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# APPLICATION OF NMR METABOLOMICS PROFILING ON QUESTIONS ARISING FROM MOLECULAR BIOLOGY

Tahereh Jafari

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# ABSTRACT

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In recent years, there has been an increasing interest in utilizing nuclear magnetic resonance (NMR) spectroscopy metabolomics to understand different biochemical mechanisms of living organisms. Metabolomic profiling can provide a comprehensive snapshot from multiple biological mechanisms in the studied subject as NMR allows simultaneous detection of a wide range of metabolites. The main aim of this thesis was to analyze, qualify and quantify the metabolites in a model plant, *Arabidopsis thaliana* (*Arabidopsis*), and two different mushroom families *Hypholoma* and *Kuehneromyces* under different circumstances. Metabolomic investigations of *Arabidopsis* included metabolic studies of distinct genotypes of *Arabidopsis* in different developmental stages and determination of the impact of light intensity on metabolomic adjustments in *Arabidopsis*. The studies revealed the essential role of these conditions on elevated levels of several metabolites. However, no metabolomic changes between different genotypes of *Arabidopsis* could be seen with NMR. The study regarding mushrooms demonstrated influences of the drying method on alteration of metabolites in fresh and herbarium mushroom families *Hypholoma* and *Kuehneromyces*. It was discovered that air-dried herbarium samples from different ages (up to 25 years) are stable and can be utilized for different purposes in the future. $^{1}$H NMR (proton NMR) was applied to distinguish and characterize metabolic profiles and key components of the study subjects under diverse conditions. In order to analyze the data, different multivariate methods were performed.

The new understanding arising from NMR metabolomic screening of *Arabidopsis* plants and mushrooms addressed in this thesis can be applied in future studies in plant genetics, investigation of different biochemical mechanisms in plant stress resistance and development as well as in further NMR based investigations of the metabolites in various taxa of mushrooms.

# TIIVISTELMÄ

TURUN YLIOPISTO
Kemian laitos/ Luonnontieteiden ja tekniikan tiedekunta

TAHEREH JAFARI: Ydinmagneettiseen resonanssispektroskopiaan perustuvan metabolomisen profiloinnin hyödyntäminen molekyylibiologisissa kysymyksissä.

Väitöskirja, 133 s.
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Ydinmagneettiseen resonanssispektroskopiaan (NMR) perustuvaa metabolomiikkaa on viime vuosina käytetty lisääntyvissä määrin erilaisissa eliöissä esiintyvien biokemiallisten mekanismien tutkimiseen. Metabolominen profilointi mahdollistaa eräänlaisen tilannekuvan muodostamisen tutkimuskohteessa tapahtuvista reaktioista, sillä NMR:n avulla voidaan näytteestä havaita yhtäaikaisesti suuri määrä eri metaboliitteja. Tämän väitöskirjan tavoitteena oli analysoida ja kvantifioida erään mallikasvina käytetyn lajin, lituruohon (*Arabidopsis thaliana*), sekä kahteen sukuun (*Hypholoma* ja *Kuehneromyces)* kuuluvien neljän eri sienilajin erilaisissa olosuhteissa muodostamat metaboliitit. Lituruohoon liittyen käytettiin tutkimuksissa lituruohon eri genotyyppejä kasvin kasvun eri vaiheissa, sekä määritettiin valon intensiteetin vaikutusta lituruohon tuottamiin metoboliitteihin. Tutkimuksissa havaittiin useilla tekijöillä olevan vaikutusta lukuisten eri metaboliittien määriin kasvissa. Merkittävää eroa metaboliiteissa lituruohon eri genotyyppien välillä ei kuitenkaan havaittu NMR:n avulla. Sieniin kohdistuvassa tutkimuksessa käsiteltiin sienien kuivausmenetelmän vaikutusta niissä esiintyviin metaboliitteihin. Tutkimus toteutettiin käyttäen sekä tuoreeltaan kuivattuja sieniä sekä museosieninäytteitä. Tutkimuksessa havaittiin ilmakuivattujen (säilytysaika maksimissaan 25 vuotta) museosieninäytteiden pysyvän stabiilina säilytyksen ajan. Tämä mahdollistaakin museosieninäytteiden käytön esimerkiksi tutkimuksessa.

Metaboliitit määritettiin eri olosuhteista peräisin olevista sieni- ja kasvinäytteistä mitatuista $^{1}$H NMR (protoni NMR) spektreistä. Saatujen tulosten analysointiin käytettiin monimuuttujamenetelmiä.

Tämän väitöskirjan lituruohon ja sienien NMR metabolomiikkaan liittyvistä osatöistä saatuja tuloksia voidaan hyödyntää tulevaisuudessa esimerkiksi kasvien genetiikkaan liittyvässä tutkimuksessa, kasvien stressinsietoon liittyvien biokemiallisten mekanismien selvittämisessä sekä eri sienilajien metaboliitteihin liittyvässä tutkimuksessa.

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# ACKNOWLEDGEMENTS

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# LIST OF ORIGINAL PUBLICATIONS

**I** Jafari, T.; Durian G.; Rahikainen, M.; Kortesniemi, M.; Kangasjärvi, S.; Sinkkonen, J. NMR study of age dependent metabolic adjustments in wild type and *pp2a-b'γ* mutant *Arabidopsis thaliana. Phytochemistry Letters.* 2017, 22, 13–20.

**II** Jafari, T.; Rahikainen, M.; Puljula, E.; Sinkkonen, J.; Kangasjärvi, S. The impact of light intensity on metabolomic profile of *Arabidopsis thaliana* wild type and *reticulata* mutant by NMR spectroscopy. Under review in the *Phytochemistry Letters*. 2018.

**III** Rahikainen, M.; Jafari, T.; Durian, G.; Winter, Z.; Alegre, S.; Pascual, J.; Sinkkonen, J.; Kangasjärvi, S. Phosphorylation of serine 91 impacts the abundance of *Arabidopsis* ACONITASE 3. Submitted into the *Physiologia Plantarum.* 2018.

**IV** Jafari, T.; Alanne, A. L.; Issakainen, J.; Pihlaja, K.; Sinkkonen, J. Suitability of dried herbarium specimens for NMR metabolomics of mushrooms. A comparison of four species of the Genera *Kuehneromyces* and *Hypholoma* (Strophariaceae). *Fungal Biology.* 2017, 122, 138–146.

# ABBREVIATIONS

| | |
|---|---|
| Acetone-$d_6$ | Deuterated acetone |
| ACO | Aconitase |
| $^{13}C$ | Carbon-13 |
| $CDCl_3$ | Deuterated chloroform |
| COSY | Correlation spectroscopy |
| 1D | One-dimensional |
| 2D | Two-dimensional |
| DMSO-$d_6$ | Deuterated dimethyl sulfoxide |
| $D_2O$ | Deuterated water |
| FID | Free Induction Decay |
| GABA | γ-aminobutyric acid |
| GSL | Glucosinolates |
| $^1H$ | Proton |
| HMBC | Heteronuclear multiple bond correlation |
| $^1H$ NMR | Proton nuclear magnetic resonance |
| $H_2O_2$ | Hydrogen peroxide |
| HSQC | Heteronuclear single quantum correlation |
| Hz | Hertz |
| MeOD-$d_4$ | Deuterated methanol |
| MS | Mass spectrometry |
| MW | Molecular weight |
| N | Nitrogen |
| NMR | Nuclear magnetic resonance |
| NOESY | Nuclear Overhauser effect spectroscopy |
| $\cdot O_2^-$ | Superoxide |
| OH | Hydroxyl |
| $p$ | Loadings matrix |
| P | Phosphorus |

| | |
|---|---|
| PCA | Principal component analysis |
| PCs | Principal components |
| PLS | Partial least squares / projection to latent structures |
| ppm | Parts per million |
| PSI | Photosystem I |
| PSII | Photosystem II |
| $Q^2$ | An estimate of predictive ability |
| $Q^2_{(cum)}$ | Cumulative $Q^2$ |
| $R^2$ | An estimate of goodness of fit |
| *re* | *Reticulata* |
| RF | Radio frequency |
| ROS | Reactive oxygen species |
| $R^2X_{(cum)}$ | Cumulative $R^2X$ |
| $R^2X$ | Fraction of $X$ variation |
| $R^2Y$ | Fraction of $Y$ variation |
| $R^2Y_{(cum)}$ | Cumulative $R^2Y$ |
| SIMCA | Soft independent modelling of class analogies |
| $t$ | Score matrix |
| TCAA | Total content of amino acids |
| TCA | Tricarboxylic acid |
| TOCSY | Total correlation spectroscopy |
| TSP | 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid |
| WATERGATE | Water suppression via gradient tailored excitation |

# 1. INTRODUCTION

The concept of metabolomics is defined as investigation of small molecules (so-called metabolites), which have a low molecular weight (MW < 1000 g $mol^{-1}$) and are produced naturally in the living organism (Harrigan and Goodacre, 2012; Lindon et al., 2011; Wang et al., 2007). Study of metabolites under different conditions contributes to better understanding of different biological aspects (Griffiths et al., 2010), such as biochemical mechanisms, diseases, and genomic features. Hence, metabolomics is a well-established and powerful tool for investigations in different fields of science, including medicine and drug discovery (Harrigan and Goodacre, 2012), food science and food chemistry (Aherne and O'Brien, 2002) and plant science (Bourgaud et al., 2001).

In plants, the exposure to various stress conditions results in modulations in metabolite contents and altered metabolic interactions that can enhance the plant defense systems. The stress may occur because of alterations in environmental parameters, such as light, temperature and water (abiotic stress; Mittler, 2006) or because of an attack by other living organisms, (biotic stress; Bolwell et al., 2002) like viruses, bacteria and fungi, which can cause damage to the targeted biological system. Plants, however, have evolved to control the stress agents through numerous response mechanisms. For instance, expression of specific genes which elicit stress resistance, enzyme activities that cause production and synthesis of metabolites (Apse and Blumwald, 2002; Park et al., 2004; Rontein et al., 2002) and transduction of signals by molecular networks which promote plant resistance (Vinocur and Altman, 2005).

Utilizing model plants can facilitate the investigation in order to study the influences of abiotic stress. In recent years, most of the studies focusing on stress-induced alterations in metabolites, as well as the genes underlying the metabolic differences, have used *Arabidopsis thaliana* (*Arabidopsis*) as a model plant (Bae et al., 2005; Corrales et al., 2017; Shi et al., 2013; Urano et al., 2003). *Arabidopsis* as an experimental tool allows genetic modification of the metabolic pathways of interest, which leads to substantial discoveries in the understanding of signaling and metabolism of plant defense.

Metabolomic studies are usually utilizing either nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) as an analytical tool, continued by proper statistical analysis, which commonly includes multivariate methods (Bundy et al., 2009; Krishnan et al., 2004). NMR is a comprehensive tool for identification of the metabolites as well as for quantification (Krishnan et al., 2004). Also, it is a reliable technique to explore a large number of primary and secondary metabolites in the targeted subject (Sekiyama et al., 2011).

NMR combined with the multivariate method can assess and quantify a wide range of chemical compounds in *Arabidopsis* and mushrooms under various conditions. Thus, NMR technique was applied for measuring and identification of the metabolites.

The main advantages of NMR include simple sample preparation, good selectivity and the possibility to identify the structure of molecules. However, the main limitation is related to the relatively low sensitivity of NMR, making it difficult or impossible to detect low abundant chemical compounds in the sample (Elipe, 2003).

This thesis and the literature review mainly concentrate on metabolomic adjustments of the model plant *Arabidopsis* under different physiological conditions, including senescence (I) and light stress (II). These conditions significantly impact on metabolomic alterations in *Arabidopsis* in order to increase plant fitness. As such, functionality of different genes, including *PP2A-B'γ* (I), *RETICULATA* (II) and *ACONITASE 3* (III) on modulation of metabolites are investigated by using different *Arabidopsis* mutants to understand their actions.

In addition, wild fresh mushrooms from *Hypholoma* and *Kuehneromyces* families (which are collected from the forest in Finland) of Strophariaceae family and their corresponding herbarium mushroom specimens, are studied. The effects of drying method on mushrooms, metabolomics alteration between fresh mushrooms and corresponding herbarium mushroom specimens, and impact of ageing (up to 25 years) on regulation of metabolites in herbarium mushroom specimens were aimed to be studied. Wild edible mushrooms are popularly consumed particularly in Europe (Kalač, 2009). Mushrooms contain high nutritional value and they are a good source of proteins and amino acids (Agrahar-Murugkar and Subbulakshmi, 2005). However, due to the seasonal limitation, collecting of the mushrooms is restricted. Hence, researchers have shown recently an increased interest in different drying methods to increase the shelf life of mushrooms (Gurtas Seyhan and Evranuz, 2000; Lee and Lee, 2008; Pei et al., 2014; Politowicz et al., 2017).

In the current mushroom study (IV) many of the identified metabolites are significant compounds for digestibility, flavor and human metabolism. Likewise, ageing is not a significant parameter to modulate the metabolites in the corresponding herbarium mushrooms. Therefore, they can be used for different purposes like biological studies.

The thesis and the corresponding literature review intent to review the impacts of mutation, abiotic stress, senescence and drying method on metabolomics regulation of the studied subjects (different *Arabidopsis* genotypes and different mushroom species) by applying NMR. In addition, the basic concept of metabolomics profiling, NMR application and multivariate method are reviewed.

# 2. LITERATURE REVIEW

## 2.1. Metabolomics

Metabolomic studies provide an important overview of the biological and physiological activities in living organisms by contributing a snapshot of the metabolites in cells of the living systems (Hong et al., 2016; Worley and Powers, 2013). Metabolomics is investigation of metabolites, including both, studies on a selected set of compounds (targeted analysis), as well as, overall fingerprinting of the metabolite alterations on a studied subject (untargeted analysis). The set of metabolites synthesized by a biological system is called metabolome (Fiehn, 2002; Oliver et al., 1998). The main aim of metabolite fingerprinting is to detect the differences between the metabolites of two or more types of biological objects to comprehend their biological relations (Worley and Powers, 2013). Thus, profiling of the metabolites can give a comprehensive perspective from the organism (Kim et al., 2011). In addition, it is a convenient method to obtain a useful overview of the metabolites in a studied subject under various biological conditions, like biotic and abiotic interactions in plants (Schauer and Fernie, 2006).

Metabolites consist of small molecules with the molecular weight of approximately 1000 g $mol^{-1}$ and they also have a large diversity from different aspects, such as size, quantity and polarity (Kim et al., 2011). Accumulation of the metabolites can be altered in the targeted subject due to various factors, for instance, environmental stress and genetic background. Metabolites are generally divided into two groups: primary (such as amino acids), which are substantial for the cell activity, and secondary (e.g. flavonoids), that are needed for the viability of the organism (Tenenboim and Brotman, 2016). In addition, metabolomics is utilized in different fields of study, for example in agriculture (Fernie and Schauer, 2009), plant biology (Schauer and Fernie, 2006; Sumner et al., 2003), food chemistry (Cevallos-Cevallos et al., 2009) and medicine (Suhre et al., 2010).

## 2.2. Abiotic stress in plants

Plant growth and production under the natural environmental circumstances usually do not take place in ideal conditions. Hence, plants face different kinds of stress parameters which restrict their growth and development. In order to increase resistance to stress, plants have evolved various adaptive mechanisms (Chinnusamy et al., 2014), for example, biochemical mechanisms, ion transport and detoxification (Wang et al., 2003).

Generally, stress refers to the conditions which have adverse influences on the development of a plant. Abiotic stress refers to the stress caused by extreme climatic conditions, such as, drought, heat, light and soil nutrition. In contrast, biotic stress occurs when the plants are under attack by herbivores or pathogens (Mithöfer et al., 2004) (generally,

another organism causes the damages). Abiotic and biotic stresses cause significant losses in agricultural production worldwide.

Environmental stress (Chalker-Scott, 1999) and biotic stress include damages from the living organism e.g. wounding, bacteria (Sekiyama et al., 2011), viruses (Choi et al., 2006) and herbivory (Leiss et al., 2009) have important effects on metabolic changes in plant.

Plant acclimation to a specific abiotic stress necessitates particular physiological and biochemical responses (Mittler, 2006). In addition, abiotic stress may lead to oxidative stress that involves production of reactive oxygen species (ROS), which are highly reactive and hold the potential to cause damage to different parts of the plant. ROS may be produced in different forms, such as free radicals like superoxide ($\cdot O_2^-$) and hydroxyl ($\cdot OH$) radicals, or non-radical, such as hydrogen peroxide ($H_2O_2$) and singlet oxygen. The main places to produce singlet oxygen and superoxide are photosystem II (PSII) and photosystem I (PSI) in the chloroplast. In general terms, accumulation of ROS may cause oxidative stress, where the toxic products are transmitted into the cells where they damage the functionality of nucleic acids, proteins and lipids. However, plants are well equipped to tolerate oxidative stress by the aid of antioxidants, which can protect against the damages (Gill and Tuteja, 2010). Currently, ROS are increasingly considered to act as signaling molecules that essentially modulate gene expression in plants, leading to alterations in different physiological responses ranging from the activation of programmed cell death to regulation of plant growth.

### 2.2.1. Light as a modulator of plant performance

Plant production and metabolic activity are tightly engaged with the effects of light intensity, photoperiod and temperature (Jaakola and Hohtola, 2010). The response of the plant to the light differs by the gradient of the light (Poorter, 1999). Darkness or dim light intensities promote etiolated plant development, the symptoms of which include reduced size of leaves, extreme elongation of stems and lack of chlorophyll (Wassink and Stolwijk, 1956), which usually causes a pale color in the plant.

Light and photoperiod (day length) have a direct effect on plant growth, flowering and seed production. Photoperiod is categorized as short photoperiods (short days) and long photoperiods (long days) (Wassink and Stolwijk, 1956). Hence, manipulation of the light conditions significantly impacts on delay or acceleration of the maturation phase and vegetative phase in the plant. This property (plant responsiveness to light) is sometimes applied to increase yield, for example in agronomy. Another aspect of light intensity is its influence on photosynthesis. Plant biomass directly relies on photosynthesis (Kangasjärvi et al., 2012).

Photosynthesis is based on the absorbance of photon energy, which is converted to biochemical energy in the photosynthetic thylakoids of chloroplasts (Miyake et al., 2005). Chloroplasts also have an essential role on the synthesis of primary and secondary

metabolites, such as hormones and vitamins. These metabolites have a significant task in inducing plant resistance against the stress parameters (Kangasjärvi et al., 2012). Changing the level of illumination in plants may alter the accumulation of metabolites (Jänkänpää et al., 2012), for example, Urbanczyk-Wochniak and Fernie (2004) explained that tomato that was grown under low light and high light intensities showed significant adjustments in concentration of amino acids, carbohydrates and organic acids.

Consequently, light influences on defensive responses of plants through multiple paths, the three most significant responses of which include: 1) effects on general energy and redox status of key intermediates, such as ATP, NADPH, and carbon skeletons 2) impacts on ROS accumulation 3) perception of signals via photoreceptors such as phytochromes (Kangasjärvi et al., 2012). By these means, plants attempt to adjust their metabolism, development and growth according to the prevailing environmental cues.

### 2.2.2. Senescence

Plant senescence (ageing) is defined as a regular degeneration and internal adjustment which causes death of cells and organs during the growth cycle (Noodén and Penney, 2001). In monocarpic plants reproduction occurs once and by the end of this process, they die (Noodén and Leopold, 1978), e.g. soybean reduces the rate of growth and development when the flowering is started and the leaves begin to turn yellow, reflecting the initial stage of senescence (Noodén, 1984). Monocarpic senescence happens when the plant displays fast and clear degeneration, thus, they are annual (biennial), whereas polycarpic plants have more than one reproductive cycle and they go slowly through a declining phase, and are hence categorized as perennials (Noodén et al., 2004). During the senescence in *Arabidopsis*, leaf development is stopped and metabolites are altered. Ageing induces the mobilization and recycling of metabolites in the plant, hence, the concentrations of metabolites are changed (Diaz et al., 2008).

