step didn't occur at least totally.

Finally a strong increase occurred in most of the amino acid contents especially phenylalanine, histidine and proline) which corresponds to the observation of Caldana et al. (2013) and means that either their biosynthesis increases or protein degradation increases or protein synthesis decreases. TOR inhibition has been shown to cause autophagy (Liu and Bassham, 2010; Dobrenel et al., 2016) so the main hypothesis is a protein degradation but as said above this process seems to be limited and could not explain alone this increase in amino acid content. However, this effect seems to disappear after 24 hours. But as explained by several groups (Deprost et al., 2007; Xiong and Sheen, 2012; Caldana et al., 2013), TOR inhibition strongly blocks translation, by lowering the number of polysomes leading to a lower protein synthesis rate. This process coupled with the protein hydrolysis could explain the diminution of the strength of the answer: there are no soluble proteins anymore to hydrolyze. It could also be that AZD 8055 has been metabolized or exported out of the cell after one day, the concentration used was indeed very low. Trehalose content increases which corresponds with the 15% reduction in TRE1 expression, underlying the important role of trehalose in stress signaling. We could have expected a stronger decrease but the system which was used is composed only from single cells which could change the responses involved in signaling. In addition trehalose is more implicated in biotic stress response (Ma et al., 2011). Finally myo-inositol content decreased strongly which seems to be contradictory at least partially with the

decrease of MIOX2 expression. Nevertheless the decrease in myo-inositol content can be explained by the fact that it is a metabolite which is used to synthesize ascorbate (Lorence et al., 2004) which is a main metabolite in stress signaling. MIOX2 is especially involved in hemicellulose and pectin pathway (Loewus and Murthy, 2000) and we showed that both of those pathways seem to be down regulated which causes the lack of stability of the cell wall (in addition of the down regulation of CESA1).

Shared targets between carbon starvation and TOR inhibition: Carbon starvation and TOR inhibition share a strong overlap. Both affect cell growth and especially cell wall integrity (by decreasing CESA1 expression). If no effect has been found on cell division in the case of TOR inhibition, it seems to be more because of the short duration or the low dose of inhibitor (Menand et al., 2002). Starvation probably slows down the growth because of a lack of availability of resources (both energy and carbon) but TOR inactivation couldn't turn it off with a lack of carbon availability (glucose content increased) so it seems possible to estimate that at least a part of the processes used for cell growth which were switched off by an energy depletion are partially controlled by TOR in optimal conditions. If starvation logically strongly decreases cellular soluble sugar content, TOR increases the glucose content. That is explained by the fact that TOR inhibition switches off glycolysis (Dobrenel et al., 2013) so, although glucose content increases, there is no effect on TCA cycle because glycolysis is probably not working very quickly. Sucrose content decreased in both cases (only slightly during TOR inhibition) although TOR inactivation should have increased it (Dobrenel et al., 2013), however glucose content increased much more: it is to considered that in both cases it was hydrolyzed to produce glucose which correspond to the idea that TOR inhibition mimics an energy depletion. Starch content never changed meaning that protein degradation could be a preferential way to generate some energy. Another explanation could be that the starch reserves are always very low (30 times less than the glucose reserve before treatment). The effect on the entry of carbon metabolism, photosynthesis, is opposite which first looks like contradictory. Several hypothesis could explain that difference, first early degradation could be a process which is carbon demanding: this could explain that it occurs only in presence of sugars and not during starvation even if TOR is probably at least partially inhibited during carbon starvation. Another hypothesis here is that the carbon starvation causes two antagonists effects: an increase of chlorophyll content caused by an arrest of growth and a decrease caused by TOR inhibition (caused by the starvation and the darkness) so it seems possible that those two effects cancel each other out. Furthermore, it seems that the late degradation of the chlorophyll in mock treatment during the starvation experiment is mostly caused by the inactivation of TOR (caused by darkness). This is supported by the fact that TOR inhibition caused a chlorosis probably by mimicking a nitrogen starvation (as it does in yeast) which seems to explain the effect on late cultures.

Both stressing conditions seem to produce signaling molecules and to increase amino acid content. Most likely those amino acids come from protein hydrolysis in both cases which corroborates the idea that TOR inhibition mimics an energy depletion.

Finally, no trace of senescence has been observed in both cases: no SAG12 and ProDH2 expression which was surprising because almost all cells died during carbon starvation. The

main hypothesis here is that CH line doesn't undergo a senescent step before dying because at least TOR inhibition is known to cause senescence (Deprost et al., 2007) Both treatments caused a really strong increase in trehalose content, especially the carbon starvation. Trehalose and T6P are normally present and can only be synthetized from glucose in plants which seems to be contradictory with the glucose depletion observed during carbon starvation. However Paul et al. (2008) showed that trehalose is present at a concentration which is between 0.1 µM and 10 µM so an increase in its synthesis seems to be still possible. Another explanation could be the fact that T6P is a very important metabolite in both cell wall synthesis and cell division (Iturriaga et al., 2009) and both processes are switched off by starvation and TOR inhibition. An accumulation of T6P could led to this strong increase of trehalose content.

General conclusion and further works

CH line is a very powerful tool and the generation of transgenic lines overexpressing AtTOR in this background is considered as a priority. Even if this project didn't manage to perform such a transformation it removed a lot of possibilities and asked a lot of new questions.

The link between TOR and carbon metabolism seems to be very strong and this project managed to set in light some shared targets in metabolism like amino acid content which generally increases in both cases, as does signaling molecules like sugar derivatives or myo inositol. Nevertheless, some metabolites seem to be controlled only by starvation (which decreases strongly the TCA cycle metabolites content quickly) although this point is subject to discussion, a strongest inhibition of AtTOR could give the same results. SAG12, MIOX2, ProDH2, ASN1 which were known to be very good stress response markers should be reconsidered in CH line cell culture.

