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THE EFFECT OF ETHYLENE, JASMONATE AND THE TRANSCRIPTION REGULATOR, AMR1, ON *ARABIDOPSIS THALIANA* ASCORBIC ACID ACCUMULATION

Master thesis (30 EAP)

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ABBREVIATIONS

AA – ascorbate

ACC - 1-aminocyclopropane-1-carboxylic acid

AMR1 – ASCORBATE MANNOSE PATHWAY REGULATOR 1

AO – ASCORBATE OXIDASE

APX1 – ASCORBATE PEROXIDASE

bHLH – basic helix-loop-helix

COI1 – coronatine insensitive 1

Col-0 – Arabidopsis thaliana Columbia accession, used as wild-type in this study

Ct – cycle threshold

CTR1 – CONSTITUTIVE TRIPLE RESPONSE 1

DHA - dehydroascorbate

DHAR – dehydroascorbate reductase

DTT – dithiotreitol

EDTA - ethylenediaminetetraacetic acid

EIN2 – ETHYLENE INSENSITIVE 2

EMS – ethylmethylsulfonate

ETR1 – ETHYLENE RECEPTOR 1

GalDH – L-GALACTOSE DEHYDROGENASE

GGP - GDP-L-galactose phosphorylase (also VTC2)

GLDH - L-GALACTONE-1,4-LACTONE DEHYDROGENASE

GME - GDP-D-mannose-3,5-epimerase

GMP – GDP-mannose pyrophosphorylase (also vtc1)

GPP - L-galactose-1-phosphate phosphatase (also VTC4)

GSH – reduced glutathione

GSH1 - γ-glutamylcysteine synthetase

gsh2 – glutathione synthetase

GR – glutathione reductase

GSSG – oxidized glutathione

HRGP – hydroxyproline-rich glycoproteins

JA-Ile – jasmonoyl-isoleucine

JAR1 – JASMONATE RESISTANT1

JAZ – jasmonate zim domain

LRR – leucine-rich repeat

MDHA - monodehydroascorbate

MDAR – MONODEHYDROASCORBATE REDUCTASE

MeJA – methyljasmonate

NADPH - nicotineamide adenine dinucleotide phosphate

NEM - N-ethylmaleimide

NLS – nuclear localization signal

NTC – no template control

OPDA - 12-oxophytodienoic acid

PMI – PHOSPHOMANNOSE ISOMERASE

PMM – PHOSPHOMANNOMUTASE

PPFD - photosynthetic photon flux density

qPCR – quantitative polymerase chain reaction

RIN – RNA integrity number

ROS – reactive oxygen species

RT-qPCR – reverse quantitative polymerase chain reaction where revertase is used

SCF complex - SKP1-CULLIN-F-BOX complex

SD – standard deviation

SOD – SUPEROXIDE DISMUTASE

TCA – trichloroacetic acid

Lowercase versions of abbreviations corresponding to plant genes refer to respective mutants.

PREFACE

Ascorbate (vitamin C; AA in abbreviation) is one abundant antioxidant in plant and animal cells. Furthermore it is the only low molecular weight antioxidant in apoplastic compartment. It reduces different oxidative agents which otherwise may cause damage to the cells. It is also a cofactor for several enzymes, and is involved in cell division and growth. Ascorbate may be found in the plant tissues in micromolar to millimolar range (Gallie 2013).

The accumulation of ascorbate is regulated by the synthesis, degradation and regeneration rates. Humans have lost the ability to synthesize vitamin C and that's why ascorbate is critical in our nutrition. Unlike mammals where ascorbate synthesis takes place only in one pathway, in plants ascorbate can be generated in multiple biosynthetic routes (Davey et al. 2000).

The main pathway, in which a significant proportion of ascorbate is synthesized, is the D-mannose/L-galactose pathway first described by Wheeler et al. (1998). In specific mutants, where some enzymes from this pathway are nonfunctional, the ascorbate concentrations are significantly reduced and some of these mutants are not viable (Conklin et al. 2000; Dowdle et al. 2007).

The knowledge of the regulation of ascorbate synthesis and regeneration is critical for pathway engineering to increase the ascorbate concentration in food. The only regulator so far identified is AMR1 (ASCORBATE MANNOSE PATHWAY REGULATOR 1). This protein is a transcriptional repressor of the expression of several genes in the ascorbate synthesis pathway (Zhang et al. 2009). Other regulators of ascorbic acid biosynthesis include the stress hormones ethylene (Gergoff et al. 2010) and jasmonate (Suza et al. 2010).

In this study experiments were done to investigate the molecular basis of regulation of the expression for the ascorbate biosynthesis enzymes. The major goal was to find the effect of jasmonate and ethylene signal transduction and effect of AMR1 to ascorbate synthesis in D-mannose/L-galactose pathway. Additionally, included in the gene expression analysis were *GSH1* and *GSH2* encoding for two biosynthetic enzymes of glutathione, another antioxidant with many roles in plants. In particular oxidized ascorbate is regenerated through the ascorbate-glutathione cycle. In the gene expression analysis also the role of ASCORBATE PEROXIDASE (APX1) was investigated. APX1 is an enzyme that oxidizes ascorbate for quenching of hydrogen peroxide.

First the ascorbate content was measured in *Arabidopsis thaliana* mutants, in which ethylene or jasmonic acid signaling was disabled or constitutively activated. Additionally the *amr1-1* mutant with disabled ascorbate synthesis regulatory properties and a *vtc1-1,* with inactive ascorbate synthesis enzyme, were used as controls.

In the second part expression of genes coding for ascorbate and glutathione biosynthetic enzymes, *AMR1* and *APX1* were measured with RT-qPCR. The altered expression of these genes might give a hint where and how ascorbate content is regulated.

1. OVERVIEW OF LITERATURE: ASCORBIC ACID AND THE REGULATORS OF ITS SYNTHESIS

1.1. Chemical properties of L-ascorbic acid

Chemically ascorbate is a C_6 sugar (aldono-1,4-lactone). It has reactive enediol groups on carbons 2 and 3, which give the compound the reducing properties – it can give away its two protons and two electrons. The π-electrons are stabilized by the conjugated enediol groups and can be delocalized. The hydrogen on the very acidic C3 hydroxyl group can readily dissociate with the pK_a of 4,13. In the physiological pH ascorbic acid is in its deprotonated form (*Figure 1*). The second hydroxyl dissociates with the pH of 11,6 (Davey et al., 2000).

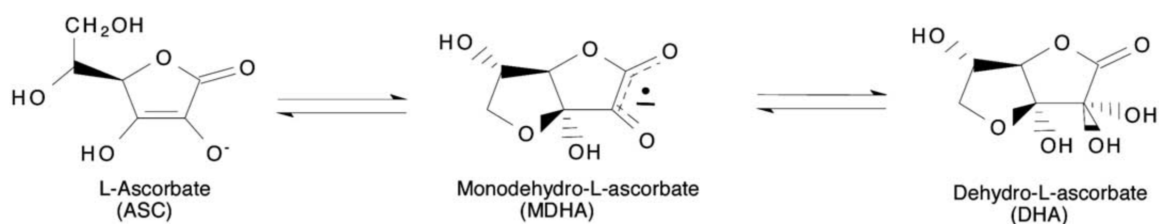

***Figure 1** The structural views of the steps of ascorbate oxidation (Potters et al., 2002).*

1.2. Redox forms, recycling and degradation of L-ascorbic acid

In optimal conditions about 90 % of ascorbate pool is in its reduced form (Pallanca and Smirnoff 2000). As a water soluble reductant, ascorbate, is stable only in dry environment – in solutions it readily oxidises.

Ascorbate oxidation takes place in two steps. The oxidation may be nonenzymatic or facilitated by specific enzymes described below. At first ascorbate loses one electron and one proton, which results in the formation of monodehydroascorbate (MDHA). Ascorbate can be regenerated from MDHA by directly accepting an electron from reduced ferredoxin (a component of the photosystem I in the thylakoid membrane) in the chloroplast or by the ascorbate-glutathione cycle (Halliwell-Foyer-Asada pathway) introduced below (Gallie 2013).

MDHA is unstable and can readily give away one more proton and electron – then it is dehydroascorbate (DHA), the fully oxidized form of ascorbate (*Figure 1*). DHA is unstable in aqueous solution above pH 7 and irreversibly delactonizes to 2,3-diketogulonate (Pallanca

and Smirnoff, 2000). The degradation byproducts are oxalate, L-threonate and L-tartrate. The enzymes involved are not yet identified (Ishikawa et al. 2006).

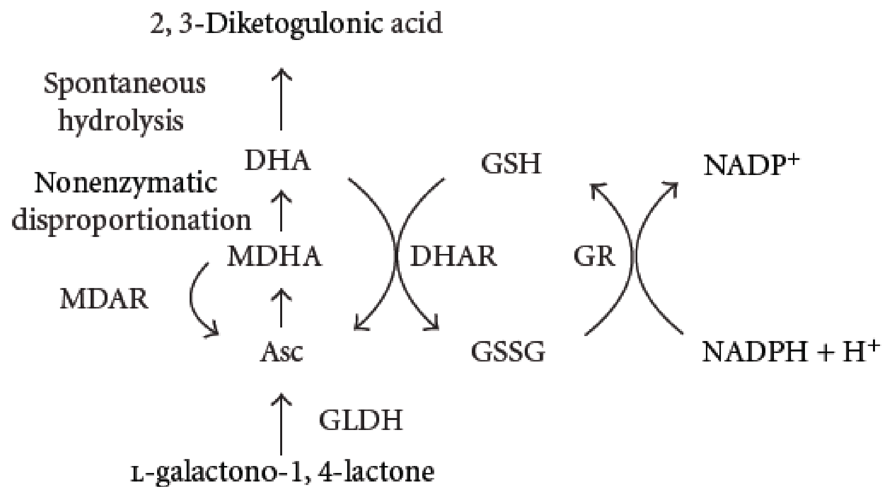

Figure 2 *The recycling of L-ascorbic acid. Ascorbate is synthesized from L- galactono-1,4-lactone in the D-mannose/L-galactose pathway. Ascorbate can be oxidized to monodehydroascorbate (MDHA) and further to dehydroascorbate (DHA). MDHA can be reduced back to ascorbate by* MONODEHYDROASCORBATE REDUCTASE (MDAR) *and DHA can be reduced with* DEHYDROASCORBATE REDUCTASE (DHAR*). The last enzyme uses the reducive power of glutathione (another antioxidant). The oxidized glutathione (GSSG) is converted back to its reduced form by* GLUTATHIONE REDUCTASE (GR) *which uses the reducive power of NADPH. The ascorbate-glutathione cycle is named Halliwell-Foyer-Asada pathway in honour of its founders. Adapted from Gallie (2013).*

The reduction of DHA to ascorbate is achieved by the action of two enzymes: DEHYDROASCORBATE REDUCTASE (DHAR) and MONODEHYDROASCORBATE REDUCTASE (MDAR) (NAD(P) dependent enzyme). Along with GLUTATHIONE REDUCTASE (GR), these enzymes comprise the Foyer-Halliwell-Asada cycle (Pallanca and Smirnoff, 2000) (*Figure 2*).

The reducing power for DHA reductase comes from the oxidation of two glutathione (GSH) molecules to gluthatione disulphide (GSSG). GSH is regenerated from GSSG by the glutathione reductase, consuming with that reaction two electrons from NADPH. The reduction of the oxidized forms of ascorbate can be done only in the cell cytoplasm, thus if the oxidation event takes place in the apoplast then the oxidized forms must be transported across plasma membrane inside to maintain the pool of active reduced ascorbate (Gallie 2013).

The active glutathione is chemically a tripeptide, which consists of glutamine, cysteine and glycine. The glutamate and cysteine are united into *γ*-glutamylcysteine in the first step catalyzed by *Γ*-GLUTAMYLCYSTEINE SYNTHETASE (GSH1) and in the second reaction glutathione is formed by GLUTATHIONE SYNTETHASE (GSH2). Glutathione and ascorbate synthesis is in negative correlation: when ascorbate pool is low, then glutathione is high and *vice versa*. This points to the strict regulation of the redox balance and compensatory effect of these two antioxidants (Foyer and Noctor 2005).

In plants two enzymes catalyse ascorbate oxidation: ASCORBATE OXIDASE (AO) and ASCORBATE PEROXIDASE (*APX*), which is the major H2O2 reducing peroxidase in plants (Potters et al. 2003). As *APX1* was included into expression analysis in this work, it is described below in detail.

APX has high affinity to hydrogen peroxide and detoxify it near the generation sites in plant cells (the enzyme is in cytosol and chloroplasts). *APX* promotes ascorbate turnover. For enzymatic ROS quenching *APX* needs ascorbate as an electron donor. *APX* is found in the cell wall, in the cytosol, the mitochondria and in the peroxysomes (Potters et al. 2003).

As the *APX1* gene has heat-shock *cis*-element in its promoter, the expression can be induced by the heat shock factor which is active during various stress conditions. The *APX1* transcript accumulates and the ascorbate peroxidase activity increases during ozone or herbicide treatment, excessive light, heat, drought, and oxidative stress. Exogenous ethylene treatment has been shown to increase the *APX1* transcript levels (Storozhenko et al. 1998).

1.3. Functions of L-ascorbic acid

Vitamin C is the most important water soluble antioxidant in plant tissues. In both plant and animal systems ascorbate quenches enzymatically and non-enzymatically (H_2O_2) and its derivatives, so-called reactive oxygen species (ROS). Reactive oxygen species such as superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) are byproducts of normal cellular metabolism in chloroplast, mitochondria and in peroxisomes. Ascorbate availability prevents oxidative damage in normal oxygenic metabolism and in stress conditions when the production of ROS is elevated (Foyer and Noctor 2009). Oxidative stress occurs when ROS accumulation exceeds that of the antioxidant defence system. During incompatible plant-pathogen interaction plants there is also a ROS oxidative burst leading to the coordinated defence response (Davey et al. 2000).

The increase in oxygenic respiration is a major response to different stress conditions like high radiation, high temperature, drought, starvation, infecton with diseases etc. In parallel with the aerobic respiration the production of ROS increases. ROS production and decomposition must be under strict control to prevent oxidation of proteins and membrane systems (ROS are highly toxic and can be also signaling substances) (Davey et al. 2000, Zhang et al. 2009). L-ascorbate has the ability to directly eliminate ROS and thus modulate the plant response against most types of stresses. Ascorbate can also terminate radical chain reactions unlike other low-molecular-weight antioxidants (α-tocopherol, carotenoids, flavonoids and glutathione) (Davey et al. 2000).

The role of ascorbate is critical in the photosynthetic aparatus, where often high-energy electrons escape from the photosynthetic electron transport chain and form superoxide ($O_2^{\bullet -}$) radicals. SUPEROXIDE DISMUTASE (SOD) transforms the superoxide into a less reactive form of ROS – H_2O_2. Ascorbate is a co-substrate for ascorbate peroxidases (APX) in the dedoxification of H_2O_2 in chloroplasts (Gallie 2013).

Ascorbate is the only antioxidant in the apoplast and has a major role in scavenging of e.g. ozone and other atmospheric pollutants before they enter the cells and damage the integrity of the cell membranes. It has been shown that the ascorbate deficient plants treated with exogenous O_3 or other oxidizing agents suffer oxidative damage more than the wild type plants (Conklin et al. 1996).

Ascorbate is also an essential cofactor in plants for several important iron containing enzymes. Ascorbic acid maintains the activity of various oxygenases by converting the prosthetic metal ions in the active center of the enzymes to their reduced forms. The biosynthesis of two essential plant hormones depends on the ascorbate availability. Ascorbic acid is a co-substrate for 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE (ACC-OXIDASE), the last enzyme in ethylene synthesis pathway; and also for GIBBERELLIN-3-DIOXYGENASE in giberellic acid synthesis (Rocklin et al. 1999; Gallie et al. 2013). Ascorbate is also a cofactor for PROLYL HYDROXYLASE, which catalyses the hydroxylation of proline of cell wall proteins like extensins and hydroxyproline-rich glycoproteins (HRGP) (Tabata et al. 2001).

The reducing power of ascorbate is also required for recycling the violaxanthin to zeaxanthin with VIOLAXANTHIN DE-EPOXIDASE (Gallie et al. 2013). Zeaxanthin dissipates the excess light energy as heat in the thylacoid membranes, preventing oxidative damage (non-

photochemical quenching) (Conklin et al. 1996). Ascorbic acid is also able to regenerate the lipophilic antioxidant α-tocopherol (vitamin E), which converts the highly damaging peroxy radicals into non-toxic compounds (Davey et al. 2000).

Ascorbate levels and redox status have positive correlation with the activity of plant cell division. The exogenous application of reducing agents like ascorbate or glutathione accelerate the cell division in maize and in *Allium*. AA/DHA and GSH/GSSG ratio determines the range of meristematic divisions by modulating the internal thiol groups of regulatory proteins of proliferation. The oxidized forms of these redox couples prevent the replication and cell divisions under conditions of oxidative stress, where DNA may be damaged (Foyer and Noctor 2009).

Decrease of AA to DHA ratio induces transition from cell division to cell elongation. Transgenic ascorbate deficient tobacco (*Nicotiana tabacum*) meristematic tissue had loose cell walls, elongated cell shapes and cells failed to divide (growth arrest in G1 phase) (Tabata et al. 2001). Cell wall protein ASCORBATE OXIDASE (AO) oxidizes ascorbate and generates MDHA in the apoplast. For ascorbate regeneration the oxidised form must be transported through the plasma membrane into the cytosol. The ascorbate is regenerated inside the cytosol by the ascorbate-glutathione cycle (*Figure 2*) (Gallie et al. 2013). Monodehydroascorbate produced by the AO activates H^+ATPase in the cell membrane and thus helps to forms acidic enviroment to the apoplast (Tabata et al. 2001).

Ascorbate inhibits formation of secondary cell walls. Ascorbate prevents cell wall lignification and stiffening by directly scavenging the monolignol radicals involved in lignin biosynthesis and by inhibiting cell wall peroxidases, which generate the monolignol radicals. Apoplastic ASCORBATE PEROXIDASES also keep the hydrogen peroxide low (H_2O_2 initiates the lignification process in the cell walls). It is proved that the balance between ascorbate and H_2O_2 in the apoplastic space determines the degree of lignification of cell walls (Davey et al. 2000).

1.4. Biosynthesis of ascorbic acid in plants

Reduced ascorbate can be found in all plant tissues in millimolar concentrations (Conklin, 2000). Ascorbate biosynthesis occurs through several biosynthetic routes with mannose, myoinositol, and galacturonic acid as principal entry points (Zhang et al, 2009). The mannose/L-galactose pathway is the only significant source of ascorbate in plants (Dowdle et

al. 2007). Hereafter only the mannose/L-galactose pathway (*Figure 3*) is described in detail, since the other pathways are not yet completely resolved.