Mobilizations and recycling of nutrients from the senescent leaves to the younger leaves or reproductive organs, such as seeds, help to delay the ageing process in plant (Masclaux-Daubresse et al., 2008). Additionally, different aspects of ageing include reduction of chlorophyll contents (Lohman et al., 1994), decreasing total amount of RNA and transforming to the nitrogen compounds with the low molecular weight, protein degradation into amino acids and metabolizing lipids to sugars. However, the content of DNA mainly remains constant and the fragmentation of DNA becomes evident during the final stages of ageing (Zimmermann and Zentgraf, 2005). Senescence occurs in leaves, flowers and fruits. Leaf senescence in plant causes changes in the structure of cells, metabolites and expression of genes (Nam, 1997). Senescence may become accelerated by external signals like cold, heat, deprivation of nutrients (especially nitrogen) and wounding (Thomas and Stoddart, 1980) or by internal parameters, like age and developing steps of

reproductive organs (Gan and Amasino, 1997; Noodén, 2012). Different stages of senescence in plant is shown in Figure 1.

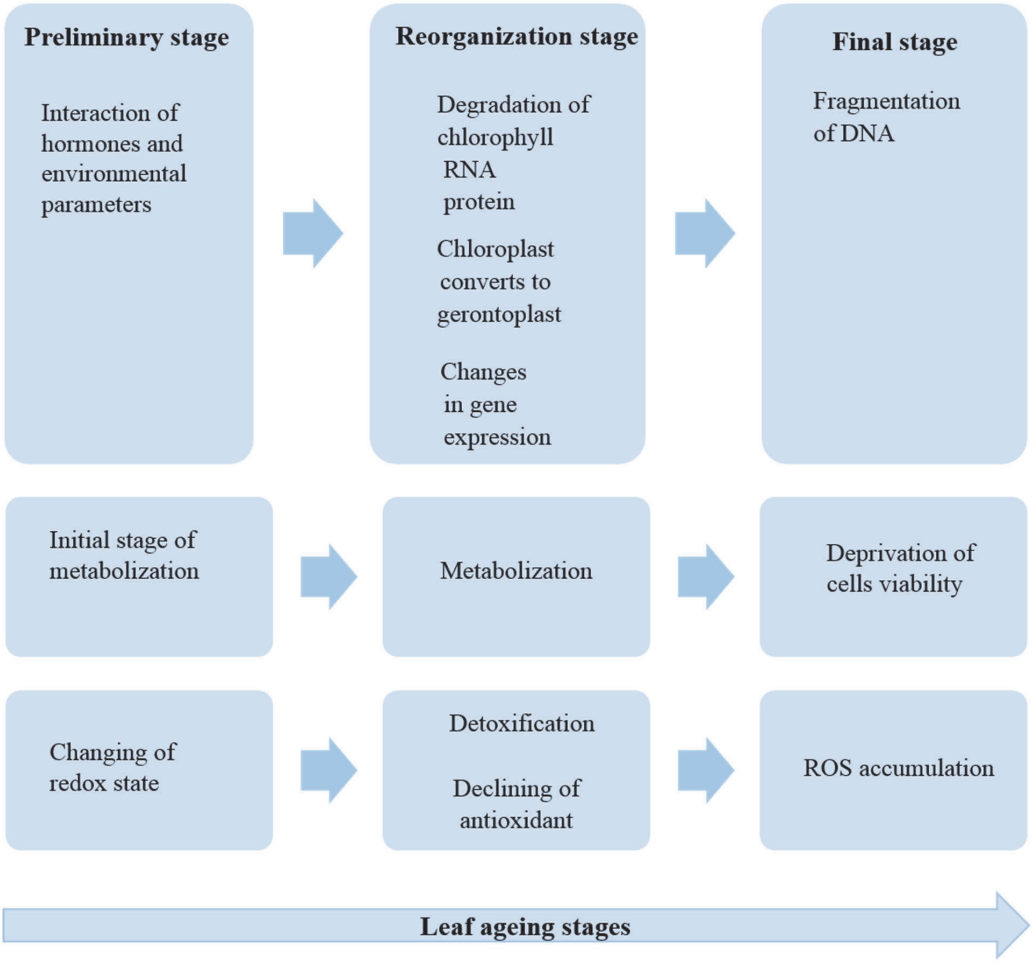



**Figure 1.** Classification of different stages of leaf ageing and their specific aspects (The Figure modified from Zimmermann and Zentgraf, 2005).

## 2.3. *Arabidopsis thaliana*

*Arabidopsis thaliana* (*Arabidopsis*) is a small, approximately 30-40 cm tall, flowering plant that belongs to the cruciferous Brassicaceae family (Meyerowitz and Pruitt, 1985). The mature plant contains small seeds (20 µg) which can be stored for a long time (years) when they are dried whithout loosing their germination functionality. The developmental rate of the plant may vary, depending on different parameters such as temperature, day length, soil nutrition and genetic background. Common ecotypes of *Arabidopsis* include Colombia

(Figure 2) and Landsberg. *Arabidopsis* has a regeneration time (from germination of seeds to maturation phase) of approximately 6 weeks (Meinke et al., 1998).

*Arabidopsis* is a model plant and it has been utilized as a biological tool to understand plant growth and development in the field of plant sciences; by focusing on genetic patways (Meinke et al., 1998; Meyerowitz and Pruitt, 1985) and ultimately biosynthetic pathways. For example, *Arabidopsis* was utilized as an experimental tool in post genomics study by Hirai et al. (2004), since the genomic squence of *Arabidopsis* was completed.

**Figure 2.** *Arabidopsis thaliana* ecotype Colombia wild type (six weeks old).

The characteristic features of the plant, such as small size, the potential to grow even in a small space, short duration of growing from seedlings to maturation phase (which is usually between 4 - 6 weeks) and high set of seed, make *Arabidopsis* a convenient tool to study various aspects, for example, genetics (Meyerowitz and Pruitt, 1985) and metabolomics (Wu et al., 2017) .

In the field of genomics, gene knockout is an important tool utilized to understand gene function (Bouché and Bouchez, 2001). There are various populations of *Arabidopsis,* which have been mutagenized to investigate phenotypic (Enders et al., 2015), morphologic (Michalko et al., 2016) and metabolomic (Böttcher et al., 2008) changes. Consequently, *Arabidopsis* is commonly utilized as a tool in molecular genetics and other related research fields (Goldberg, 1988).

### 2.3.1. *Arabidopsis PP2A-B'γ*

Protein phosphatase 2A (PP2A) belongs to the serine / threonine protein phosphatases. It has an important role in the regulation of plant growth and development (Konert, 2014; Trotta et al., 2011). PP2A is trimeric and contains a catalytic subunit C, a scaffold subunit A, and a regulatory subunit B. *Arabidopsis* contains 17 B subunits which are classified into B, B' and also B" (Trotta et al., 2011).

The B subunits significantly affect the PP2A functionality by controlling determination of the target of PP2A holoenzyme. B'γ subunit of PP2A was distinguished as an effective component to maintain acclimation to light (Konert et al., 2015a), controlling response to the pathogens and impacting on plant resistance (Durian et al., 2016) and ageing in *Arabidopsis* (Trotta et al., 2011). In addition, regulatory B'*γ* subunit of PP2A influences on inducing resistance against stress. Knockdown *pp2a-b'γ* mutant showed senescence and premature yellowing in their leaves (Trotta et al., 2011). In addition, PP2A-B'*γ* interacts with ACONITASE 3 (ACO3) (Konert et al., 2015b) in order to control ROS signaling and impacts on indole glucosinolates (GSL) concentration in *Arabidopsis* (Rahikainen et al., 2017). GSL contains large groups of secondary components which operate as deterrents against pathogens. *Arabidopsis pp2a-b'γ* mutant phenotypically contains smaller leaves size compared to *Arabidopsis* wild type (Figure 3).

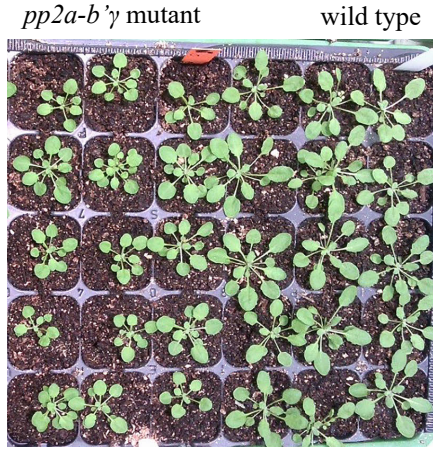



**Figure 3**. Four weeks old *Arabidopsis*; *pp2a-b'γ* mutant (left) and wild type (right).

### 2.3.2. *Arabidopsis RETICULATA*

There are several types of reticulated *Arabidopsis* mutants that show a distinction in leaf vascular architechture by exhibiting green reticulation on a paler lamina (González-Bayón et al., 2006; Kinsman and Pyke, 1998; Li et al., 1995). For the first time one of the reticulate mutants was called *reticulata* (*re*) by Rédei and Hirono (1964), after that, the classical genetic of these types of mutants was named *reticulata*. *Re* mutant leaves contain smaller and also fewer mesophyll cells in the interveinal regions (Pérez-Pérez et al., 2013). Vascular tissues have essential roles in transportation of micronutriants and metabolites between different cells and organs in plant. In *Arabidopsis* as a C3 plant, the veins are flanked with the layer of cells, that contain choloroplast and are called bundele sheath cells (Kangasjärvi et al., 2009). Thus, because of alteration of bundel sheat cells, the mesophyll cells are different (Kinsman and

Pyke, 1998). In addition, in the early stages of growth in some *re* mutants, there are less number of essential metabolites in plant because of constraining of growth and development. That may cause abnormal structures of mesophyll cells.

*Arabidopsis re* mutants have shown alterations in the metabolites and morphology of leaves under long-day photoperiods (Pérez-Pérez et al., 2013). Hence, there is a correlation between the day length and appearing of reticulation in the leaves of *re* mutant (Barth and Conklin, 2003; Overmyer et al., 2008). The picture of *re* mutant is shown in Figure 4.

There are different mutant allels of *RE* gene which have been applied in different biological studies. For example, González-Bayón et al. (2006) examined 6 different allels of *RE* gene in order to investigate the biological features of leaf reticulation.


**Figure 4.** *Reticulata* (*re 8*) mutant, grown under (130 µmol photons $m^{-2}s^{-1}$ and 16 hours light/ 8 hours dark), shows leaf reticulation.

### 2.3.3. *Arabidopsis ACONITASE*

Aconitase (ACO) is an enzyme that catalyses the transformation of citrate to isocitrate in tricarboxylic acid (TCA) cycle by the aid of *cis*-aconitate which acts as an intermediate (Moeder et al., 2007). ACO contains two different isoforms: 1) the mitochondrial, which is a component of the citric acid cycle (Moeder et al., 2007), 2) the second isoform is discovered in the cytosol (Courtois-Verniquet and Douce, 1993; Hayashi et al., 1995). The cytosolic ACO consists of two different forms, of which the active form of enzyme includes a cluster of iron–sulfur (Moeder et al., 2007). In specific conditions, such as oxidative stress, low

concentration of iron and existence of nitric oxide can lead to disappearing of the cluster after which the protein loses its enzymatic property and converts to a protein that binds to RNA (Hentze and Kühn, 1996; Hirling et al., 1994; Klausner et al., 1993).

*Arabidopsis* contains three genes encoding ACO1-3. However, the role of each isoenzyme in plant growth is not well understood. Investigation on *Arabidopsis* mutants with a knock-down in these genes individually showed that ACO3 is mainly responsible for citrate metabolism (Hooks et al., 2014). They demonstrated that ACO3 was cytosolic in early stages of seedling development and operated in citrate catabolism (Hooks et al., 2014). Additionally, Moeder et al. (2007) suggested that aconitases have an essential role in interfering with oxidative stress and moderating cell death in plant. Konert et al. (2015b) demonstrated that ACO3 is a main target for PP2A-B'$\gamma$ toward controlling oxidative streess and metabolic regulations in *Arabidopsis.*

## 2.4. Why mushrooms?

Wild edible mushrooms contain proteins, minerals and vitamins, such as riboflavin, ascorbic acid and vitamin $D_2$, as well as low amounts of fat and fibers (Agrahar-Murugkar and Subbulakshmi, 2005; Toledo et al., 2016; Valentão et al., 2005). Thus, they are a popular source of food consumption worldwide. Previous studies have demonstrated that the nutritional values of mushrooms are comparable with milk, egg and meat (Mdachi et al., 2004). Reproductive part or fruiting body of mushroom is significant because of texture, nutritional value, flavour and chemical characteristics. Likewise, vegetative part or mycelia is benefical for the ecosystem because of its decomposing feature (Manzi et al., 2001). Mushrooms can obtain the required nutrients from the substance such as wood logs and also they are categorized as heterotrophs (O'Gorman, 2010).

Mushrooms do not perform photosynthesis, thus, the requirment nutritions are absorbed from substrance (Walde et al., 2006). However, some mushrooms colonize plant roots and form mycorrhiza to obtain photosynthetic products from the plant and return N and P instead. Therefore, significant symbiosis constructs between them which can impact on ecosystems, especially in nutrient poor forests.

Another advantage of mushrooms is their medicinal feature, as some of them can be utilized as a useful remedy for different illnesses like cancer (Zaidman et al., 2005) and hypercholesterolemia (Bobek and Galbavý, 1999). Some species of mushrooms contain a good source of antioxidants, for instance, tocopherols and phenols (Barros et al., 2008; Elmastas et al., 2007; Heleno et al., 2015; Heleno et al., 2009, 2010; Lo and Cheung, 2005; Mau et al., 2002; Reis et al., 2011).

### 2.4.1. Drying methods of mushrooms

The importance of collecting herbarium mushrooms has been known for decades, as they are significant from different aspects, like studing the genetic background and taxa of mushrooms. For example, taxonomical problems of different mushroom specimens can be recognized by the type of corresponding DNA (Hosaka and Uno, 2011; Nagy et al., 2011). Also, they are good collections of unknown lineages from which the taxa can be identified in the future (Hosaka and Uno, 2011).

Mushrooms are perishable goods, they start to decay very fast after collecting, and usually one day after harvesting the process of degeneration is started. Gooday (1974) illustrated that re-distrubution of carbohydrates occurs during the postharvest time in cultivated mushrooms and that affects on differing the quality of mushroom. Therefore, in order to extend the shelf life of mushrooms, various techniques can be applied, such as drying (Walde et al., 2006) and conserving (Valentão et al., 2005). In fact, the storage conditions significantly affect the chemical components and the taste of mushrooms. There are different methods to dry mushrooms, such as air drying, using microwave, freeze drying, vacuum (Li et al., 2015). Studies of different drying methods on *P. eryngii* by Li et al. (2015) showed that drying techniques significantly impact on the non-volatile chemical components and the concentrations of organic acids.

### 2.4.2. Strophariaceae family

Wood decaying fungi *Kuehneromyces mutabilis* (Fritsche, 1997) and sheathed woodtuft, *Kuehneromyces lignicola*, are two edible mushroom species that belong to the family of Strophariaceae. *K. mutabilis* and *K. lignicola* are wild mushrooms growing in the forest (Figures 5 and 6). *K. mutabilis's* growing season ranges usually from the end of spring to the beginning of autumn. The *Kuhneromyces* species include caps with the cinnamon brown color and mild taste (Knudsen, 2012). *K. lignicola* grows on dead woody stems of trees as clusters. They have edible fruiting bodies and their growing regions are Europe and West part of Asia. *K. lignicola* is rare in Finland. However, in China it is cultivated widely and they are exported worldwide. The morphology of this mushroom is similar to the poisonous mushroom Funeral Bell (*Galerina marginata*) (Roberts and Evans, 2011).

**Figure 5.** *Kuehneromyces mutabilis,* grown in the forest in the Southwestern part of Finland.

**Figure 6.** *Kuehneromyces lignicola* which grew up in the Southwestern part of Finland.

Edible *Hypholoma capnoides* and poisonous *Hypholoma fasciculare* belong to the *Hypholoma* species of the family Strophariaceae (Figures 7 and 8). In general, they grow on decaying wood.

**Figure 7.** *Hypholoma capnoides* which grew in a forest (Southwestern part) in Finland.

**Figure 8.** *Hypholoma fasciculare,* grew up in the Southwestern partof Finland.

The *Hypholoma* species generally have an indistinct smell and the taste is mild or bitter depending on the type of specimens. The color of caps of wood decaying fungus *H. capnoides* is between olive brown to greyish brown and it is growing from summer to autumn (Knudsen, 2012). *H. capnoides* can be found in all parts of Europe.

*H. fascicular* is a poisonous and bitter fungus which grows wild in the forests, in Finland, Germany (Krieglsteiner, 1991) and Netherlands (Verhagen et al., 1998), for example. *H. fasciculare* is mainly known for one specific toxic chemical compond, called fasciculol. Several derivatives of this compound have been distinguished. They are called as fasciculic

acids A – F and H – K. Fasiculic acids have allelochemical properties, prevention of plant growth (Akasaka and Shiono, 2005) and antitumor features (Ding et al., 2009).

## 2.5. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is commonly utilized for determining the structures of chemical compounds. The phenomenon of NMR was observed for the first time in 1945–1946 by two independent groups of physicists (Purcell and Bloch) in the United States (Günther, 2013). NMR is a widely applicable tool in different fields of science, for example, in medicine (Bottomley, 1989) food science (Hills, 2006) plant science (Ishida et al., 2000) and chemistry (Stothers, 2012). In order to observe the NMR signal, the spin quantum number of the specific nucleus has to be non-zero. For instance, proton ($^{1}$H), carbon-13 ($^{13}$C) and nitrogen-15 ($^{15}$N) contain spin quantum number of $^{1}/_{2}$. The principle of NMR experiment (Figure 9) relies on exciting the certain nuclei in a sample placed in a magnetic field with a radio frequency (RF) pulse and measuring the signal frequency that is emitted by the nuclei (Günther, 2013).

The excitation occurs when the resonance frequency of the nuclei is close to the carrier frequency (excitation pulse which contains certain frequency). The energy levels of nuclei depend on the magnetic field ($B_0$) and on the nucleus(Günther, 2013).

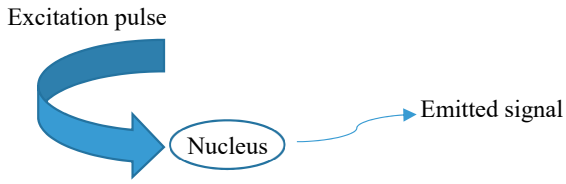



**Figure 9.** Schematic illustration of principle of NMR technique.

The RF transmitter distinguishes the level of energy absorption and displays it as a signal as function of time (Figure 10). Hence, the NMR raw data is created (Günther, 2013), and it is called free induction decay (FID). Fourier-transformation (Mo and Raftery, 2008) is applied to convert time domain signal into the frequency domain spectrum. The resonance signals are separated from each other by chemical shift ($\delta$), which is represented as either hertz (Hz) or parts per million (ppm) (Keeler, 2011). Difference in the levels of energy mainly impacts on the chemical shift.