Further experimentation will be necessary to fulfill this project and the first one will be a RNA sequencing on both experiments to find more reliable stress response genes. A study of the influence of nitrogen starvation could be mandatory too because it seems to interfere with the carbon one. A study of the eventual senescent step or process seems to be also important to explain the end of this stress procedure. TOR inhibition duration will be changed both shortly (to determine the presence of very short time effects) and longer (to determine if cells reach the effects described in the literature).

Finally, SnRK1 could be included in this study to determine which targets really belong to TOR targets and which don't because TOR and SnRK1 share some targets but some are specific to each pathway.

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List of supplementary figures:

List of supplementary tables :

Gene ID	Primer sense	Length (bp)	Primer sequence (5'➔ 3')	Product size
AT1G27450 (APT1)	Forward	20	GAGACATTTTGCGTGGGATT	124
	Reverse	20	CGGGGATTTTAAGTGGAACA	
AT4G34270 (TIP41)	Forward	20	TCCATCAGTCAGAGGCTTCC	117
	Reverse	20	GCTCATCGGTACGCTCTTTT	
AT4G05050 (UBQ11)	Forward	19	GCAGATTTTCGTTAAAACC	167
	Reverse	17	CCAAAGTTCTGCCGTCC	
AT1G30230 (EF1)	Forward	21	CTGGAGGTTTTGAGGCTGGTA	53
	Reverse	21	CAAAGGGTGAAAGCAAGAAGA	
AT3G46520 (Actin-12)	Forward	21	GATGCCCAGAAGTCTTGTTCC	86
	Reverse	20	GACCTGCCTCATCATACTCG	
AT5G23860 (Tub8)	Forward	27	GAGCCTTACAACGCTACTCTGTCTGTC	104
	Reverse	27	ACACCAGACATAGTAGCAGAAATCAAG	
AT5G08290 (YLS8)	Forward	20	AGAGCGTCTCGTCGTCATTC	71
	Reverse	20	CAAGCACCTCATCCATCTGC	
AT1G62930 (RPF3)	Forward	21	AAGAAATGAGGAGTTGCGGGT	146
	Reverse	21	GTTGCTCCCCAATTCGTTTCT	
AT5G27630 (ACBP5)	Forward	20	TGGTGTGACGATTGGGGAAA	70
	Reverse	20	AGTCTTAGATGCCCCGCTTT	
AT5G46630 (AP2M)	Forward	20	GAGTCCACGCTAAGTGCAGA	145
	Reverse	22	TCTCCCAAACCTTGAGAAAACG	
AT1G69960 (PP2A)	Forward	20	TAACGTGGCCAAAATGATGC	78
	Reverse	20	GTTCTCCACAACCGCTTGGT	
AT4G36800 (UBI)	Forward	20	CTGTTCACGGAACCCAATTC	133
	Reverse	20	GGAAAAAGGTCTGACCGACA	
AT4G32410 (CESA1)	Forward	20	AATCTGATGGCGGGACCAAA	131
	Reverse	20	ACACACAGGGAAGGCACATT	
AT5G38710 (ProDH2)	Forward	20	GTGCTTTAGACCGCCAACTT	66
	Reverse	21	TGTGTCTTCACCAAGCCATAA	
AT1G48470 (GLN1;5)	Forward	21	AGCAAAGCCAGGACATTACCA	96
	Reverse	19	ATCTCCGGCAGCTTGATCG	
AT4G24040 (TRE1)	Forward	21	ATGTTCACTACCGCCAAAGGT	111
	Reverse	21	AGGTGGTTGGCTTCTGTTAGT	
AT5G45890 (SAG12)	Forward	24	TCCTTACAAAGGCGAAGACGCTAC	87
	Reverse	22	ACCGGGACATCCTCATAACCTG	
AT2G19800 (MIOX2)	Forward	22	GAGGAGAGGGATAATGAATTGG	204
	Reverse	24	CTTCTTCACAAAGTCATAGGTCTG	
AT3G47340 (ASN1)	Forward	20	AACTTGTCGCCAGATCAAGG	162
	Reverse	20	GGAACACGTGCCTCTAGTCC	