Mannose/L-galactose pathway pathway starts from D-glucose via mannose and galactose. The conversion of D-glucose to ascorbate does not involve inversion of the hexose carbon scelecton vise versa to the glucuronate pathway characterised in vertebrates. The first enzyme, which converts sugars (fructose-6-phosphate) from glycolysis into secondary metabolites in the mannose/L-galactose pathway, is PHOSPHOMANNOSE ISOMERASE (PMI). The D-mannose metabolism continues with conversion of D-mannose-6-phosphate to D-mannose-1-phosphate. The reaction is catalysed by the enzyme PHOSPHOMANNOSE MUTASE (PMM) (Ishikawa et al. 2006).

The substrate mannose-phosphate sugar must be activated with GTP for the next conversions. The GDP-D-mannose synthesis is catalysed by GDP-MANNOSE PYROPHOSPHORYLASE (GMP or VTC1). The vitamin C deficient mutant *vtc1* has a reduced GMP activity caused by the mutation in the gene At2g39770 (*VTC1*) (Conklin et al. 1999). The antisense supression of the gene shows similar decrease of ascorbate content as in *vtc1* mutant (Keller et al, 1999).

GDP-D-MANNOSE-3,5-EPIMERASE (GME) converts GDP-D-mannose into GDP-L-galactose. The double epimerization is the last undedicated step in ascorbate synthesis through mannose/L-galactose pathway. GDP-D-mannose and GDP-L-galactose are also substrates for polysaccharide synthesis (rhamnogalacturonan II is a component of pectin in extracellular matrix) and for protein glycosylation (Ishikawa et al. 2006).

The specific steps for ascorbate synthesis start from the GDP-L-galactose hydrolysis to L-galactose-1-P catalysed by GDP-L-GALACTOSE PHOSPHORYLASE (GGP). The GDP-L-GALACTOSE PHOSPHORYLASE is encoded by two conditionally expressed genes *VTC2* and *VTC5.* Functional *VTC2* and *VTC5* genes are obligatory for ascorbate biosynthesis and seedling viability. This first commited step is suggested to be main regulatory point of ascorbate synthesis (Dowdle et al. 2007; Linster et al. 2007).

The inorganic phosphate is released from L-galactose-1-phosphate by the enzyme L-GALACTOSE-1-PHOSPHATE PHOSPHATASE (GPP; coded by the gene *VTC4*) (Conklin et al. 2006). The last steps involve L-galactose oxidation. The cytosolic NAD-dependent L-GALACTOSE DEHYDROGENASE (GalDH) oxidises galactose at C1 resulting in production of L-galactono-1,4-lactone (Gatzek et al. 2002). The last enzyme, L-

GALACTONO-1,4-LACTONE DEHYDROGENASE (GLDH) turns L-galactono-1,4-lactone into ascorbic acid by oxidizing the position C2/C3 (Wheeler et al., 1998). GLDH is localized in the mitochondrion, where it uses cytochrome c as an electron acceptor. The GLDH catalytic region is oriented toward the outer side of the inner mitochondrial membrane where also mitochondrial electron transport chain complexes are located. Bartoli et al. (2000) suggested that GLDH may feed the complexses III and IV with electrons through the reduction of cytochrome c, while L-galactono-1,4-lactone is converted into L-ascorbate (Bartoli et al. 2000). GLDH is activated in plants by light (Mastropasqua 2012) and lycorine specifically inhibits this enzyme activity (Potters et al. 2003).

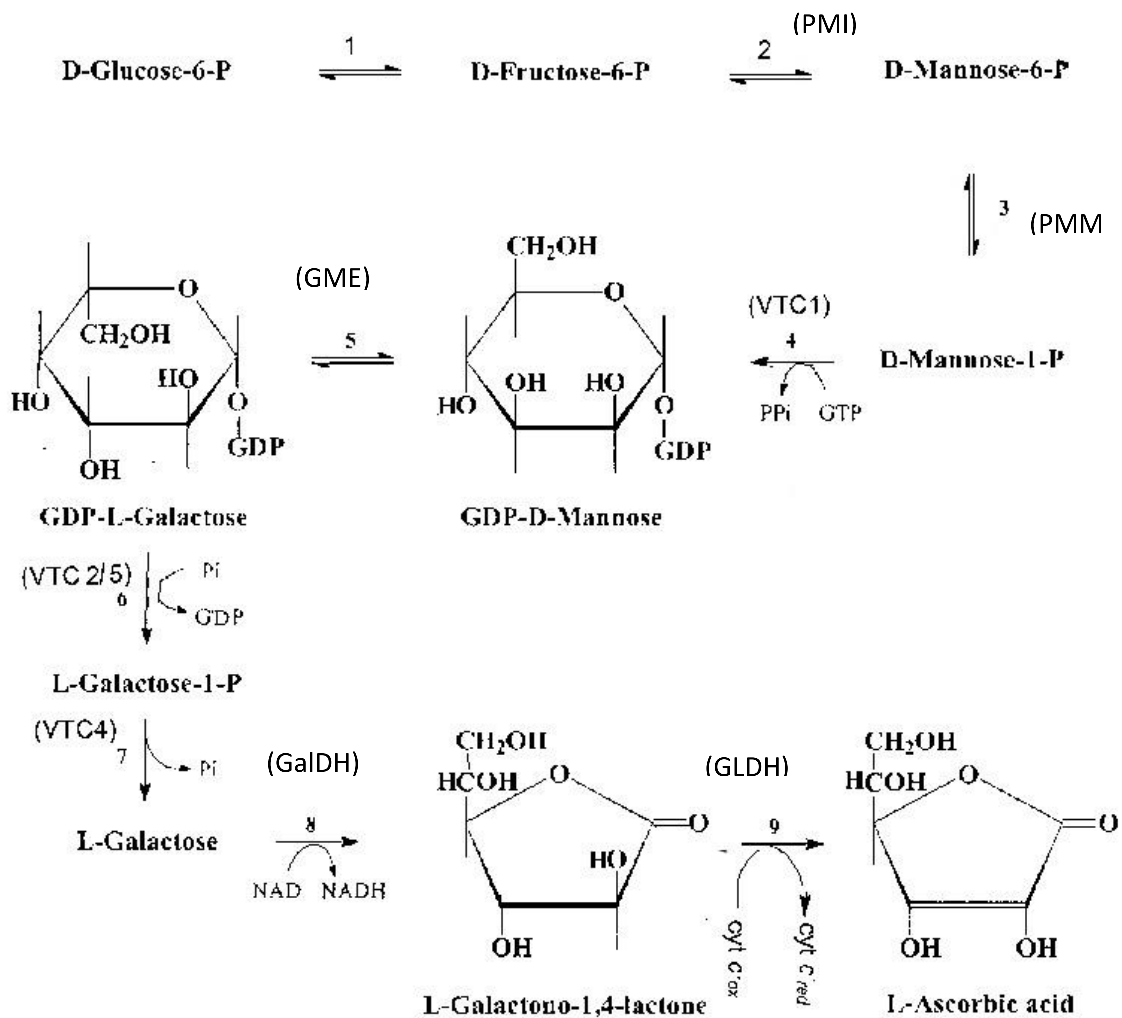

***Figure 3** Mannose/ L-galactose pathway for L-ascorbic acid biosynthesis in Arabidopsis thaliana first proposed by Wheeler et al. (1998). Enzymes: 1.* GLUCOSE-6-PHOSPHATE ISOMERASE *(glycolysis step); 2.* PHOSPHOMANNOSE ISOMERASE (PMI)*; 3.* PHOSPHOMANNOMUTASE (PMM)*; 4.* GDP-D-MANNOSE PYROPHOSPORYLASE (VTC1)*; 5.* GDP-D MANNOSE-3,5-EPIMERASE (GME)*; 6.* GDP-GALACTOSE PHOSPHORYLASE (VTC2)*; 7.* L-GALACTOSE-1-

PHOSPHATE PHOSPHATASE (VTC4) *8.* L-GALACTOSE DEHYDROGENASE (GALDH); *9.* L-GALACTONO-1,4-LACTONE DEHYDROGENASE (GLDH). *Adapted from Wheeler et al. (1998).*

1.5. Ascorbate transport

Ascorbate is synthesized on the inner membrane of mitochondria, but it must be distributed allover the cell and also to the apoplast compartment to be functional. No transporter for mitochondrial inner membrane is needed as the catalytic site of the last enzyme in the ascorbate synthesis faces the intermembrane space of the mitochondria. The precursor of ascorbate – L-galactonolactone – can readily diffuse through the outer mitochondrial membrane, but for ascorbate the lipid bilayer is impermeable (Bartoli et al. 2000). As the molecule is charged in cells and cannot penetrate the lipid membranes, there must be special transport systems for ascorbate and DHA. As the transport of ascorbic acid into the chloroplast and mitochondria follow Michaenis-Menten kinetics and have saturation threshold there may be assumed that ascorbate is transported with a facilitated or active transport system through the membranes of these compartments. Only the uptake of ascorbate into the vacuole does not show saturation kinetics and thus do not have specific transporters (Rautenkranz et al. 1994). In animals the transport of dehydroascorbate is facilitated by glycose transporters (Bartoli el al. 2000). In plants the specific transporter proteins of ascorbate and genes associated with this transport activity are not yet eluciated. Horemans et al. (2000) hypothesis that there is a specific AA/DHA exchange carrier in the plasma membrane has not yet have been proven.

1.6. The regulation of ascorbate content

The ascorbate pool size is regulated by external and internal cues. The ascorbate concentration is developmentally controlled: it is at its maximum level after germination and decreases during the development being the lowest in the senescencing tissues (Bartoli et al. 2000). There is evidence that there must be several regulatory mechanisms that control ascorbate accumulation, recycling and degradation.

There is a good positive correlation between ascorbate concentrations and the activity of several biosynthetic or ascorbate recycling enzymes. The experiments with ascorbate-deficient mutants which have defects in the genes coding enzymes for ascorbate biosynthesis or recycling have drastically reduced ascorbate concentrations compared to wild type plants. As ascorbate is essential for plant growth, loss-of-function mutants unable to synthesise this antioxidant at all are not viable without ascorbate supply in the growth medium (Dowdle et al.

2007). Furthermore overexpression of some of these genes gives increased ascorbate accumulation.

The mutants *vtc1*, *vtc2* and *vtc5 (*for *vitamin c 1, 2 and 5* respectively) contain about 25 %, 20 % and 80 % ascorbate respectively to the wild-type level (Conklin et al. 1999; Dowdle et al. 2007). These defects can be reversed by L-galactose supply, which is a principal precursor of ascorbate. The antisense supression of GDP-D-MANNOSE PYROPHOSPORYLASE, GalDH and GLDH also lead to decline of ascorbate pool size (Keller et al. 1999; Gatzek et al. 2002; Tabata et al. 2001). The remaining ascorbate content in mutants with nonfunctional mannose/L-galactose pathway, suggest that the alternative pathways also contribute to ascorbate biosynthesis (Gatzek et al. 2002; Conklin et al. 2006).

Wheeler et al. (1998) suggested that the rate of ascorbic acid synthesis may be regulated in the conversion of the D-mannose into L-galactose. The enzymes that contribute to this conversion are in order PHOSPHOMANNOMUTASE (PMM), GDP-D-MANNOSE PYROPHOSPORYLASE (VTC1), GDP-D MANNOSE-3,5-EPIMERASE (GME), GDP-GALACTOSE PHOSPHORYLASE (VTC2 OR VTC5) and L-GALACTOSE-1-P PHOSPHATASE (VTC4).

GDP-D mannose-3,5-epimerase (GME) catalyses two epimerisation reactions which have two distinct products GDP-L-galactose and GDP-L-gulose. Both branches lead to ascorbate synthesis, but as previously mentioned GDP-L-galactose is also needed in cell wall/glycoprotein synthesis. GDP-L-gulose is proposed to be channeled directly into the vitamin C pathway, but the enzymes catalysing this step are not yet purified. The epimerisation reaction is tightly controlled. As the epimerase is purified in *Arabidopsis* only together with heat shock protein 70.3 (Hsp70.3) it can be hypotezised that this chaperon might interact with the enzyme and increase its activity and/or favor the formation of GDP-L-gulose. The fact that HSP70 proteins are stress inducible links the vitamin C synthesis to stress responses (Wolucka and van Montagu, 2003). Concomidant with these results ascorbate concentrations are increased in response to various stresses (Wolucka et al. 2005) like for example high light intensity (Bartoli et al. 2000).

The first step specific to ascorbate synthesis is the GDP-L-galactose hydrolysis to L-galactose-1-phosphate catalysed by GDP-L-galactose phosphorylase encoded by the Arabidopsis genes *VTC2* and *VTC5*. These genes partly compensate each other in the single mutants (Dowdle et al. 2007). The major regulation point for D-mannose/L-galactose pathway

is suggested to be in this step (Linster et al. 2007). Both the *VTC2* and *VTC5* transcripts and the activity of GDP-L-galactose phosphorylase are highly responsive to light having a diurnal cycle. *VTC2* and *VTC5* expression peak in the first few hour of the light cycle and may be controlled by the circadian clock. The enzyme activity is highest at the end of the light period. There is also ascorbate feedback inhibition in the transcription level to the *VTC2* (Dowdle et al. 2007).

L-galactose dehydrogenase (GalDH) is the penultimate enzyme in the D-man/L-gal pathway. Although asorbate concentration does not affect *GalDH* expression, it has negative effect on its activity indicating reversible negative feedback regulation of ascorbate synthesis in this point (Mieda et al. 2004). The product (ascorbate) accumulation down-regulates its own synthesis by inhibiting also other enzymes in this pathway like GME (Wolucka and Montagu, 2003) PMI1 (Maruta et al. 2008) and VTC2 (Dowdle et al. 2007).

Light intensity is the major environmental factor affecting leaf ascorbate accumulation. In some plant species (for example in Arabidopsis) ascorbate concentration fluctuates in a diurnal rythm which correlates with the light availability. There is evidence that galactonolactone dehydrogenase (GLDH) activity and transcript fluctuate in parallel to ascorbate accumulation in green tissues (Tamaoki et al. 2003). As GLDH is localized in the mitochondrion and uses oxidized cytochrome c as an electron acceptor, it can be concluded that the last step in ascorbate synthesis is dependent on respiratory chain capacity and the redox balance of cytochrome c. The inhibition of cytochrome c oxidase by KCN inhibits ascorbate synthesis, because the reduced cytochrome c cannot accept electrons (Bartoli et al. 2000). The reduced product of photosynthesis NADPH is needed in mitochondrial respiratory electron transport chain as a substrate for generating proton gradient and driving the ATP synthesis. This explains why light availability affects positively GLDH activity. The activity is also affected by substrate (carbohydrate) bioavalialbility directly from photosynthesis and associated reactions (Tamaoki et al. 2003). There is also evidence that transcript levels of *VTC1, VTC2* and *VTC4 (GPP)* increase under continuous light and decrease under darkness, indicating that photosynthetic electron transport chain largely controls the synthesis of ascorbate and photosynthesis is not merely the carbon source (Yabuta et al. 2007). The PMI1 activity and the gene expression follows the diurnal cycle, where the peak is at the end of the light period (Maruta et al. 2008).

Ascorbate mannose pathway regulator 1 (AMR1) is the only negative regulator of the D-mannose/ L-galactose pathway known today. An activation tagged mutant with increased *AMR1* expression resulted in decreased ascorbate concentration (about 60 % less than in wild type) and coordinated reduction in the expression of genes encoding enzymes in the D-mannose /L-galactose pathway. In the *amr1-1* mutant expression of *AMR1* is absent due to a T-DNA insertion in the coding sequence. In *amr1-1* the foliar ascorbate concentration is two fold higher than in wild type plants. The inverse relationship of *AMR1* expression and ascorbate content supports the hypothesis that the AMR1 is the negative regulator of ascorbate. AMR1 regulate negatively the transcription of *GME* and *VTC2*. The expression of *AMR1* correlate positively with the leaf age and is also negatively regulated by the light activity (Zhang et al. 2009).

The AMR1 protein has a conserved F-box in its N-terminus, suggesting that it recognizes the E3 ligase complex in the SCF-ubiquitin complex. The ubiquitin complex targets the substrate selectively for degradation by ubiquitin-proteasome pathway. The target recognition sequence of AMR1 is not yet confirmed, but it is suggested to be in the unique C-terminal motif DUF295 (Zhang et al. 2009).

In senescencing leaves increase in ethylene production correlates with decrease in ascorbate and subsequent increase in ascorbate oxidized redox state. Gergoff et al (2010) showed that ethylene modifies ascorbate accumulation without affecting oxidative stress status of the senescencing tissue. They showed that the *ctr1* mutant that constitutively activate the ethylene signal pathway has decreased total ascorbate content (66 % compared to wild type) and this result was comparable to results from experiments where the ethylene analogue etephon was applied to wild type plants. On the contrary in the ethylene insensitive mutant *ein2,* where the ethylene response is disabled, the ascorbic acid concentration was doubled compared to wild type (Gergoff et al. 2010). In the plants with high ascorbate, the senescence is delayed and the ascorbate deficient mutants are senescencing prematurely (Zhang et al. 2009).

Jasmonate application increase ascorbate and glutathione accumulation providing resistance to oxidative stress (Suza et al. 2010, Sasaki-Sekimoto et al. 2005). The ascorbate accumulation is largely caused by the increased activity of the ascorbate recycling enzymes DEHYDROASCORBATE REDUCTASE (DHAR) and MONODEHYDROASCORBATE REDUCTASE (MDHAR). The ingrease in glutathione after jasmonate treatment is due to the transcriptional induction of its genes coding for two biosynthetic enzymes GSH1 (Γ-

GLUTAMYLCYSTEINE SYNTHETASE) and GSH2 (GLUTATHIONE SYNTHETASE) (Sasaki-Sekimoto et al. 2005). Jasmonate also regulates some steps in the ascorbate synthesis pahtway through L-galactose (Suza et al. 2010). Microarray experiments provided evidence that the expression of *VTC1*, *VTC2* and *VTC5* is increased after treatment with exogenous jasmonates (Sasaki-Sekimoto et al. 2005). It is not known if the ascorbate synthesis regulator AMR1 itself is under the control of jasmonates or not (Suza et al. 2010). It has been shown that defects in jasmonate signaling may disrupt the activation of ascorbate synthesis and recycling in stress situation (Sasaki-Sekimoto et al. 2005).

1.7. The functions of ethylene and its signal transduction pahway

Ethylene is a gaseus plant hormone which has a simple hydrocarbon structure (C_2H_4). Ethylene affects development of plants throughout their life-cycle and regulates responsiveness to a variety of stresses. Ethylene is needed for seed germination, cell elongation, sex determination, fruit ripening, leaf abscission, flower senescence, defence against pathogens and responses to mechanical trauma. The triple response reaction has been useful for *Arabidopsis* mutant screens in finding ethylene signal transduction mutants (Alonso et al. 1999). The triple response comprises radial swelling of the hypocotyl, exaggeration of the apical hook and inhibition of root and hypocotyl growth. The curved structure of apical hook protects the meristematic tissues in the tip of the seedling shoot while the stem is emerging from the ground to the atmosphere (Guzman and Ecker, 1990).