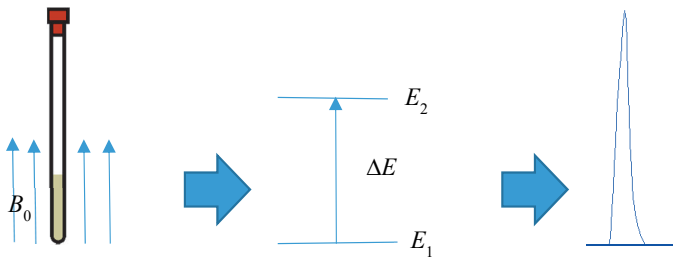



**Figure 10.** Establishment of NMR signal (The Figure modified from Günther, 2013).

To maximize the RF signal transmission, tuning is applied, which contains regulation of tune and match capacitors. The procedure is usually automated. Further step is shimming which is significant in order to achieve a good quality spectrum. Bad shimming produces broad and non-symmetric signals in the spectrum. In shimming minor adjustment to the magnetic field are applied to enhance the field homogeneity (Topgaard et al., 2004). Proton displays a high sensitivity in NMR because of its high natural abundancy of 99.98% (Günther, 2013). In contrast, carbon-13 requires longer experimental time due to the low natural abundancy (1.1%). In order to gain high-resolution NMR, liquid samples are required.

The solvents applied for the NMR samples are deuterated solvents, due to optimizing resolution for the sample and to provide deuterium lock signal for the stability of the magnetic field (Schripsema, 2010). Frequently, $D_2O$, MeOD-$d_4$, $CDCl_3$ and DMSO-$d_6$ are utilized as solvents. Quantification analysis can be accomplished by utilizing internal standard compounds like, 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid (TSP). These standard compounds can also be used to calibrate the chemical shift. Coupling constant (J-coupling) in hertz can be determined from the distance between individual proton peaks in the frequency unit which indicates the groups of coupled protons. Identification of the chemical compounds requires usually the aid of different sets of one-dimensional (1D) and two-dimensional experiments (2D). For example, $^1$H, $^1$H correlation spectroscopy (COSY), shows the chemical shifts of proton nuclei that are *J*-coupled to each other, heteronuclear single quantum correlation (HSQC) displays one bond correlation of $^1$H and corresponding $^{13}$C and $^1$H–$^{13}$C heteronuclear multiple bond correlation (HMBC) determines long-range connections of proton and carbon, usually over 2–3 bonds.

### 2.5.1. Data pre-processing

In order to optimize and reduce variation of NMR metabolomics data, specific procedures need to be performed. The data is usually sensitive to the impacts of water signal and pH alteration (Kortesniemi, 2016). In general, pre-processing of the data contains Fourier transformation, either manual or automatic corrections of phase and baseline, alignment and integration of the signals (Larive et al., 2014). Phase correction is utilized to remove

the phase errors (Mäkelä, 2016). Baseline correction eliminates the distortions which may have a negative impact on statistical analysis (Brennan, 2014). Integration of signals is applied typically to proton spectra and the integral values correspond to the number of magnetically equivalent protons responsible for that specific signal. However, in complex sample materials, such as plant extracts, the signals are often overlapping and the integration is complicated. To facilitate analysis of metabolomics data, the number of unwanted variables are reduced in the dataset by bucketing of the spectra (Kortesniemi, 2016).

To remove the unwanted variation, normalization and scaling are applied. Normalization of the data makes the data from the samples comparable to each other by minimizing impacts of dilution. Normalization can be achieved by normalizing the integrals to the total intensity of signals or to a reference region (Craig et al., 2006). The main aim of the scaling is to reduce partly the systematic bias in the spectral intensities. In NMR metabolomic studies, preferred scaling selection is Pareto scaling system, which gives the variable a variance equal to the standard deviation and mainly emphasizes on the medium to small trends in the data (Kortesniemi, 2016). Consequently, scaling and normalization are essential for the analysis of metabolomics data (Craig et al., 2006; Eriksson et al., 2013).

### 2.5.2. Multivariate data analysis

One of the main approaches to evaluate metabolomics data is multivariate analysis (Worley and Powers, 2013). Multivariate data analysis can be used to analyze the selected observations and variables in order to find systematic differences in the data set (Eriksson et al., 2013). Also, it can give a comprehensive overview, distinguishing of pattern, prediction of model and classification between the groups (Kortesniemi, 2016). Three of the most common models that are applied for the analysis of metabolites data are principal component analysis (PCA) (Hotelling, 1933; Pearson, 1901), partial least squares projection to latent structures (PLS) (Wold et al., 2001) and partial least squares discriminant analysis (PLS-DA) (Brereton and Lloyd, 2014). PCA is an unsupervised model that without any predefined grouping displays systematic trends and patterns in the data matrix ($X$) that consists of observations and variables in the rows and columns, respectively. Linear combinations of the variables are principal components (PCs) that represent the variation in the dataset.

The PCA scores plot shows the responsible observations as a function of the PCs. The loadings plot determines the variables that cause differences in the dataset, based on the corresponding scores plot. The vectors ($p$) in the loadings plot contributes the relationship between the variables. In the model the values of goodness-of-fit ($R^2$) (elucidated variation) and predictive ability ($Q^2$) represent validity of the model (Eriksson

et al., 2013; Kortesniemi, 2016). PLS is a supervised technique which displays differences among predefined groups. Also, it is a regression method, and characterized by the content of matrix *Y* (Kortesniemi, 2016). It detects the maximum correlation between *X* and *Y* variables.

Additionally, PLS-DA is a supervised model also creates *Y* variables and (dummy *Y* variables) includes discriminating information and attempts to find the discriminator line (Brereton and Lloyd, 2014) between two groups. Also presents $R^2X$, $Q^2$ and $R^2Y$ (fraction of the *Y* variation). Generally, it is utilized after defining the observation in two or more classes. SIMCA software, is frequently utilized for the evaluation of NMR data in metabolomics studies.

### 2.5.3. NMR metabolomics

Metabolomics is defined as identification and quantification of the metabolites in a biological sample, as well as evaluation of changes among them under different circumstances (Larive et al., 2014). Identification and quantification of the metabolites are highly dependent on the selected methods and instruments. There are various types of instrumentations and methodologies, for instance, NMR spectroscopy and MS. MS techniques include gas chromatography-MS (GC-MS), liquid chromatography-MS (LC-MS), capillary electrophoresis–MS (CE-MS) and Fourier transform ion cyclotron resonance–MS (FT-ICR-MS) (Khakimov et al., 2014; Okazaki and Saito, 2012). All the above tools are suitable to analyze complex mixtures of samples (Hong et al., 2016).

NMR metabolomics has been accomplished in various types of research, such as plant cell line studies (Schripsema and Verpoorte, 1991), fingerprinting of metabolites in *Arabidopsis thaliana* (Ward et al., 2003), tobacco (Choi et al., 2004a), mushrooms (Cho et al., 2007) and in food chemistry, for instance, berries (Kortesniemi et al., 2014) and honey (Kortesniemi et al., 2016).

The main advantages of NMR compared to MS methods are simple sample preparation, good selectivity and identification of the compound structure, fast analysis as well as its non-destructiveness of the samples (Aretz and Meierhofer, 2016). However, the main limitation of NMR is the low sensitivity compared to MS. In addition, relatively large amounts of sample materials are required (Beluhan and Ranogajec, 2011; Elipe, 2003). Thus, NMR is not well applicable for identification of the low concentration metabolites in a sample. Also, the signals of different compounds may appear at the same spectral region, causing overlapping of the signals. This influences on the interpretation and identification of the metabolites (Kim et al., 2010). Finally, the initial investment costs of an NMR instrument are high and the identification of compounds demands special interpretation skills.

Metabolite fingerprinting aims to identify a large number of metabolites in a sample. Metabolite profiling concentrates more on a specific set of metabolites as well as identification and quantification of them. Also, metabolite profiling can be utilized to recognize metabolic alterations. Profiling of the metabolites can be divided into targeted and untargeted analysis. Targeted profiling consists of quantitative screening of certain metabolites in a sample (Weljie et al., 2006). In contrast to that, untargeted metabolomics study mainly focuses on general discovery of the chemical compositions in samples without requirement of identification and qualification of specific metabolites.

General procedures of an NMR metabolomics study, such as preparation of sample, data processing, statistical analysis and interpretation (Larive et al., 2014), are shown in Figure 11.

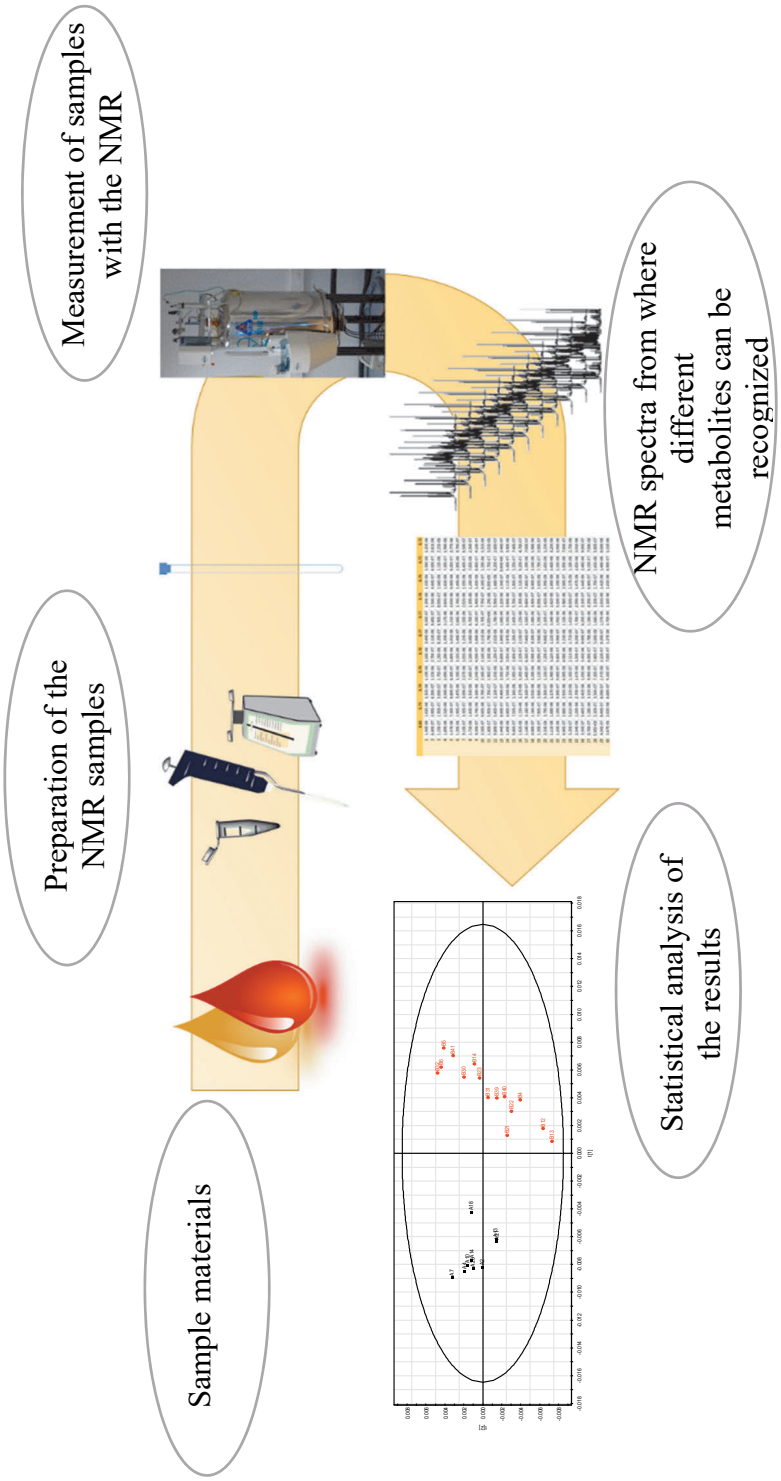



**Figure 11.** Schematic model of NMR metabolomics process.

In NMR metabolomic studies involving plant material, some parameters are needed to be taken into consideration in the experimental design, for example: 1) Developmental stage of the plant is important, since concentrations of metabolites may change in different growth stages of the plant. 2) Another significant factor is related to the quantity of plant material available, because at the beginning stage of plant development the seedlings are small. Thus, sufficient amount of plant material is required for an NMR sample (usually in case of plant extract 50 mg of dry powder). On the other hand, utilizing larger number of samples lead to more reliable statistical analyses (Abdel-Farid et al., 2007; Keurentjes et al., 2006). 3) An appropriate method to store fresh plant material after harvesting has to be utilized to avoid degradation. Hence, cooling immediately after harvesting material is highly recommended to reduce enzymatic degradation. This can be achieved by transferring the harvested material into a container filled with liquid nitrogen, for example. Harvested plant material can be stored in a freezer in –80 ºC approximately from one week to a month before extraction (Kim et al., 2010). 4) Sample material pre-processing, which includes drying, weighting and extracting of the sample material.

The most common drying method is freeze drying. However, there are many different methods that can be applied. Drying of the plant material inhibits enzymatic reactions that can be induced in the presence of water, but it also reduces the overlapping of the water signal in the NMR spectrum and shifting of resonance due to pH alterations. After drying, in order to prepare fine powder, pestle and mortar can be used to grind the small sized and fine plant material. For woody or bigger plant tissues, a specific blender can be used. The weighting of a sample before extracting is significant and usually 50-100 mg of dry weight material is sufficient. However, depending to the sample material and type of the experiment, less than this amount can also be used (Kim et al., 2010, 2011).

Sample extraction is critical, because depending on the solvent choice, different types of metabolites in the sample can be recognized. Usually non-polar solvent, like chloroform or a mix of chloroform and methanol have been applied in GC-MS, while more polar solvents are used for LC-MS and NMR (Kim and Verpoorte, 2010). In addition, a combination of several solvents can be utilized in order to identify wider range of metabolites. Usually, a mixture (1:1) of MeOD-$d_4$ and phosphate buffer (pH 6.0, $D_2O$) can give a good overview of both primary and secondary metabolites (Kim and Verpoorte, 2010). It is also important to consider the pH of the extract to prevent shifting of the NMR signals. Thus, different types of buffer can be utilized to keep the pH constant, such as phosphate buffer, which is the most common choice (Kim et al., 2010). Finally, it is important to minimize all types of variation in all stages of the study, such as sample selection, sample storage and preparation and during the measurement process (Maher et al., 2007).

Proton NMR ($^1$H NMR) spectroscopy is usually fast and simple and it can also supply hundreds of signals (Figure 12) of the corresponding metabolites. Metabolite concentrations

can be measured by comparing the signal intensity with an internal standard signal, such as TSP. TSP can be added into the sample in order to perform quantification of the metabolites, but it is also used for the calibration of NMR chemical shifts (Kim et al., 2010, 2011). NMR spectrum can be roughly divided into three main regions of amino acid, sugar and aromatic respectively (Figure 12).

The residual water signal can be suppressed to avoid unwanted signal overlapping. Some of the most common suppression methods are 1D-NOESY, presaturation (pre-sat) (Hoult, 1976) that is based on a weak irradiation of radiofrequency and water suppression via gradient tailored excitation (WATERGATE) which is based on gradient methods (Sklenar et al., 1993). However, pre-sat is applied extensively in metabolomics because it is simple and robust (Kim et al., 2010).

Identification of the metabolites is performed after distinguishing the signals that are responsible for the differences between samples based on the applied statistical model. For example, unsupervised multivariate data analysis was utilized to determine the metabolic changes in *Catharanthus roseus* leaves which were infected by phytoplasmas (Choi et al., 2004b) and supervised multivariate method was used to study genetic modifications in transgenic maize (Manetti et al., 2004).

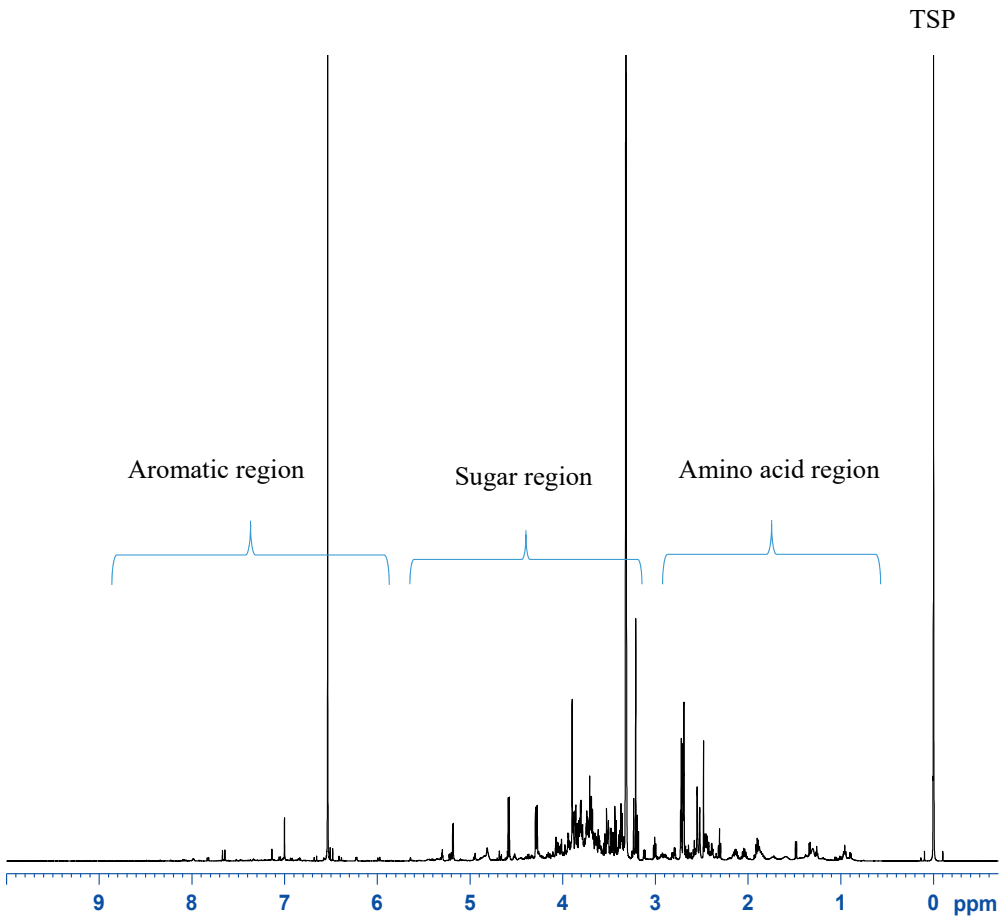



**Figure 12.** $^{1}$H NMR spectrum of four weeks old *Arabidopsis thaliana,* wild type, under high light stress. Three main regions of amino acids, sugars and aromatic are shown in the spectra; 3-(trimethylsilyl) propionic acid sodium salt (TSP) was used as an internal refrence.

Usually identification of the metabolites is accomplished by adding a corresponding reference compound to the sample and comparing the specific signals from the spectra with the signals belonging to the added reference compound (so called spiking), by using databases and by performing a set of 2D experiments to validate the identification. Databases include NMR or MS spectra of known metabolites, which can be utilized for identification of chemical compounds. For instance, for NMR data, the Human Metabolome Database (HMDB: www.hmdb.ca) (Wishart et al., 2012) and the Spectral Database for Organic Compounds (SDBS: http://sdbs.db.aist.go.jp/sdbs/cgi-bin/cre_index.cgi) are commonly used in metabolomic studies (Kim et al., 2011).

Also, identification can be performed by the aid of different 1D and 2D experiments, such as total correlation spectroscopy (TOCSY) that represents spin systems of coupled protons arising from the same molecule. It is very helpful in the identification of carbohydrates and amino acids.