Table SS 1: Full primer list for q-PCR

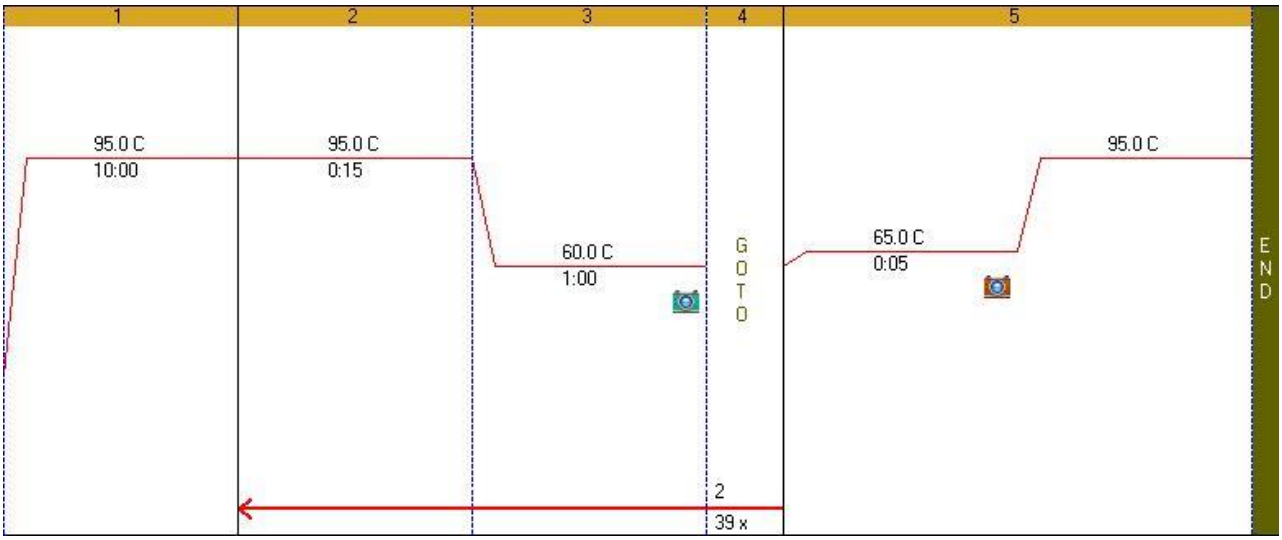

Figure SS 1: q-PCR program

Macro elements concentration (mg/L)		
Element name	Gamborg	MS
$CaCl_2$	113.23	332.02
KNO_3	2500.00	1900.00
$MgSO_4$	121.56	180.54
NaH_2PO_4	130.44	0
$(NH_4)2SO_4$	134.00	0
KH_2PO_4	0	170.00
NH_4NO_3	0	1650.00
Micro elements concentration (mg/L)		
Element name	Gamborg	MS
$CoCl_2.6H_2O$	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025
FeNaEDTA	36.70	36.70
H_3BO_3	3.00	6.20
KI	0.75	0.83
$MnSO_4.H_2O$	10.00	16.90
$Na_2MoO_4.2H_2O$	0.25	0.25
$ZnSO_4.7H_2O$	2.00	8.60
Vitamins concentration (mg/L)		
Vitamin name	Gamborg	MS
myo-Inositol	100.00	100.00
Nicotinic acid	1.00	0.50
Pyridoxine HCl	1.00	0.50
Thiamine HCl	10.00	0.10
Glycine	0	2.00
Total nutrient concentration	**3163.98**	**4405.19**

Table SS 2: Culture medium composition

Figure SS 2: Effect of carbenicilin on *Agrobacterium tumefaciens* growth (LB medium) after 4 days.
200 mg/L seems to be enough to inhibit all Agrobacterium tumefaciens development but for more safety 500 mg/L were chosen as long it does not affect CH line development

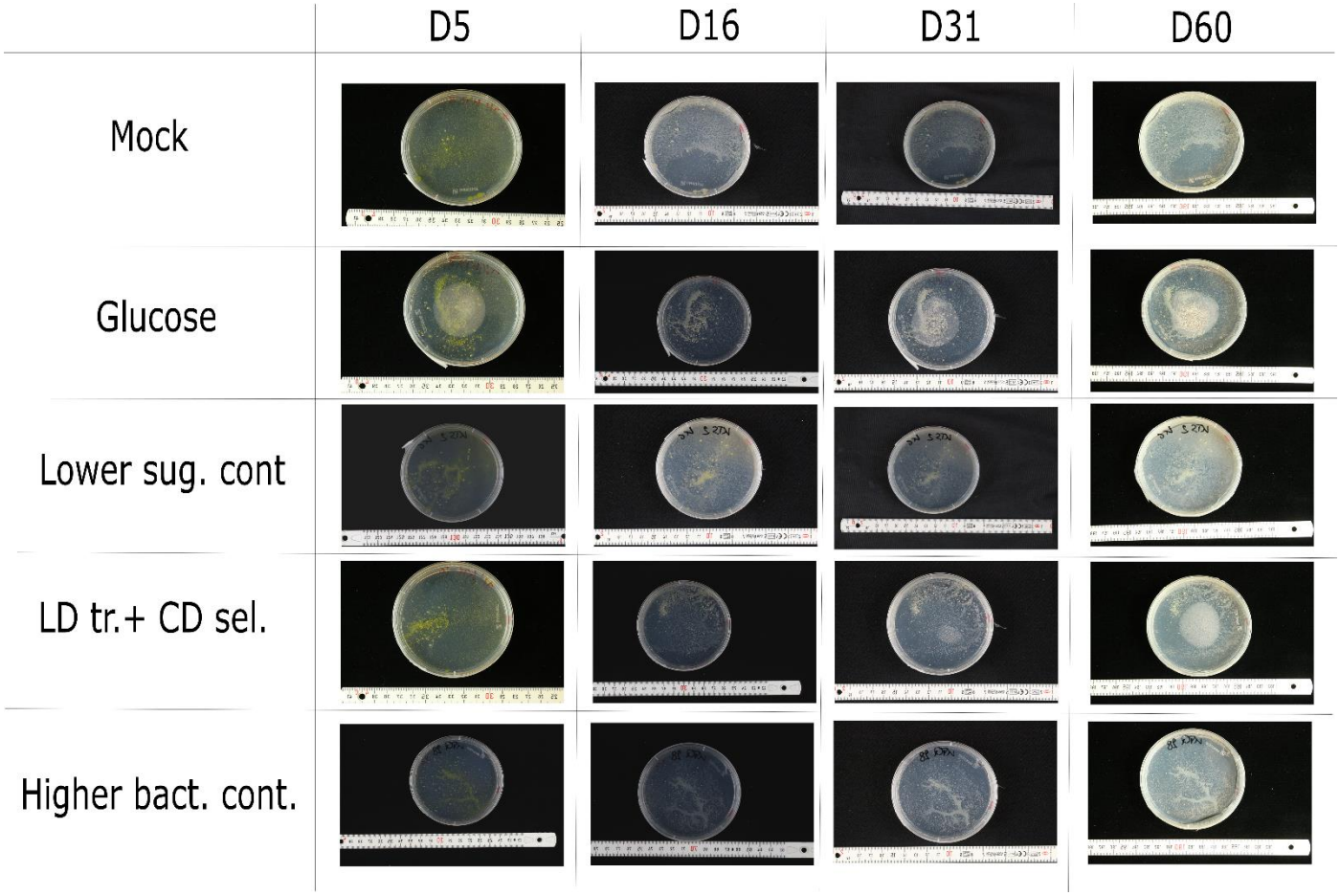

Figure SS 3: Examples of tranformation evolution during 60 days. Mock: standard protocol, Glucose: glucose as carbon source, lower sug. content: no sugar during transformation and selection, LD tr. + CD sel.: transformation performed in long day and selection in continuous day, Higher bact. cont.: 400 µL of Agrobacterium tumefaciens. Time is in days above the table. After 60 days the plates were dry and have been discarded as no callus was observed.