The ethylene signal is perceived by a family of endoplasmatic reticulum membrane anchored receptors (*Figure 4*). The receptor ETHYLENE RECEPTOR 1 (ETR1) is related to histidine kinases in the procaryotic two component signal transduction systems. In the absence of ethylene the receptor is active and physically associated with the receptor-associated Raf-like kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) a negative regulator (Clark et al. 1998, Ju et al. 2012). Activation of ethylene signaling relies on inactivation of the inhibitor CTR1. After binding ethylene, receptor ETR1 is prevented from activation of the kinase CTR1. Subsequently, inhibition of CTR1 triggers dephosphorylation of ETHYLENE INSENSITIVE 2 (EIN2; Huang et al. 2003), an endoplasmatic reticulum membrane-localized Nramp metal ion transporter homologue, with a cytosolic C- terminal domain that has a conserved nuclear localization signal (NLS) (Ju et al. 2012). Following activation by ethylene binding, EIN2 is cleaved near its C-terminus (after Ser 645) resulting in nuclear localization of its carbocyl-terminal EIN2 fragment (CEND) (Qiao et al. 2012) (*Figure 4*). CEND

probably acts as a co-regulator of gene expression and activation of the ethylene signal pathway resulting in altered expression of several ethylene responsive genes (Ju et al. 2012). Without ethylene, EIN2 is targeted for 26S proteasomal degradation by F-box proteins ETB1/2 (Qiao et al. 2012).

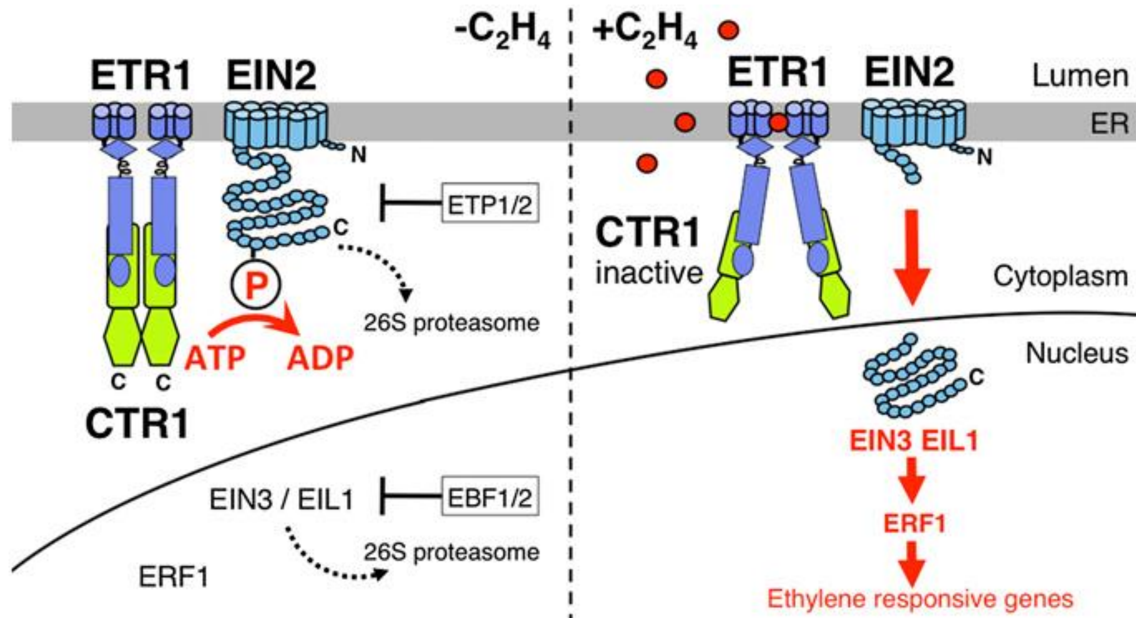

Figure 4 *The inactive and active ethylene signal transduction pathway. The signal transduction mechanism is described in the text below. Adapted from Ju et al. 2012.*

Figure 5 *Phenotypes of four weeks old ctr1-1 (left) compared to wild type Col0 (right).*

The *ctr1-1* mutant exhibits constitutive expression of ethylene-regulated genes and its morphology is similar to phenotypes observed in plants treated with ethylene (Kieber et al. 1993). As cell elongation is inhibited, the plants are significantly smaller and the leaf plates are tiny compared to wild type *Arabidopsis* plants in the same age (*Figure 5*). The

serine/threonine specific kinase activity of CTR1 is abolished in the *ctr1-1* mutant caused by point mutation. In *ctr1* the EIN-2 C-terminal peptide constitutively accumulates in the nucleus triggering the ethylene response even in the absence of ethylene (Qiao et al. 2012).

Figure 6 *Phenotypes of four weeks old ein2-1 (left) compared to wild type Col0 (right).*

The *ein-2-1* mutant is similar in size to wild type and leafplates have slightly curved edges (*Figure 6*). The e*in2-1* mutant was a found in the triple response assay mutant screening after ethylmethylsulfonate (EMS) treatment (Guzman and Ecker, 1990). The *ein-2* mutant has a premature stop codon and has disabled ethylene response since the functional CEND-region in the C-terminus is missing (Alonso et al. 1999, Qiao et al 2012).

1.8. The triple respone assay

The ethylene triple response assay screening is a powerful tool for isolation of ethylene signaling mutants (Guzman and Ecker, 1990). The visual features of triple response in the ethylene treated etiolated seedlings (*Figure 7*) of the normal ethylene signaling are:

a) inhibiton of root and hypocotyl elongation,
b) exaggregated tightening of the apical hook,
c) radial swelling of hypocotyl.

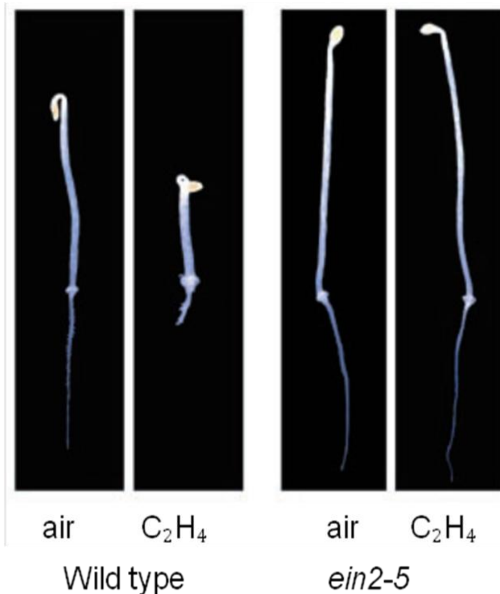

Figure 7 *The growth of 3-days-old etiolated wild type and ein-2-5 Arabidopsis thaliana seedlings grown in pure air or with ethylene in it (10 ppm). Adapted from Alonso et al. (1999).*

The curved structure of apical hook protects the meristematic tissues in the tip of the seedling shoot while the stem is emerging from the ground to the atmosphere (Guzman and Ecker, 1990). The hypocotyl hook is caused by the diferential rates of cell expansion between the outer and inner cells of the hook (Ellis and Turner, 2002). The triple response phenotype is positively regulated by ethylene. By germinating a mutant seed collection in the presence of ethylene (or its pre-cursor ACC) it is possible to find mutants insensitive to ethylene, or in reverse identify mutants with a constitutive triple response in the absence of ethylene (Guzman and Ecker, 1990).

1.9. The functions of jasmonates and their signal transduction pahway

Most jasmonates originate from the oxidation of linolenic acid (18:3). The main source of linolenic acid is the chloroplast membrane. The intermediate cyclic product 12-oxophytodienoic acid (OPDA) is converted to linear jasmonic acid (JA) in the proccess of β-oxidation in peroxisomes. JA can be metabolized further and thus the term jasmonates is often applied to diverse collection of jasmonic acid relates oxylipins, which have signaling roles in plants (Staswick 2007). Jasmonates regulate plant responses to pathogen attack, wounding, ozone exposure and water deficit. They also regulate gamete development, cell cycle, root growth inhibition and senescence. Jasmonates largely determine if the resources are allocated to growth or for defence (Fernandez-Calvo et al. 2011).

The bioactive form of jasmonic acid is a conjugate with the amino acid isoleucine (Ile). Jasmonoyl-isoleucine (JA-Ile) is formed with the aid of JA:amino synthetase JASMONATE RESISTANT1 (JAR1). In *jar1-1* mutant the JA-Ile production is impaired and the jasmonate response is cut off (Suza et al. 2010).

The key component in jasmonate signaling is the SCF^{COI1} ubiquitin ligase complex which acts as a receptor for JA-Ile. The COI1 protein has a leucine-rich repeat (LRR) and a conserved F-box. F-box proteins commonly act as specific receptors that recruit regulatory proteins as substrates for ubiquitin mediated destruction in the proteasome (Devoto et al. 2002). JA-Ile (and to a lesser extent JA-Leu and JA-Val) promote the SCF^{COI1} complex interaction with jasmonate ZIM domain protein (JAZ protein) and target the latter to degradation (Thines et al. 2007) (*Figure 8*). The C-terminal Jas motif of JAZ protein (with a consensus sequence SLX2FX2KRX2RX5PY) is essential for JA/Ile-dependent interaction with COI1 (Melotto et al. 2008).

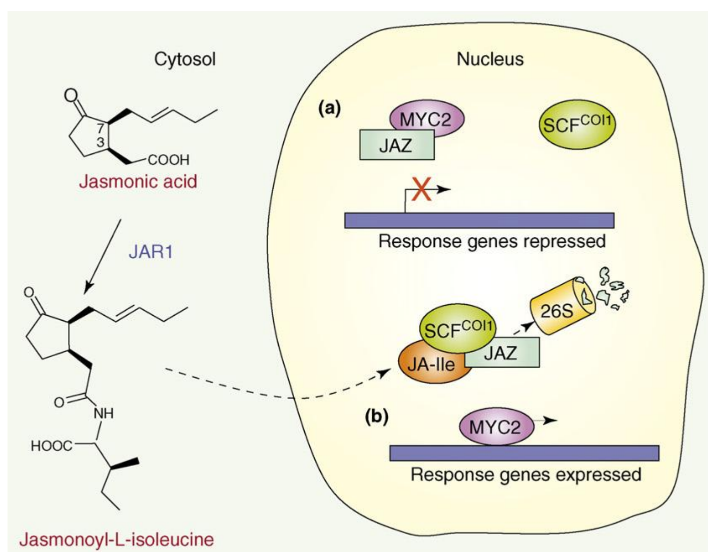

Figure 8 *Jasmonate activation and the signal transduction mechanism. In the absence of the hormone (a), JAZ repressor binds to the transcriptional activators (e.g. MYC2) and prevents the activation of jasmonate dependent transcription. Jasmonate-dependent destruction of JAZ transcriptional repressors (b) lead to the activation of downstream response genes. Adapted from Staswick 2007.*

JAZ protein functions as a repressor of jasmonate signaling (Thines et al. 2007). In the absence of jasmonates JAZ repressor recruits transcription activators and prevents their activity (Fernandez-Calvo et al. 2011) (*Figure 7*). Jasmonate-dependent destruction of JAZ transcriptional repressors leads to the activation of downstream response genes (Thines et al. 2007). Several basic helix-loop-helix (bHLH) transcription factors MYC2, MYC3 and MYC4 are direct targets of JAZ. The transcriptional activators act additively in the regulation of

jasmonate response by binding DNA in the sequence specific manner. MYC2, MYC3 and MYC4 partly compensate each other functions, with MYC2 affect mostly root development and MYC3 and MYC4 are expressed in the plant organs aboveground (Fernandez-Calvo et al. 2011). MYC2 increases the expression of genes involved in wound and/or insect response (Suza et al., 2010), oxidative stress response and flavonoid synthesis and downregulates genes that activate pathogen defence (Staswick 2007).

2. AIM OF THE EXPERIMENTAL WORK

Arabidopsis thaliana (L.) Heynh. wildtype Columbia (Col-0) and *A. thaliana* mutants described in **Error! Reference source not found.** were used for all experiments. The *coi1-16xein2-1* double mutant was construsted in our own lab and verified to be homozygous for both mutations by testing the triple response for ethylene insensitivity and methyl jasmonate inhibition of root growth for jasmonate insensitivity (see below for results).

The first part of the project focus on the role of ethylene and jasmonates in regulation of ascorbate accumulation, using ascorbate measurements in ethylene and jasmonate deficient mutants and in the double mutant where both signaling pathways were disabled. Additionally the *amr1-1* mutant, with disabled ascorbate synthesis regulatory properties, and *vtc1-1,* with inactive ascorbate synthesis enzyme, were used as controls. The plant lines used are described below in detail (**Error! Reference source not found.**).

In the second part of the project real time quantitative polymerase chain reaction (RT-qPCR) was performed on the same mutants to identify where ascorbate biosynthesis genes are regulated. For expression analysis primers for all genes in ascorbic acid biosynthesis and glutathione biosynthesis were used. The relative expression pattern for these genes would uncover where and how the inhibitor of ascorbate synthesis AMR1 and the two hormones (jasmonate and ethylene) affect the ascorbate accumulation.

Table 1 *A. thaliana mutant lines used in this study.*

Plant line	Protein function	Mechanism of action	Place of mutation	Reference
ein2-1	Required for ethylene induced developmental and stress responses.	Mediates ethylene induced signal propagation between CTR1 and EIN3/EIL.	EMS induced mutant: in *EIN-2* gene is a premature stop codon (protein contains only transmembrane NH2 terminal portion).	Guzman and Ecker 1990 and Alonso et al. 1999.
vtc1-1	Enzyme in cell wall carbohydrate and ascorbate synthesis and protein glycosylation.	Codes the enzyme GDP-mannose pyrophosphorylase which produces GDP-mannose.	EMS missense mutation that converts a conserved Pro to a Ser at amino acid 22 (cytosine to thymine at position +64).	Conklin et al. 1996 and Conklin et al. 1999.

Plant line	**Protein function**	**Mechanism of action**	**Place of mutation**	**Reference**
ctr1	Negative regulator of ethylene response pathway	ETR1 family of ethylene receptors activate the CTR1 kinase activity in the absence of ethylene. Active CTR1 inhibits the activation of downstream signaling component EIN2.	EMS mutant where Asp residue 694 in kinase catalytic domain is changed to a Glu (abolished Ser/Thr specific kinase activity).	Kieber et al. 1993, Huang et al. 2003.
amr1-1	Negative regulator of ascorbic acid biosynthesis.	Downregulates the genes of the enzymes from mannose/ L-galactose pathway in response to developmental and environmental cues.	T-DNA mutant from the SALK collection. T-DNA from the vector pROK2 inserted into the predicted At1g65770 promoter region.	Zhang et al. 2009.
coi1-16	Required for wound and jasmonates-induced defence and germination.	In a SCF complex binds substrate proteins targeted for ubiquitination.	EMS mutant, where Phe is substituted for a Leu (L245F) in a leucine-rich repeat (region responsible of binding target proteins).	Ellis and Turner 2002
ein2-1 x coi1-16	Look at the single mutants descriptions.	Look at the single mutants descriptions.	Look at the single mutants descriptions.	This work

3. METHODOLOGY

3.1. *Plant material preparation*

Seeds were vernalized in water for two days in darkness at 4°C. Seeds were sown into separate small pots on a soil mixture with 1/3 of vermiculite (No 2 by Vermipu OY, Finland) and 2/3 of peat (Brown 025W, Kekkilä OY, Finland). Plants were grown in 12 h/12 h light/darkness conditions in a climate controlled growth chamber (Sneijder chamber). In the growth chamber the photosynthetic photon flux density (PPFD) was 125 μmol photons m^{-2} s^{-2}, relative humidity 70 % and temperature 23 °C in day time and 18 °C at night.

Samples for ascorbate measurements and qPCR were grown at the same time in the same conditions. For the ascorbate measurments the same mutant were grown in a repeat of 6 to 8 pots (one plant per a pot) and for qPCR analysis five plants per mutant were pooled for RNA isolation. The plants were watered regularly until harvesting the plants after four weeks for the analyses described below.

3.2. *Ascorbate and dehydroascorbate absorbance measurment with spectrophotometer*

The contents of ascorbate and its oxidized form, dehydroascorbate, were determined by spectrofotometric procedure as described in Gillespie and Ainsworth, Nature Protocols, 2007.

1. For the 10 mM ascorbate standard-solution 0,076 g of pure ascorbate (AA; Sigma, kat. nr. A5960) was dissolved in 10 ml 6 % trichloroaceticacid (TCA; Sigma, kat. nr. T6399). For more diluted solutions the proportions of 6 % TCA and primary solution taken is shown below (Table 2). The ascorbate standards were kept in the ice bath.

Table 2 *Proportions of 6 % TCA and primary solution taken*

Ultimate concentration of solution	Primary solution (μl)	6 % TCA (μl)
5,0 mM	500 μl	500 μl
2,5 mM	250 μl	750 μl
1,0 mM	100 μl	900 μl
0,5 mM	50 μl	1000 μl
0,15 mM	20 μl	1320 μl
0,075 mM	10 μl	1320 μl

2. The plant material were harvested, weighted (recorded for further data analysis) and put into screw-capped 2-ml microtubes with 2 mm Ø SiO_3 granules. 1 ml of 6 % TCA was added. The samples were kept in the ice bath.

3. The 2-ml tubes with the plant tissues were homogenized in the homogenisator (6,5 m/s for 1 min). Homogenates were centrifuged at 13,000 × g for 5 min at 4 ºC. The supernatants were carefully transfered to new 2-ml microtubes. All extracts were kept on ice to avoid ascorbic acid pool degradation.

4. The 1-ml Eppendorf tubes were labeled for the ascorbate assay. The assay contained blanks, standards and samples in duplicate for both reduced AA and total AA.

5. 50 µl 75 mM phosphate buffer and 100 µl of either 6 % TCA (blanks), AA standards (0,075 – 5 mM) or samples was added to the labbeled tubes.

6. 50 µl 10 mM dithiotreitol (DTT) was added to the total AA tubes and incubate at room temperature for 10 min. This reduced the pool of oxidized AA. 50 µl 0,5 % N-ethylmaleimide (NEM) was added to the total AA tubes to remove the excess DTT.

7. 100 µl water was added to the reduced AA assay tubes to acount for the volume of DTT and NEM in the total AA assay tubes.

8. 250 µl 10 % TCA, 200 µl 43 % H_3PO_4, 200 µl 4 % α-α`-bipyridyl (Spectrum, kat. nr. BI120) and 100 µl 3 % $FeCl_3$ were pipetted into all the assay tubes. After $FeCl_3$ was added, the mixture was immediately shaked with Vortex in capped tubes (it avoided formation of a white precipitate that would interfere with the absorbance reading).

