Chapter 12
Pollen Metabolome Dynamics: Biochemistry, Regulation and Analysis

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Abstract The metabolome of an organism represents the readout of its biochemistry comprising numerous and tightly regulated metabolic pathways. Experimental analysis of the metabolome and its interpretation in a biochemically and physiologically meaningful context is focused by the research field of metabolomics which has become an integral part of many systems biological studies. Pollen development, germination and tube growth comprise numerous steps of metabolic regulation resulting in significant metabolome dynamics. To unravel involved regulatory molecular processes and to promote the understanding of developmental reprogramming and stress tolerance mechanisms in pollen, it is crucial to quantitatively resolve dynamics in the pollen metabolome. Since these dynamics affect various substance groups with different physico-chemical properties, different experimental platforms are needed for robust compound identification and quantification. It has been shown that developmentally and stress-induced metabolic reprogramming in pollen significantly affects the redox homeostasis as well as metabolism of carbohydrates, amino acids, lipids, polyamines, flavonoids and phytohormones. In this chapter, mechanisms of metabolic reprogramming are summarized and discussed in the context of pollen development and stress exposure. Finally, it is discussed how these metabolome dynamics can be resolved methodologically in order to unravel molecular physiological mechanisms of pollen development.

Keywords Metabolomics • Pollen development • Biochemistry • Metabolic network • Primary metabolism • Secondary metabolism

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Abbreviations

ABA	Abscisic acid
ATP	Adenosine triphosphate
GA	Gibberellic acid
GABA	γ-Aminobutyric acid
GC	Gas chromatography
HXK	Hexokinase
Inv	Invertase
LC	Liquid chromatography
MS	Mass spectrometry
$NAD^+/NADH+H^+$	Nicotinamide adenine dinucleotide (oxidized and reduced form)
STP	Sugar transport protein
SuSy	Sucrose synthase
UV	Ultraviolet

12.1 Pollen Development and Tube Growth: A Brief Overview

Development of pollen, which represents the mature male gametophyte, is a complex and sequential process occurring in the anthers of flowers. In particular, two phases, microsporogenesis and microgametogenesis, can be differentiated (Owen and Makaroff 1995; Scott et al. 2004). The meiotic division of diploid pollen mother cells results in tetrads of haploid microspores during microsporogenesis. Subsequently, microspores are released from the tetrad, enlarge and produce a large vacuole (vacuolation) in the stage of microgametogenesis. This polarized microspore is asymmetrically divided during Pollen Mitosis I resulting in a bicellular stage of development with a vegetative and a germ cell. Finally, during Pollen Mitosis II, twin sperm cells are produced from the germ cell (for a detailed overview of molecular mechanisms, see, e.g. Borg et al. 2009). Depending on the species, the step of Pollen Mitosis II might occur within the pollen grain before anthesis (tri-cellular pollen, e.g. *Arabidopsis thaliana*) or within the growing pollen tube (bi-cellular pollen, e.g. *Lilium longiflorum*).

Following the phase of vacuolation and Pollen Mitosis I, the pollen grain undergoes a dehydration phase before anther opening (Pacini 2000). Prior to anther opening, starch reserves, which have been built during pollen growth and development, are degraded and interconverted to soluble sugars, such as sucrose, fructose and glucose as well as to pectins or polysaccharides (Pacini et al. 2006). According to the species, the degree of starch hydrolysis may vary significantly and even allows for the differentiation between starchy and starchless ripe pollen (Baker and Baker 1979). The composition of reserve, structural and soluble carbohydrates plays a central role in the determination of pollen water content and viability