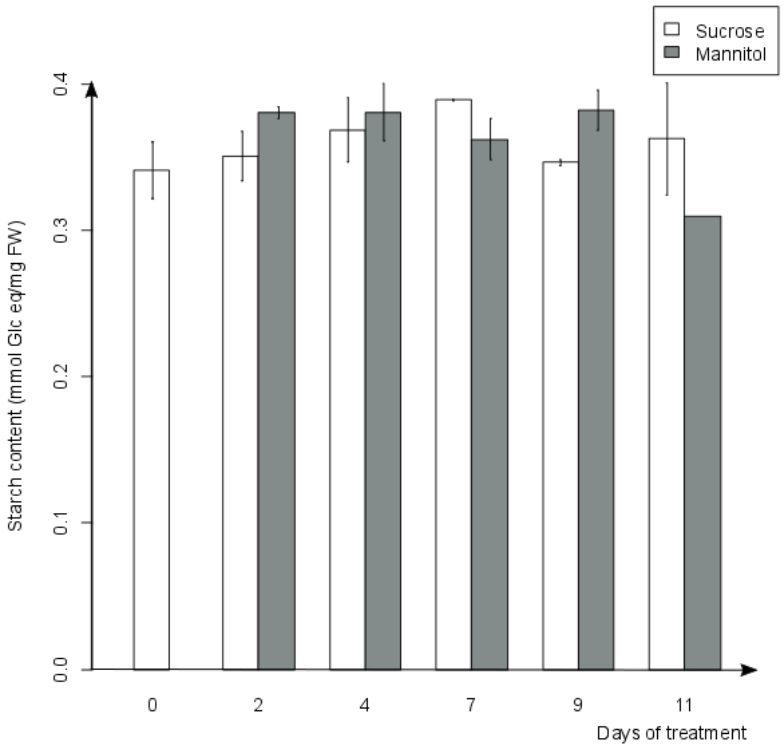

Figure SS 4: Starch content in cells under carbon starvation. Mean value ±SD are shown for 2 independent cultures for each condition.

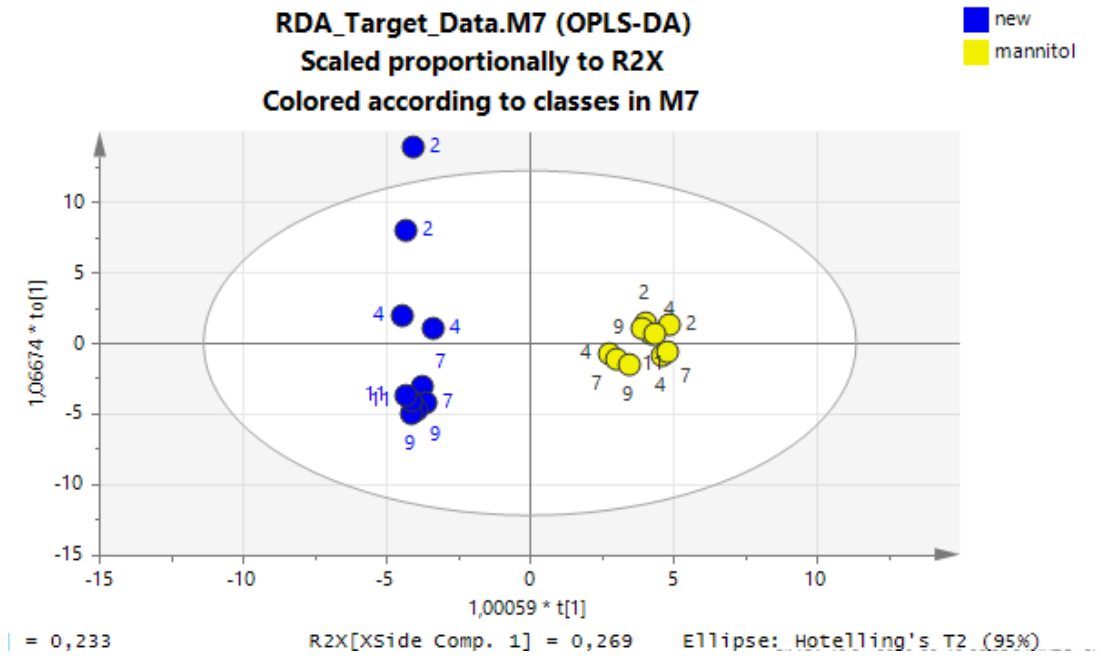

Figure SS 5: PCA analyze of metabolite content of both cells growing with sugars and starving cells. Abscissa axis explains 23,3% of the variation and ordinate axis explains 26,9% of the variation. Dots labels correspond to the time in days.