9. The assay tubes were incubated at 37 °C for 1 hour.

10. 200 µl of the sample, standard and blank reaction solutions were transfered from the assay tubes to a 96-well microplate wells and read the absorbance of each well at 525 nm with Thermo Scientific Multiscan FC spectrophotometer. The data were saved.

3.3. Ascorbate and dehydroascorbate concentration analysis

Total ascorbate pool, and reduced ascorbate content calculation were done with Microsoft Exel. The average of two repeats of the blank results was calculated. The average of blank results was subtracted from the two repeats of the sample and standard values. The averages were calculated for blank-corrected standard and sample values. For the ascorbate

concentration calculation from the 525 nm light absorbance data the regression line were constructed from the standardized samples (table 2). The regression line function y=ax+b gives the concentration (nmol) per a well. To get the concentration per a g fresh weight the nmol per a well concentration were divided with the fresh weight of the sample (take the dilution into account). The final results are given in the form mg/g (the ascorbate molarity is 176,12 g/mol). The amount of oxidized ascorbate was obtained by substracting the amount of reduced form from that of total ascorbate.

3.4. Harvesting the plant material and RNA isolation

After harvesting the leafs were put in a aluminium foil and deep-freezed in liquid nitrogen and stored at – 83º C. *Arabidopsis* RNA isolation was carried out using Sigma Spectrum™ Plant Total RNA Kit (Sigma manual, Sigma Chemical Co., St. Louis, Mo.).

The whole plant material was grinded in mortar in liquid nitrogen which prevents RNA-degrading enzymatic activity. Before grinding the plant material, mortar and pestle were frozen. Aproximately 0,08 g of the fine powder from every sample were put into the 2 ml Eppendorf tubes with 500 µl lysis buffer and 5 µl β-merkaptoetanol and vortexed immediately and vigorously for at least 30 seconds. Lysis solution broke down the cells and released RNA and at the same time inactivated ribonucleases and interfering secondary metabolites, such as polyphenolic compounds. All the samples were incubated at 56º C for 3 minutes and after that centrifugated 4 min at 13 000 rpm to pellet cellular debris. The suspension was isolated from the pellet by pipeting it to next tubes with membranefilter and the pellet, which contained cell walls was thrown away.

The suspension still contained unneccesary cell contents, which were removed from the probes by filtrating with two different filtersystems. First membranefilter called filtration column excluded the last particles from the lysate. The second membrane bound the RNA from the solution with the aid of special RNA-binding buffer (500 µl binding solution was added briefly before filtrating with binding-column). After both filtrating-procedures the tubes with samples were centrifugated 1 min at 13000 rpm. The RNA-binding membrane was washed three times with wash solutions (Wash Solution 1 and Wash Solution 2) to prevent occasionally membrane-bound chlorophyll molecules interfering the futher proccess. After every wash the probes were centrifugated at maximum speed and flow-thru was discarded.

Binding columns with RNA were transfered to new collection tubes. 50 µl of sterile water was added directly onto the centre of binding filters to get the purified RNA. The elution was repeated with 30 µl of water to increase the yield. The binding columns were thrown away and the tubes with RNA solution were stored at -83ºC.

The concentrations of RNA samples were measured with Nanodrop spectrophotometer. The spectrophotometer was calibrated against sterile water.

3.5. The RNA quality control

The RNA purity and quality was tested with Agilent 2100 Bioanalyzer, which measures the integrity of total RNA in the samples. The device uses a microchannel based electrophoretic cell that allows a sensitive (5-500 ng/µl) investigation of nucleic acid samples. The procedure followed Agilent 2100 Bioanalyzer protocol for Plant RNA (Agilent 2100 Bioanalyzer protocol, 2004).

3.6. DNAse I treatment

Most types of RNA isolation leave trace amount of genomic DNA in the isolated RNA. This DNA can give false results in qPCR and needs to be removed. An equal concentration (3 µg/ µl) of RNA solution was used for each sample. The DNAse treatment mastermix for every sample contained 2 µl of DNAse buffer, 0,25 µl of RNAse inhibitor and 1 µl of RNAse free DNAse. The final reaction volume was 20 µl. All samples were mixed briefly with Vortex, centrifugated some seconds and incubated for 30 minutes at 37ºC in the heat block.

2 µl of 50 mM EDTA was added to inactivate the DNAse. The samples were heated for 10 min at 65ºC in the heat block to denature secondary structure of the RNA, placed on ice for 1 min and spinned down.

3.7. cDNA synthesis

The ultimate goal in this step was to synthesise cDNA from mRNA by the aid of reverse transcriptase (RT). The proccess was done by the instructions of the manufacturer (Fermentas). Oligo-dT (Microsynth) was used as a primer to initiate the synthesis reactions.

For each sample 9,5 µL of the mastermix was added (the components and their volumes are shown in table 3). The samples were incubated at 50°C for 2 hour to synthesize the cDNA. After cDNA synthesis was finished the microtubes were put immediately on ice bath. 70 µl of H_2O was added and the samples with cDNA were stored at -20 ºC overnight.

Table 3 *cDNA synthesis mix composition (added to the samples) and the amounts of the components*

Component	Volume for one sample (µl)	Volume for 8 samples (µl)
oligo-dT	1	8
buffer	6,2	49,6
dNTP	1,5	12
RNAse inhibitor	0,25	2
Premium reverse-transcriptase	0,5	4

3.8. qPCR preparation

qPCR preparations begun with labelling tubes and making the mastermixes which contained all the final components except the samples. Each of the 16 mastermix tubes contained 5*EvaGreen, sterile water and one primerpair (left and right primer) from all the sixteen primerpairs. The components of the mastermixes and their volumes are shown below (***Table 4***).

Table 4 *The components of the qPCR mix and their volumes.*

Component	Volume for one sample (µl)	Volume for 8 samples (µl)
5*EvaGreen	6	120
Primer (10 µM)	1.5	12
Sterile water	20.5	164

There were 16 different primers: *APX1, AMR1, GSH1, GSH2, VTC1, VTC2, GPP, VTC5, GME, GalDH, GLDH, PMM, PMI, YLS8, TIP41* and *SAND* (Table 5). The last three were reference primers.

Table 5 *Primers for the qPCR. Primers are adapted from Czechowski et al. 2005 and Zhang et al. 2009. The primers for APX1, GSH1 and GSH2 were designed ourselves. The reference primers used for normalization were taken from Czechowski et al., 2005. The true primer efficiencies were determined using a standard curve of diluted cDNA.*

Gene name	Accession number	Forvard primer	Reverse primer	Primer efficiency (%)
APX1	At1g07890	GCACTATTGGACGACCCTGT	AGCAAACCCAAGCTCAGAAA	90,7
AMR1	At1g65770	TTCACAAAGGGCAAACATACG	CACAACATTCCACAAGTCTCC	not determined
GSH1	At4g23100	CGGAGAAGCTCTTGGAGATG	CCTTTTGTTCACGTCCCATT	91,8
GSH2	At5g27380	ATGTTCTTGGTGCGAGAAGG	TTCGTCTTTGCTCCTGAGGT	92,4
PMI1	At3g02570	TGTTCCTGCAGATACCGAGA	AAAACCTACTGTTGATTCCTGCTC	89,1
PMM	At2g45790	TTTCTCAATTGGGGGACAGA	GTCCTCGAGGTATTGCAAGC	91,1
VTC1	At2g39770	TTGTTGACGAAACCGCTAC	TGCCACCCGATGATACTG	91,1
GME	At5g28840	CGATGAGTGTGTTGAAGG	AGATTGTTGTCTGAGTTACG	93,6
VTC2	At4g26850	CAATGTTAGTCCGATAGAGTATGG	TGTAACCGAGTCTGAAGTATGG	91,7
VTC5	At5g55120	AATGTGAGTCCGATTGAGTATGG	AGTAAGCCTGAAAGTGAAGATGG	91,2
VTC4	At3g02870	ACATTAGACGATACAACCAACAG	GCTTCTTTCACGATAACAATTCC	91,4
GalDH	At4g33670	GGTGTGGGTGTGATAAGTG	GACGAAATCTCCTTGTTTGC	90,2
GLDH	At3g47930	CAGCAGATTGGTGGTAT	GACCTCAGCAACAACTCC	90,5

The mastermix with primers were divided to a new series of labeled tubes, and 3 µl cDNA template added. Each of the 16 primers and each of the seven samples (plus no template control) were represented in a single tube only once. The no template control (NTC) contained 3 µl of water instead of a sample. The qPCR mixes were mixed with Vortex and spinned down. 10 µl of the new mix was pipetted to each well on the 384-well qPCR plate

(from one qPCR mix three technical repeats). The plate was sealed and centrifuged for 5 minutes at 3000 rpm.

3.9. qPCR program

The quantitative PCR was done in the ABI7900HT Fast RT PCR System for 384 well plates. The program was as follows:

1. 2 min at 50 °C;
2. initial denaturation, 10 min at 95 °C;
3. denaturation, 15 sec at 95 °C;
4. annealing, 30 sec at 60 °C;
5. extension, 30 sec at 72 °C;
6. returning to step 3 for 40 additional cycles.

After the last annealing step in the dissotiation step the temperature was 95 °C. The qPCR proccess took 2 hours. The mechanism of qPCR is as the same as in PCR, but the difference lies at the detection – the fluorecence of a DNA binding dye is measured at each cycle as the amplification proceeds. In contrast, in standard PCR the product is measured once e.g. on an agarose gel stained with a dye for example ethidium bromide.

The commercial qPCR mixed from Solid Biodyne includes the DNA-binding dye EvaGreen I. When free in the solution, EvaGreen I displays relatively low fluorescence, but when bound to double-stranded DNA its fluorescence increases by over 1000-fold. The more double-stranded DNA that is present, the more binding-sites there are for the dye, thus fluorescence increases proportionately to DNA concentration. This property of the dye provides the mechanism that allows it to be used to track the accumulation of PCR product. As the target is amplified, the increasing concentration of double-stranded DNA in the solution can be directly measured by the increase in fluorescence signal

One limitation of this assay is the inherent non-specificity of this method. EvaGreen I will increase in fluorescence when bound to any double-stranded DNA. Therefore, the reaction specificity is determined solely by the primers (Stratagene, 2004).

3.10. Analysis

The raw data provided by the SDS2.4 software are the cycle threshold (Ct) values. The Ct value is the number of reaction cycles necessary to reach a fixed concentration of an amplicon

which correlates with the threshold fluorescence. Threshold fluorescence is the first significant fluorescence detected by the laser in the certain reaction well of the 384 well plate where the cDNA amplification takes place. The fluorescence comes from the Eva Green I dye bound to the double-stranded DNA. Fluorescence intensity values correlate with the concentration of the PCR products. The Ct values for the target and the normalizer should be within about ten cycles of each other. There must not appear any fluorescence in NTC.

The Ct values were further analyzed in a program specific for expression analysis - qBase PLUS (Biogazelle). The Ct values were calculated into relative expression in arbitary units and normalized against the reference genes. The reference genes *YLS8, TIP41* and *SAND* were used for correcting the variations in the Ct of the genes of interest that is not due the changes in expression level. The normalizing target must be a gene whose expression is constant in all the samples. Any variation in the Ct of the normalizer can be attributed to other sources of variation, such as efficiency of the reverse transcription reaction, yield of the RNA purification, or variation in the number of cells from which the RNA was isolated. These sources of variation would affect the normalizer and the genes of interest equally. The quality control in qBase PLUS was used to exclude the technical repeat wells where Ct values deviated more than 0,5. Figures, tables and statistics were made in Microsoft Excel.

3.11. Phenotyping

Ethylene and jasmonic acid insensitive mutants have altered growth responses in the presence of the corresponding hormone and can be identifyed by these features. The mutants screened on phenotyping plates were *ein2, coi1-16* and *ein2-1xcoi1-16* with the wild type Col-0 as control. The features examined on these agar plates were:

a. the triple response assay with plates containg 10 µM ACC.
b. MeJA inhibition of root growth was screened with 50 µM MeJA.

3.12. Sterilizing and plating seeds

500 µl of sterilisation solution (70 % ethanol, 0,1% Triton-X and ETDA) was added into the labeled Eppendorff tubes with seeds and shaked 15 min. The next steps must be sterile and done under laminar.

The liquid was thrown away and the seeds were washed through with 70 % ethanol three times (between the steps the solution was thrown away). After washing steps 800 µl of sterile Milli-Q water was added and the surface sterilized seeds were plated on 1- % agar growth

medium. Half of the plates contained in addition to 1 % agar also 10 µl ACC (the precursor of ethylene) and the other half had 50 µl MeJA. The lid was put on the plates and the phenotyping plates were cold treated at 4 °C for 4 days in darkness before germination. The ACC containing plates were grown 3 days in darkness at 23 °C (Guzman and Ecker 1990). The MeJA plates with seedlings were grown 3 to 4 days in the Sanyo growth chamber. In the growth chamber the relative humidity was 70 %, temperature at 23 °C and photosynthetic photon flux density (PPFD) was 125 µmol photons m^{-2} s^{-2}.

4. RESULTS AND DISCUSSION

4.1. Examination of the phenotypes of the mutants used

The *coi1-16xein2-1* double mutant was constructed in our lab. To show that *coi1-16xein2-1* is homozygous for both alleles it was phenotyped on plate assays. As controls the *coi1-16* and *ein2-1* single mutants and Col-0 wild type were grown on the same plates.

The triple response assay with plates containg ACC (precursor of ethylene) showed that neither the double mutant nor the *ein2* single mutant plants were responsive to ethylene treatment. Thus, they are homozygous for the *ein2* mutation. In contrast wildtype Col-0 and *coi1-16* showed the previously described features of classic triple response (*Figure 9* and *Figure 10*).

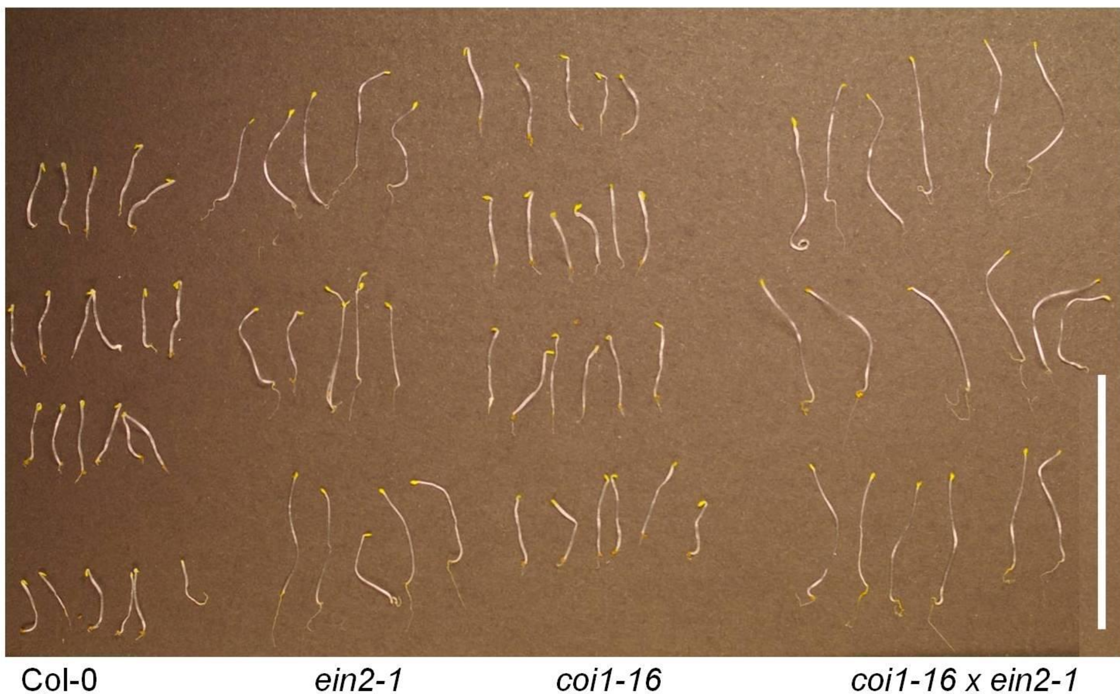

Figure 9 *Morphological features of the triple response in wild type Arabidopsis and coi1-16 seedlings and in the ethylene insensitive mutants ein2 and coi1-16 x ein2. In the ethylene insensitive mutants there was no triple response phenotype (described in text). The scalebar length is 3 cm.*

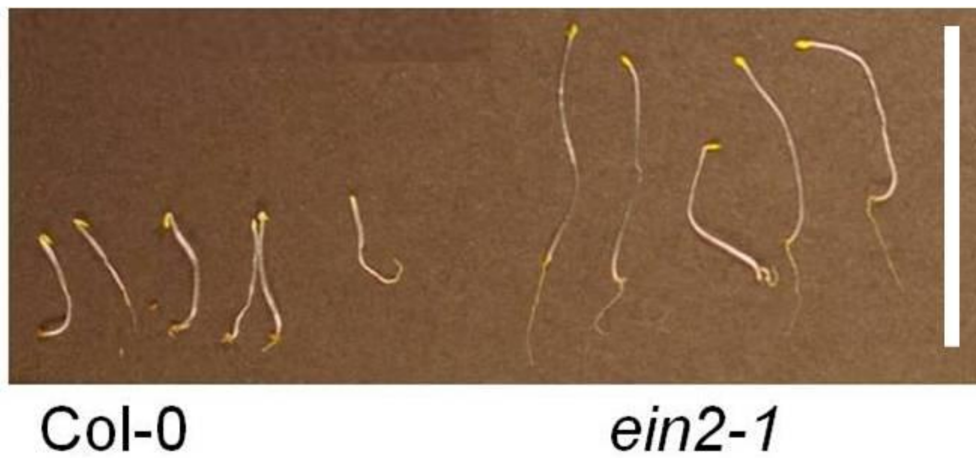

Figure 10 *Comparison of the triple respone (wild type Col on the left picture) and the absent triple response phenotype (ein2 on the right picture). The scalebar length is 2 cm.*

In wild type plants MeJA aplication on seedlings inhibits root growth (Ellis and Turner, 2002). To demonstrate the effect of *coi1-16* mutation on jasmonate signaling the phenotyping plates (contained 50 µM MeJA) were done with seedlings from Col-0, *coi1-16, ein2-1* and *coi1-16* x *ein2-1*. MeJA severly inhibited the root elongation of Col-0 and *ein2-1*, whereas the *coi1-16* and *coi1-16* x *ein2* seedlings were longer and less sensitive (*Figure 11*).

The results confirmed that the constructed double mutant *coi1-16xein2-1* has defect in both ethylene and jasmonate signaling pathway. This double mutant proved to be a useful tool in the investigation of the regulation ascorbic acid content.