In a 1D version of TOCSY experiment, a specific signal (with a good intensity) in the spectrum is chosen and the other corresponding signals can be determined by applying excitation pulse on the selected signal. COSY displays proton correlations that have spin-spin couplings. HSQC (Kim et al., 2010) represents the single-bond correlations of protons and carbons. HMBC shows long range correlations (normally two or three bond correlations) of protons and carbons in the spectra. It is helpful for the identification of signals that belong to the same molecule.

Identification of primary metabolites such as sugars, amino acids and organic acids is usually easier than the identification of secondary metabolites, e.g. phenols, flavonoids and alkaloids, because the structures of secondary metabolites are often more complicated (Kim et al., 2011). Sometimes, identification of metabolites is restricted due to overlapping of the signals with other signals related to different metabolites at the same regions in the spectrum. In that case, to enhance identification of metabolites, both NMR and HPLC methods can be utilized (Lambert et al., 2007).

# 3. AIMS OF THE STUDY

The main aim of this study was to perform NMR metabolomic investigations on the model plant, *Arabidopsis thaliana,* under various physiological conditions, including abiotic stress and metabolic alterations caused by mutations in genes related to metabolic activities. Additionally, metabolic comparison of different wild fresh mushroom species to their corresponding herbarium species was performed. Thus, the specific aims of my thesis were:

1. To analyze alterations of metabolites during senescence in *Arabidopsis* wild type and *pp2a-b'γ* mutant in two different ages, four and six weeks old (Article **I**).

2. To investigate the effects of two different light intensities (growth light and high light) and short photoperiods, on the metabolomics adjustments in *Arabidopsis* wild type and *reticulata* mutant (Article **II**).

3. To assess metabolomic changes in different genotypes of *Arabidopsis,* wild type and *aconitase* mutants (Article **III**).

4. To find metabolomic differences between wild fresh mushroom species from two different genera, *Kuehneromyces* and *Hypholoma,* of the family Strophariaceae and their corresponding herbarium mushroom species, as such usability of NMR metabolomics to the fungi was tested. Additionally, to examine the stability of the metabolites in herbarium mushroom species from different ages, in order to consider utilizing them as research material in future mushroom studies. (Article **IV**).

# 4. MATERIALS AND METHODS

## 4.1. Samples

The set of *Arabidopsis* samples in the study I, II, and III were grown in the chamber placed at Molecular Plant Biology (Department of Biochemistry, University of Turku). In study IV the wild fresh mushrooms from two different species *Kuehneromyces* and *Hypholoma* were collected from the South Western Finland, Turku area; and their corresponding herbarium species were obtained from the Herbarium, Department of Biology, University of Turku and Åbo Akademi University.

### 4.1.1. *Arabidopsis*

Three of the sub-studies in this thesis utilized mainly *Arabidopsis* wild type (ecotype Colombia) and its various mutants.

(I) Wild type, a homozygote *pp2a-b'γ* mutant (SALK_039172 for At4g15415) (Alonso et al., 2003; Trotta et al., 2011) and complementation line *pp2a-b'γ 35S::PP2A-B'γ* which was complemented by 35S-driven expression of the *PP2A-B'γ* gene (Trotta et al., 2011) were grown for the experiment; the complementation line was used as a control. The plants were harvested in two different ages, four and six weeks old. The plants were grown under 130 µmol photons $m^{-2}s^{-1}$/22 ºC with 50% humidity at an 8-hour light period conditions in the trays. Each tray contained three lines (wild type, *pp2a-b'γ* and *pp2a-b'γ 35S::PP2A-B'γ*), (Figure 13). In this experiment, approximately thousand plants were grown, since the size of the plant was small and after freeze drying the mass of the plant was low. Therefore, in order to obtaining enough dry powder for NMR samples, large numbers of targeted plants were grown.

(II) *Arabidopsis* wild type, homozygote *re* mutant (Figure 14) and transgenic $RE_{pro}$*:GUS* (Pérez-Pérez et al., 2013) were grown under growth light (130 µmol photons $m^{-2}s^{-1}$) and short days for two weeks, next the trays of seedlings were transferred under either growth light or high light (500-600 µmol photons $m^{-2}s^{-1}$) for two more weeks. The total age of plants under two different light intensities was four weeks. The humidity was 50 % at 22 ºC and the plants were grown at 8-hours light and 16 hours dark.

**Figure 13.** The trays contain seedlings of *Arabidopsis* wild type, *pp2a-b'γ* mutant and *pp2a-b'γ 35S::PP2A-B'γ* complementation line.

(III) The seedlings of *Arabidopsis* wild type and different *aconitase* (Salk_013368; Arnaud et al., 2007) mutants (*aco3*, *aco3 pACO3::ACO3*, *aco3 pACO3::ACO3*$^{S91A}$ and *aco3 pACO3::ACO3*$^{S91D}$), were grown in a chamber under 130 µmol photons $m^{-2}s^{-1}$, 22 ºC, 50% humidity and at 8/16 hours light period. Total age of plants was eight weeks.

*Arabidopsis* under high light *Arabidopsis* under growth light

**Figure 14.** *Arabidopsis* wild type and *reticulata* mutant, grown under high light (left) and growth light (right).

The plants were immediately transferred into the liquid nitrogen after harvesting to avoid any enzymatic activity. In addition, freeze dried sample materials (solely, wild type and *re* mutant) were kept in the freezer at −80 °C temperature.

### 4.1.2. Mushrooms

(IV) *Hypholoma* and *Kuehneromyces* species, including wild edible *K. mutablis and H. capnoides*, were collected freshly from ten different locations in Southwest Finland (Turku area) during summer-autumn 2015. The collecting sites were chosen by the aid of the mycologist of the group. Likewise, *K. lignicola* and poisonous *H. fasciculare* were collected from 1 to 2 different locations, as they are rare in Turku region. The fruiting bodies (caps + stipes) were transferred into the liquid nitrogen tank immediately at the collection site to be frozen and preventing alteration of chemical compositions.

The corresponding herbarium mushroom species (*K. mutablis, K. lignicola, H. capnoides* and *H. fasciculare*) were obtained from the combined Herbarium, Department of Biology, University of Turku and Åbo Akademi University during January 2016. In general, the method to store mushrooms in the Herbarium is following: after collecting the mushrooms, they are air-dried with a fan in +20 - +40°C. However, depending to the field conditions, the method may differ slightly. After obtaining the corresponding herbarium mushrooms, they were transferred into liquid nitrogen and freeze dried afterwards to follow similar sample preparation method as fresh mushroom specimens.

In addition, to study deterioration of herbarium mushroom species, samples from the following periods of time were also obtained from the Herbarium: *K. mutablis and H. capnoides* (1990-1994, 2000-2004 and 2010-2014), *K. lignicola* (1995-1999, 2000-2004 and

2010-2014) and *H. fasciculare* (1995-1999, 2000-2004 and 2005-2009). The samples were obtained from 5-years long collection periods and the total storage time was up to 25 years. The collection was based on the availability of the herbarium mushroom specimens in the specific period of time.

## 4.2. Methods

### 4.2.1. Sample preparation

In the studies I, II and III, the *Arabidopsis* plants were frozen in liquid nitrogen after harvesting and they were kept in the freezer at –80 ºC. In the next step the plants were freeze-dried and ground with a mortar and pestle to prepare a fine powder. For the study IV, the mushroom samples were frozen in liquid nitrogen immediately after collection and they were kept there before applying freeze dryer. Freeze dried sample material (mushrooms) were grounded to powder and kept in –18 ºC freezer before extraction.

In the study I, 25 mg of the corresponding powder was mixed with 0.8 ml of MeOD-$d_4$. *Arabidopsis* samples vortexed (15 min) and centrifuged (Eppendorf centrifuge 5424 R, Hamburg, Germany) for 10 min at 20 °C and at 15000 rpm. In order to obtain 25 mg of *Arabidopsis* dry powder, plant material was combined; 15 individual plants for four weeks old and 5 individual plants for six weeks old. 27 replicates for four weeks old plants and 44 replicates for six weeks old plants were provided respectively.

For the second study (II), 15 mg (obtained by pooling three plants) of the *Arabidopsis* powder was mixed with 0.8 ml solution (1:1 V/V) of deuterated methanol and 0.1 M phosphate buffer in $D_2O$ (pH 6.2, 3 mM TSP, 2 mM $NaN_3$). The samples were vortexed (15 min) and centrifuged (9000 rpm, 15 min, 22 ºC). For *Arabidopsis* wild type and *re* mutant 26 and 21 biological replicates were provided respectively.

The experiment III was performed by applying fine powder (40 mg) of *Arabidopsis* in 0.8 ml of buffer (0.1 M phosphate buffer in $D_2O$, pH 6.2, 3 mM TSP, 2 mM $NaN_3$). The mixture was vortexed (15 min) and centrifuged (20 ºC, 15000 rpm, 10 min). The age of plants were eight weeks old and in order to achieving 40 mg powder, three plants were pooled together. In addition, 3 replicates per plant line (plant lines= wild type, *aco3*, *aco3 pACO3::ACO3*, *aco3 pACO3::ACO3*$^{S91A}$ and *aco3 pACO3::ACO3*$^{S91D}$) were prepared.

In the study IV 50 mg of dry powder of mushroom was extracted with 0.5 ml solution (1:1 V/V) of MeOD-$d_4$ and 0.1 M phosphate buffer in $D_2O$ (pH 7.0, 2 mM $NaN_3$, 5 mM TSP). Mushroom samples were vortexed (15 min) and centrifuged (21130 g, 15 min). In total, 182 fresh mushroom samples used for NMR analysis consisted of 79 *K. mutabilis*, 90 *H. capnoides*, 5 *K. lignicola* and 8 *H. fasciculare* samples. Additionally, the number of herbarium mushroom samples per collection period contained; (1900-1994): 10 *K. mutabilis* and 10 *H. capnoides*; (1995-1999): 1 *K. lignicola* and 12 *H. fasciculare*; (2000-2004): 10 *K.*

*mutabilis* 10 *H. capnoides,* 8 *K. lignicola* and 1 *H. fasciculare*; (2005-2009): 2 *H. fasciculare*; (2010-2014): 10 *K. mutabilis,* 10 *H. capnoides* and 2 *K. lignicola.* In total, 86 herbarium mushroom samples were obtained.

For all experiments (I, II, III and IV), 600 µl of the supernatant was transferred to the 5 mm NMR tube to perform the analysis.

### 4.2.2. Nuclear magnetic resonance (NMR) spectroscopy

The instrument utilized in the sub-studies I and IV was a Bruker Avance 500 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) equipped with a broadband inverse (BBI-5 mm-Zgrad-ATM) autotune probe, operating at 500.13 MHz for $^{1}H$ for (I and IV) and at 125.76 MHz for $^{13}C$ (I).

The NMR instrument for studies (II) and (III) was a Bruker Avance III 600 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) with a triple head Prodigy TCI 5 mm inverse nitrogen cooled cryprobe operating at 600.16 MHz for $^{1}H$ and at 150.92 MHz for $^{13}C$ and equipped with precooled SampleJet sample changer. Both instruments were placed in Instrument Centre, Department of Chemistry, University of Turku.

In the study I, double suppressed (*lc1pnf2* pulse programme) proton spectra was measured to suppress residual signals of $CHD_2OD$ and $CD_3OH$ at 25 ºC; containing: 1024 scans, an acquisition time 3.27 s, a relaxation delay 4 s and a 90° pulse of 6.9 µs. Free induction decays (FIDs) included 64 k data points and spectral width was 10,000 Hz.

In the study II and III, pre-sat proton experiment (*zgpr* pulse programme) was performed to suppress residual signal of $H_2O$ at 4.80 ppm. It was continued with: 256 scans at 25º C, an acquisition time of 5.45 s, a relaxation delay of 5.0 s and a 90º pulse of 8 µs. FIDs contained 128 K data points and spectral width was 12,000 Hz.

Study IV was performed by applying double presaturation (*lc1pnf2* pulse sequence) proton experiment to suppress the residual signals of $CHD_2OD$ and $H_2O$.The acquisition consisted of 256 scans, contained 64 k data points, an acquisition time 3.28 s and a relaxation delay 5 s.

In the studies I, II and IV, a set of 1D and 2D experiments, such as DQF-COSY (*cosygpmfqf*), 1D-TOCSY(*selmlgp*), NOESY (*noesygp*), $CH_2$-edited $^{1}H$-$^{13}C$ HSQC (*hsqcedetgpsisp2*) and $^{1}H$-$^{13}C$ HMBC (*hmbcgplpndqf*), were applied for the specific samples, to help the identification of the metabolites.

### 4.2.3. Spectral processing

In the studies I, II, III and IV, the spectra were pre-processed in TopSpin 3.2 software (Bruker BioSpin GmbH, Rheinstetten, Germany), continued with utilizing AMIX software (Bruker BioSpin GmbH) to bin the spectra. Baseline and phase were corrected either manually (I) or automatically (II, III and IV) in TopSpin. Bin widths in the studies were: 0.01 ppm (I), 0.02

ppm (II), 0.05 ppm (III) and 0.005 (IV) respectively. Generally, only the solvent regions were excluded.

The studies I, III and IV were accomplished by performing, integration mode of positive intensities to bin integrals and normalization achieved by scaling to the total intensity. In the study II, normalization was achieved by scaling the spectra to the reference region of TSP and the integration mode was selected as positive intensity.

In the study I the spectral alignment was performed by *i*coshift (Savorani et al., 2010) tool in the Matlab R2016a software (Kortesniemi et al., 2014), in order to correct peak shifting in the regions of 6.74 and 8.38 ppm respectively.

In the studies II, III and IV, the data was calibrated to the TSP signal at 0.00 ppm. Likewise, in the study I, the calibration was performed manually to the signal of α-glucose (the most intense signal in the corresponding spectra) at 5.102 ppm.

The identification of the metabolites was accomplished by utilizing literature, the metabolite databases HMDB (http://www.hmdb.ca/) (Wishart et al., 2007) and SDBS (http://sdbs.db.aist.go.jp/sdbs/cgi-bin/cre_index.cgi; coordinated by National Institute of Advanced Industrial Science and Technology (AIST), Japan) (Johnson and Lange, 2015), by arranging sets of 1D and 2D experiments for selected number of samples, by using Chenomx NMR Suite 8.1 software (evaluation version, Chenomx Inc., Edmonton, Canada) and by the addition of reference compounds to the NMR sample and comparing the proton spectra before and after adding the reference compound to the selected sample (spiking).

### 4.2.4. Statistics and multivariate data analysis

In order to execute multivariate data analyses, SIMCA-P+ 12.0.1 software (Umetrics AB, Umeå, Sweden), was used. For the scaling Pareto-scaling (II, III and IV) and mean-centering (I) were selected.

Principal component analysis (PCA) was applied as an unsupervised model in the studies I, II, III and IV and partial least squares discriminant analysis (PLS-DA) was tested in the studies I and II as a supervised model. In general, when a separation between the observations in the scores plot was observed based on the PCA model, also the differences were dominant on the PLS-DA model.

A classification of the supervised model (PLS-DA) in I was utilized based on two different ages (four weeks old and six weeks old) and in II two different light intensities (growth light and high light).

# 5. RESULTS AND DISCUSSION

## 5.1. NMR metabolomics reveals age dependent metabolomic alterations in *Arabidopsis*

*Arabidopsis* wild type, *pp2a-b'γ* mutant and *pp2a-b'γ 35S:PP2A-B'γ* complementation line (applied as a control line in the experiment) were grown into two different ages of four and six weeks old. *PP2A-B'γ* interacts with metabolic enzymes, and it is known to have an essential role in regulating GLS profiles (Rahikainen et al., 2017) and cytoplasmic ACO3 (Konert et al., 2015b). Thus, *PP2A-B'γ* regulatory operation impacts on the defense mechanisms in *Arabidopsis* leaves. Therefore, *pp2a-b'γ* mutant was chosen for the analysis to better understand the metabolic alterations in comparison with in actively growing or senescent wild type plants.

During the growth and development of *Arabidopsis* plants, different stages can be defined: at two weeks of age the seedlings are actively growing, at four weeks of age they are defined broadly as being in the vegetative phase (rosette leaves have grown) and at six weeks of age they are in the maturation phase (initial stage of producing seeds). Thus, in order to compare vegetative phase and maturation phase or developing stage, these ages were selected. Additionally, the impact of mutation on the modulation of the metabolites was one aim of the study (metabolic comparison of wild type and *pp2a-b'γ* mutant).

*Arabidopsis* wild type and *pp2a-b'γ* mutant showed morphological difference (Figure 15); *pp2a-b'γ* mutant contains smaller leaf size compared to the wild type. Therefore, based on the morphological difference between wild type and mutant, the metabolic analysis of the plant was performed to understand metabolic adjustments between wild type and mutant plants.

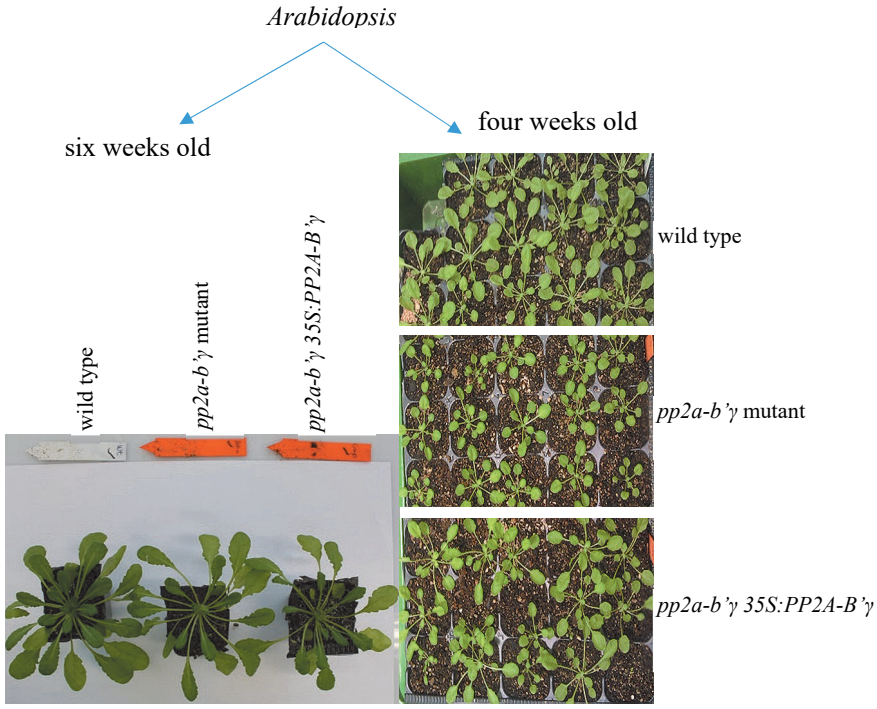



**Figure 15.** *Arabidopsis* four weeks (right) old (wild type, *pp2a-b'γ* mutant and *pp2a-b'γ 35S:PP2A-B'γ*); Six weeks age (left) *Arabidopsis*: wild type, *pp2a-b'γ* and *pp2a-b'γ 35S:PP2A-B'γ*.

To distinguish the metabolic alterations in two different ages, the NMR analysis was applied as an analytical tool. In the preliminary stage, different solvents for the extraction were tested, the proton spectra of some of the tested solvents are shown in Figure 16. Selection of the suitable solvent was based on the observation of large number of signals and also on the good quality of signals and quantitative tests. Therefore, deuterated methanol was selected as the solvent. After measuring the $^{1}$H NMR of all samples, the data was analyzed with multivariate methods.