during phases of pollen ripening, presentation and dispersal (as reviewed in Pacini et al. 2006). For example, pollen water content and turgor pressure depends on carbohydrate reserves and their interconversion which reduces or prevents water loss. Hence, although mature pollen grains from the same flower may differ significantly in their water and carbohydrate content (Firon et al. 2012), a tight regulation of carbohydrate metabolism is a crucial part of pollen development. This also comprises the carbohydrate supply within the anthers, for which it has been shown that disturbance may lead to male sterility (Dorion et al. 1996; Goetz et al. 2001; Hedhly et al. 2016). Anthers are structured heterogeneously and consist of the anther wall, locular fluid and the pollen grains. The anther wall includes the connective tissue as well as the locule surrounding cell layers which are the epidermis, endothecium, middle layers and tapetum. This fraction has been shown to be the site of sugar synthesis, storage, mobilization and secretion during pollen development (Reznickova 1983; Reznickova and Dickinson 1982; Reznickova and Willemse 1980; Clément et al. 1998; Staiger et al. 1994). Following the ripening process, pollen grains are presented and released after the locular fluid has disappeared (Keijzer 1987). When landed on the stigma surface, they germinate to produce a pollen tube. The pollen tube penetrates the stigma and grows through the style towards the ovary. Finally, it invades the embryo sac and releases two sperm cells. One of these sperm cells fertilizes the egg, while the other one forms the triploid endosperm together with two polar nuclei of the central cell.

The pollen-stigma interaction and the (fast) growth of the pollen tube represent tightly coordinated processes comprising numerous molecular entities and a complex signalling network (Liu et al. 2014; Cheung and Wu 2008, 2016; Reichler et al. 2009; Šamaj et al. 2006; Hiscock and Allen 2008). As outlined previously, the pollen-stigma interaction comprises pollen-pistil interaction ranging from the pollen capture to the growth of the pollen tube through the transmitting tissue of the stigma and the entry into the style (Hiscock and Allen 2008). Lipids have been found to be an essential factor needed for the penetration of the stigma by the pollen tube (Wolters-Arts et al. 1998). In particular, *cis*-unsaturated triacylglycerols were shown to be required for penetration of the stigma by tobacco pollen tubes (Wolters-Arts et al. 1998). As summarized recently, polar and neutral lipids, e.g. triacylglycerols, accumulate during the late phase of pollen development (Ischebeck 2016). Pollen lipids play diverse roles in development, germination and tube growth. In addition to their protective role during dehydration, they are further involved in the attachment of the pollen to the stigma as well as in membrane signalling during pollen tube growth (Murphy 2006). Furthermore, metabolism of lipids, fatty acids and waxes plays a central role in pollen wall development (Jessen et al. 2011; Jung et al. 2006; Qin et al. 2013; Wu et al. 2014; Quilichini et al. 2015). In general, mature pollen grains contain three wall layers, the outer exine, the inner intine and the tryphine, which is also known as the pollen coat consisting of lipids, flavonoids, proteins and aromatic compounds (for a detailed overview, see Shi et al. 2015). The tapetum layer in the anthers predominantly contributes to the formation of the exine and tryphine, while the microspore contributes to the intine formation. Typically, the intine consists of cellulose, hemicellulose and pectin, while the exine

is composed of sporopollenin, a polymer of covalently linked phenylpropanoid and lipidic monomers (Heslop-Harrison 1968; Shi et al. 2015). In summary, it is obvious that pollen wall development comprises various substances derived from numerous biochemical pathways which are regulated by a large number of enzymes, transcription factors and metabolites. Particular complexity of regulation arises from the interconnection of lipid and polysaccharide metabolism that needs a tight regulation in order to provide an adequate developmental reprogramming and, probably, also stress response during pollen development.