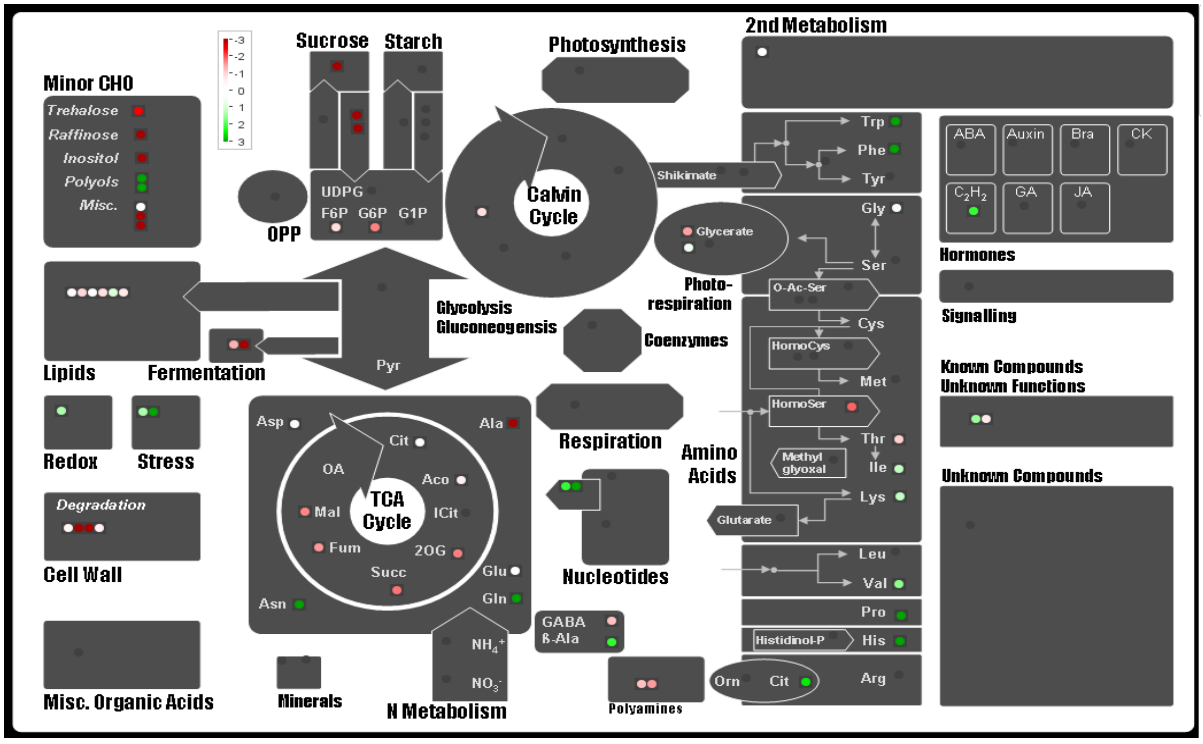

Figure SS 6: First metabolic response to a carbon starvation (D2) Mean value ±SD are shown for 2 independent cultures for each condition. *Data are shown in log (2) fold change, red color corresponds to a decrease, green color to an increase*

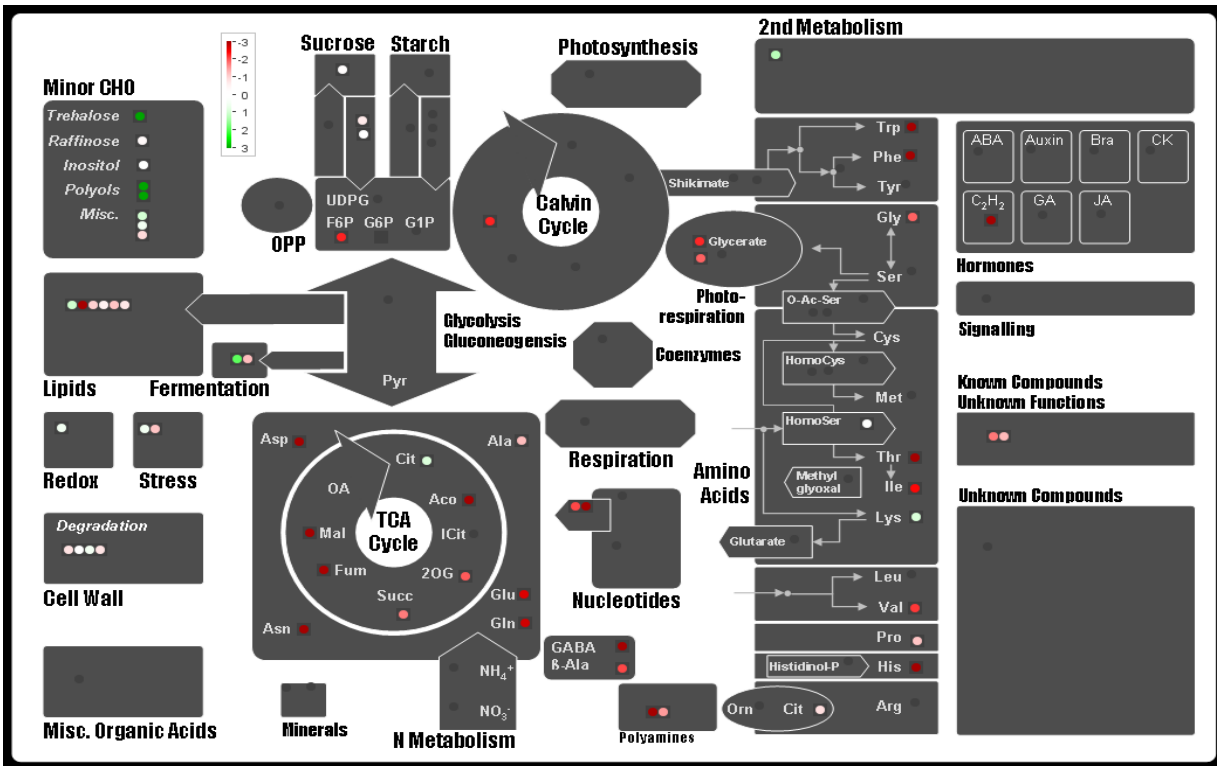

Figure SS 7: Late metabolic response to a carbon starvation (D11) Mean value ±SD are shown for 2 independent cultures for each condition. *Data are shown in log (2) fold change, red color corresponds to a decrease, green color to an increase*

Duration	Mannitol	Malic acid	Citric acid	Succinic acid	Aconitic acid	Ascorbic acid	Asp	Asn	Gln	Glu
2	7,00	0,26	0,68	0,29	0,67	3,46	0,67	19,44	4,40	0,96
4	7,00	0,18	0,55	0,47	0,38	4,29	0,40	5,36	1,20	0,92
7	43,00	0,13	0,38	0,40	0,10	23,33	0,09	0,71	0,30	0,27
9	14,00	0,13	0,10	0,29	0,04	16,67	0,05	0,29	1,00	0,43
11	80,00	0,13	0,13	0,28	0,03	4,10	0,02	0,05	0,16	0,02