The PCA was demonstrating 71.4 % of the total variation via the first two principal components (Figure 2A, Study I). Also it showed a great goodness-of-fit ($R^2X_{(cum)} = 0.95$) and predictive ability ($Q^2_{(cum)} = 0.85$ %). Additionally, clear separation was observed between the *Arabidopsis* wild type, *pp2a-b'γ* mutant and *pp2a-b'γ 35S:PP2A-B'γ* complementation line in two different ages of four and six weeks old based on the PCA model. However, the PCA model of *Arabidopsis* wild type and *pp2a-b'γ* mutant at the same age (either four or six weeks old) showed similar discrimination of the metabolites (refer to the section 5.3).

In the $^{1}$H NMR spectra of *Arabidopsis* samples, accumulation of two compounds (two signals contained higher intensities) was clearly dominant, even before applying statistical analysis. The intense signals belonged to choline (3.39 ppm) in the four weeks old plants and

fumaric acid (6.69 ppm) in the six weeks old plants (Figure 17). Fumaric acid is one of the components involved in the TCA cycle. Metabolization of this compound provides the required energy for production of the other compounds in the developing and reproduction stage of plant. In addition, the concentration of fumaric acid in *Arabidopsis* is increased (Chia et al., 2000).

In contrast, increasing abundancy of choline in four weeks age (vegetative phase) plant is mainly due to choline's anti-ageing (Zheng et al., 2008) and enzymatic properties (BeGora et al., 2010) and because of the essential role of choline to increase total chlorophyll content in plants. Meanwhile, several studies have reported the significant role of choline to protect the plant against different abiotic stress; for instance the impact of choline when the plant is under drought stress (Gou et al., 2015). Another discovery in four weeks old *Arabidopsis* was observation of γ-aminobutyric acid (GABA) which was devoid in the six weeks old plants.

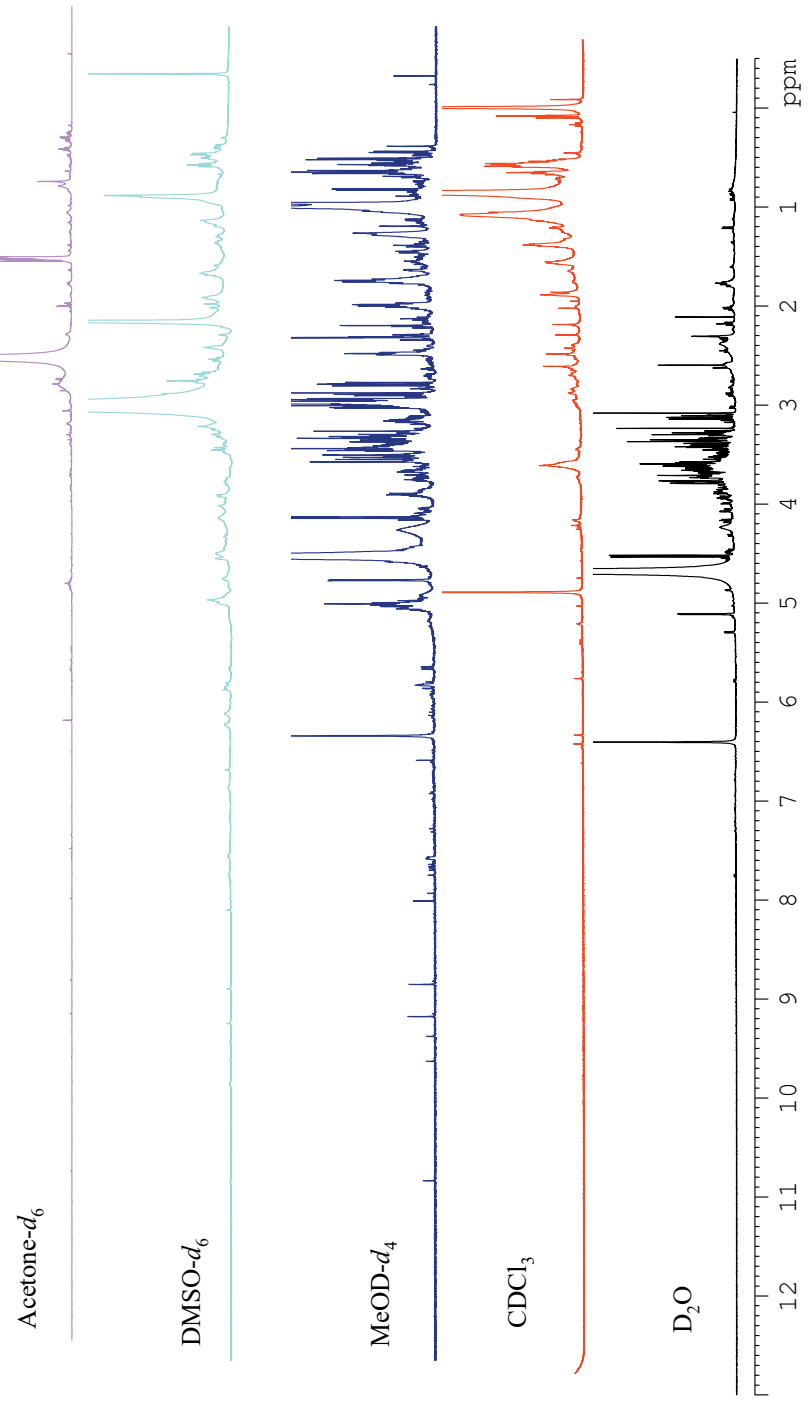



**Figure 16.** $^1$H NMR spectra of *Arabidopsis*; different solvents were applied.

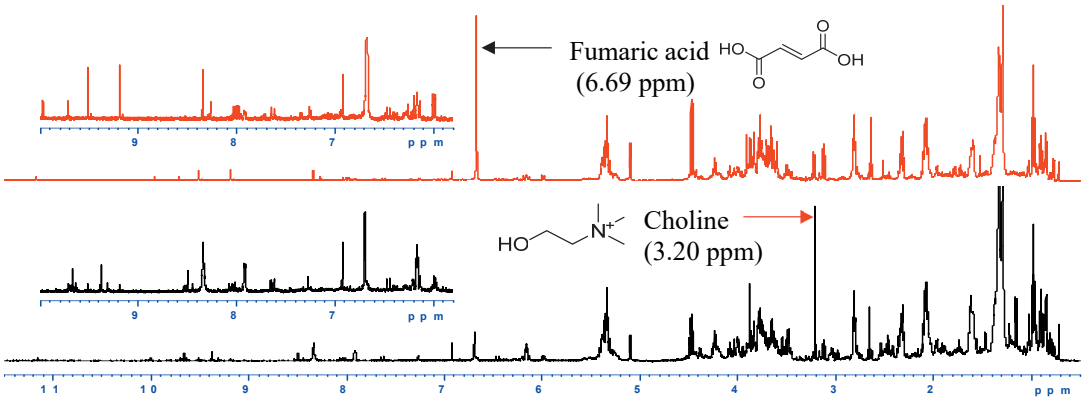



**Figure 17.** $^{1}$H NMR spectra of *Arabidopsis*: Black colored 4 weeks old wild type; red colored 6 weeks old wild type.

In the proton spectra of *Arabidopsis* the signals in the region between 8 – 11.30 ppm belonged to the chlorophylls. Previously, precise identification of the chlorophyll was performed by purification of the compound separately by using liquid chromatography (Khachik et al., 1986).

Identification of chlorophyll by NMR with no prior purification step of the crude sample was complicated. Nevertheless, in the chlorophyll region, the number of signals were more abundant in four weeks old plants because of the accumulation of total chlorophyll content (which consists of chlorophylls a and b). In contrast, in six weeks old plants, the number of signals were reduced (due to the degradation of total chlorophyll content at the maturation stage) but the intensity of signals increased. Generally, degradation procedure starts from the chlorophyll a (Hörtensteiner, 2006). Therefore, in the initial stage of chlorophyll breakdown the concentration of chlorophyll a is induced because of converting of chlorophyll b to a type which caused higher intensity signals in six weeks old *Arabidopsis.*

Consequently, based on NMR experiment in this study a number of metabolites were identified without applying fractionation or derivatization, which are the advantages of NMR. However, because of lower sensitivity of NMR (Elipe, 2003) to distinguish low abundant compounds, no metabolite discrimination was observed between the wild type and mutant. In addition, distinguishing the fatty acid signals in the spectra was complicated with the NMR, due to the overlapping of the signals in the spectra, similarity of the structures and low sensitivity of fatty acids signal.

Identification of some fatty acids (such as, linolenic acid) in this study was mainly achieved by the aid of HSQC and HMBC.

## 5.2. Effects of light intensity and photoperiods on *Arabidopsis* wild type and *reticulata*

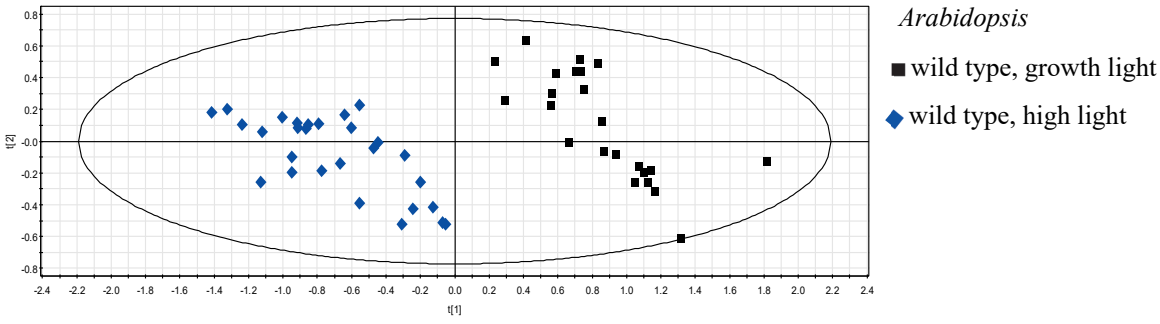
Light and photoperiod have significant roles in metabolic regulation, growth and development of plants. Day length has been shown to condition the process of leaf development in various *Arabidopsis* mutants. Previous studies showed that while long days were significantly effective on defense mechanism of *Arabidopsis catalase 2* (*cat2*) mutant deficient in a major antioxidant enzyme (Queval et al., 2007; Chaouch et al., 2010), short days did not impact the defensive systems of the mutants, because the response is controlled through regulatory functions by *PP2A-B'γ* (Chaouch et al., 2010; Li et al., 2014). Despite of extensive research efforts, the mechanisms underlying photoperiodic leaf development and metabolic regulation are still awaiting discovery. In order to understand the aspects of light intensity and short days on metabolic adjustment and leaf development in *Arabidopsis* wild type and *re* mutant the current experiment was designed.

*Arabidopsis re* mutant contains a green vascular reticulation on a paler lamina as illustrated in (González-Bayón et al., 2006; Kinsman and Pyke, 1998; Li et al., 1995; Mollá-Morales et al., 2011). Pérez-Pérez et al. (2013) demonstrated that leaf reticulation in reticulate phenotypes is induced under long days. In the current study, the impacts of short photoperiods and different light intensities including high light (500-600 µmol photons $m^{-2}s^{-1}$) and growth light (130 µmol photons $m^{-2}s^{-1}$) on *Arabidopsis* wild type (ecotype Colombia) and *re* were examined.

According to the NMR metabolomic investigations of wild type and *re* by applying PCA model, a clear trend of difference was observed between the wild type and *re* in two different light intensities. The model revealed ($R^2X_{(cum)} = 0.89$ and $Q^2_{(cum)} = 0.84$) separation in the first and the second component, which covered 81.75 % of the total variance (Figure 4A, Study II). However, no difference was observed between the metabolites of wild type and *re* at the same light intensities based on the PCA model (refer to the section 5.3).

Likewise, the data was tested by the PLS-DA model by defining two groups of light intensities, growth light and high light either for wild type (Figure 18) or *re* mutant (Figure 19). The PLS-DA (Figure 18 A) scores plot t[2] vs. t[1] of wild type at two different illuminations (growth light and high light) displayed $R^2X_{(cum)} = 0.88$, $R^2Y_{(cum)} = 0.97$ and $Q^2_{(cum)} = 0.95$ and a clear discrimination was observed between wild type plants in two different light intensities. The first two components explained 64.01 % of the total variation. The corresponding loadings plot (Figure 18 B) showed the main variables that caused the difference on the first principal component p[1].

**A)**




**B)**

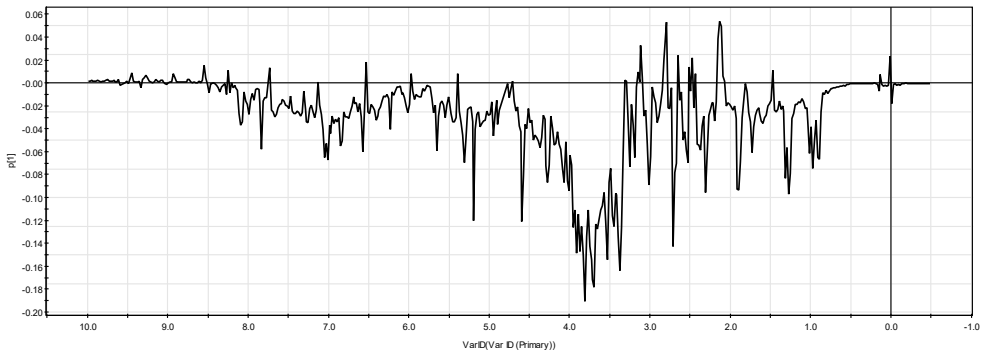
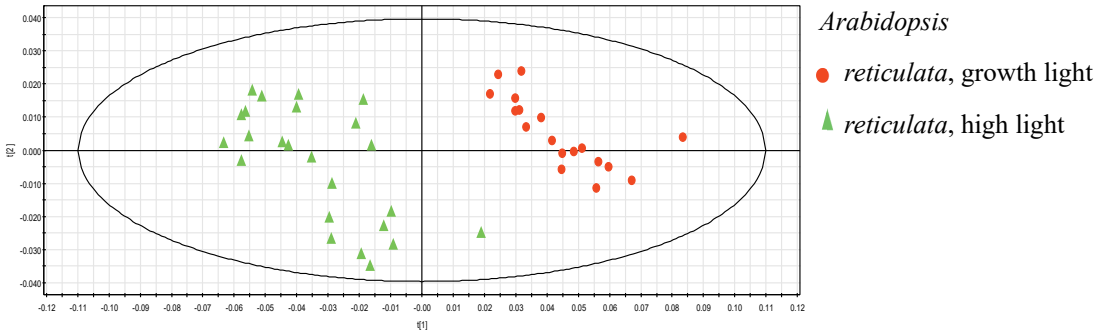



**Figure 18. (A)** The PLS-DA scores plot t[2] vs. t[1]; $R^2X_{(cum)} = 0.88$, $R^2Y_{(cum)} = 0.97$ and $Q^2_{(cum)} = 0.95$; observations colored according to *Arabidopsis* wild type under growth light and high light **(B)** The corresponding loadings plot p[1] vs. VarID (ppm shifts).

The PLS-DA model (Figure 19 A) of *re* under growth light and high light showed $R^2X_{(cum)} = 0.91$, $R^2Y_{(cum)} = 0.98$ and $Q^2_{(cum)} = 0.93$. Both $R^2X_{(cum)}$ and $R^2Y_{(cum)}$ illustrated excellent goodness-of-fit and the predictive ability ($Q^2_{(cum)}$). The first two components explained 73.83 % of the total variance. The model represented separation clearly in t[1] axis. The loadings line plot (Figure 19 B) of the PLS-DA model showed the variables that caused distinctions on the p[1].

**A)**




**B)**

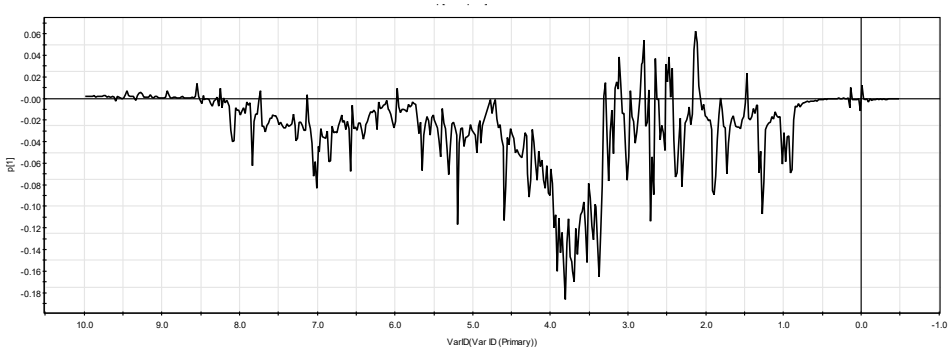



**Figure 19. (A)** PLS-DA scores plot; $R^2X_{(cum)} = 0.91$, $R^2Y_{(cum)} = 0.98$ and $Q^2_{(cum)} = 0.93$; observations colored according to *Arabidopsis re* under growth light and high light. **(B)** The corresponding loadings plot p[1] vs. VarID.

The corresponding results are similar to the obtained results from the PCA model in the article III. Loadings line plot of PLS-DA and PCA displayed similar metabolites (variables) causing the differences. The concentrations of all metabolites were higher in the wild type and *re* mutant that were grown under high light, except for glutamine, alanine, glutamic and aspartic acids. Consequently, light intensity significantly impacts on metabolic modulations in *Arabidopsis.* A large number of metabolites were more characteristics under high light due to the plant defense responses to the stress parameter. Prior studies have shown that under high light stress the chemical compounds involved in the TCA cycle as well as the sugar content are increased, due to induction of photosynthetic activity in the plant and to prevent oxidative stress (Jänkänpää et al., 2012; Wulff-Zottele et al., 2010). Also, short day photoperiods are not the key factor on leaf reticulation in the reticulate phenotypes.

## 5.3. Similar metabolomic discriminations in different *Arabidopsis* genotypes under the same conditions based on the NMR

The NMR data and multivariate method analysis of *Arabidopsis* wild type, *pp2a-b'γ* mutant and *pp2a-b'γ 35S:PP2A-B'γ* complementation line (study I) at the same ages (four and six weeks old) showed similar metabolomic changes between wild type and mutant. The PCA model of wild type and *pp2a-b'γ* mutant at four weeks of age displayed $R^2X_{(cum)} = 0.89$ and $Q^2_{(cum)} = 0.69$ and the first two components explained 55.1 % of the total variation (Figure 1A, Study I). The PCA model of wild type and *pp2a-b'γ* mutant at six weeks old represented $R^2X_{(cum)} = 0.84$ and $Q^2_{(cum)} = 0.56$, which covered 68.7 % of the total variance (Figure 1B, Study I). Examples of $^1$H NMR and PCA model of wild type and *pp2a-b'γ* mutant (control line was excluded) at two different ages are displayed in Figure 20.