Following the germination of pollen and the successful penetration of the stigma, the pollen tube grows through the stigma and style towards the ovary and the ovule. The pollen tube is guided through the female tissue along its growth path in a process termed pollen tube guidance. This guidance process has been shown to involve multiple steps of control (Dresselhaus and Franklin-Tong 2013) and was suggested to be separated in a preovular and ovular phase (Higashiyama and Takeuchi 2015). In *Arabidopsis*, female tissue was found to provide brassinosteroids to pollen tubes along the growth path during preovular guidance (Vogler et al. 2014). In detail, the authors showed that the promoter of one of the key enzymes in brassinosteroids biosynthesis, CYP90A1/CPD, is highly active in cells of the tract that form the pathway for pollen tubes. In addition, they observed that pollen growth was significantly reduced in the reproductive tract of a CYP90A1-deficient mutant, *cyp90a1* (Vogler et al. 2014). Also other phytohormones were shown to affect pollen germination and growth. For example, auxin was shown to stimulate in vitro pollen tube growth (Chen and Zhao 2008), and a gibberellin-deficient *reduced pollen elongation1* was observed to exhibit reduced pollen tube elongation (Chhun et al. 2007). In addition to phytohormones, also other metabolic compounds were determined to play a role in pollen tube growth and guidance, e.g. the non-proteinogenic amino acid γ-aminobutyric acid, GABA. The *Arabidopsis POP2* gene was shown to encode a transaminase degrading GABA and contributing to a gradient leading up to the micropyle (Palanivelu et al. 2003). *pop2* flowers accumulated significantly higher levels of GABA, finally resulting in infertility due to growth arrest of the pollen tube or misguided pollen tubes in the *pop2* pistil (Palanivelu et al. 2003). A mechanism of GABA-mediated communication between style and pollen tube was suggested by Yu and colleagues who observed that exogenous GABA modulates putative Ca^{2+}-permeable plasma membrane channels of pollen grains and tubes (Yu et al. 2014), and recently the GABA receptor was found to be the anionic channel ALMT, with effects on pollen tube growth (Ramesh et al. 2015).

Although the described processes being involved in pollen development, germination and tube growth are far from being complete, they already indicate a complex interplay of numerous metabolic pathways and regulatory mechanisms. Particularly, with respect to stress exposure and a multifaceted plant-environment interaction, it is an experimental and theoretical challenge to resolve dynamics of pathway regulation and its metabolic output. Due to the wide range of metabolic compounds being involved, various experimental techniques, methods and platforms are needed which can cope with the wide range of the physico-chemical properties and tissue-specific abundance. The following chapters aim at exemplarily discussing the biological

function of central primary metabolites, secondary metabolites and phytohormones. Finally, for each compound class, applied techniques and experimental workflows are discussed with respect to their capability of resolving metabolome dynamics.

12.2 Primary Metabolome Dynamics During Pollen Development and Stress Exposure

Pollen development, pollen tube growth and the response towards environmental fluctuations involve and affect metabolism of sugars, organic acids, amino acids, polyamines as well as energy metabolism. Previous studies have clearly demonstrated that disturbance of primary metabolism, in particular of carbohydrates, severely affects pollen development and may even lead to male sterility (Dorion et al. 1996; Datta et al. 2002; Goetz et al. 2001; Zhu et al. 2015; David-Schwartz et al. 2013). Photoassimilates for the carbohydrate demands of the anther and the pollen are supplied by leaves and also by floral organs including the anther itself (Vu et al. 1985; Kirichenko et al. 1993; Clément et al. 1997). For *Lilium* it was shown that anther wall layers regulate pollen sugar nutrition during maturation (Clément and Audran 1995). Sucrose was determined to be the main sugar in young filaments as well as in desiccating mature pollen grains (Clement et al. 1996; Speranza et al. 1997). Metabolism of sucrose in sink organs crucially involves the cleavage catalysed either by sucrose synthase (SuSy) or invertase (Inv) enzymes. Sucrose synthase is a glycosyl transferase, converting sucrose, for example, into UDP-glucose and fructose, while invertase represents a hydrolase releasing only free hexoses, i.e. glucose and fructose. Invertase enzymes have been shown to exist in different isoforms which possess different biochemical properties and are located in diverse subcellular compartments (Sturm 1996, 1999; Tymowska-Lalanne and Kreis 1998). Soluble and cell wall-bound invertase isoforms have also been detected in lily anthers (Miller and Ranwala 1994; Singh and Knox 1984). Furthermore, when pollen were cultured in sucrose-containing medium, it was observed that sucrose was quickly converted into equimolar amounts of glucose and fructose indicating the presence of cell wall-bound invertase in the growing pollen tube (Ylstra et al. 1998). Together with starch also soluble sugars, which are stored in the mature pollen, are used for pollen tube growth. Previous studies have shown that pollen tubes can grow at a very high rate. For maize, growth rates of approximately 1 cm h^{-1} have been reported (Mascarenhas 1993; Barnabas and Fridvalszky 1984), while in *Arabidopsis* growth rates of 5.3 $\mu m\ min^{-1}$, i.e. 0.3 mm h^{-1}, were observed (Wilhelmi and Preuss 1996). This rapid growth rate directly implies the need for energy supply, e.g. by stored carbohydrates or by carbohydrate secretions from the stylar canal (Labarca and Loewus 1973), to support respiration and cell wall growth. Due to its symplastic isolation from surrounding tissue, membrane transporters are required for nutrient import into the pollen tube. While the *Arabidopsis* sucrose transporter AtSUC1, which is localized to the plasma membrane of pollen tubes,