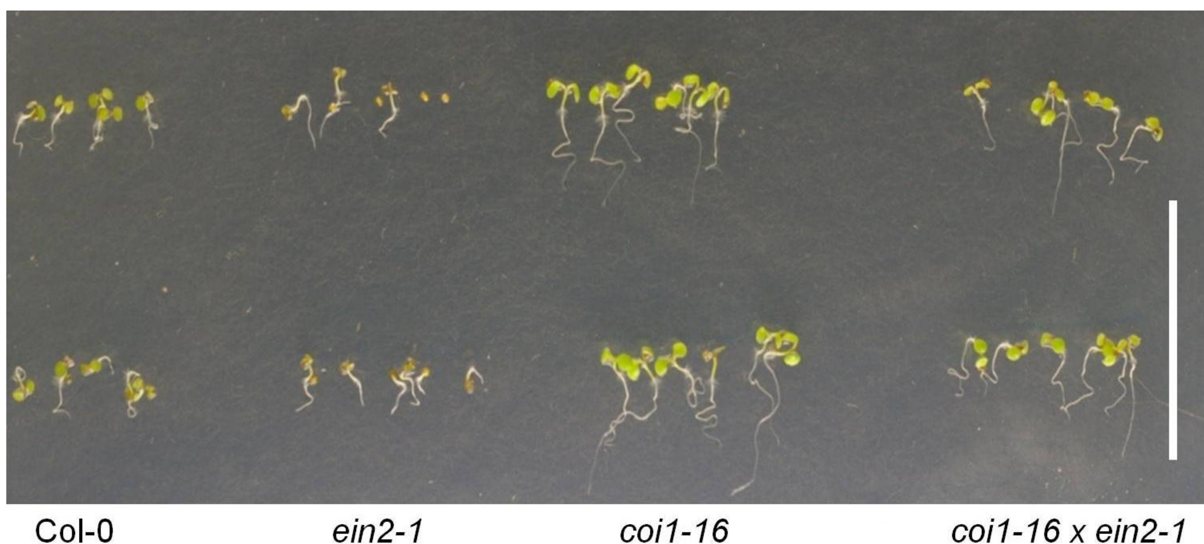

Figure 11 *Morphological features of the response to MeJA treatment in wild type Arabidopsis and ein2 seedlings and in the jasmonate insensitive mutants coi1-16 and coi1-16* x *ein2. In the jasmonate insensitive mutants the root is elongated despite the MeJA application to the 1 % agar growth medium. The scalebar length is 3 cm.*

4.2. *Ascorbate measurments in wild type and in mutants*

Very little is known about regulation of ascorbate biosynthesis. There is some evidence that ethylene and jasmonates have a role in ascorbic acid biosynthesis regulation (Gergoff et al 2009, Suza et al. 2010). Furthermore, one negative regulator AMR1 has been identified, which downregulates the expression of ascorbic acid biosynthesis enzymes in the mannose/L-galactose ascorbic acid biosynthetic pathway (Zhang et al. 2009).

The comparative measurment of the reduced and oxidized ascorbate content in 0,08 g fresh plant material was done by using wild type Col-0 plants as control and several mutants, which were defective in ethylene (*ein2, ctr1*) and jasmonic acid signaling responses (*coi1-16*) and also the double mutants with two nonfunctional genes from ethylene and jasmonate signaling (*ein2xcoi1-16*) to confirm the effects of these hormones on ascorbate accumulation. Additionally, one ascorbate synthesis enzyme deficient mutant *vtc1-1* and the previously mentioned negative regulator defective mutant (*amr1*) were used. The features of these mutants are introduced in the table 1 in previous section. The results from three independent measurments are given in *Table 6* and on *Figure 12*.

Table 6 *Ascorbate total and reduced concentrations (µmol/g), standard deviation and % from wild type (Col-0) total. Oxidized AA (DHA) is the difference between the total pool and the reduced pool. The results are the average from three independent experiments.*

Sample	Ascorbate conc. (µmol/g)	SD	% from wt total	DHA conc. (µmol/g)
Col-0 total	4,72	0,37	100,0	0,87
Col-0 reduced	3,84	0,51	81,5	
amr-1 total	7,10	0,56	150,6	1,18
amr-1 reduced	5,92	0,45	125,6	
coi1-16 total	3,71	0,54	78,7	0,61
coi1-16 reduced	3,10	0,46	65,8	
coi1-16 x ein-2 total	4,11	0,40	87,2	0,76
coi1-16 x ein-2 reduced	3,35	0,40	71,1	
vtc-1 total	1,72	0,30	36,5	0,35
vtc-1 reduced	1,37	0,24	29,1	
ein-2 total	6,82	0,36	144,5	1,35
ein-2 reduced	5,47	0,44	116,0	
ctr-1 total	2,83	0,31	60,1	0,67
ctr-1 reduced	2,17	0,32	45,9	

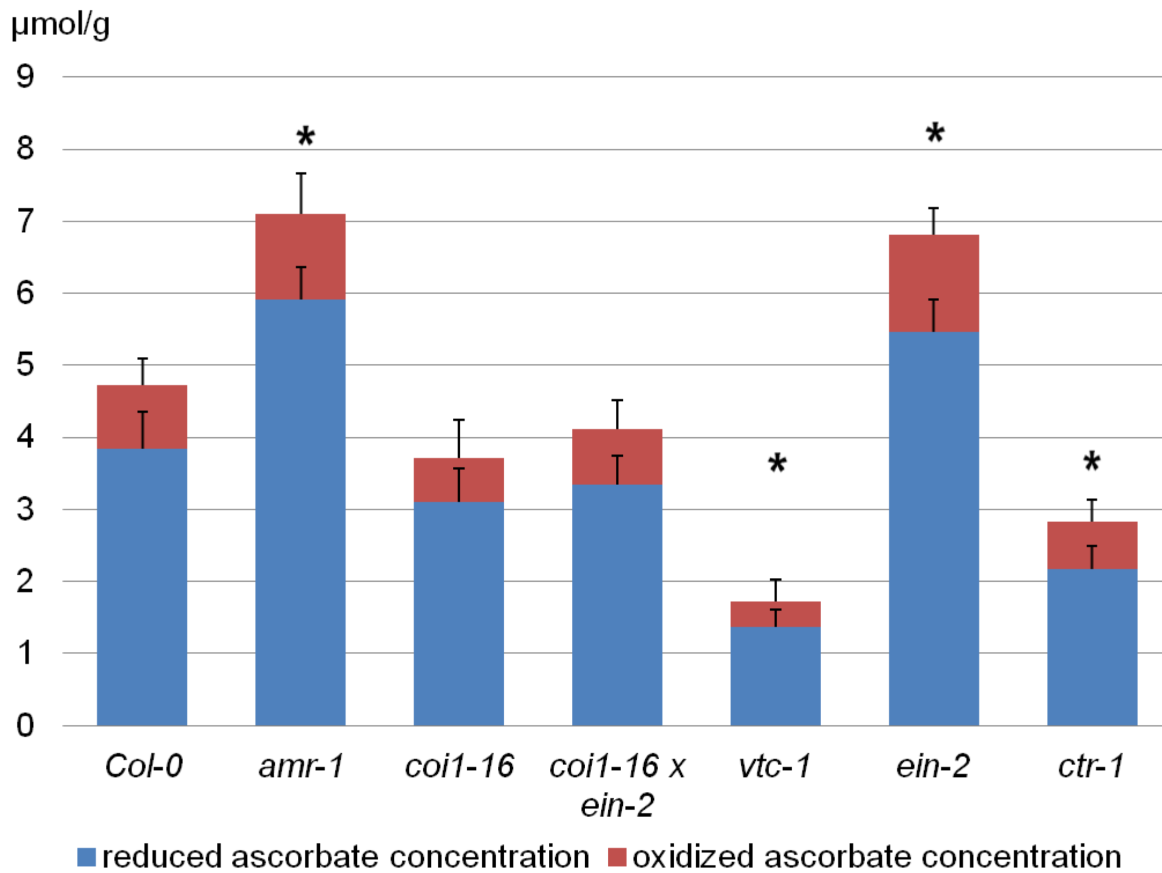

Figure 12 *Ascorbate total and reduced concentrations (µmol/g fresh weight) and standard deviation (over three independent measurments). The statistical significance was calculated with t-test (marked significant with one asterisk, when t ≤ 0,05 (95 % probability).*

The ascorbate measurment statistics shows that in *amr-1* and *ein-2* the ascorbate content is increased and in *ctr1* and *vtc1* significantly decreased compared to the wild type Columbia (Col-0) total and reduced concentrations. Both in *coi1-16* and in *ein2xcoi1-16* the average values are slightly lower than in Col-0, but are not statistically significant. The decrease or increase of ascorbate accumulation may be caused from changed biosynthesis, degradation or recycling rates.

Table 7 *The approximate total ascorbic acid content in wild type and in mutants (µmol/g fresh weight) after references.*

Arabidopsis line	**AA content µmol/g fresh weight**	**Reference**
Col-0	2,6 - 3,9	Pavet et al. 2009, Conklin et al. 2000
vtc1-1	1,278	Pavet et al. 2009
amr1-1	7,91	Zhang et al. 2009
ctr1-1	1,554	Gergoff et al. 2010
ein2-1	7,24	Gergoff et al. 2010
coi1-16	Indirect data	Suza et al. 2010
*coi1-16*ein2-1*	No data	-

There is evidence that ethylene downregulates ascorbate accumulation in *Arabidopsis thaliana* by lowering both its *de novo* synthesis rate and its recycling from its oxidized form (Gergoff et al. 2010). As EIN2 is a positive regulator of ethylene responses, the higher concentration of ascorbate in *ein2-1* mutant (144,5 % from total Col-0 ascorbate pool, statistically significant) are consistent with previous published results (Table 7). The constitutive triple response mutant *ctr1-1* showed a constitutive ethylene response even without ethylene treatment (Kieber et al. 1993). In *ctr1-1*, the ascorbate is significantly (t-test; $P \leq 0,05$) decreased (60 % from total Col-0 ascorbate pool – the results are consistent with previous observations (table 7). The oxidized/reduced forms ratio of ascorbic acid was similar for *ein-2* compared to wild type and was slightly increased for *ctr1-1* (Table 8).

In *amr1-1* mutant the ascorbate concentration was significantly higher (50 %) than in wild type. Zhang et al. (2009) have measured doubled ascorbate accumulation in *amr1* compared to Col-0. The results confirm previous observations that AMR1 specifically inhibits the ascorbate biosynthesis in D-mannose/ L- galactose pathway (Zhang et al. 2009). In *amr1-1* the ratio of ascorbate oxidized and reduced form was similar to wild type (Table 8).

Table 8 *The oxidized/reduced forms ratio of ascorbic acid.*

Arabidopsis line	DHA/AA ratio
Col-0	0,227
amr-1	0,199

coi1-16	0,196
coi1-16 x ein-2	0,236
vtc-1	0,253
ein-2	0,247
ctr-1	0,310

In *Arabidopsis* exogenous MeJA treatment enhances ascorbate accumulation (Suza et al. 2010). In jasmonate signaling mutant *coi1-16* the concentration of ascorbate are slightly decreased compared to wild type plants (*Table 6* and *Figure 12*). However, this reduction was not significantly different compared to wild type. The dehydroascorbate ratio to ascorbate was similar to wild type *(Table 8).*

Vtc1-1 mutant is defective in ascorbate synthesis (Conklin et al. 1999) and has about one third of ascorbate compared to wild type Col-0. It has been shown that lack of triggers the jasmonate-dependent signaling pathway (Kerchev et al. 2011). Jasmonate in turn induces the transcription of ascorbate synthesis enzymes coding genes (Suza et al. 2010). It means, that in stress situations, where ascorbate (antioxidant) is critically exploited the stress induced jasmonate signaling attempt to rescue the redox status of the cells. Jasmonates also stimulate the synthesis of the other low molecular weight antioxidant – glutathione. High levels of glutathione in turn represses the jasmonate signaling through antagonistic salicylic acid dependent pathway (Kerchev et al. 2011). It can be concluded that the antioxidant status of the cells is strictly controlled by different plant hormones and that there must be a balance between ascorbate and glutathione antioxidant pool sizes.

The *ein2-1xcoi1-16* double mutant constructed for this project has both disabled jasmonate and ethylene responses (Alonso et al, 1999; Ellis and Turner, 2002). The ascorbate concentration was slightly lower, however not significantly different compared to wild type. These results indicated a possible epistatic relationship between the examined genes. The regulator of jasmonate response COI1 masks the effect of EIN2, which means that *coi1-16* is epistatic to *ein2-1*. Epistasis occurs when one gene affects the expression of another masking one′s presence or combine to produce a new trait. As the ascorbate concentrations were similar with *coi1-16*, there can be concluded, that the jasmonate regulation point for ascorbate is downstream from ethylene and as a consequence jasmonate insensitivity diminishes the effect of ethylene on ascorbate accumulation. Both hormones mainly alterate the activity of the ascorbic acid biosynthetic enzymes from the mannose/ L-galactose pathway (Suza et al.

2010). Thus the hypothesis is that these hormones mainly regulate ascorbate accumulation through modulation of its synthesis, possibly via altered expression of the biosynthesis enzymes.

4.3. Expression analysis of ascorbate biosynthetic genes and genes involved in its recycling

To complement the ascorbic acid concentration measurements, gene expression analysis using real time quantitative RT-PCR were done on the previously mentioned mutants (table 1), with primers for all genes in the ascorbate biosynthesis in mannose/ L-galactose pathway. In addition, genes in the synthesis of glutathione, which is the second important antioxidant, and the repressor of ascorbate synthesis enzymes – *AMR1* were included (table 5).

Before synthesising cDNA from the RNA samples the quality of the total RNA were measured. The purified RNA quality control with Agilent 2100 Bioanalyzer showed good RIN (RNA Integrity Number) values (table 9). The RIN algorithm of the Plant RNA assay measures the integrity of total plant RNA (Babu and Gassmann, 2011). The RIN value >7 showed that the RNA in the sample has a good quality for further RT-PCR step.

Table 9 *The RNA integrity numbers (RIN) for the RNA samples from the RNA quality control with Agilent 2100 Bioanalyzer.*

RNA sample	**RIN value**
Col-0	8,9
ein2-1	8,6
vtc1-1	8,9
ctr1-1	9,1
amr1-1	9
coi1-16	8,8
coi1-16 x *ein2-1*	8,8

The hormone (ethylene, jasmonate) and ascorbate repressor signaling mutants were tools for assessing the effect of them on precise steps in the ascorbate synthesis. The quantitative measurment of cDNA by RT-PCR allows us to predict where ascorbic acid biosynthesis is regulated at the transcription level.

The *ctr1-1* mutant is severly dwarfed which may complicate the interpretation of the results from this mutant. As the leafplates were tiny and the stem comprised significantly larger

proportion of the biomass of the shoot compared to other samples, then the amount of bioactive molecules which are mostly found in the leaves may be smaller in the *ctr1-1*. This feature needs to be kept in mind while intepreting the gene expression results.

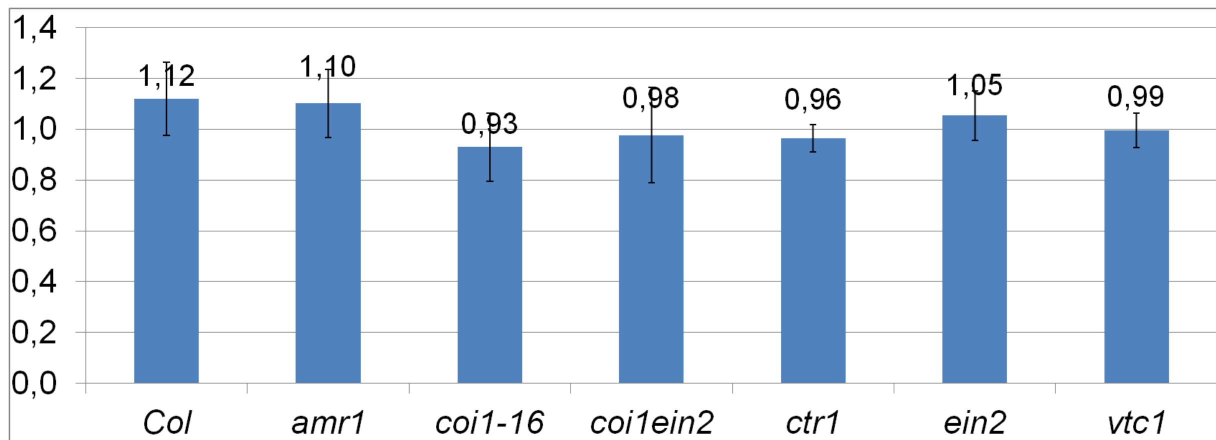

Figure 13 *The expression of PMI1 (At3g02570; PHOSPHOMANNOSE ISOMERASE) in arbitary units. Error bars indicate standard deviation over five repeats. No significant changes were found.*

The first enzyme which is not part of glycolysis is PHOSPHOMANNOSE ISOMERASE (PMI). This enzyme catalyses the isomerization of D-fructose-6-phosphate to D-mannose-6-phosphate. Only the PMI1 isoenzyme is involved in ascorbate biosynthesis, the second isoenzyme PMI2 does not affect ascorbate synthesis in any way (Maruta et al 2008).

Previously, *PMI1* has been shown to be induced by jasmonate application (Suza et al. 2010). Here is demonstrated that in mutants where jasmonate response is disabled, the *PMI1* transcript levels were slightly but not significantly lower than in wild type (*Figure 13*). This indicates only a weak effect of jasmonate. Generally the results showed that *PMI1* expression is independent of endogenous jasmonate, ethylene and AMR1.