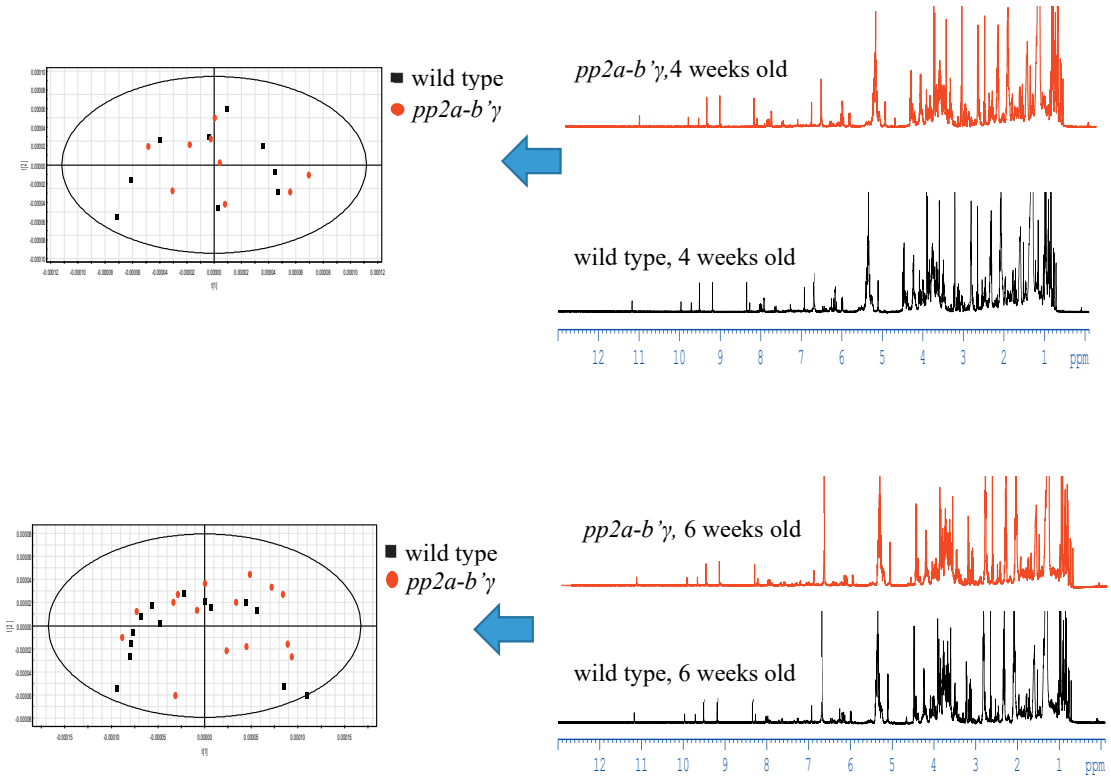



**Figure 20.** Examples of $^1$H NMR of four and six weeks old *Arabidopsis*, wild type and *pp2a-b'γ* mutant; PCA model of wild type and *pp2a-b'γ* mutant at the same ages (either four or six weeks old).

NMR metabolomic study (II) of *Arabidopsis* wild type and *re* mutant under the same light intensities (growth light or high light) revealed no distinction between the metabolites in wild type and *re* at the same light conditions. The corresponding PCA model of wild type and *re* under growth light showed $R^2X_{(cum)} = 0.90$ and $Q^2_{(cum)} = 0.78$ which covered 76.17 % of the

total variation (Figure 6A, Study II). Likewise, PCA model of wild type and *re* under high light demonstrated $R^2X_{(cum)} = 0.84$ and $Q^2_{(cum)} = 0.74$. No changes were observed between the metabolites and the first two principal components explained 69.39% of the total variation (Figure 6B, Study II). PCA model and illustrations of $^1$H NMR spectra of wild type and *re* mutant at the same light intensities are displayed in Figure 21.

In order to understand the main role of ACO3 phosphorylation in metabolic regulations of plant, *Arabidopsis* wild type and the aconitase phosphodeletion and phosphomimic mutants (*aco3*, *aco3 pACO3::ACO3*, *aco3 pACO3::ACO3*$^{S91A}$ and *aco3 pACO3::ACO3*$^{S91D}$) were analyzed by NMR (study III). No metabolic differences were observed between different genotypes of *Arabidopsis* (wild type and *aconitase* mutants). The PCA model of this study showed $R^2X_{(cum)} = 0.79$ and $Q^2_{(cum)} = 0.58$, which covered 67.49 % by the first two principal components (Figure 4B, Study III).

Neither studies I and II nor study III could distinguish discriminations between the metabolites of *Arabidopsis* wild type and different mutants at the similar conditions based on the NMR. In general, NMR is one of the main method for identification of the chemical structures of the metabolites (Elipe, 2003). However, one of the most significant challenge in order to applying NMR as an analytical tool is related to the low sensitivity of NMR (Elipe, 2003; Emwas, 2015) to identify low concentration compounds in the sample. To improve this problem and increasing NMR sensitivity, numbers of adjustment can be performed. For instance, increasing the strength of magnetic field (Gruetter et al., 1998) and utilizing specific probes, such as microprobes (Grimes and O'Connell, 2011), have enormously elevated the sensitivity of NMR.

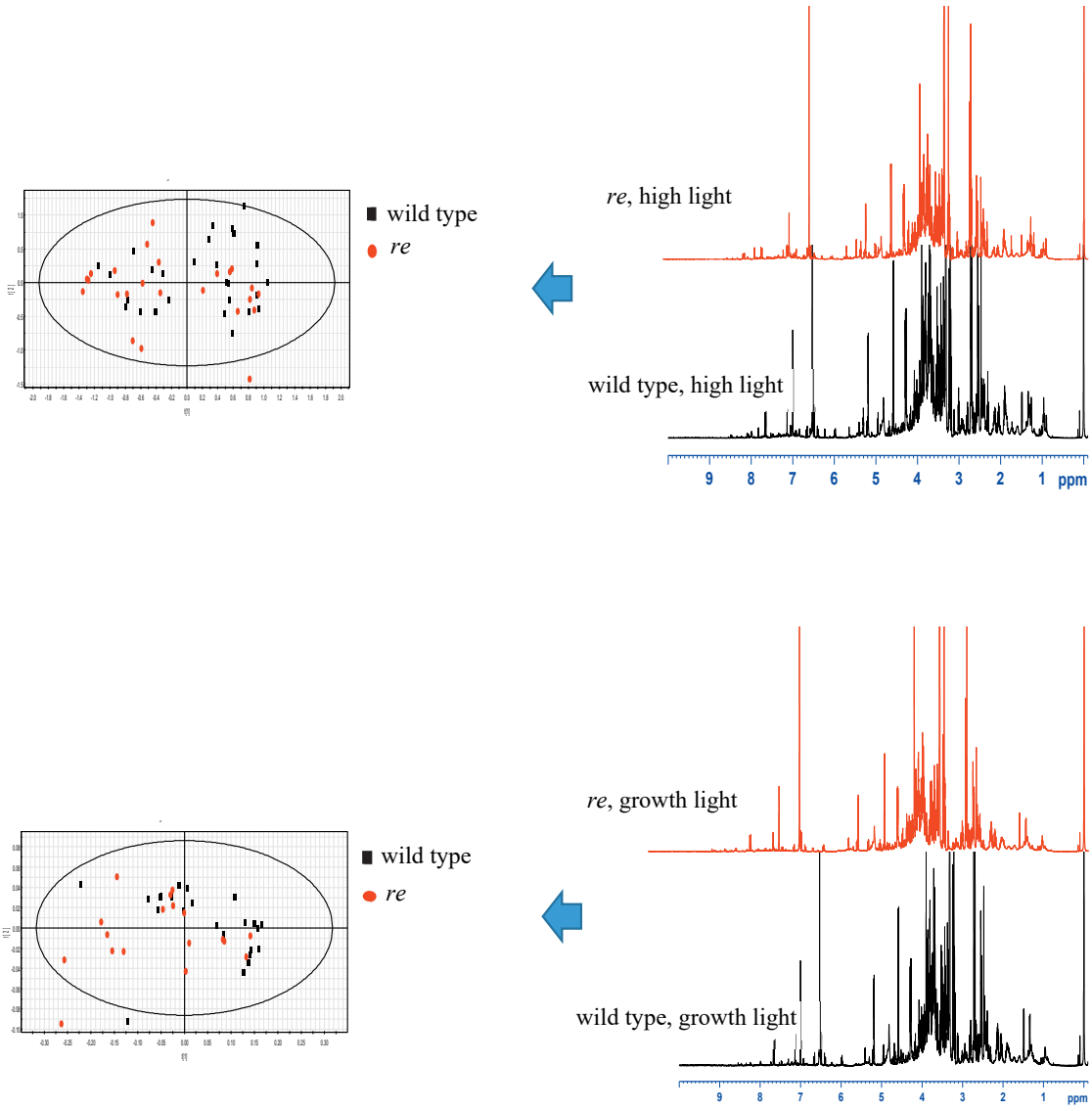



**Figure 21.** Illustrations of $^{1}$H NMR of *Arabidopsis*, wild type and *reticulata* (*re*) mutant under growth light and high light; PCA model of wild type and *re* at the same illuminations (either growth light or high light).

## 5.4. Fresh and herbarium mushroom species and NMR metabolomics

The fresh mushroom species *Kuehneromyces mutabilis*, *Hypholoma capnoides*, *Kuehneromyces lignicola* and *Hypholoma fasciculare* were collected from ten different collecting sites in South Western part of Finland. In addition, their corresponding herbarium mushroom specimens were obtained from the combined Herbarium

(Department of Biology, University of Turku and Åbo Akademi University). Herbarium samples were collected from different periods of time (1990 – 2014), the time scale covering approximately 25 years.

The result of this study demonstrated that concentration of α-α-trehalose was significantly higher compared to the other sugars (β-glucose and β-xylose) among the fresh mushroom specimens, which were easily recognized due to their high intensity. Comparison of freshly freeze-dried mushroom samples and air dried herbarium mushroom samples which were freeze-dried after collection, revealed that the sugar content was increased in fresh mushroom specimens.

Total content of amino acids (TCAA) was more abundant in herbarium mushroom samples compared to the fresh mushroom specimens, which might be due to the degradation of some proteins in herbarium specimens. However, previous studies have shown the content of protein remained constant in air-dried (at + 40 ºC) or by freeze-dried (at – 20 ºC) mushrooms, unless they were boiled (Barros et al., 2007; Kalač, 2009) since boiling declines the content of protein significantly. Generally, the method of drying and the time of harvesting directly impact on the TCAA. In addition, storage conditions have a significant impact on chemical compounds and nutrients in mushrooms (Manzi et al., 1999; Mattila et al., 2002).

While organic acids, including fumaric and malic acids, displayed more abundancy in fresh mushroom samples, succinic acid and GABA were more characteristic in herbarium mushroom specimens. Malic acid as a main organic acid provides the umami taste in mushroom. GABA also produces the sour taste in mushroom (Rotzoll et al., 2005). Succinic acid impacts on inducing flavor in mushrooms (Baines and Seal, 2012).

The signals in $^{1}$H NMR, which belonged to the fatty acids were higher in herbarium mushrooms compared to the fresh mushroom samples. However, the precise identification of fatty acids was not accomplished due to the low sensitivity of NMR to identify them.

Among different derivatives of fasciculic acids (Akasaka and Shiono, 2005); fasciculic acid E (Figure 22) was identified in both fresh and herbarium *H. fascicular*. Fasciculic acid E was more concentrated in fresh mushroom specimens.

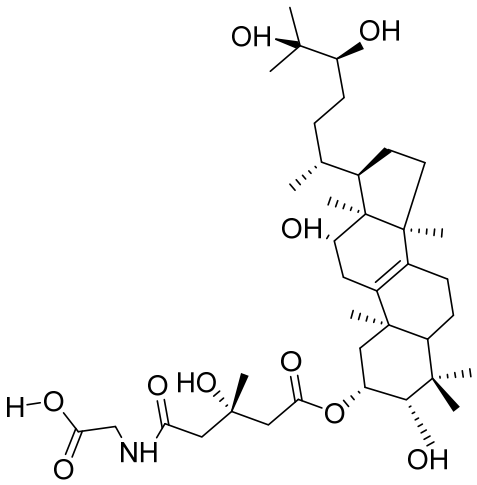



**Figure 22.** The chemical structure of fasciculic acid E; the compound was identified in *H. fascicular* fresh and corresponding herbarium mushroom samples.

The statistical analysis of fresh and herbarium mushroom species also demonstrated clear distinction between them (fresh samples vs. herbarium samples) based on the PCA model. The model showed $R^2X_{(cum)} = 0.97$ and $Q^2_{(cum)} = 0.92$ and explained 57.56 % of the total variance by the first two principal components (Figure 2A, Study IV).

Other interesting discovery was observation of no metabolic changes between the herbarium samples which were collected at different time scale (up to 25 years). Hence, air-drying technique can conserve the nutrients and chemical compositions in mushroom and could be utilized as a good method to store mushrooms.

The PCA models of: *K. mutabilis* herbarium into different ages (1990-1994, 2000-2004 and 2010-2014) represented excellent goodness-of-fit ($R^2X_{(cum)} = 0.88$) and predictive ability ($Q^2_{(cum)} = 0.67$) which covered 62.7% of the total variation (Figure 5A, Study IV). *H. capnoides* showed $R^2X_{(cum)} = 0.90$ and $Q^2_{(cum)} = 0.77$, that explained 57.49% of the total variance (Figure 5B, Study IV).

Consequently, it seems herbarium mushroom species can be used as a research materials in the future studies of mushrooms due to the stability of chemical compounds in air dried mushrooms, which can open a lot of possibility to utilize herbarium mushroom samples worldwide. As such, NMR is shown to be a promising tool to screen a large number of chemical components of fungal species.

# 6. CONCLUSIONS

In this thesis the NMR profiling method that can discriminate the impact of environmental cues on plants has been developed. Simultaneously, the results of different sub-studies highlight the advantage and disadvantage of NMR to identify different metabolites. NMR technique allows to detect the vast range of metabolites with setting a single experiment, for instance, proton measurement. However, the results indicate the disadvantage of using NMR to distinguish less-abundant metabolites in targeted biological systems; e.g. investigation of the functions of different genes on metabolomic adjustments of *Arabidopsis* (mutants) by NMR. Nevertheless, the chemical composition of the plant does not change dramatically upon mutation. Thus, these results can provide better understanding for future metabolomics analysis by NMR.

Fingerprinting of metabolites in different growing stages including vegetative and developing (maturation) phases influence on adjustment of metabolites in *Arabidopsis*. Metabolic profiling of *Arabidopsis* wild type and *re* mutant under light stress shows significant impacts of high-light on modulating of metabolites in *Arabidopsis.* Nevertheless, based on the NMR technique no clear trends were observed between the metabolites in various genotypes of *Arabidopsis* due to the low sensitivity of NMR to distinguish less concentrated compounds in the sample.

NMR metabolomic study of fresh and herbarium mushrooms revealed air drying method is a convenient technique to conserve the nutritional value of herbarium mushrooms.

The multivariate methods discussed in the thesis provided comprehensive overview of the studied subjects including different genotypes of *Arabidopsis* (wild type, *pp2a-b'γ, reticulata* and *aconitase*) and also fresh and herbarium mushroom species (*Kuehneromyces mutabilis*, *Kuehneromyces lignicola, Hypholoma capnoides* and *Hypholoma fasciculare*).

All the sub-studies in this thesis indicated that NMR based metabolomics analysis is a remarkable technique to comprehend metabolomics profiling of plant and mushroom under different conditions.

# REFERENCES


Abdel-Farid, I. B.; Kim, H. K.; Choi, Y. H.; Verpoorte, R. Metabolic characterization of Brassica rapa leaves by NMR spectroscopy. *Agricultural and Food Chemistry*. **2007**, *55* (19), 7936–7943.

Agrahar-Murugkar, D.; Subbulakshmi, G. Nutritional value of edible wild mushrooms collected from the Khasi hills of Meghalaya. *Food Chemistry*. **2005**, *89* (4), 599–603.

Aherne, S. A.; O'Brien, N. M. Dietary flavonols: chemistry, food content, and metabolism. *Nutrition*. **2002**, *18* (1), 75–81.

Akasaka, H.; Shiono, Y. Fascicularones H–K, Four new sesquiterpenoids from the cultured mycelia of the fungus *Hypholoma fasciculare*. *Helvetica chimica acta*. **2005**, *88* (11), 2944–2950.

Alonso, J. M.; Stepanova, A. N.; Leisse, T. J.; Kim, C. J.; Chen, H.; Shinn, P.; Stevenson, D. K.; Zimmerman, J.; Barajas, P.; Cheuk, R. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*. **2003**, *301* (5633), 653–657.

Apse, M. P.; Blumwald, E. Engineering salt tolerance in plants. *Current Opinion in Biotechnology*. **2002**, *13* (2), 146–150.

Aretz, I.; Meierhofer, D. Advantages and pitfalls of mass spectrometry based metabolome profiling in systems biology. *International journal of molecular sciences*. **2016**, *17* (5), 632.

Arnaud, N.; Ravet, K.; Borlotti, A.; Touraine, B.; Boucherez, J.; Fizames, C.; Briat, J.-F.; Cellier, F.; Gaymard, F. The iron-responsive element (IRE)/iron-regulatory protein 1 (IRP1)–cytosolic aconitase iron-regulatory switch does not operate in plants. *Biochemical Journal*. **2007**, *405* (3), 523–531.

Bae, H.; Herman, E.; Bailey, B.; Bae, H. J.; Sicher, R. Exogenous trehalose alters *Arabidopsis* transcripts involved in cell wall modification, abiotic stress, nitrogen metabolism, and plant defense. *Physiologia Plantarum*. **2005**, *125* (1), 114–126.

Baines, D.; Seal, R. Natural food additives, ingredients and flavourings. *Elsevier*. 2012; pp 90–92.

Barros, L.; Baptista, P.; Correia, D. M.; Sá Morais, J.; Ferreira, I. C. Effects of conservation treatment and cooking on the chemical composition and antioxidant activity of Portuguese wild edible mushrooms. *Journal of Agricultural and Food Chemistry*. **2007**, *55* (12), 4781–4788.

Barros, L.; Cruz, T.; Baptista, P.; Estevinho, L. M.; Ferreira, I. C. Wild and commercial mushrooms as source of nutrients and nutraceuticals. *Food and Chemical Toxicology*. **2008**, *46* (8), 2742–2747.

Barth, C.; Conklin, P. L. The lower cell density of leaf parenchyma in the *Arabidopsis thaliana* mutant *lcd1-1* is associated with increased sensitivity to ozone and virulent *Pseudomonas syringae*. *The Plant Journal*. **2003**, *35* (2), 206–218.

BeGora, M. D.; Macleod, M. J.; McCarry, B. E.; Summers, P. S.; Weretilnyk, E. A. Identification of phosphomethylethanolamine N-methyltransferase from *Arabidopsis* and its role in choline and phospholipid metabolism. *Journal of Biological Chemistry*. **2010**, *285* (38), 29147–29155.

Beluhan, S.; Ranogajec, A. Chemical composition and non-volatile components of Croatian wild edible mushrooms. *Food Chemistry*. **2011**, *124* (3), 1076–1082.

Bobek, P.; Galbavý, Š. Hypocholesterolemic and antiatherogenic effect of oyster mushroom (*Pleurotus ostreatus*) in rabbits. *Molecular Nutrition & Food Research*. **1999**, *43* (5), 339–342.

Bolwell, G. P.; Bindschedler, L. V.; Blee, K. A.; Butt, V. S.; Davies, D. R.; Gardner, S. L.; Gerrish, C.; Minibayeva, F. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of Experimental Botany*. **2002**, *53* (372), 1367–1376.

Böttcher, C.; von Roepenack-Lahaye, E.; Schmidt, J.; Schmotz, C.; Neumann, S.; Scheel, D.; Clemens, S. Metabolome analysis of biosynthetic mutants reveals a diversity of metabolic changes and allows identification of a large number of new compounds in *Arabidopsis*. *Plant physiology*. **2008**, *147* (4), 2107–2120.

Bottomley, P. A. Human in vivo NMR spectroscopy in diagnostic medicine: Clinical tool or research probe? *Radiology*. **1989**, *170* (1), 1–15.