has previously been shown to play a crucial role in pollen germination (Sivitz et al. 2008), sucrose might also be hydrolysed by cell wall-associated invertase releasing the free hexoses fructose and glucose (Singh and Knox 1984). Recently, a member of the SUGAR TRANSPORT PROTEIN (STP) family in *Arabidopsis*, AtSTP10, was functionally characterized (Rottmann et al. 2016). Analysis of in vitro-grown pollen tubes showed a glucose concentration-dependent downregulation of *STP10* expression which was found to disappear in pollen tubes lacking the sugar sensor hexokinase 1 (HXK1) indicating a regulatory link between the glucose uptake system and the hexokinase pathway (Rottmann et al. 2016).

Significant dynamics of the sucrose-hexose ratio were also observed to differentiate the metabolic signature before and after pollen germination (Obermeyer et al. 2013). Obermeyer and colleagues found hexose levels to increase significantly during pollen growth, while sucrose concentration clearly decreased. These sugar dynamics were also persistent after application of antimycin A, an inhibitor of the mitochondrial electron transport chain limiting ATP production. Similar to previous studies (Mellema et al. 2002; Gass et al. 2005), the authors observed an increase of ethanol concentration immediately after application of antimycin A being due to rerouting of pyruvate to ethanol fermentation (Obermeyer et al. 2013). In addition, GABA dynamics were found to change immediately after antimycin A application. Inhibition of the mitochondrial transport chain and related ATP production resulted in an increase in GABA levels which the authors discussed in the context of deregulation of GABA biosynthesis. Based on their experimental observations, Obermeyer and co-workers hypothesized that the inhibition of the mitochondrial transport chain leads to an accumulation of reducing equivalents, i.e. $NADH/H^+$. This accumulation might be prevented by inducing ethanol formation via pyruvate decarboxylase and alcohol dehydrogenase explaining the reduced pyruvate levels (Obermeyer et al. 2013). Finally, to sustain pyruvate levels, biosynthesis of GABA would contribute to its regeneration and, at the same time, would consume reducing equivalents, thereby affecting the reprogramming of the redox homeostasis in growing pollen tubes.

GABA has been shown to be involved in numerous metabolic processes in plants, comprising roles in stress-induced signalling, pH and redox regulation and maintaining the energy as well as the carbon/nitrogen homeostasis (for an overview, see Fait et al. 2008). Together with proline, which represents another glutamate derivative, the role of GABA in sexual reproduction of angiosperms was summarized recently (Biancucci et al. 2015). While the specific role of proline during pollen development still has to be evaluated, several authors suggest that it plays a central role in protection of mature pollen during dehydration (Székely et al. 2008). Interestingly, Obermeyer and co-workers observed a clearly distinct dynamic behaviour of glutamate and proline concentrations, i.e. substrate and product of proline biosynthesis, when inhibiting the mitochondrial electron transport chain: after 3 h of inhibition, glutamate levels were reported to be similar to that of non-treated pollen tubes, while proline levels were found to dramatically increase due to antimycin A application (Obermeyer et al. 2013). This hints towards a possible regulatory interaction of GABA, proline and glutamate metabolism, possibly

comprising a previously discussed GABA shunt (Fait et al. 2008; Obermeyer et al. 2013).