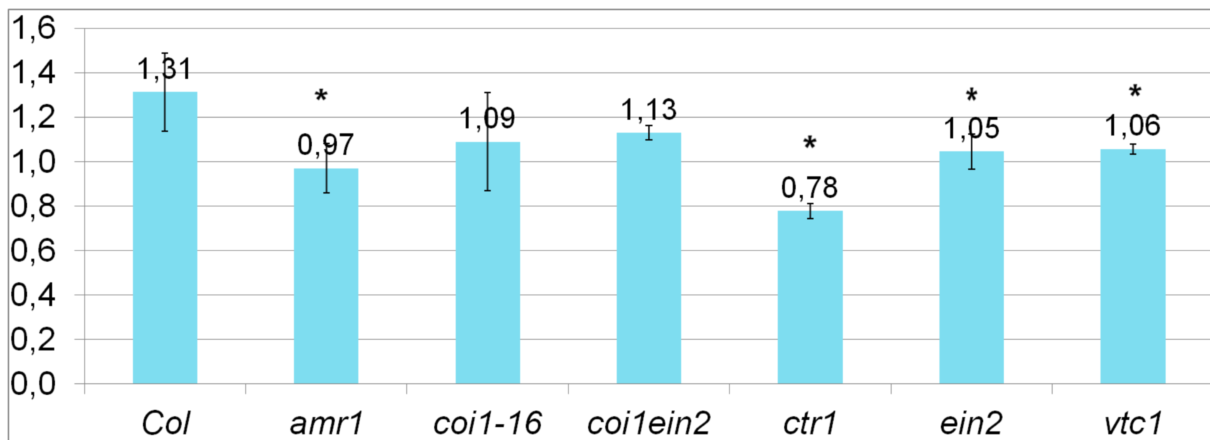

Figure 14 *The expression of PMM (At2g45790; PHOSPHOMANNOMUTASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant with asterisk when t ≤ 0,05 (95 % probability).*

Phosphomannomutase (PMM) mediates the interconversion of D-mannose-6-phosphate and D-mannose-1-phosphate. As the later is the substrate for synthesising GDP-mannose – a structural component of many derivatives in the cell – thus beside ascorbate synthesis PMI and PMM also provide building blocks for the cell wall carbohydrates and for protein glycosylation (Qian et al. 2007). Arabidopsis conditional heat sensitive mutant *pmm-12* has impaired protein glycosylation and has severely reduced ascorbate content if the mutant is exposed to higher temperatures (> 28°C). Mutant seedlings grown at the 16 – 18°C show similar appearance as wild type and grow normally (Hoeberichts et al. 2008). It is impossible to obtain a mutant with permanently decreased *PMM*, because seeds with mutation in this gene are not viable (Qian et al. 2007).

In the *PMM* expression analysis the highest transcript level is in wild type Col-0 samples (*Figure 14*). Interesting is to point out that *amr1-1* and *ein2-1* with increased ascorbate concentration, showed lower *PMM* expression than wild type. This means that AMR1 has a positive effect on *PMM* transcript accumulation. Also the *ctr1-1* mutant with elevated ethylene response appears to have significantly depressed *PMM* transcript accumulation. It may be possible that both the shortage and the excess ethylene affect negatively the *PMM* transcript accumulation (directly or indirectly). Qian et al. (2007) observed, that there is no great correlation between ascorbic acid content and *PMM* expression. Their hypotesis was that PMM and the following enzyme activity or gene expression may be co-regulated, but the mechanism and involved molecules remain to be determined.

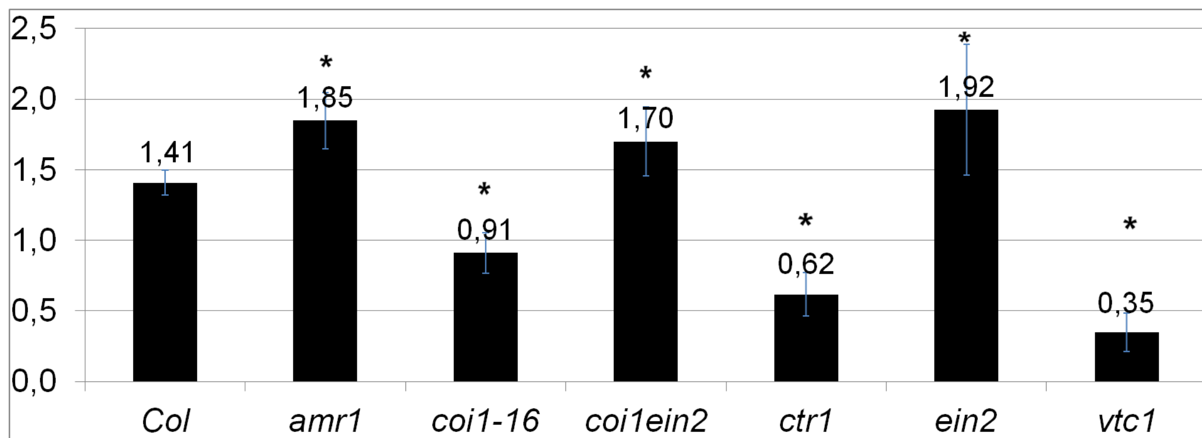

Figure 15 *The expression of VTC1 (GDP-D-MANNOSE PYROPHOSPORYLASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant with asterisk when t ≤ 0,05 (95 % probability).*

The results for GDP-D-mannose pyrophosphorylase indicated that this step might be the main regulation point for D-mannose/ L-galactose pathway (*Figure 15*). All the mutants have significant difference in the expression values compared to the wild type. *VTC1* had higher expression in *ein2-1, amr1-1* and *coi1-16xein2* mutants and decreased expression in *coi1-16*, *ctr1-1* and *vtc1-1*. The expression pattern in the mutants and in wild type highly correlated with the ascorbate concentration (*Figure 12*).

As ethylene inhibits the ascorbic acid accumulation, it was proposed that the key regulation point is in this step (Gergoff et al. 2010). The data clearly shows that the transcription is highly responsive to ethylene signaling (constitutive signal down-regulates and ethylene insensitivity up-regulates *VTC1* expression). Jasmonate also slightly induces this gene which confirms the previous results reported by Suza et a. (2010). Here, (*Figure 15*) in *coi1-16* mutant the *VTC1* transcript levels are lower than in wild type. In *coi1-16xein2-1* double mutant the gene is in intermediate level compared to the respective single mutants, indicating that both hormones – ethylene and jasmonate – have regulatory effects on this gene.

Zhang et al. (2009) demonstrated that knocking-out the only known inhibitor of ascorbate synthesis genes AMR1, results in significant increase in ascorbate concentration. In *amr1-1* mutant several of the ascorbate biosynthetic genes are induced compared to wild type. As shown in *Figure 15 VTC1* transcript is also induced in this mutant, pointing out the regulatory effect of AMR1 on this gene.

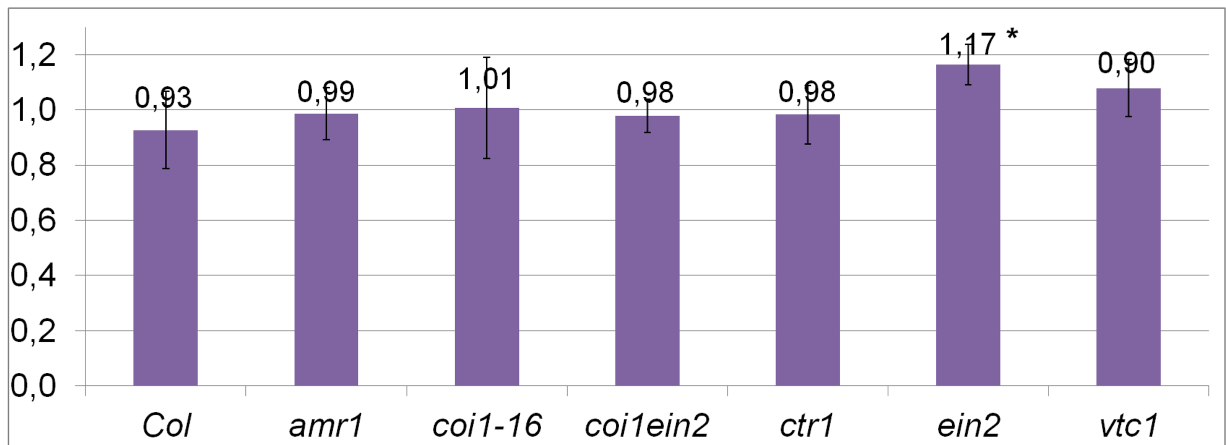

Figure 16 *The expression of GME (GDP-D-MANNOSE-3,5-EPIMERASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant when t ≤ 0,05 (95 % probability)).*

GDP-D-MANNOSE-3,5-EPIMERASE produces GDP-galactose and in lesser extent GDP-gulose from the GDP-mannose. The product is formed in the same active reaction centre in three steps which cover oxidation, epimerisation and reduction. The NAD(P)+ cofactor is essential for the hydride transfer (Major et al. 2005). The second product GDP-gulose is mainly produced in stress conditions – this observation proves the stress responsiveness of this enzyme (Wolucka and Montagu 2003).

Previously *GME* expression has been shown to be inhibited by AMR1 (Zhang et al. 2009). This could not be reproduced in our growth conditions (*Figure 16*). Surprisingly, there was an increase in the *GME* expression in the ascorbate deficient mutant *vtc1-1*. Li et al. (2013) explained this unexpected behavior with the attempt to rescue the ascorbate content in the leaves by re-directing the GDP-mannose to the alternative L-gulose pathway, which is also proposed to produce ascorbate as a final product (Wolucka and Montagu, 2003).

While Ioannidi et al. (2009) found that ethylene and jasmonate does not affect *GME* transcription, results shown in *Figure 16* indicated that in *ein2-1 GME* expression was significantly induced. However in *ctr1-1* there was no difference with Col-0 indicating that constitutive ethylene signaling does not alter this gene transcription. In jasmonate mutant *coi1-16* and in the ethylene and jasmonate double mutant *coi1-16xein2-1* there was no difference in this gene expression confirming previous observations in tomato (Ioannidi et al. 2009). In tobacco (*Nicotiana tabacum)* BY-2 cells exogenous methyl-jasmonate treatment highly induces the *GME*, but not in Arabidopsis (Wolucka et al. 2005).

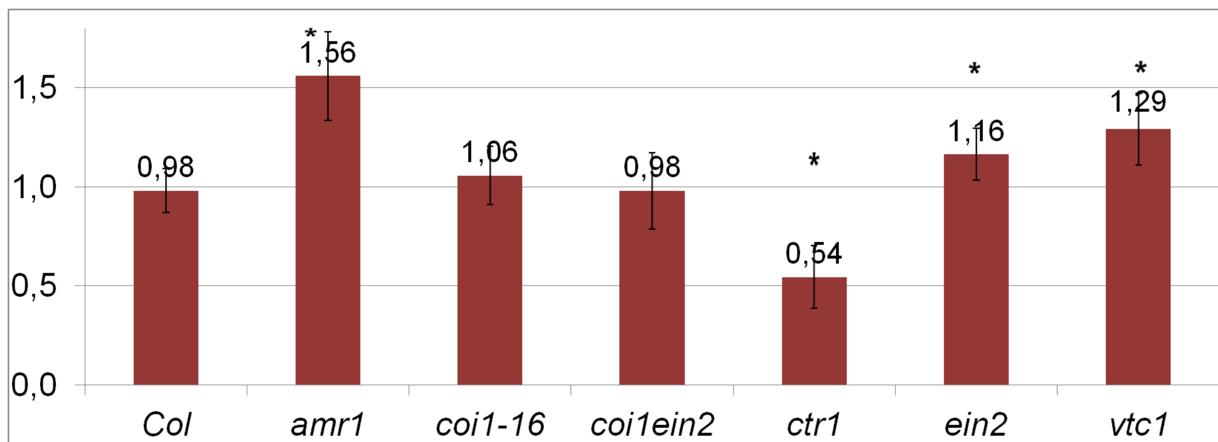

Figure 17 *The expression of VTC2 (GDP-L-GALACTOSE PHOSPHORYLASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant with asterisk when t ≤ 0,05 (95 % probability)).*

The *Arabidopsis thaliana* genes *VTC2* and *VTC5* are paralogs, which both code for the same enzyme with two different activities: it has both GDP-L-GALACTOSE PHOSPHORYLASE and L-GALACTOSE GUANYLYLTRANSFERASE activity. The enzyme variants coded by these genes partly compensate each others activity in the respective single mutants, the *vtc2xvtc5* double mutant is not viable. While *VTC2* is largely expressed in the shoot, where is photosynthetic tissue, the *VTC5* transcript is mainly found in the roots (Müller-Moule 2008; Dowdle et al. 2007). *VTC2* is activated by light, in contrast *VTC5* is responsive only to circadian clock. It is thought that the GDP-L-galactose phosphorylase coding genes may be the also important regulation points in the D-mannose/ L-galactose pathway (Dowdle et al. 2007, Linster et al. 2007).

Previous results have been shown that externally applied jasmonate induces both the *VTC2* and *VTC5* transcription (Sasaki-Sekimoto et al. 2005). Expression of *VTC2* in jasmonate signaling mutants *coi1-16* and *coi1-16xein2-1* were the same as in *Arabidopsis* Col-0 (*Figure 17*), which indicates that jasmonate may have only minor effect on this gene in transcription level.

The ethylene effect on this gene was significant (*Figure 17*). The *ctr1-1* mutant with constitutive ethylene response had reduced *VTC2* transcript levels and on the contrary *ein-2-1* had increased *VTC2* transcript accumulation.

As espected from previous observations where *VTC2* transcript levels highly correlate with ascorbate accumulation (Conklin et al. 2000), the *amr1-1* mutant has higher levels of ascorbate and also highly expressed *VTC2* (Zhang et al. 2009). Surprisingly in *vtc1-1* mutant

expression of *VTC2* was significantly higher than in wild type. This suggests that there may be some salvation pathway active in this mutant with reduced ascorbate synthesis. This result may approve the existence of the energy-conserving VTC2 hub that links photosynthesis with the biosynthesis of vitamin C and the cell wall polysaccharides first proposed by Wolucka and van Montagu (2007). The VTC2 transferase reaction in the VTC2 cycle results in a conversion of the glycolysis intermediate D-glucose-1-phosphate into ascorbate synthesis precursor L-galactose-1-phosphate using GDP-L-galactose as guanylyl donor (Wolucka and van Montagu 2007; Linster et al. 2007). This way using different substrates the the first enzymes in the proposed D-mannose/L-galactose vitamin C synthesis pathway (PHOSPHOMANNOSE ISOMERASE (PMI), PHOSPHOMANNOMUTASE (PMM) AND GDP-D-MANNOSE PYROPHOSPORYLASE (VTC1)) can be bypassed. The only problem with this hypothesis is that the VTC2 cycle require two more enzymes in the recycling the GDP-D-glucose back into the guanylyl donor GDP-L-galactose, from which one is hypothetical (Wolucka and van Montagu 2007). GDP-D-MANNOSE-3,5-EPIMERASE (GME) responsible for the second reaction in the recycling hub is well characterized (Wolucka and van Montagu 2003; Major et al. 2005), but the first proposed enzyme GDP-D-MANNOSE-2''-EPIMERASE is still unidentified (Wolucka and van Montagu 2007).

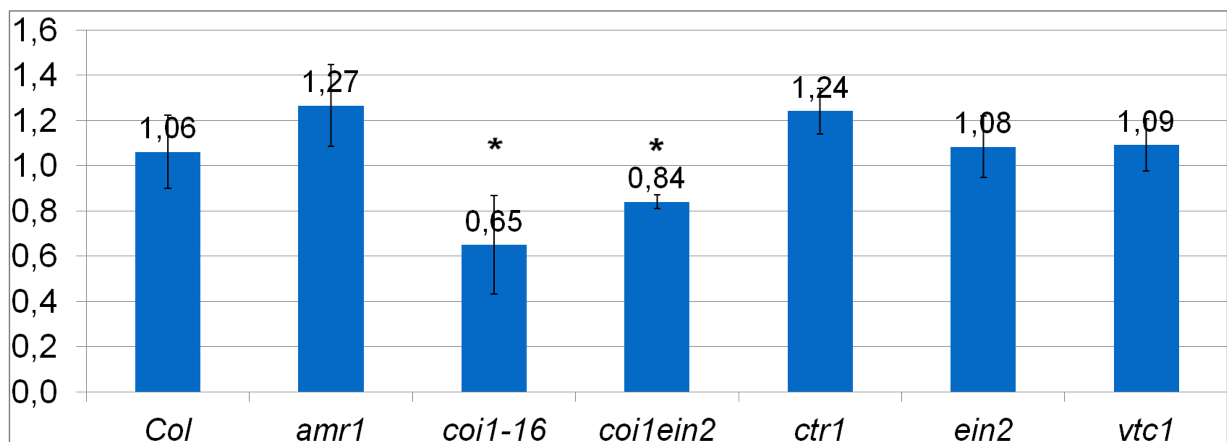

Figure 18 *The expression of VTC5 (GDP-L-GALACTOSE PHOSPHORYLASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant when t ≤ 0,05 (95 % probability)).*

Expression of *VTC5* was significantly reduced in the *coi1-16* mutant and in the double mutant *coi1-16xein2-1* (*Figure 18*). This indicated a connection between *VTC5* transcript levels and the jasmonate response. The hypothesis is supported by the fact that exogenously applied MeJA enchances the expression *VTC5* transcript (Suza et al. 2010). As the ethylene mutants

had wild type expression of *VTC5* it may be concluded that ethylene does not affect this enzyme at its transcription level.

Comparing the expression patterns of the paralogous *VTC2* and *VTC5* it can be concluded that the genes are differentially regulated (*Figure 17* and *Figure 18*). While *VTC2* is significantly responsive to ethylene, the transcription of *VTC5* is induced by jasmonates. Regulation by AMR1 appears to be stronger for *VTC2*.

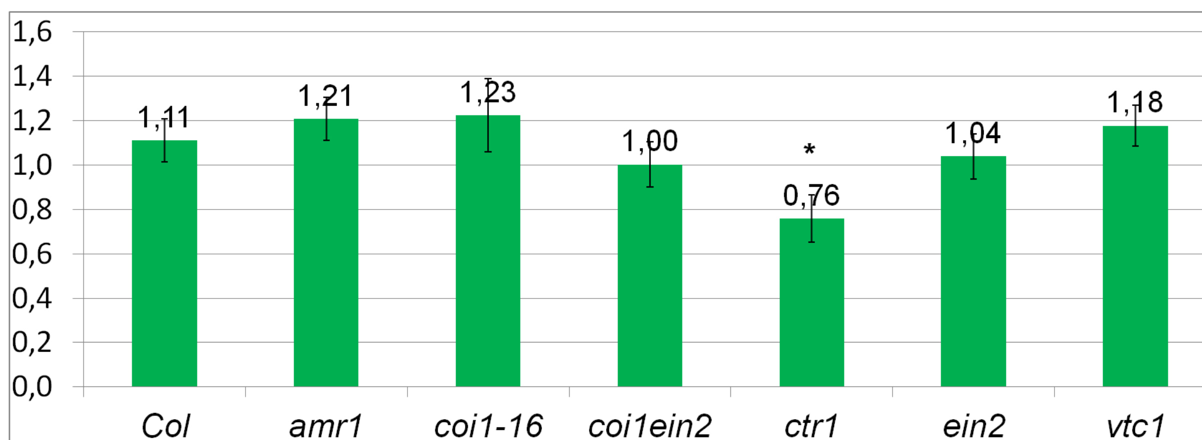

Figure 19 *The expression of VTC4 (L-GALACTOSE-1-P PHOSPHATASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant with asterisk when t ≤ 0,05 (95 % probability)).*

The expression of *VTC4* decreased in the constitutive ethylene signaling *ctr1-1* mutant, but was similar to wild type in *ein2-1* (*Figure 19*). These results show that increased ethylene reduced this gene expression, but the lack of ethylene response had no effect.