Bouché, N.; Bouchez, D. *Arabidopsis* gene knockout: phenotypes wanted. *Current opinion in plant biology*. **2001**, *4* (2), 111–117.

Bourgaud, F.; Gravot, A.; Milesi, S.; Gontier, E. Production of plant secondary metabolites: a historical perspective. *Plant science*. **2001**, *161* (5), 839–851.

Brennan, L. NMR-based metabolomics: from sample preparation to applications in nutrition research. *Progress in nuclear magnetic resonance spectroscopy*. **2014**, *83*, 42–49.

Brereton, R. G.; Lloyd, G. R. Partial least squares discriminant analysis: taking the magic away. *Journal of Chemometrics*. **2014**, *28* (4), 213–225.

Bundy, J. G.; Davey, M. P.; Viant, M. R. Environmental metabolomics: a critical review and future perspectives. *Metabolomics*. **2009**, *5* (1), 3.

Cevallos-Cevallos, J. M.; Reyes-De-Corcuera, J. I.; Etxeberria, E.; Danyluk, M. D.; Rodrick, G. E. Metabolomic analysis in food science: a review. *Trends in Food Science & Technology*. **2009**, *20* (11), 557–566.

Chalker-Scott, L. Environmental significance of anthocyanins in plant stress responses. *Photochemistry and photobiology*. **1999**, *70* (1), 1–9.

Chaouch, S.; Queval, G.; Vanderauwera, S.; Mhamdi, A.; Vandorpe, M.; Langlois-Meurinne, M.; Van Breusegem, F.; Saindrenan, P.; Noctor, G. Peroxisomal hydrogen peroxide is coupled to biotic defense responses by ISOCHORISMATE SYNTHASE1 in a daylength-related manner. *Plant Physiology*. **2010**, *153* (4), 1692–1705.

Chia, D. W.; Yoder, T. J.; Reiter, W.-D.; Gibson, S. I. Fumaric acid: an overlooked form of fixed carbon in *Arabidopsis* and other plant species. *Planta*. **2000**, *211* (5), 743–751.

Chinnusamy, V.; Dalal, M.; Zhu, J. K. Epigenetic regulation of abiotic stress responses in plants. *Plant Abiotic Stress, Second Edition*. **2014**, 203–229.

Cho, I. H.; Kim, Y.-S.; Choi, H. K. Metabolomic discrimination of different grades of pine-mushroom (*Tricholoma matsutake Sing.*) using $^{1}$H NMR spectrometry and multivariate data analysis. *Journal of pharmaceutical and biomedical analysis*. **2007**, *43* (3), 900–904.

Choi, H.-K.; Choi, Y. H.; Verberne, M.; Lefeber, A. W.; Erkelens, C.; Verpoorte, R. Metabolic fingerprinting of wild type and transgenic tobacco plants by $^{1}$H NMR and multivariate analysis technique. *Phytochemistry*. **2004a**, *65* (7), 857–864.

Choi, Y. H.; Kim, H. K.; Linthorst, H. J.; Hollander, J. G.; Lefeber, A. W.; Erkelens, C.; Nuzillard, J.-M.; Verpoorte, R. NMR Metabolomics to Revisit the Tobacco Mosaic Virus Infection in *Nicotiana t abacum* Leaves. *Journal of Natural Products*. **2006**, *69* (5), 742–748.

Choi, Y. H.; Tapias, E. C.; Kim, H. K.; Lefeber, A. W.; Erkelens, C.; Verhoeven, J. T. J.; Brzin, J.; Zel, J.; Verpoorte, R. Metabolic discrimination of *Catharanthus roseus* leaves infected by phytoplasma using $^{1}$H-NMR spectroscopy and multivariate data analysis. *Plant Physiology*. **2004b**, *135* (4), 2398–2410.

Corrales, A. R.; Carrillo, L.; Lasierra, P.; Nebauer, S. G.; Dominguez-Figueroa, J.; Renau-Morata, B.; Pollmann, S.; Granell, A.; Molina, R. V.; Vicente-Carbajosa, J. Multifaceted role of cycling Dof Factor 3 (CDF3) in the regulation of flowering time and abiotic stress responses in *Arabidopsis*. *Plant, cell & environment*. **2017**, *40* (5), 748–764.

Courtois-Verniquet, F.; Douce, R. Lack of aconitase in glyoxysomes and peroxisomes. *Biochemical Journal*. **1993**, *294* (1), 103–107.

Craig, A.; Cloarec, O.; Holmes, E.; Nicholson, J. K.; Lindon, J. C. Scaling and normalization effects in NMR spectroscopic metabonomic data sets. *Analytical chemistry*. **2006**, *78* (7), 2262–2267.

Diaz, C.; Lemaître, T.; Christ, A.; Azzopardi, M.; Kato, Y.; Sato, F.; Morot-Gaudry, J.-F.; Le Dily, F.; Masclaux-Daubresse, C. Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in *Arabidopsis* under low nitrogen nutrition. *Plant Physiology*. **2008**, *147* (3), 1437–1449.

Ding, Y.; Bao, H. Y.; Bau, T.; Li, Y.; Kim, Y. H. Antitumor components from *Naematoloma fasciculare*. *Journal of microbiology and biotechnology*. **2009**, *19* (10), 1135–1138.

Durian, G.; Rahikainen, M.; Alegre, S.; Brosché, M.; Kangasjärvi, S. Protein phosphatase 2A in the regulatory network underlying biotic stress resistance in plants. *Frontiers in plant science*. **2016**, *7*.

Elipe, M. V. S. Advantages and disadvantages of nuclear magnetic resonance spectroscopy as a hyphenated technique. *Analytica Chimica Acta*. **2003**, *497* (1), 1–25.

Elmastas, M.; Isildak, O.; Turkekul, I.; Temur, N. Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. *Journal of Food Composition and Analysis*. **2007**, *20* (3), 337–345.

Emwas, A. H. M. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Metabonomics: Methods and Protocols*. **2015**, 161–193.

Enders, T. A.; Oh, S.; Yang, Z.; Montgomery, B. L.; Strader, L. C. Genome sequencing of *Arabidopsis* abp1-5 reveals second-site mutations that may affect phenotypes. *The Plant Cell*. **2015**, *27* (7), 1820–1826.

Eriksson, L.; Byrne, T.; Johansson, E.; Trygg, J.; Vikström, C. Multi-and megavariate data analysis basic principles and applications. Umetrics Academy, 2013; pp 22–500.

Fernie, A. R.; Schauer, N. Metabolomics-assisted breeding: a viable option for crop improvement? *Trends in Genetics*. **2009**, *25* (1), 39–48.

Fiehn, O. Metabolomics—the link between genotypes and phenotypes. Functional Genomics. Springer, 2002; pp 155–171.

Fritsche, W. Enhancement of pyrene mineralization in soil by wood-decaying fungi. *FEMS Microbiology Ecology*. **1997**, *22* (1), 77–83.

Gan, S.; Amasino, R. M. Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). *Plant physiology*. **1997**, *113* (2), 313–319.

Gill, S. S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant physiology and biochemistry*. **2010**, *48* (12), 909–930.

Goldberg, R. B. Plants: novel developmental processes. *Science*. **1988**, *240* (4858), 1460.

González-Bayón, R.; Kinsman, E. A.; Quesada, V.; Vera, A.; Robles, P.; Ponce, M. R.; Pyke, K. A.; Micol, J. L. Mutations in the RETICULATA gene dramatically alter internal architecture but have little effect on overall organ shape in *Arabidopsis* leaves. *Journal of experimental botany*. **2006**, *57* (12), 3019–3031.

Gooday, G. Control of development of excised fruit bodies and stipes of *Coprinus cinereus*. *Transactions of the British Mycological Society*. **1974**, *62* (2), 391–399.

Gou, W.; Tian, L.; Ruan Zh, Z. P.; Chen, F.; Zhang, L.; Cui Zh, Z. P.; Li Zh, G. M.; Shi, W.; Zhang, L.; Liu, J. Accumulation of choline and glycinebetaine and drought stress tolerance induced in maize (*Zea mays*) by tree plant growth promoting Rhizobacteria (PGPR) strains. *Pak J Bot*. **2015**, *47* (2), 581–586.

Griffiths, W. J.; Koal, T.; Wang, Y.; Kohl, M.; Enot, D. P.; Deigner, H. P. Targeted metabolomics for biomarker discovery. *Angewandte Chemie International Edition*. **2010**, *49* (32), 5426–5445.

Grimes, J. H.; O'Connell, T. M. The application of micro-coil NMR probe technology to metabolomics of urine and serum. *Journal of biomolecular NMR*. **2011**, *49* (3-4), 297–305.

Gruetter, R.; Weisdorf, S. A.; Rajanayagan, V.; Terpstra, M.; Merkle, H.; Truwit, C. L.; Garwood, M.; Nyberg, S. L.; Ugurbil, K. Resolution Improvements inin Vivo $^{1}$H NMR Spectra with Increased Magnetic Field Strength. *Journal of magnetic resonance*. **1998**, *135* (1), 260–264.

Günther, H. NMR spectroscopy: basic principles, concepts and applications in chemistry. John Wiley & Sons, 2013; pp 734.

Gurtas Seyhan, F.; Evranuz, Ö. Low temperature mushroom (*A. bisporus*) drying with desiccant dehumidifiers. *Drying technology*. **2000**, *18* (1-2), 433–445.

Harrigan, G. G.; Goodacre, R. Metabolic profiling: its role in biomarker discovery and gene function analysis. Springer Science & Business Media, 2012; pp 2–334.

Hayashi, M.; De Bellis, L.; Alpi, A.; Nishimura, M. Cytosolic aconitase participates in the glyoxylate cycle in etiolated pumpkin cotyledons. *Plant and cell physiology*. **1995**, *36* (4), 669–680.

Heleno, S. A.; Barros, L.; Martins, A.; Queiroz, M. J. R.; Morales, P.; Fernández-Ruiz, V.; Ferreira, I. C. Chemical composition, antioxidant activity and bioaccessibility studies in phenolic extracts of two *Hericium* wild edible species. *LWT-Food Science and Technology*. **2015**, *63* (1), 475–481.

Heleno, S. A.; Barros, L.; Sousa, M. J.; Martins, A.; Ferreira, I. C. Study and characterization of selected nutrients in wild mushrooms from Portugal by gas chromatography and high performance liquid chromatography. *Microchemical Journal*. **2009**, *93* (2), 195–199.

Heleno, S. A.; Barros, L.; Sousa, M. J.; Martins, A.; Ferreira, I. C. Tocopherols composition of

Portuguese wild mushrooms with antioxidant capacity. *Food Chemistry*. **2010**, *119* (4), 1443–1450.

Hentze, M. W.; Kühn, L. C. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proceedings of the National Academy of Sciences*. **1996**, *93* (16), 8175–8182.

Hills, B. Applications of low-field NMR to food science. *Annual reports on NMR spectroscopy*. **2006**, *58*, 177–230.

Hirai, M. Y.; Yano, M.; Goodenowe, D. B.; Kanaya, S.; Kimura, T.; Awazuhara, M.; Arita, M.; Fujiwara, T.; Saito, K. Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. *Proceedings of the National academy of Sciences of the United States of America*. **2004**, *101* (27), 10205–10210.

Hirling, H.; Henderson, B.; Kühn, L. Mutational analysis of the [4Fe-4S]-cluster converting iron regulatory factor from its RNA-binding form to cytoplasmic aconitase. *The EMBO journal*. **1994**, *13* (2), 453.

Hong, J.; Yang, L.; Zhang, D.; Shi, J. Plant metabolomics: an indispensable system biology tool for plant science. *International journal of molecular sciences*. **2016**, *17* (6), 767.

Hooks, M. A.; Allwood, J. W.; Harrison, J. K.; Kopka, J.; Erban, A.; Goodacre, R.; Balk, J. Selective induction and subcellular distribution of ACONITASE 3 reveal the importance of cytosolic citrate metabolism during lipid mobilization in *Arabidopsis*. *Biochemical Journal*. **2014**, *463* (2), 309–317.

Hosaka, K.; Uno, K. Assessment of the DNA quality in mushroom specimens: effect of drying temperature. *Bull. Natl. Mus. Nat. Sci.* **2011**, *37*, 101–111.

Hotelling, H. Analysis of a complex of statistical variables into principal components. *Journal of educational psychology*. **1933**, *24* (6), 417.

Hoult, D. Solvent peak saturation with single phase and quadrature Fourier transformation. *Journal of Magnetic Resonance (1969)*. **1976**, *21* (2), 337–347.

Hörtensteiner, S. Chlorophyll degradation during senescence. *Annu. Rev. Plant Biol.* **2006**, *57*, 55–77.

Ishida, N.; Koizumi, M.; Kano, H. The NMR microscope: a unique and promising tool for plant science. *Annals of Botany*. **2000**, *86* (2), 259–278.

Jaakola, L.; Hohtola, A. Effect of latitude on flavonoid biosynthesis in plants. *Plant, cell & environment*. **2010**, *33* (8), 1239–1247.

Johnson, S. R.; Lange, B. M. Open-access metabolomics databases for natural product research: present capabilities and future potential. *Frontiers in bioengineering and biotechnology*. **2015**, *3*.

Jänkänpää, H. J.; Mishra, Y.; Schröder, W. P.; Jansson, S. Metabolic profiling reveals metabolic shifts in *Arabidopsis* plants grown under different light conditions. *Plant, cell & environment*. **2012**, *35* (10), 1824–1836.

Kalač, P. Chemical composition and nutritional value of European species of wild growing mushrooms: A review. *Food chemistry*. **2009**, *113* (1), 9–16.

Kangasjärvi, S.; Neukermans, J.; Li, S.; Aro, E. M.; Noctor, G. Photosynthesis, photorespiration, and light signalling in defence responses. *Journal of experimental botany*. **2012**, *63* (4), 1619–1636.

Kangasjärvi, S.; Nurmi, M.; Tikkanen, M.; A. R. O, E. Cell-specific mechanisms and systemic signalling as emerging themes in light acclimation of C3 plants. *Plant, cell & environment*. **2009**, *32* (9), 1230-–1240.

Keeler, J. Understanding NMR spectroscopy. John Wiley & Sons, 2011; pp 2–211.

Keurentjes, J. J.; Fu, J.; De Vos, C. R.; Lommen, A.; Hall, R. D.; Bino, R. J.; van der Plas, L. H.; Jansen, R. C.; Vreugdenhil, D.; Koornneef, M. The genetics of plant metabolism. *Nature genetics*. **2006**, *38* (7), 842–849.

Khachik, F.; Beecher, G. R.; Whittaker, N. F. Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *Journal of Agricultural and Food Chemistry*. **1986**, *34* (4), 603–616.

Khakimov, B.; Bak, S.; Engelsen, S. B. High-throughput cereal metabolomics: Current analytical technologies, challenges and perspectives. *Journal of Cereal Science*. **2014**, *59* (3), 393–418.

Kim, H. K.; Choi, Y. H.; Verpoorte, R. NMR-based plant metabolomics: where do we stand, where do we go? *Trends in biotechnology*. **2011**, *29* (6), 267–275.

Kim, H. K.; Choi, Y. H.; Verpoorte, R. NMR-based metabolomic analysis of plants. *Nature protocols*. **2010**, *5* (3), 536–549.

Kim, H. K.; Verpoorte, R. Sample preparation for plant metabolomics. *Phytochemical Analysis*. **2010**, *21* (1), 4–13.

Kinsman, E.; Pyke, K. Bundle sheath cells and cell-specific plastid development in *Arabidopsis* leaves. *Development*. **1998**, *125* (10), 1815–1822.

Klausner, R. D.; Rouault, T. A.; Harford, J. B. Regulating the fate of mRNA: the control of cellular iron metabolism. *Cell*. **1993**, *72* (1), 19–28.

Knudsen, H. Funga nordica: agaricoid, boletoid, clavarioid, cyphelloid and gastroid genera. Nordsvamp. 2012; pp 2–1083.

Konert, G.; Rahikainen, M.; Trotta, A.; Durian, G.; Salojärvi, J.; Khorobrykh, S.; Tyystjärvi, E.; Kangasjärvi, S. Subunits B′ γ and B′ ζ of protein phosphatase 2A regulate photo-oxidative stress responses and growth in *Arabidopsis thaliana*. Plant, cell & environment. **2015a**, 38 (12), 2641–2651.

Konert, G.; Trotta, A.; Kouvonen, P.; Rahikainen, M.; Durian, G.; Blokhina, O.; Fagerstedt, K.; Muth, D.; Corthals, G. L.; Kangasjärvi, S. Protein phosphatase 2A (PP2A) regulatory subunit B′ γ interacts with cytoplasmic ACONITASE 3 and modulates the abundance of AOX1A and AOX1D in *Arabidopsis thaliana*. New Phytologist. **2015b**, 205 (3), 1250–1263.

Konert, G. PP2A: At The Crossroads Between Growth and Defence In Plants. 2014. Doctoral dissertation, University of Turku, ISBN 978-951-29-5935-8.

Kortesniemi, M. NMR Metabolomics of Foods–Investigating the Influence of Origin on Sea Buckthorn Berries, Brassica Oilseeds and Honey. 2016. Doctoral dissertation, University of Turku, ISBN 978-952-93-8154-8.

Kortesniemi, M.; Sinkkonen, J.; Yang, B.; Kallio, H. $^{1}$H NMR spectroscopy reveals the effect of genotype and growth conditions on composition of sea buckthorn (*Hippophae rhamnoides L.*) berries. *Food chemistry*. **2014**, *147*, 138–146.

Kortesniemi, M.; Slupsky, C. M.; Ollikka, T.; Kauko, L.; Spevacek, A. R.; Sjövall, O.; Yang, B.; Kallio, H. NMR profiling clarifies the characterization of Finnish honeys of different botanical origins. *Food Research International*. **2016**, *86*, 83–92.

Krieglsteiner, G. J. Verbreitungsatlas der Großpilze Deutschlands (West). Ulmer, 1991; pp 596.

Krishnan, P.; Kruger, N.; Ratcliffe, R. Metabolite fingerprinting and profiling in plants using NMR. *Journal of experimental botany*. **2004**, *56* (410), 255–265.

Lambert, M.; Wolfender, J. L.; Stærk, D.; Christensen, S. B.; Hostettmann, K.; Jaroszewski, J. W. Identification of natural products using HPLC-SPE combined with CapNMR. *Analytical chemistry*. **2007**, *79* (2), 727–735.

Larive, C. K.; Barding Jr, G. A.; Dinges, M. M. NMR spectroscopy for metabolomics and metabolic profiling. *Analytical chemistry*. **2014**, *87* (1), 133–146.

Lee, J. H.; Lee, M. J. Effect of drying method on the moisture sorption isotherms for Inonotus obliquus mushroom. *LWT-Food Science and Technology*. **2008**, *41* (8), 1478–1484.

Leiss, K. A.; Choi, Y. H.; Abdel-Farid, I. B.; Verpoorte, R.; Klinkhamer, P. G. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in Senecio hybrids. *Journal of chemical ecology*. **2009**, *35* (2), 219–229.

Li, H.; Culligan, K.; Dixon, R. A.; Chory, J. CUE1: a mesophyll cell-specific positive regulator of light-controlled gene expression in *Arabidopsis*. *The Plant Cell*. **1995**, *7* (10), 1599–1610.

Li, S.; Mhamdi, A.; Trotta, A.; Kangasjärvi, S.; Noctor, G. The regulatory subunit B′γ of protein phosphatase 2A acts downstream of oxidative stress in the control of salicylic acid responses by peroxisomal $H_2O_2$. *New Phytologist*. **2014**, *202*, 145–160.