Glutamate metabolism and associated concentration dynamics are crucially involved in the coordination of plant carbon/nitrogen metabolism. In addition to serving as a substrate for the above-mentioned derivatives, proline and GABA, glutamate participates in numerous further metabolic interconversions, e.g. as an amino group donor for the biosynthesis of other amino acids. Furthermore, glutamate serves as a metabolic substrate for the biosynthesis of ornithine and arginine which are precursors for the biosynthesis of the polyamines putrescine, spermidine and spermine being involved in many plant stress tolerance reactions (Alcázar et al. 2010; Alcázar and Tiburcio 2016; Sengupta et al. 2016). Also for pollen development and tube growth, it was shown that polyamines play various physiologically relevant roles. Polyamines are involved in regulation of developmental steps occurring during microsporogenesis; they play a role in the context of the quiescent state, pollen viability, rehydration and tube emergence as well as in the pollen-pistil interaction during fertilization and self-incompatibility. A more detailed summary of these various molecular and regulatory roles can be found elsewhere (Aloisi et al. 2016). During pollen tube growth, polyamines were shown to be involved in the organization and assembly of the cytoskeleton as well as cell wall deposition (Del Duca et al. 2009; Di Sandro et al. 2010) which seems, at least partly, to be mediated by transglutaminase activity. In pollen tubes of *Arabidopsis*, spermidine was found to be involved in the activation of Ca^{2+} channels, thus directly affecting the polarized growth of the pollen tube apex (Wu et al. 2010). Further, spermine was found to inhibit pollen tube elongation, and it was suggested that the observed degradation of nuclear DNA, and related cell death, was induced by Ca^{2+}-activated signalling or by an alteration of the cellular redox homeostasis (Aloisi et al. 2015).

12.3 The Interface of Primary and Secondary Metabolism in Pollen: A Central Role for Flavonoids

The secondary metabolome of plants comprises a vast variety of chemical structures that are characterized by diverse physico-chemical properties, physiological functions and ranges of concentrations challenging current bioanalytical approaches. In the context of pollen development, fertility and stress response, flavonoids have been shown to be crucially involved. Flavonoids comprise diverse classes of compounds such as flavonols, flavones, anthocyanins and isoflavonoids (Winkel-Shirley 2002). Flavonols were found to play a central role in pollen germination. Mo and colleagues observed that a lack of chalcone synthase, which catalyses the first step of the phenylpropanoid pathway leading to flavonoids, disrupts pollen fertility and flavonoid synthesis in maize and petunia (Mo et al. 1992). In another study, Ylstra and co-workers described a high abundance of flavonols and mRNA

of chalcone synthase in male and female reproductive organs of *Petunia hybrida* (Ylstra et al. 1994). The authors could show that due to anti-sense inhibition of the chalcone synthase gene activity, flavonol biosynthesis was blocked completely rendering these plants self-sterile. Such a central role of flavonoid biosynthesis was also shown for tomato where RNA interference silencing of chalcone synthase resulted in parthenocarpic fruits and impaired pollen tube growth (Schijlen et al. 2007). Interestingly, in *Arabidopsis* fertility was not affected by the disruption of the synthesis of active chalcone synthase indicating a species-specific effect of flavonoids (Burbulis et al. 1996). Yet, as summarized previously (Taylor and Grotewold 2005), these plants were affected in seed set and in vitro pollen tube growth (Ylstra et al. 1996; Kim et al. 1996), suggesting that flavonoids might enhance pollen tube growth but lacking flavonols can be compensated in their function by other metabolic compounds. In a recent study, this idea was supported by the finding that *Arabidopsis* plants lacking pollen-specific flavonols due to a defective glycosyltransferase were still fertile (Yonekura-Sakakibara et al. 2014).