Duration	GABA	2OG	Proline	Thr	Alanine	His	Trehalose
2	0,77	0,83	8,67	0,54	0,05	16,67	0,44
4	0,29	0,89	0,91	0,11	0,33	4,35	17,00
7	0,11	0,75	1,50	0,21	0,28	1,48	22,22
9	0,20	0,81	2,25	1,12	0,95	0,89	65,22
11	0,08	0,81	1,00	0,37	1,00	0,05	764,71

Table SS 3: Metabolite changes in starved cells Mean ratio $\frac{Cells\ growing\ without\ sugar\ content}{Cell\ growing\ with\ sugar\ content}$ value are shown for 2 independent cultures for each condition. Asp: aspartic acid, asn: asparagine, gln: glutamine, glu: glutamic acid, 2OG: 2-oxyglutarate, thr: threonine, his: histidine. Duration of the transformation is in days.

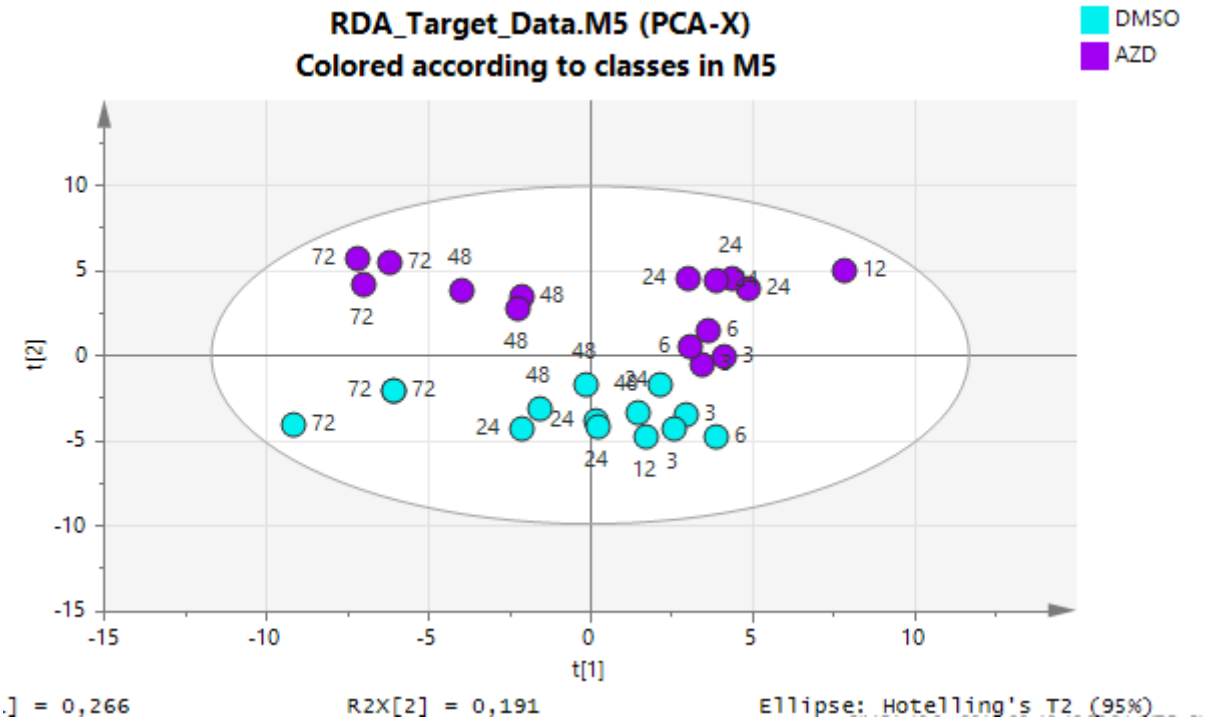

Figure SS 8: PCA analyze of metabolite content of both cells with or without TOR activity. Abscissa axis explains 26,6% of the variation and ordinate axis explains 19,1% of the variation. Dots labels correspond to the time in hours.