Li, X.; Feng, T.; Zhou, F.; Zhou, S.; Liu, Y.; Li, W.; Ye, R.; Yang, Y. Effects of drying methods on the tasty compounds of *Pleurotus eryngii*. *Food chemistry*. **2015**, *166*, 358–364.

Lindon, J. C.; Nicholson, J. K.; Holmes, E. The handbook of metabonomics and metabolomics. Elsevier, 2011; pp 572.

Lo, K.; Cheung, P. C. Antioxidant activity of extracts from the fruiting bodies of *Agrocybe aegerita var*. alba. *Food Chemistry*. **2005**, *89* (4), 533–539.

Lohman, K. N.; Gan, S.; John, M. C.; Amasino, R. M. Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Physiologia Plantarum*. **1994**, *92* (2), 322–328.

Maher, A. D.; Zirah, S. F.; Holmes, E.; Nicholson, J. K. Experimental and analytical variation in human urine in $^{1}$H NMR spectroscopy-based metabolic phenotyping studies. *Analytical Chemistry*. **2007**, *79* (14), 5204–5211.

Manetti, C.; Bianchetti, C.; Bizzarri, M.; Casciani, L.; Castro, C.; D'Ascenzo, G.; Delfini, M.; Di Cocco, M. E.; Laganà, A.; Miccheli, A. NMR-based metabonomic study of transgenic maize. *Phytochemistry*. **2004**, *65* (24), 3187–3198.

Manzi, P.; Aguzzi, A.; Pizzoferrato, L. Nutritional value of mushrooms widely consumed in Italy. *Food chemistry*. **2001**, *73* (3), 321–325.

Manzi, P.; Gambelli, L.; Marconi, S.; Vivanti, V.; Pizzoferrato, L. Nutrients in edible mushrooms: an inter-species comparative study. *Food chemistry*. **1999**, *65* (4), 477–482.

Masclaux-Daubresse, C.; Reisdorf-Cren, M.; Orsel, M. Leaf nitrogen remobilisation for plant development and grain filling. *Plant Biology*. **2008**, *10* (s1), 23–36.

Mattila, P.; Salo-Väänänen, P.; Könkö, K.; Aro, H.; Jalava, T. Basic composition and amino acid contents of mushrooms cultivated in Finland. *Journal of Agricultural and Food Chemistry*. **2002**, *50* (22), 6419–6422.

Mau, J. L.; Lin, H. C.; Song, S. F. Antioxidant properties of several specialty mushrooms. *Food Research International*. **2002**, *35* (6), 519–526.

Mdachi, S. J.; Nkunya, M. H.; Nyigo, V. A.; Urasa, I. T. Amino acid composition of some Tanzanian wild mushrooms. *Food Chemistry*. **2004**, *86* (2), 179–182.

Meinke, D. W.; Cherry, J. M.; Dean, C.; Rounsley, S. D.; Koornneef, M. *Arabidopsis thaliana*: a model plant for genome analysis. *Science*. **1998**, *282* (5389), 662–682.

Meyerowitz, E. M.; Pruitt, R. E. *Arabidopsis thaliana* and plant molecular genetics. *Science*. **1985**, *229* (4719), 1214–1218.

Michalko, J.; Glanc, M.; Perrot-Rechenmann, C.; Friml, J. Strong morphological defects in conditional *Arabidopsis abp1* knock-down mutants generated in absence of functional ABP1 protein. *F1000Research*. **2016**, *5* (86), 1–13.

Mithöfer, A.; Schulze, B.; Boland, W. Biotic and heavy metal stress response in plants: evidence for common signals. *FEBS letters*. **2004**, *566* (1-3), 1–5.

Mittler, R. Abiotic stress, the field environment and stress combination. *Trends in plant science*. **2006**, *11* (1), 15–19.

Miyake, C.; Miyata, M.; Shinzaki, Y.; Tomizawa, K. I. $CO_2$ response of cyclic electron flow around PSI (CEF-PSI) in tobacco leaves—relative electron fluxes through PSI and PSII determine the magnitude of non-photochemical quenching (NPQ) of Chl fluorescence. *Plant and Cell Physiology*. **2005**, *46* (4), 629–637.

Mo, H.; Raftery, D. Solvent signal as an NMR concentration reference. *Analytical chemistry*. **2008**, *80* (24), 9835–9839.

Moeder, W.; Del Pozo, O.; Navarre, D. A.; Martin, G. B.; Klessig, D. F. Aconitase plays a role in regulating resistance to oxidative stress and cell death in *Arabidopsis* and *Nicotiana benthamiana*. *Plant molecular biology*. **2007**, *63* (2), 273–287.

Mollá-Morales, A.; Sarmiento-Mañús, R.; Robles, P.; Quesada, V.; Pérez-Pérez, J. M.; González-Bayón, R.; Hannah, M. A.; Willmitzer, L.; Ponce, M. R.; Micol, J. L. Analysis of *ven3* and *ven6 reticulata* mutants reveals the importance of arginine biosynthesis in *Arabidopsis* leaf development. *The Plant Journal*. **2011**, *65* (3), 335–345.

Mäkelä, V. Automated Analysis of Quantitative NMR Spectra. 2016. Doctoral dissertation, University of Helsinki, ISBN 978-951-51-1834-9.

Nagy, L. G.; Petkovits, T.; Kovács, G. M.; Voigt, K.; Vágvölgyi, C.; Papp, T. Where is the unseen fungal diversity hidden? A study of Mortierella reveals a large contribution of reference collections to the identification of fungal environmental sequences. *New Phytologist*. **2011**, *191* (3), 789–794.

Nam, H. G. The molecular genetic analysis of leaf senescence. *Current Opinion in Biotechnology*. **1997**, *8* (2), 200–207.

Noodén, L.; Leopold, A. Phytohormones and the endogenous regulation of senescence and abscission. *Phytohormones and related compounds: a comprehensive treatise*. **1978**, *2*, 329–369.

Noodén, L. D. Integration of soybean pod development and monocarpic senescence. *Physiologia plantarum*. **1984**, *62* (2), 273–284.

Noodén, L. D. Senescence and aging in plants. Elsevier, 2012; pp 3–512.

Noodén, L. D.; Guiamét, J. J.; John, I. Whole plant senescence. Plant Cell Death Processes. Elsevier, 2004; pp 227–244.

Noodén, L. D.; Penney, J. P. Correlative controls of senescence and plant death in *Arabidopsis thaliana* (Brassicaceae). *Journal of Experimental Botany*. **2001**, *52* (364), 2151–2159.

O'Gorman, A. Metabolic profiling and fingerprinting for the detection and discrimination of mechanical damage in mushrooms (*Agaricus bisporus*) during storage. 2010. Doctoral dissertation, Aoife O'Gorman, *Dublin Institute of Technology*, doi:10.21427/D7DK5P.

Okazaki, Y.; Saito, K. Recent advances of metabolomics in plant biotechnology. *Plant biotechnology reports*. **2012**, *6* (1), 1–15.

Oliver, S. G.; Winson, M. K.; Kell, D. B.; Baganz, F. Systematic functional analysis of the yeast genome. *Trends in biotechnology*. **1998**, *16* (9), 373–378.

Overmyer, K.; Kollist, H.; Tuominen, H.; Betz, C.; Langebartels, C.; Wingsle, G.; Kangasjärvi, S.; Brader, G.; Mullineaux, P.; Kangasjärvi, J. Complex phenotypic profiles leading to ozone sensitivity in *Arabidopsis thaliana* mutants. *Plant, cell & environment*. **2008**, *31* (9), 1237–1249.

Park, E. J.; Jeknić, Z.; Sakamoto, A.; DeNoma, J.; Yuwansiri, R.; Murata, N.; Chen, T. H. Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. *The Plant Journal*. **2004**, *40* (4), 474–487.

Pearson, K. LIII. On lines and planes of closest fit to systems of points in space. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science*. **1901**, *2* (11), 559–572.

Pei, F.; Yang, W. J.; Shi, Y.; Sun, Y.; Mariga, A. M.; Zhao, L. Y.; Fang, Y.; Ma, N.; An, X. X.; Hu, Q. H. Comparison of freeze-drying with three different combinations of drying methods and their influence on colour, texture, microstructure and nutrient retention of button mushroom (*Agaricus bisporus*) slices. *Food and bioprocess technology*. **2014**, *7* (3), 702–710.

Pérez-Pérez, J. M.; Esteve-Bruna, D.; González-Bayón, R.; Kangasjärvi, S.; Caldana, C.; Hannah, M. A.; Willmitzer, L.; Ponce, M. R.; Micol, J. L. Functional redundancy and divergence within the *Arabidopsis* RETICULATA-RELATED gene family. *Plant physiology*. **2013**, *162* (2), 589–603.

Politowicz, J.; Lech, K.; Sánchez-Rodríguez, L.; Figiel, A.; Szumny, A.; Grubor, M.; Carbonell-Barrachina, Á. A. Volatile Composition and Sensory Profile of Oyster Mushroom as Affected by Drying Method. *Drying Technology*. **2017**,(just-accepted).

Poorter, L. Growth responses of 15 rain-forest tree species to a light gradient: the relative importance of morphological and physiological traits. *Functional ecology*. **1999**, *13* (3), 396–410.

Queval, G.; Issakidis-Bourguet, E.; Hoeberichts, F. A.; Vandorpe, M.; Gakiere, B.; Vanacker, H.; Miginiac-Maslow, M.; Van Breusegem, F.; Noctor, G. Conditional oxidative stress responses in the *Arabidopsis* photorespiratory mutant *cat2* demonstrate that redox state is a key modulator of daylength-dependent gene expression, and define photoperiod as a crucial factor in the regulation of $H_2O_2$-induced cell death. *The Plant Journal*. **2007**, *52* (4), 640–657.

Rahikainen, M.; Trotta, A.; Alegre, S.; Pascual, J.; Vuorinen, K.; Overmyer, K.; Moffatt, B.; Ravanel, S.; Glawischnig, E.; Kangasjärvi, S. PP2A-B′ γ modulates foliar trans-methylation capacity and the formation of 4-methoxy-indol-3-yl-methyl glucosinolate in *Arabidopsis* leaves. *The Plant Journal*. **2017**, *89* (1), 112–127.

Rédei GP, Hirono Y. Linkage studies. *Arabidopsis* Information Service. **1964**, 1, 9–10.

Reis, F. S.; Heleno, S. A.; Barros, L.; Sousa, M. J.; Martins, A.; Santos-Buelga, C.; Ferreira, I. C. Toward the antioxidant and chemical characterization of mycorrhizal mushrooms from Northeast Portugal. *Journal of Food Science*. **2011**, *76* (6).

Roberts, P.; Evans, S. The Book of Fungi. University of Chicago Press, Chicago, 2011; pp 656.

Rontein, D.; Basset, G.; Hanson, A. D. Metabolic engineering of osmoprotectant accumulation in plants. *Metabolic engineering*. **2002**, *4* (1), 49–56.

Rotzoll, N.; Dunkel, A.; Hofmann, T. Activity-guided identification of (S)-malic acid 1-O-d-glucopyranoside (morelid) and γ-aminobutyric acid as contributors to umami taste and mouth-drying oral sensation of morel mushrooms (*Morchella deliciosa Fr.*). *Journal of agricultural and food chemistry*. **2005**, *53* (10), 4149–4156.

Savorani, F.; Tomasi, G.; Engelsen, S. B. icoshift: A versatile tool for the rapid alignment of 1D NMR

spectra. *Journal of Magnetic Resonance*. **2010**, *202* (2), 190–202.

Schauer, N.; Fernie, A. R. Plant metabolomics: towards biological function and mechanism. *Trends in plant science*. **2006**, *11* (10), 508–516.

Schripsema, J. Application of NMR in plant metabolomics: techniques, problems and prospects. *Phytochemical Analysis*. **2010**, *21* (1), 14–21.

Schripsema, J.; Verpoorte, R. Investigation of extracts of plant cell cultures by proton nuclear magnetic resonance spectroscopy. *Phytochemical Analysis*. **1991**, *2* (4), 155–162.

Sekiyama, Y.; Chikayama, E.; Kikuchi, J. Evaluation of a semipolar solvent system as a step toward heteronuclear multidimensional NMR-based metabolomics for $^{13}$C-labeled bacteria, plants, and animals. *Analytical chemistry*. **2011**, *83* (3), 719–726.

Shi, H.; Ye, T.; Chen, F.; Cheng, Z.; Wang, Y.; Yang, P.; Zhang, Y.; Chan, Z. Manipulation of arginase expression modulates abiotic stress tolerance in *Arabidopsis*: effect on arginine metabolism and ROS accumulation. *Journal of experimental botany*. **2013**, *64* (5), 1367–1379.

Sklenar, V.; Piotto, M.; Leppik, R.; Saudek, V. Gradient-tailored water suppression for $^{1}$H-$^{15}$ N HSQC experiments optimized to retain full sensitivity. *Journal of Magnetic Resonance, Series A*. **1993**, *102* (2), 241–245.

Stothers, J. Carbon-13 NMR Spectroscopy: Organic Chemistry, A Series of Monographs. Elsevier, 2012; pp 574.

Suhre, K.; Meisinger, C.; Döring, A.; Altmaier, E.; Belcredi, P.; Gieger, C.; Chang, D.; Milburn, M. V.; Gall, W. E.; Weinberger, K. M. Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. *PloS one*. **2010**, *5* (11), e13953.

Sumner, L. W.; Mendes, P.; Dixon, R. A. Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry*. **2003**, *62* (6), 817–836.

Tenenboim, H.; Brotman, Y. Omic relief for the biotically stressed: metabolomics of plant biotic interactions. *Trends in plant science*. **2016**, *21* (9), 781–791.

Thomas, H.; Stoddart, J. L. Leaf senescence. *Annual review of plant physiology*. **1980**, *31* (1), 83–111.

Toledo, C. V.; Barroetaveña, C.; Fernandes, Â.; Barros, L.; Ferreira, I. C. Chemical and antioxidant properties of wild edible mushrooms from native Nothofagus spp. Forest, Argentina. *Molecules*. **2016**, *21* (9), 1201.

Topgaard, D.; Martin, R. W.; Sakellariou, D.; Meriles, C. A.; Pines, A. "Shim pulses" for NMR spectroscopy and imaging. *Proceedings of the National Academy of Sciences of the United States of America*. **2004**, *101* (51), 17576–17581.

Trotta, A.; Wrzaczek, M.; Scharte, J.; Tikkanen, M.; Konert, G.; Rahikainen, M.; Holmström, M.; Hiltunen, H. M.; Rips, S.; Sipari, N. Regulatory subunit B′ γ of protein phosphatase 2A prevents unnecessary defense reactions under low light in *Arabidopsis*. *Plant physiology*. **2011**, *156* (3), 1464–1480.

Urano, K.; Yoshiba, Y.; Nanjo, T.; Igarashi, Y.; Seki, M.; Sekiguchi, F.; YAMAGUCHI-SHINOZAKI, K.; Shinozaki, K. Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. *Plant, Cell & Environment*. **2003**, *26* (11), 1917–1926.

Urbanczyk-Wochniak, E.; Fernie, A. R. Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically-grown tomato (*Solanum lycopersicum*) plants. *Journal of Experimental Botany*. **2004**, *56* (410), 309–321.

Valentão, P.; Andrade, P. B.; Rangel, J.; Ribeiro, B.; Silva, B. M.; Baptista, P.; Seabra, R. M. Effect of the conservation procedure on the contents of phenolic compounds and organic acids in chanterelle (*Cantharellus cibarius*) mushroom. *Journal of Agricultural and Food Chemistry*. **2005**, *53* (12), 4925–4931.

Verhagen, F. J.; Van Assema, F. B.; Boekema, B. K.; Swarts, H. J.; Wijnberg, J. B.; Field, J. A. Dynamics of organohalogen production by the ecologically important fungus *Hypholoma fasciculare*. *FEMS microbiology letters*. **1998**, *158* (2), 167–178.

Vinocur, B.; Altman, A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Current opinion in biotechnology*. **2005**, *16* (2), 123–132.

Walde, S.; Velu, V.; Jyothirmayi, T.; Math, R. Effects of pretreatments and drying methods on dehydration of mushroom. *Journal of food engineering*. **2006**, *74* (1), 108–115.

Wang, W.; Vinocur, B.; Altman, A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*. **2003**, *218* (1), 1–14.

Wang, Y.; Ludwig, C.; Lilley, D. M.; Gunther, U. L.; Clore, M.; Campbell, S.; Han, X.; Neidle, S.; Postle, T.; Topliff, C. Metabolomics, metabonomics and metabolite profiling. Royal Society of Chemistry, 2007; pp 6–314.

Ward, J. L.; Harris, C.; Lewis, J.; Beale, M. H. Assessment of $^{1}$H NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopsis thaliana*. *Phytochemistry*. **2003**, *62* (6), 949–957.

Wassink, E.; Stolwijk, J. Effects of light quality on plant growth. *Annual Review of Plant Physiology*. **1956**, *7* (1), 373–400.

Weljie, A. M.; Newton, J.; Mercier, P.; Carlson, E.; Slupsky, C. M. Targeted profiling: quantitative analysis of $^{1}$H NMR metabolomics data. *Analytical chemistry*. **2006**, *78* (13), 4430–4442.

Wishart, D. S.; Jewison, T.; Guo, A. C.; Wilson, M.; Knox, C.; Liu, Y.; Djoumbou, Y.; Mandal, R.; Aziat, F.; Dong, E. HMDB 3.0—the human metabolome database in 2013. *Nucleic acids research*. **2012**, *41* (D1), D801–D807.

Wishart, D. S.; Tzur, D.; Knox, C.; Eisner, R.; Guo, A. C.; Young, N.; Cheng, D.; Jewell, K.; Arndt, D.; Sawhney, S. HMDB: the human metabolome database. *Nucleic acids research*. **2007**, *35* (suppl_1), D521–D526.

Wold, S.; Sjöström, M.; Eriksson, L. PLS-regression: a basic tool of chemometrics. *Chemometrics and intelligent laboratory systems*. **2001**, *58* (2), 109–130.

Worley, B.; Powers, R. Multivariate analysis in metabolomics. *Current Metabolomics*. **2013**, *1* (1), 92–107.

Wu, S.; Tohge, T.; Cuadros-Inostroza, Á.; Tong, H.; Tenenboim, H.; Kooke, R.; Méret, M.; Keurentjes, J. B.; Nikoloski, Z.; Fernie, A. R. Mapping the *Arabidopsis* Metabolic Landscape by Untargeted Metabolomics at Different Environmental Conditions. *Molecular Plant*. **2017**, *11* (1), 118–134.

Wulff-Zottele, C.; Gatzke, N.; Kopka, J.; Orellana, A.; Hoefgen, R.; Fisahn, J.; Hesse, H. Photosynthesis and metabolism interact during acclimation of *Arabidopsis thaliana* to high irradiance and sulphur depletion. *Plant, cell & environment*. **2010**, *33* (11), 1974–1988.

Zaidman, B. Z.; Yassin, M.; Mahajna, J.; Wasser, S. P. Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Applied Microbiology and Biotechnology*. **2005**, *67* (4), 453–468.

Zheng, D. F.; Zhao, L. M.; Feng, N. J. Effects of Plant Growth Regulators (PGRs) on Endogenous Hormone Contents and Activities of Protective Enzymes in Soybean Leaves. J. *Acta Agronomica Sinica*. **2008**, *7*, 020.

Zimmermann, P.; Zentgraf, U. The correlation between oxidative stress and leaf senescence during plant development. *Cellular and Molecular Biology Letters*. **2005**, *10* (3), 515.

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