Flavonoids are, together with substances like alkanes, steryl esters and oleosins, abundant in the coat of mature pollen. One possible function of flavonoids on the pollen coat might be the protection against damage due to UV radiation (Winkel-Shirley 2001; Pacini and Hesse 2005). Among the substances of this pollen coat, the structural characteristics of the phenylpropanoids hydroxycinnamic acid amides and flavonol glycosides are highly conserved in angiosperm pollen (Fellenberg and Vogt 2015). For *Zea mays* and *Petunia hybrida*, coat flavonoids have been shown to be involved in pollen germination and tube growth processes (Napoli et al. 1999; Mo et al. 1992). They are discharged on the pollen surface upon cell death of tapetum cells which accumulate endoplasmic reticulum-derived flavonoids (Hsieh and Huang 2007).

12.4 Signalling and Developmental Regulation: Phytohormones in Pollen

The regulatory networks which are involved in metabolic adjustment as well as the coordinate response of pollen metabolism during different developmental stages and environmental stresses comprise a remarkable number of molecular instances and signalling mechanisms. Particularly in the context of pollen tube/tip growth, the second messenger Ca^{2+} has emerged as a central regulator (for a detailed overview, see Franklin-Tong 1999; Steinhorst and Kudla 2013). Comprehensive models have been suggested integrating concentration dynamics of cytosolic Ca^{2+}, apical exocytosis of cell wall material, regulation of stretch-activated Ca^{2+} channels, inositol polyphosphates, reactive oxygen species, etc. (Steinhorst and Kudla 2013). Further, for *Arabidopsis*, a recent summary and comparison of transcriptome studies on pollen revealed an average value of more than 6000 genes being expressed in mature pollen (Rutley and Twell 2015). Hence, bringing together this multigenic

attribute with the comprehensive and highly interlaced structure of signalling networks and with the highly dynamic physiological output of developing pollen reveals a complex picture of regulation. Phytohormones have been shown to play a central role in the coordination and regulation of these networks affecting various steps of pollen development, tube growth and stress response.

Auxin belongs to the very central phytohormones playing an important role in promotion of cell elongation and cell division. Also in the context of pollen development, auxin was described to play a crucial role. Feng and colleagues found auxin flow in anther filaments to be critical for pollen grain development (Feng et al. 2006). In another study, auxin synthesized in anthers was suggested to coordinate anther dehiscence and pollen maturation using auxin receptor triple and quadruple mutants (Cecchetti et al. 2008). Furthermore, external application of auxin was observed to stimulate in vitro pollen tube growth (Chen and Zhao 2008; Wu et al. 2008) and, hence, seems to directly affect the signalling network of tube growth regulation. Mutant pollen being defective in PIN8, a pollen-specific auxin transporter, was claimed to be affected in germination when compared to the wild type (Bosco et al. 2012; Ding et al. 2012). Due to the fact that PIN8 is localized in the endoplasmic reticulum, this may indicate a link of the intracellular auxin homeostasis to the development of the male gametophyte.

Another phytohormone which has been described to play a central role in pollen development and stress response is gibberellic acid (GA). GA is involved in tapetum differentiation and initiation in tapetum programmed cell death which was described recently to occur via a GA-regulated transcriptional activator (Plackett et al. 2014; Aya et al. 2009). Singh and colleagues provided evidence that GAs are required for normal growth of the pollen tube by ectopically expressing a pea GA 2-oxidase2 cDNA in *Arabidopsis* resulting in reduced pollen growth (Singh et al. 2002). Based on studies in rice (*Oryza sativa*), it was suggested that GA de novo synthesis is preliminary for pollen germination and elongation (Chhun et al. 2007). While these observations clearly indicate a central role of GAs in pollen development and tube growth, they were also found to be involved in temperature stress response. During exposure to continuous heat stress, GA content was observed to positively correlate with pollen viability in two rice cultivars with a differential stress susceptibility (Tang et al. 2008). In contrast, cold stress was found to result in a reduction of bioactive GAs, particularly in susceptible anthers by the repression of GA biosynthesis genes (Sharma and Nayyar 2016; Sakata et al. 2014).