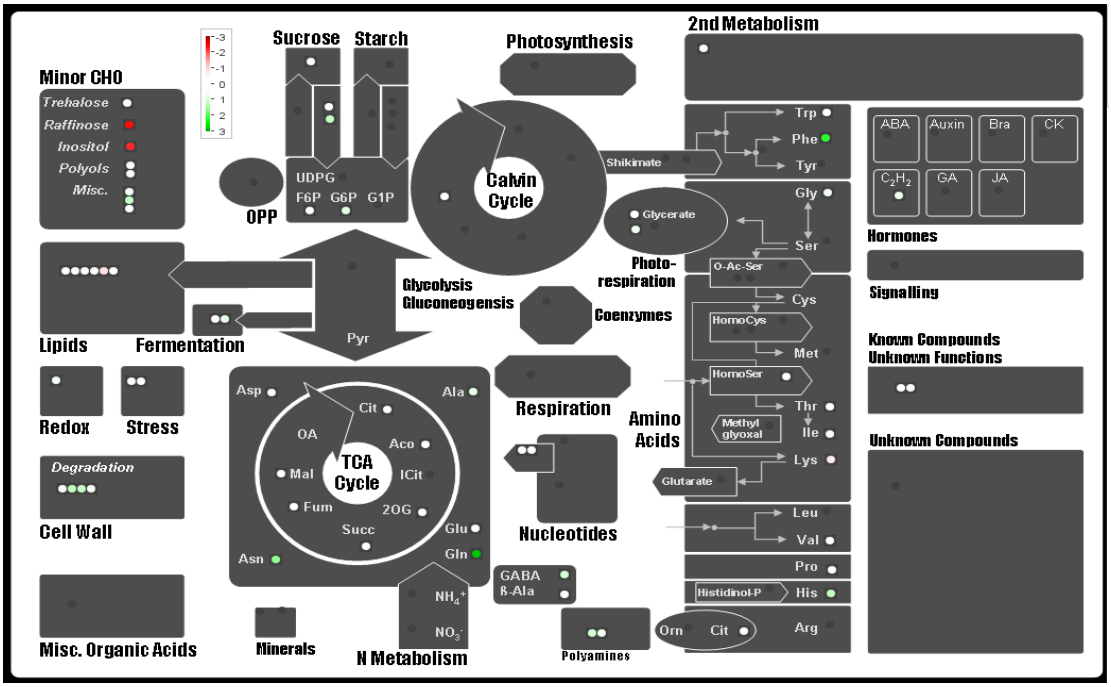

Figure SS 9: Metabolic recovery after 48h of TOR inhibition (48 hours). Mean value ±SD are shown for 3 independent cultures for each condition. Data are shown in log (2) fold change, red color corresponds to a decrease, green color to an increase

Duration	Phe	His	Pro	Myo inositol	Raffinose	Glycine	Asn	Spermidine	Trp	Succinic acid
3	2,14	2,50	3,00	0,96	0,78	1,50	1,83	0,84	4,38	0,85
6	2,29	3,80	3,40	0,87	0,58	1,38	1,32	1,12	4,17	0,77
12	7,00	6,75	3,82	1,00	0,86	1,91	4,70	2,39	9,33	0,92
24	3,80	6,00	1,20	0,61	0,59	1,25	4,80	1,95	2,67	0,67
48	6,86	2,31	0,96	0,22	0,22	1,50	2,93	1,33	1,00	1,00
72	15,00	1,93	1,67	0,29	0,13	1,57	2,70	1,35	1,75	0,67
Duration	**Val**	**Ala**	**Suc**	**Lys**	**Glc+ Man**	**Iso**	**Glu**	**Gln**	**Tréhalose**	**Sorbitol**
3	3,00	1,48	1,00	7,50	1,05	6,09	0,94	1,55	0,81	1,20
6	3,58	1,79	1,10	10,79	1,79	6,05	0,99	2,42	0,83	1,67
12	7,64	2,05	0,97	21,72	1,87	8,20	1,21	9,29	1,00	2,53
24	3,13	1,36	0,88	6,25	1,59	2,80	1,00	11,88	0,72	1,24
48	1,00	1,81	0,86	0,62	1,67	0,70	1,23	6,06	1,58	0,74
72	1,77	1,93	0,91	1,48	1,27	1,80	1,22	4,24	1,98	1,00

Table SS 4: Metabolites changes caused by TOR inhibition Mean ratio $\frac{Cells\ without\ TOR\ activity\ content}{Cell\ with\ TOR\ activity\ content}$ value are shown for 2 independent cultures for each condition under 24h, then for 3 independent cultures. Phe: phenylalanine, his: histidine, pro: proline, asn: asparagine, trp: tryptophan, val: valine, ala: alanine, suc: sucrose, glc+man: glucose + mannose, iso: isoleucine, glu: glutamic acid, gln: glutamine. Duration is in hours

Duration	Phe	His	Pro	Myo inositol	Raffinose	Glycine	Asn	Spermidine	Trp	Succinic acid
24	4,80E-03	6,32E-06	4,60E+00	1,18E+00	4,00E-01	4,60E-01	8,54E-04	3,81E-01	8,20E-02	1,50E+00
48	8,60E-02	2,98E-03	6,80E+00	2,40E-01	1,06E-01	8,80E-02	6,54E-03	6,29E-02	8,40E+00	4,20E+00
72	8,78E-04	7,03E-03	1,81E+00	1,21E+00	4,20E-02	8,40E-02	2,28E-02	5,08E-01	5,40E-02	9,80E-01
Duration	**Val**	**Ala**	**Suc**	**Lys**	**Glc+ Man**	**Iso**	**Glu**	**Gln**	**Tréhalose**	**Sorbitol**
24	2,00E-01	2,54E+00	7,02E-02	9,40E-01	3,52E-01	1,95E-01	3,16E+00	2,20E-04	3,15E+00	2,16E+00
48	1,02E+01	1,74E-03	2,68E+00	7,93E-01	9,80E-02	4,00E+00	9,38E-01	3,20E-01	5,04E-01	2,35E-01
72	2,80E-01	5,40E-01	9,40E-01	1,78E+00	2,40E-01	3,40E-01	3,61E+00	7,27E-03	2,42E+00	1,34E+01

Table SS 5: Statistical significance of metabolites changes caused by TOR inhibition. Student test with n = 3 for all conditions. P-values are given after a Bonferroni correction according to the following code : yellow: not significant (α=0.05), light green: 0.05 < p-value < 0.01, middle green: 0.001<p-value<0.01, dark green: p-value< 0.001. Not statistical analyze has been performed on times shorter than 24 hours because of the weak number of replicates. Phe: phenylalanine, his: histidine, pro: proline, asn: asparagine, trp: tryptophan, val: valine, ala: alanine, suc: sucrose, glc+man: glucose + mannose, iso: isoleucine, glu: glutamic acid, gln: glutamine. Duration is in hours.