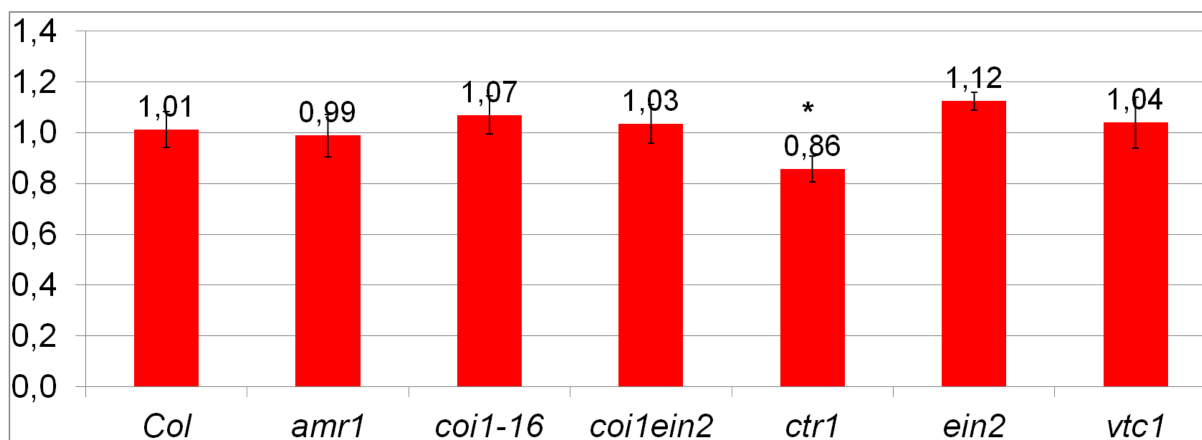

Figure 20 *The expression of GalDH (L-GALACTOSE DEHYDROGENASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant with asterisk when t ≤ 0,05 (95 % probability)).*

The penultimate enzyme L-GALACTOSE DEHYDROGENASE oxidizes C1 of L-galactose to L-galactono-1,4-lactone (Wheeler et al., 1998). As the reaction is irreversible (Gatzek et al. 2002) Mieda et al. (2004) proposed that GalDH enzyme activity or gene expression might be the regulation point for this ascorbate synthesis pathway. The experiments showed that the known regulators (ethylene, jasmonates and AMR1) did not have little effect on the expression of *GalDH,* although in *ctr1-1* the expression was significantly lower compared to wild type (*Figure 20*). This effect might be indirect due to the *ctr1-1* severly dwarfed phenotype or that constitutive ethylene signaling downregulated almost all genes in the ascorbate synthesis pathway. It is unlikely that the pathway is regulated at its last two steps. Furthermore, the changes in the *GalDH* expression do not affect ascorbate accululation (Gatzek et al. 2002), but high ascorbate concentration inhibits GalDH enzyme acticvity – the enzyme is under negative feedback control (Mieda et al. 2004).

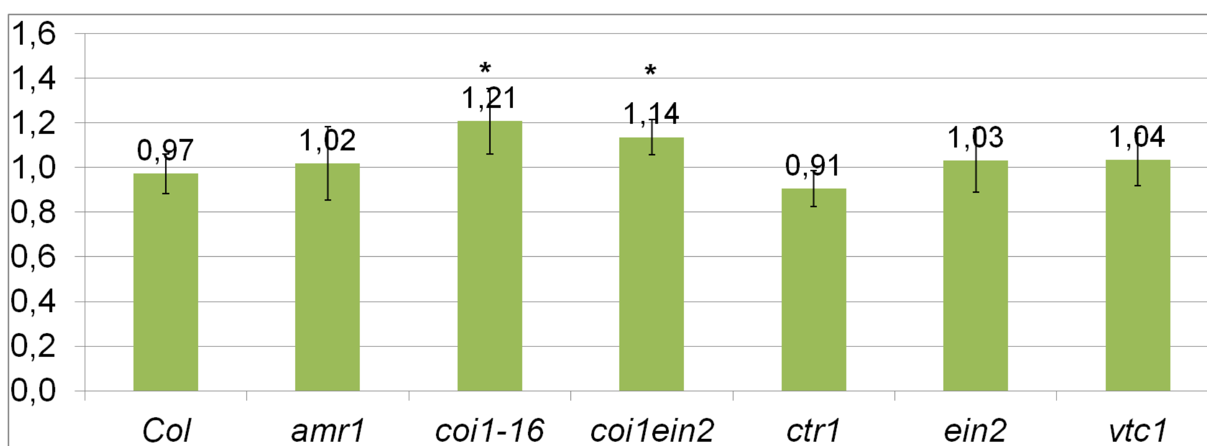

Figure 21 *The expression of GLDH (L-GALACTONO-1,4-LACTONE DEHYDROGENASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant with asterisk when t ≤ 0,05 (95 % probability)).*

The L-GALACTONO-1,4-LACTONE DEHYDROGENASE is the last step in ascorbate biosynthesis (Imai et al. 2009). The expression data showed that in jasmonate mutants *coi1-16* and *coi1-16xein2-1* the *GLDH* was increased (*Figure 21*). This indicates that jasmonate may be a negative regulator of this gene.

Over-expression of this gene does not elevate the ascorbate concentrations and it has been tought that instead substrate galactonolactone availability is crucial for raising the ascorbate pool (Imai et al. 2009). The ascorbate content in different developmental stages correlates with GLDH enzyme activity – both degrease with leaf age (Bartoli et al. 2000).

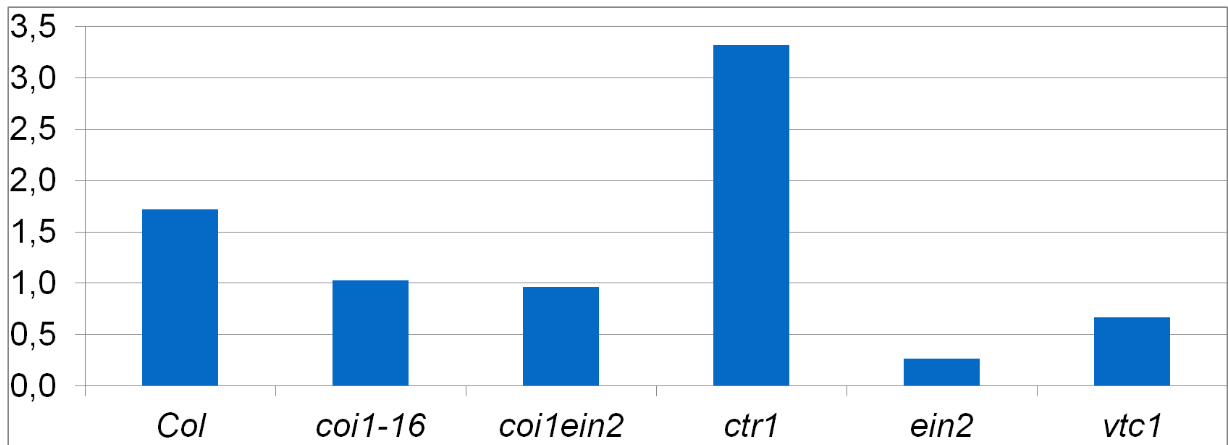

Figure 22 *The expression of AMR1 (ASCORBATE MANNOSE PATHWAY REGULATOR 1) in arbitary units. The data includes only one experiment. The expression in amr1 mutant was not detected.*

AMR1 is a negative regulator of transcription of several genes in the D-mannose/ L-galactose pathway (Zhang et al. 2009). Although only one repeat was available, ethylene may have an important role in regulating *AMR1* at the transcriptional level (*Figure 22*). In the ethylene signal deficient mutant *ein2-1* the *AMR1* expression was supressed and the constitutive ethylene signaling *ctr1-1* the transcript level was highly increased. Unexpectedly, in the ascorbate deficient mutant *vtc1-1* the transcript level of *AMR1* was decreased. This may indicate positive feedback regulation from the product (ascorbate) or some intermediate substance accumulation (D-mannose, L-galactose).

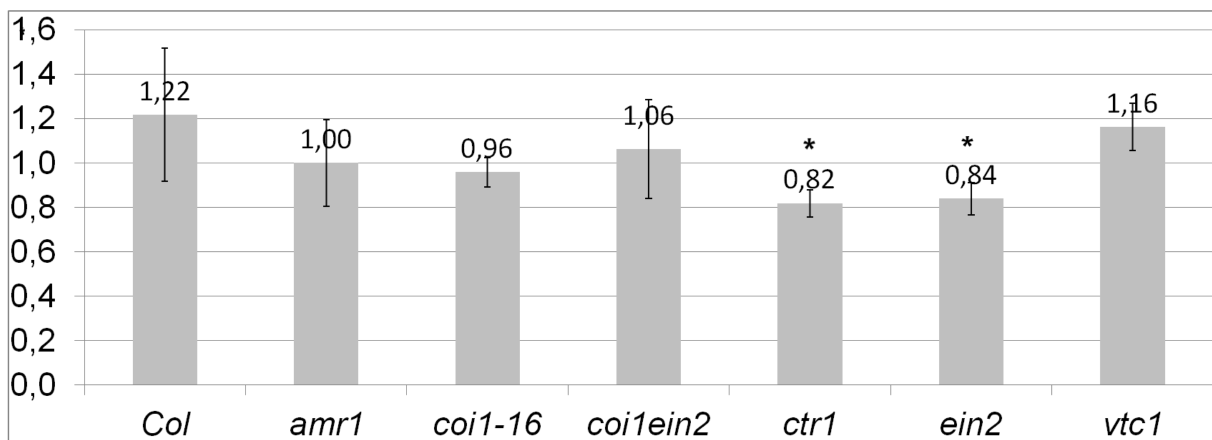

Figure 23 *The expression of APX1 (ASCORBATE PEROXIDASE) in arbitary units. Error bars indicate standard deviation over four repeats. The statistical significance was calculated with t-test (marked significant with asterisk when $t \leq 0{,}05$ (95 % probability)).*

APX1 is the main enzyme (beside CATALASE), which detoxifies hydrogen peroxide (H_2O_2) with the aid of reducing power from ascorbate, which is an essential cofactor for this enzyme (Maruta et al. 2008). The ethylene signaling mutant *ctr1-1* had decreased *APX1* transcript

accumulation compared to wild type (*Figure 23***Error! Reference source not found.**). There was less *APX1* mRNA also in the ethylene insensitive mutant *ein2-1*. Yoshida et al. (2009) performed transcriptome analysis in *ein2-1* and in Col-0 as a control and these results for *APX1* expression were comparable to the results presented here. These results suggest that an appropriate level of ethylene signaling is needed for correct *APX1* transcript accumulation. The other mutants did not show significant difference in *APX1* expression compared to wild type.

APX1 transcript accumulated in the wild type and in *vtc1* mutant equally, but there is evidence that ascorbate peroxidase activity is doubled in the *vtc1* mutant. Under optimal conditions only ascorbate peroxidase increased activity compensates ascorbate deficiency leaving glutathione and α-tocopherol pool unchanged. On the contrary, in stress conditions (like high light intensity) glutathione pool size increases in the *vtc1* mutant (Colville and Smirnoff, 2008).

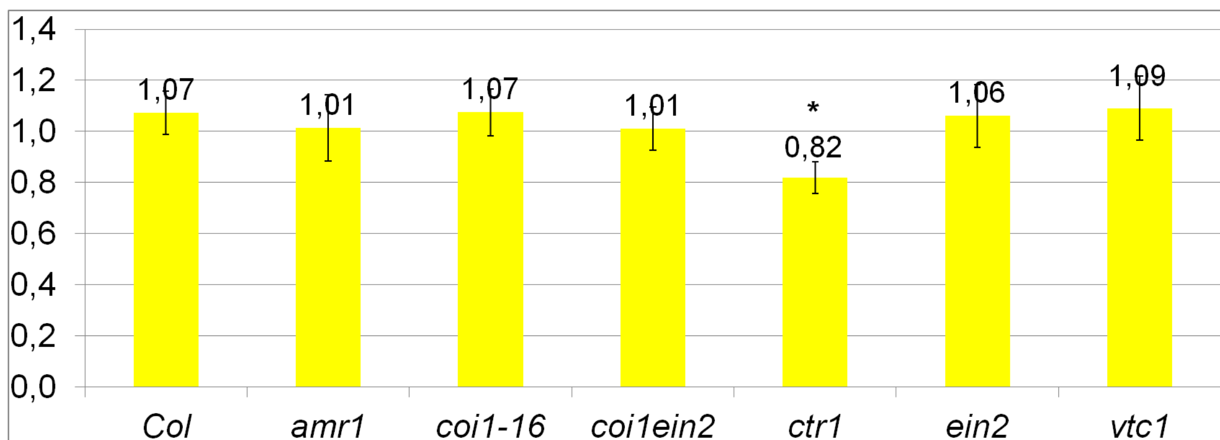

Figure 24 *The expression of GSH1 (Γ-GLUTAMYLCYSTEINE SYNTHETASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant with asterisk when t ≤ 0,05 (95 % probability)).*

Γ-GLUTAMYLCYSTEINE SYNTHETASE is an enzyme that catalyzes the first step in glutathione synthesis: glutamate and cysteine is united into *γ*-glutamylcysteine (Yoshida et al. 2009). The *GSH1* mRNA levels were decreased in *ctr1-1* and similar to wildtype in the other mutants (*Figure 24*).

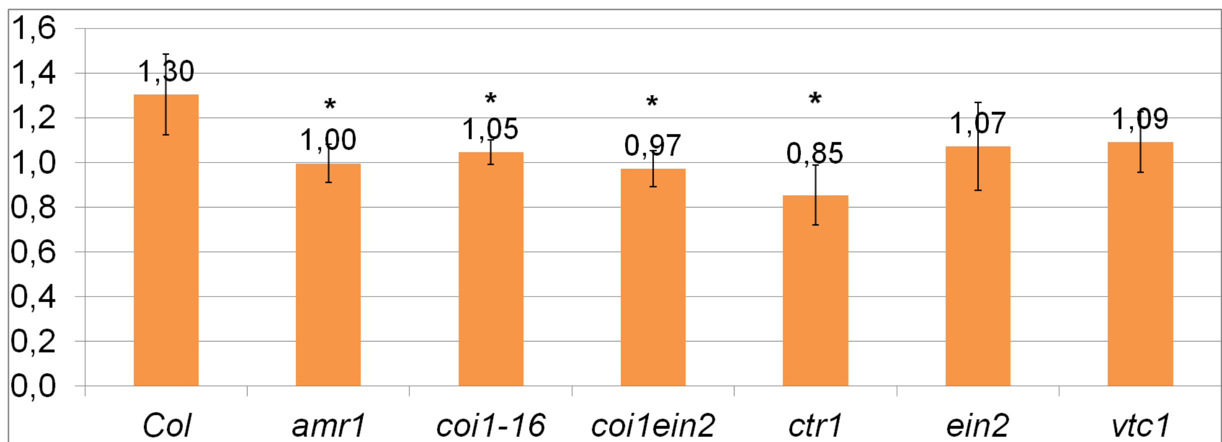

Figure 25 *The expression of GSH2 (GLUTATHIONE SYNTHETASE) in arbitary units. Error bars indicate standard deviation over five repeats. The statistical significance was calculated with t-test (marked significant with asterisk when t ≤ 0,05 (95 % probability)).*

GLUTATHIONE SYNTHETASE (GSH2) is responsible for the synthesis of glutathione from the γ-glutamylcysteine and glycine and it could be the regulation point for glutathione synthesis (Yoshida et al. 2009). In the constitutive ethylene signaling mutant *ctr1-1* both the *GSH2* and *GSH1* were lowly expressed (*Figure 24* and *Figure 25*). As the transcript levels of *GSH2* were significantly reduced also in *amr1-1* and in the jasmonate signaling mutants *coi1-16* and in *coi1-16xein2-1*, it may be suggested that ethylene, jasmonate and *AMR1* cause altered transcription of *GSH2*.

The ascorbate and glutathione concentrations are in inverse relationship (Müller-Moule, 2008). Lower levels of glutathione in *ein2-1* are compensated by the increase in ascorbate synthesis and recycling (Gergoff et al. 2010). The same was seen in the *amr1-1* mutant – the *GSH2* transcription was reduced compared to wild type and although the glutathione itself was not measured it can be predicted that also glutathione content is reduced.

Sasaki-Sekimoto et al. (2005) showed that MeJA application induces glutathione synthesis and defects in jasmonate signaling disrupt the activation of antioxidant synthesis. Here the results demonstrated that jasmonate signaling is necessary for glutathione biosynthesis. Tamaoki et al. (2008) demonstrated that jasmonate signaling defective mutant *jar1* had reduced *GSH1* and *GSH2* transcription (comparable with the results from *coi1-16* and *coi1-16xein2-1*).

Ethylene has several roles in plants, including promoting senescence and cell death (Tamaoki et al. 2003), but it has been revealed that ethylene under stress conditions may protect the plant from oxidative damage by activating the glutathione biosynthesis, which triggers the

stress response and quenches ROS. Yoshida et al. (2009) showed that in *ein2-1* mutant the glutathione content was reduced and it might be due to the reduced synthesis activity. The lower levels of glutathione in *ein2-1* are compensated by the increase in ascorbate synthesis and recycling (Gergoff et al. 2010). While Col-0 had strong induction of *GSH2* transcript synthesis after 3 hours ozone exposure, the ozone induced increase in the expression of *GSH2* was much lower in the *ein2-1* mutants (Yoshida et al. 2009). On the contrary, Tamaoki et al. (2008) demonstrated that in *ein2-1* the transcript accumulation of *GSH1* and *GSH2* was similar to wild type under nonstress conditions. Here in the constitutive ethylene signaling mutant *ctr1-1* the reduced *GSH2* expression may indicate that the constitutive ethylene signal also triggers sress and the antioxidative system cannot handle it. This is consistent with Tamaoki et al. (2003) who demonstrated that ethylene enhances the injuries caused by ozone treatment.

Some research papers have shown, that jasmonate also regulate ethylene response itself and *vice versa* (Ellis and Turner, 2002). The ascorbate concentration measurment results revealed from the double mutant *coi1-16xein2-1* that jasmonate have dominant effect over ethylene. In other words the regulation point for ethylene in the ascorbate synthesis pathway through L-galactose is before jasmonate.

The expression data from the ethylene signaling mutants was consistent with the data from the ascorbic acid measurment experiments. Constitutive ethylene signalling was in negative correlation with ascorbate concentrations. *Coi1-16xein2-1* showed the properties of both the ethylene and jasmonate mutants and intermediate gene expression pattern.

Exogenously applied MeJA enchances the expression of *VTC1* and *VTC2* and *GME* transcripts (Suza et al. 2010; Wolucka et al 2005). Consistent with these results the jasmonate signaling mutant *coi1-16* has reduced transcript level of *VTC1.* Although *VTC2* expression was not affected by the defect in the jasmonate signaling, the paralogous *VTC5* had significantly less transcript levels in *coi1-16* and in the jasmonate and ethylene signaling deficite double mutant *coi1-16xein2-1.* This result reveals that although the enzymatic functions of VTC2 and VTC5 are the same, the regulation of these enzyme coding genes is different.