In addition to the GA-associated regulatory effects on different levels of molecular organization, it is also the crosstalk with other phytohormones that increases the coverage and complexity of phytohormone-based signalling to a very comprehensive level. One example of such a crosstalk is the DELLA-mediated interaction with signalling components of several phytohormones (Claeys et al. 2014). DELLA proteins represent transcriptional regulators and are conserved repressors of growth. GAs have been shown to regulate gene expression by promoting degradation of DELLA proteins (Murase et al. 2008). Other phytohormones, e.g. auxin, jasmonic acid or brassinosteroids, are interacting either directly with GAs, e.g. by transcriptional regulation, or indirectly, e.g. by post-translational regulation of

DELLAs (Claeys et al. 2014). Brassinosteroids were shown to promote *Arabidopsis* pollen germination and growth in a dose-dependent manner (Vogler et al. 2014). By expression analysis, Vogler and colleagues observed a highly active promoter of a key enzyme in brassinosteroid synthesis in cells of the reproductive tract forming the pathway from the stigma to the ovule (Vogler et al. 2014). An interesting regulatory interaction between hormone and carbohydrate metabolism was suggested for jasmonic acid, which was proposed to control water transport into the anther, perhaps via induction of the *AtSUC1* gene (Ishiguro et al. 2001). A strong interaction and influence on the central carbohydrate metabolism has also been unravelled for abscisic acid (ABA), which, together with GA, is relevant for the carbohydrate supply to the tapetum and microspores (De Storme and Geelen 2014). Particularly with regard to stress response, several studies have indicated a strong interaction between sugar- and ABA-mediated signalling (Gibson 2004; Dekkers et al. 2008). For example, ABA was shown to repress the expression of anther cell wall-associated invertase in wheat (*Triticum aestivum*) which resulted in a perturbation of sugar metabolism and a reduced level of hexoses in developing spores (Ji et al. 2011). This ABA-induced perturbation was directly related to an observed increase in drought sensitivity, exemplifying the central role of ABA in stress response of pollen. In rice, increased ABA levels in cold-stressed anthers were shown to interfere with apoplastic sugar transport inducing pollen abortion (Oliver et al. 2007). In accordance with the observations in wheat, exogenous ABA treatment revealed an effect on gene expression of cell wall invertase and monosaccharide transporter, which was accompanied by increased pollen sterility.

Although these examples of chosen regulatory interactions are only a very small part of the whole hormonal network, which has been unravelled in numerous studies, they clearly indicate the comprehensive impact on pollen physiology, development and stress tolerance. Finally, to be able to draw a reliable picture of those processes and to unravel interactions between hormonal regulation and observed dynamics in the metabolome, adequate bioanalytical techniques and integrative platforms with a comprehensive metabolic coverage are desirable.

12.5 Experimental Analysis of Metabolome Dynamics in Pollen

Although the discussed metabolic pathways and the involved substance classes represent only a part of the metabolism, they are highly interlinked, and, hence, their interdependent dynamics will significantly shape experimental results. This necessitates accurate and, at the same time, comprehensive experimental approaches to reveal a reliable picture of pollen metabolome dynamics during pollen development, tube growth and stress exposure. Particularly with regard to stress exposure, which very often leads to a strong accumulation or decrease of metabolite concentrations, suitable methodological workflows and analytical platforms are needed to enable the

reliable resolution of those dynamics. During the last two decades, the development and application of hyphenated high-throughput technologies, the so-called 'omics' technologies, have initiated the idea of the experimental multilevel analysis of an organism's molecular organization (Weckwerth 2011). The functional interpretation of the resulting high-throughput data represents the motivation for systems biology research ultimately aiming at the development of comprehensive and physiologically reliable models of molecular organization. Metabolites and their concentration dynamics play a crucial role in the development and validation of such models as they represent the output of regulation in biological systems. In their entirety, those metabolites constitute the metabolome of an organism. Finally, information about metabolome constitution and dynamics is essential to interpret the response of an organism to environmental changes or genetic perturbation (Fiehn 2002).