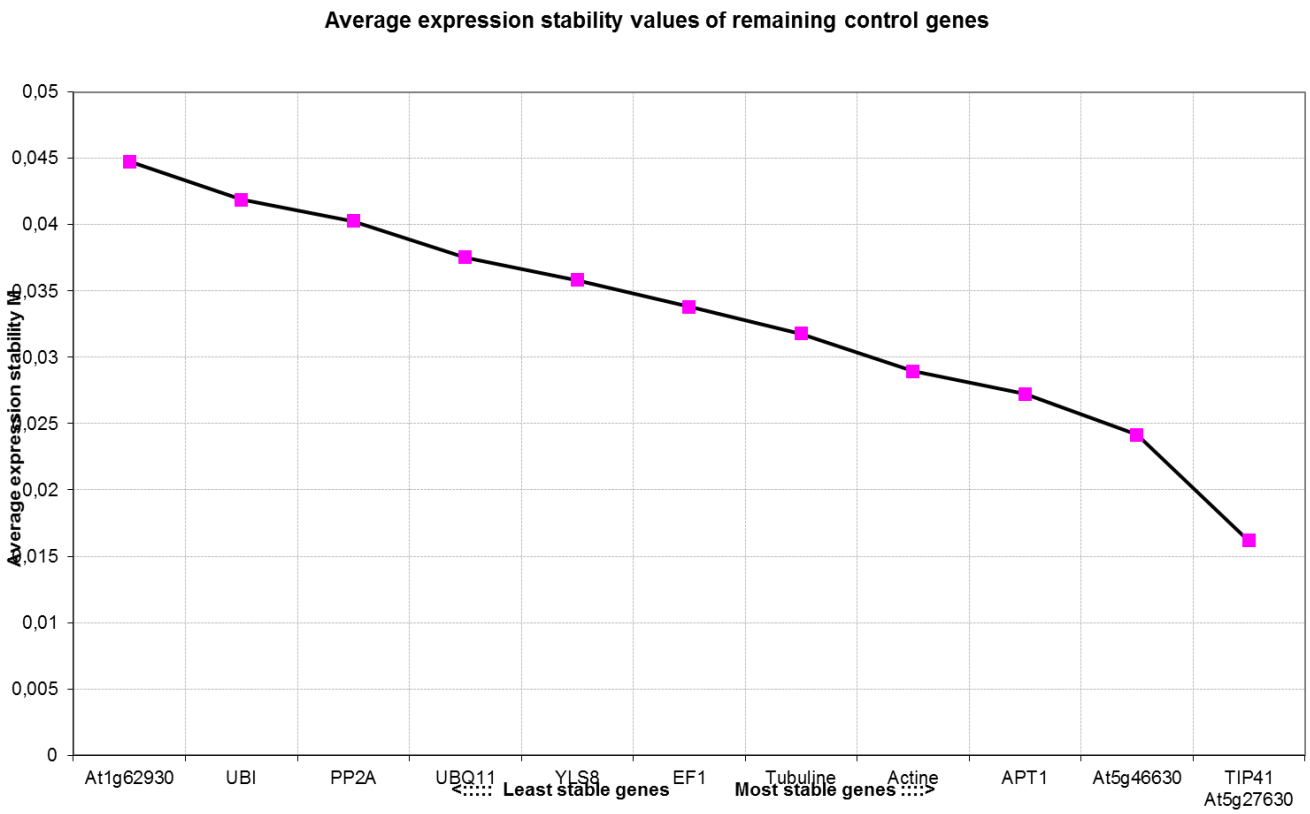

Figure SS 10: Stability of several genes after an AZD 8055 treatment. GeNorm was used to determine the most stable gene on a pre-selection of 14 made by GeneInvestigator. TIP41 was not conserved because of a too strong expression which needed a 1:63 dilution before getting a linear expression so At5g46630 and At5g27630 were chosen.

Abstract – English:

Carbohydrates are major molecules in plant development. They have multiple roles: they have a vital energy producing role, a structural importance by contributing to the cell wall synthesis and a signaling function. This signaling role is crucial and helps plants to adapt their metabolism to varying conditions in their environment. To relay this information some sensors are required. One of them is a kinase called TOR (Target Of Rapamycin) which is known to be upregulated by the presence of sugars. TOR activity stimulates growth, transcription and nutrient uptake. This kinase is activated in favorable condition and switched off by any kind of stress. Nevertheless TOR is not the only kinase which take part of carbon signaling and some responses to a carbon starvation are totally independent from it. This works propose to determine the metabolic and transciptomic responses to a carbon starvation and TOR inhibition and beyond them which are connected together. *Arabidopsis thaliana* cell cultures were used as model for their homogeneity and their ability to grow fast. A study of a new protocol to transform those cells is included. This study would lead to a better understanding of this TOR-sugars interaction. It could be use in the future to increase metabolite content in cell cultures like microalgae by adjusting the activity of TOR which could help for energy production.

Résumé – Français:

Les sucres sont des molécules majeures pour le développement des plantes. Ils jouent de multiples rôles : ils ont une importance vitale dans la production d'énergie, possèdent un rôle structural en contribuant à la synthèse de la paroi et ont une fonction de signalisation. Ce rôle de signal est crucial et permet aux plantes d'adapter leurs métabolismes aux conditions changeantes de leur environnement. Ces signaux sont relayés par des senseurs qui sont requis pour transmettre l'information. L'un d'eux est une kinase appelée TOR (Target Of Rapamycin), est connu pour être stimulé en présence de sucre. Son activité favorise la croissance, la transcription et l'assimilation de nutriments. Cette kinase est activée en conditions favorables mais est désactivée par tout type de stress. Cependant TOR n'est pas la seule kinase qui participe au métabolisme carboné et certaines réponses causées par la carence carbonée sont totalement indépendantes de son activité. Ce travail a pour objectif de déterminer les réponses à la fois métaboliques et transcriptomiques provoquées par la carence carbonée et par une inhibition de TOR afin de pouvoir déterminer lesquelles sont communes parmi elles. Des cultures cellulaires d'*Arabidopsis thaliana* ont été utilisées comme modèle pour garantir une homogénéité dans le système d'études et pour leur capacité à pousser rapidement. Une étude d'un nouveau protocole pour transformer les cellules en question est inclue. Cette étude pourrait permettre une meilleure compréhension de l'interaction entre TOR et les sucres. Cela pourrait être utilisé dans le futur pour augmenter le contenu de certains métabolites d'intérêt dans des cultures cellulaires telles que les micro-algues en ajustant l'activité de TOR ce qui pourrait favoriser la production d'énergie.