The different regulation of *VTC2* and *VTC5* is further confirmed with the fact, that ethylene signaling downregulates *VTC2*, but it had no effect on *VTC5* transcript levels. The

constitutive ethylene signaling in *ctr1-1* mutant also downregulated *PMM, VTC1, VTC2, VTC4* and *GalDH* transcript levels. When ethylene signal is disabled (*ein2-1* mutant) then the *VTC1, GME* and *VTC2* expression was significantly induced. This means that for some genes the lack of ethylene signal has also an effect and that result may be used, when fruits and vegetables are storaged in the controlled environment where there is no ethylene. This could potentially increase the ascorbate content.

In our growth conditions AMR1 was a negative regulator of *VTC1* and *VTC2* and a positive regulator of *PMM* and *GSH2*. In *amr1-1* mutant the ascorbate concentration was 50 % higher compared to wild type and the *VTC1* and *VTC2* transcripts were significantly induced. The reduction of *GSH2* in *amr1-1* indicates the inverse relationship of ascorbate and glutathione.

Plants have evolved a mechanism to compensate the deficiency of one antioxidant by increasing the levels of others. Mutant plants which have reduced levels of ascorbate (*vtc1, vtc2, vtc5*) show higher enzyme activity in glutathione synthesis (*GSH2*) and increased glutathione content (Müller-Moule, 2008).

The data obtained in this thesis can be used to form new ideas and experiments on how the regulation of ascorbate biosynthesis is achieved. For example the regulation of *AMR1* by ethylene should be studied in more detail.

SUMMARY

Ascorbate is an important antioxidant in plants and animals (Suza et al. 2010). Although the main ascorbate biosynthetis pathway (D-mannose/ L-galactose pathway) in plants was proposed already in 1998 (Wheeler et al.), the regulation of ascorbate content is still poorly understood. Recently an inhibitor of ascorbate synthesis AMR1 was described (Zhang et al. 2009). Furthermore, in 2010 Gergoff et al. demonstrated that gaseous plant hormone ethylene reduces plant ascorbate concentration and Suza et al. published a paper (2010) about an inductive effect of jasmonate on ascorbate.

The aim of this study was to find the effect of jasmonate and ethylene signal transduction and *AMR1* on ascorbate synthesis in D-mannose/L-galactose pathway in the model organism *Arabidopsis thaliana*. To achieve this, ascorbate concentrations were measured spectorphotometrically in *Arabidopsis* mutants (*amr1-1, ein2-1, coi1-16, coi1-16xein2-1, ctr1-1)* and in wildtype Col-0 plants. Then to elucidate the specific steps where jasmonates, ethylene or the negative regulator AMR1 regulate this pathway the gene expressions was measured. The altered expression of these genes gave a hint where and how ascorbate content is regulated.

The obtained results from ascorbate concentration measurements were consistent with values previously published in scientific literature. The mutant *coi1-16xein2-1* (ethylene and jasmonate signaling deficient) was constructed in our lab for analyzing the effect of ethylene and jasmonate on ascorbate accumulation. The double mutant showed that COI1 (activator of jasmonate signal transduction) masks the effect of EIN2 (activator of ethylene signal transduction), which means that *coi1-16* is epistatic to *ein-2.* As the ascorbate concentration in the double mutant was similar with *coi-1-16,* it can be concluded, that the jasmonate regulation point for ascorbate biosynthesis pathway is downstream from ethylene. As a consequence jasmonate insensitivity diminishes the effect of ethylene on ascorbate accumulation.

Both jasmonate and ethylene alter the activity of the ascorbic acid biosynthetic enzymes from the mannose/ L-galactose pathway (Suza et al. 2010). The expression of genes coding ascorbate and glutathione biosynthesis enzymes, *AMR1* and *APX1* were measured with RT-qPCR in the mutants mentioned above. Instead of a single regulation point for these genes there appeared to be several places for regulation of ethylene and jasmonate.

The expression analysis of *amr1-1* mutant suggested that ethylene is important in regulating *AMR1* at the transcriptional level. Jasmonate induced and ethylene and *AMR1* reduced the transcription of genes coding several enzymes in ascorbate biosynthesis pathway. This indicates that ethylene inhibited at least two steps in the pathway – *VTC1* and *VTC2* expressions. These genes were both inhibited by constitutive ethylene signaling and induced, when ethylene signal was absent. Jasmonate might induce the expression of *VTC1* and *VTC5*.

The expression data suggests that the most important regulation point was the transcription of *VTC1* – both hormones and the inhibitor *AMR1* significantly altered its expression. The *AMR1* and ethylene coregulation in the regulation of ascorbate content needs further investigation. The effects of these plant hormones on the enzyme activities of the ascorbate synthesis D-mannose/ L-galactose pathway remains to be unravelled in the future.

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Etüleeni, jasmonaad ja transkriptsiooni regulaatori AMR1 mõju askorbaadi sünteesile ja tasemele harilikus müüriloogas (Arabidopsis thaliana)

Agnes Alev

Resümee

Askorbaati ehk C vitamiini ei suuda inimorganism ise sünteesida ning peab seda saama toidust. Peamisteks C vitamiini allikateks on taimed. Nii nagu loomades on askorbaat ka taimedes peamine antioksüdant. Antioksüdandid teevad organismis kahjutuks oksüdeerivaid kahjulikke ühendeid (hapnikuradikaale). Askorbaat on lisaks ka ensüümidele kofaktoriks ning oluline rakkude jagunemises ning kasvus. Taimedes sünteesitakse seda C_6 suhkrut peamiselt D-mannoosi/ L-galaktoosi biosünteesi rajas. Askorbaadi tase rakus sõltub sünteesi, regeneratsiooni ning degradatsiooni kiirusest. Askorbaati on rohkem noortes taimedes ning taimede generatiivsetes organites. (Gallie 2013).

Askorbaadi kontsentratsiooni on üritatud erinevates toidutaimedes suurendada, kuid seni, kuni selle antioksüdandi sünteesiraja kontrollmehanisme ja mõjutavaid tegureid ei teatud, on olnud pingutused tulutud (Ishikawa jt. 2006). Ainuke teadaolev transkriptsiooni inhibiitor AMR1 spetsiifiliselt takistab D-mannoosi/ L-galaktoosi raja ensüüme kodeerivate geenide transkriptsiooni (Zhang jt. 2009). Lisaks on tõestatud, et jasmonaat suurendab (Suza jt. 2010) ja etüleen vähendab askorbaadi kontsentratsiooni (Gergoff jt. 2009). Nii etüleen kui ka jasmonaat on taimsed hormoonid, millel on palju erinevaid funktsioone (Gergoff jt. 2009; Suza jt. 2010).

Antud magistritöö eesmärgiks oli uurida jasmonaadi, etüleeni ning transkriptsiooni regulaatori AMR1 mõju askorbaadi biosünteesile D-mannoosi/ L- galaktoosi rajas kasutades mudelorganismi *Arabidopsis thaliana*. Esiteks määrati spektrofotomeetriliselt askorbaadi kontsentratsioonid Col-0 metsiktüübis ning järgnevates mutantides: *amr1-1, ein2-1, coi1-16, coi1-16xein2-1, ctr1-1*. *Amr1-1* mutandis ei sünteesita askorbaadi sünteesi inhibiitorit ning *vtc1-1* mutandis on üks askorbaadi biosünteesis osalev ensüüm mittefunktsionaalne. *Ein2-1* mutandis on etüleeni signaali ülekanne takistatud ning vastupidi *ctr1-1* mutandis on etüleeni signaalirada alaliselt aktiivne (metsiktüübis indutseerib signaaliülekande vaid etüleeni olemasolu). *Coi1-16* mutandis seevastu oli defektne jasmonaadi signaalirada. Meie oma laboris konstrueeritud *coi1-16xein2-1* topeltmutandil on mõlema ühe geeni mutandi fenotüüp

ning see aitas selgitada jasmonaadi ja etüleeni koosmõju askorbaadi tekkele. Lisaks määrati kvantitatiivse PCR-iga ära geeniekspressiooni tasemed nendes mutantides, selleks, et saada teada, kuidas jasmonaat, etüleen ja AMR1 mõjutavad D-mannoosi/ L-galaktoosi raja ensüüme kodeerivate geenide transkriptsiooni.

Askorbaadi kontsentratsiooni mõõtmiste tulemused olid vastavuses varasemalt avaldatud teaduskirjanduses saadud tulemustega. Konstrueeritud *coi1-16xein2-1* mutandis oli askorbaati sama palju kui *coi1-16* mutandis ning oluliselt vähem, kui *ein2-1* mutandis. Sellest võis järeldada, et askorbaadi sünteesil domineerib COI1 (jasmonaadi signaaliülekande aktivaator) mõju ning maskeerib EIN2 efekti askorbaadi sünteesile (etüleeni signaaliülekande aktivaator). Seega askobaadi biosünteesirajas reguleerib jasmonaat hilisemaid etappe kui etüleen.

Selleks, et välja selgitad jasmonaadi, etüleeni ning AMR1 mõju askorbaadi ja glutatiooni biosünteesis olulisi ensüüme kodeerivate geenide transkriptsioonile, mõõdeti kvantitatiivse PCR-i abil nende geenide ekspressioonitaset eelpool kirjeldatud mutantides. Leiti, et geeniregulatsioon on kompleksne – nii etüleen, jasmonaat kui ka AMR1 mõjutavad mitmete ensüüme kodeerivate geenide avaldumist.

Amr1-1 mutandi ekspressioonianalüüsil leiti, et etüleeni signaal indutseerib tugevasti askorbaadi biosünteesi inhibiitorit kodeeriva geeni *AMR1* transkriptsiooni. Jasmonaat suurendab ja etüleen ning AMR1 vähendavad askorbaadi biosünteesil osalevate ensüüme kodeerivate geenide transkriptsiooni. Etüleen inhibeerib vähemalt kahe ensüümi kodeeriva geeni ekspressiooni – *VTC1* ja *VTC2*. Jasmonaat aga aktiveerib *VTC1* ja *VTC5* transkriptsiooni.

Tulemuste põhjal püstitati hüpotees, et askorbaadi biosünteesi mõjutab transkriptsiooni tasemel enim *VTC1* regulatsioon. Nii etüleen, jasmonaat kui ka AMR1 mõjutasid oluliselt selle geeni ekspressiooni. Etüleeni ja jasmonaadi mõju askorbaadi biosünteesis D-mannoosi/ L-galaktoosi rajas osalevate ensüümide aktiivsusele selgitavad välja edasised uuringud.

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APPENDIX 1. The qPCR expression data of target genes in selected mutants (samples). The fold change is given in arbitary units. SD means standard deviation. There was done five independent experiments. The statistical significance was calculated with t-test (marked significant with when t ≤ 0,05 (95 % probability).

	APX1						
Samples	repeat 2	repeat 3	repeat 4	repeat 5	average	SD	t-test
Col	1,32	1,20	1,53	0,82	1,22	0,30	
amr1	1,29	0,94	0,90	0,87	1,00	0,20	0,270
coi1-16	0,93	1,05	0,96	0,89	0,96	0,07	0,143
coi1ein2	0,95	1,07	0,86	1,37	1,06	0,22	0,441
ctr1	0,88	0,83	0,73	0,83	0,82	0,06	0,040
ein2	0,74	0,90	0,89	0,82	0,84	0,07	0,049
vtc1	1,02	1,14	1,25	1,23	1,16	0,11	0,740

	GLDH							
Samples	repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,05	0,92	1,09	0,91	0,89	0,97	0,09	
amr1	0,89	1,09	1,22	1,09	0,81	1,02	0,16	0,599
coi1-16	1,27	1,40	1,00	1,22	1,15	1,21	0,15	0,015
coi1ein2	1,18	1,26	1,09	1,09	1,07	1,14	0,08	0,015
ctr1	0,85	0,90	0,85	0,88	1,04	0,91	0,08	0,244
ein2	0,92	1,23	1,04	0,88	1,09	1,03	0,14	0,450
vtc1	0,91	1,13	0,98	0,98	1,19	1,04	0,12	0,370

	GME							
Samples	repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	0,95	0,77	0,82	1,13	0,99	0,93	0,14	
amr1	0,82	0,91	1,01	0,97	1,06	0,99	0,09	0,794
coi1-16	1,33	1,15	0,84	1,05	0,99	1,01	0,18	0,209
coi1ein2	1,08	0,95	0,98	1,04	0,94	0,98	0,06	0,358
ctr1	0,92	1,05	1,11	0,92	0,85	0,98	0,11	0,637
ein2	1,02	1,17	1,20	1,19	1,11	1,17	0,07	0,020
vtc1	0,96	1,24	1,10	1,02	1,08	1,08	0,10	0,097

	GPP							
Samples	repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,28	1,07	1,10	1,03	1,08	1,11	0,10	
amr1	1,25	1,17	1,36	1,14	1,12	1,21	0,10	0,151
coi1-16	1,03	1,44	1,18	1,14	1,35	1,23	0,16	0,219
coi1ein2	1,07	1,09	0,83	1,02	1,00	1,00	0,10	0,120
ctr1	0,63	0,91	0,74	0,81	0,72	0,76	0,11	0,001
ein2	1,10	1,16	0,91	0,96	1,06	1,04	0,10	0,436
vtc1	1,28	1,27	1,16	1,10	1,08	1,18	0,09	0,115

Samples	GSH1 repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,17	1,07	1,11	1,08	0,94	1,07	0,08	
amr1	0,97	0,98	1,18	1,09	0,84	1,01	0,13	0,790
coi1-16	1,15	1,18	1,07	1,00	0,97	1,07	0,09	0,903
coi1ein2	1,06	1,09	0,87	1,01	1,03	1,01	0,09	0,438
ctr1	0,82	0,84	0,76	0,77	0,91	0,82	0,06	0,004
ein2	0,92	1,23	1,09	0,96	1,09	1,06	0,12	0,501
vtc1	0,95	1,09	1,00	1,15	1,27	1,09	0,13	0,290

Samples	GSH2 repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,32	1,46	1,18	1,48	1,07	1,30	0,18	
amr1	0,90	1,00	1,08	1,08	0,92	1,00	0,09	0,008
coi1-16	1,02	1,13	1,05	0,98	1,05	1,05	0,05	0,015
coi1ein2	0,91	1,04	0,87	0,97	1,06	0,97	0,08	0,005
ctr1	0,90	1,00	0,86	0,63	0,87	0,85	0,13	0,002
ein2	0,89	1,40	1,04	0,96	1,07	1,07	0,20	0,087
vtc1	0,99	1,30	0,97	1,08	1,12	1,09	0,13	0,066

Samples	GalDH repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,01	0,90	1,04	1,10	1,00	1,01	0,07	
amr1	1,01	0,87	1,10	0,99	0,98	0,99	0,08	0,655
coi1-16	1,11	1,10	1,05	1,13	0,95	1,07	0,07	0,240
coi1ein2	1,05	1,09	0,92	1,11	1,01	1,03	0,08	0,616
ctr1	0,90	0,92	0,84	0,81	0,82	0,86	0,05	0,004
ein2	1,14	1,15	1,16	1,09	1,08	1,12	0,04	0,012
vtc1	0,94	1,00	1,03	1,02	1,21	1,04	0,10	0,615

Samples	PMI repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,27	0,99	1,19	0,95	1,20	1,12	0,14	
amr1	1,31	1,12	1,10	1,01	0,97	1,10	0,13	0,843
coi1-16	0,75	1,07	0,99	0,83	1,00	0,93	0,13	0,062
coi1ein2	0,87	1,07	0,72	1,01	1,21	0,98	0,19	0,213
ctr1	1,04	0,94	0,96	0,90	0,97	0,96	0,05	0,053
ein2	0,94	1,18	1,12	1,04	0,98	1,05	0,10	0,419
vtc1	0,94	1,02	0,98	1,10	0,95	0,99	0,07	0,117

Samples	PMM repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,41	1,02	1,27	1,44	1,42	1,31	0,17	
amr1	0,86	0,98	1,04	0,86	1,11	0,97	0,11	0,006
coi1-16	1,05	1,11	0,98	0,86	1,45	1,09	0,22	0,114
coi1ein2	1,14	1,10	1,16	1,16	1,09	1,13	0,03	0,051
ctr1	0,80	0,74	0,75	0,78	0,82	0,78	0,03	0,000
ein2	0,94	1,14	1,03	1,01	1,11	1,05	0,08	0,015
vtc1	1,04	1,08	1,06	1,03	1,07	1,06	0,02	0,012

Samples	VTC1 repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,42	1,25	1,43	1,45	1,48	1,41	0,09	
amr1	1,94	2,04	1,93	1,82	1,52	1,85	0,20	0,002
coi1-16	0,94	1,00	0,75	1,08	0,78	0,91	0,14	0,000
coi1ein2	1,46	1,83	1,42	1,85	1,95	1,70	0,24	0,036
ctr1	0,73	0,53	0,40	0,79	0,62	0,62	0,15	0,000
ein2	1,41	2,03	1,83	1,71	2,64	1,92	0,46	0,039
vtc1	0,11	0,39	0,37	0,42	0,45	0,35	0,14	0,000

Samples	VTC2 repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	1,13	1,05	0,91	0,97	0,85	0,98	0,11	
amr1	1,60	1,83	1,34	1,71	1,32	1,56	0,22	0,001
coi1-16	0,99	1,25	1,15	1,04	0,86	1,06	0,15	0,387
coi1ein2	1,22	0,99	0,73	0,87	1,09	0,98	0,19	0,997
ctr1	0,41	0,54	0,82	0,45	0,50	0,54	0,16	0,001
ein2	1,35	1,16	1,00	1,20	1,10	1,16	0,13	0,045
vtc1	1,08	1,56	1,21	1,26	1,34	1,29	0,18	0,011

Samples	VTC5 repeat 1	repeat 2	repeat3	repeat 4	repeat 5	average	SD	t-test
Col	0,88	1,07	0,94	1,29	1,13	1,06	0,16	
amr1	1,41	1,08	1,49	1,23	1,11	1,27	0,18	0,096
coi1-16	0,67	0,87	0,65	0,76	0,30	0,65	0,22	0,010
coi1ein2	0,79	0,85	0,84	0,87	0,86	0,84	0,03	0,017
ctr1	1,30	1,39	1,19	1,14	1,20	1,24	0,10	0,067
ein2	0,94	1,05	1,16	0,99	1,28	1,08	0,14	0,820
vtc1	0,95	1,18	1,22	1,12	0,99	1,09	0,12	0,741

Samples	AMR1		
Col	1,72	ctr1	3,32
coi1-16	1,03	ein2	0,26
coi1ein2	0,97	vtc1	0,67

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