The experimental analysis of metabolome constitution and dynamics is focused by the research field of metabolomics, comprising steps of metabolite extraction, detection, identification and quantification. Metabolome analysis of pollen or pollen tube samples represents a challenge in several aspects. First, sampling of pollen or pollen tubes within the same or similar developmental and nutritional stage represents a difficult task. In contrast to leaves or roots for which developmental and nutritional states can often be estimated by eye, optical tools are necessary for pollen. Especially for the early stages of pollen development, it is essential to find a practicable and reliable method to separate, e.g. pollen mother cells from tetrads without the risk of changes in the metabolome. Recently, Dupl'áková and colleagues developed a protocol for separation of four pollen developmental stages, the uninucleate microspore, bi-cellular pollen, tri-cellular immature pollen and mature pollen grain applying a discontinuous Percoll concentration gradient (Dupl'akova et al. 2016). Using a Percoll gradient is advantageous because it does not penetrate the pollen grain, does not change the osmotic pressure, is not metabolized and, as a consequence, does not affect the biological function of the separated pollen.

A second challenge in pollen metabolomics is the washing step before metabolite extraction. It is necessary in order to remove pollenkitt and other hydrophobic compounds on the pollen coat. This washing step may be performed using hexane as described previously (Obermeyer et al. 2013).

Third, due to their small size and the (relatively) robust pollen wall, the grinding process has to be performed in a very thorough manner. Again, in contrast to other plant material, the control by eye is very limited in this step. A further very critical step is the metabolite extraction itself. While the targeted analysis of a special class of substances might facilitate the extraction procedure, e.g. using ethanol extraction for soluble sugars (Nägele et al. 2012) or hot water extraction for sugar phosphates (Sekiguchi et al. 2004), the combined and quantitative extraction of as many as possible hydrophilic and hydrophobic substances from one sample is much more challenging. Although a basic procedure for the integrated extraction of metabolites, proteins and RNA was developed already more than a decade ago (Weckwerth et al. 2004; Valledor et al. 2014) and has previously been applied to extract the primary metabolome of lily pollen (Obermeyer et al. 2013), such extraction methods need to be continuously developed and improved to increase the metabolic coverage. After

a successful extraction of metabolites, the fourth challenge is the very high dynamic range of metabolites within a tissue but also between developmental stages, which are differentially metabolic active. For instance, the polar fraction of an extract can contain low abundant primary metabolites, like amino acids and 100-fold higher levels of sucrose. This effect is even much stronger if very low abundant signalling molecules, like phytohormones, are analysed.

For the unbiased analytical assessment of the metabolome, chromatography coupled to mass spectrometry has become a central approach (Weckwerth 2003). Focusing on the quantitative analysis of dynamics in the parts of the metabolome which were discussed in the previous chapters, i.e. primary metabolites, flavonoids and phytohormones, a combination of gas chromatographic (GC) and liquid chromatographic (LC) separation coupled to mass spectrometric (MS) detection represents a suitable approach (Weckwerth 2003; Scherling et al. 2010; Doerfler et al. 2013). While chemical derivatization enables the GC-MS-based identification and quantification of central primary metabolites comprising soluble sugars, carboxylic acids and amino acids (Fragner et al. 2014), for larger and thermally instable molecules, e.g. secondary metabolites like flavonoids, LC-MS is the method of choice (Stobiecki and Kachlicki 2013; Doerfler et al. 2013; Scherling et al. 2010). An interesting combination of methods has been highlighted recently for the quantification of phytohormones. As summarized by Fu and colleagues, although LC-MS can directly analyse plant hormones, a previous chemical derivatization, which is usually applied for GC-MS, can increase the sensitivity up to 1000-fold improving the quantification of low concentrated phytohormones (Fu et al. 2011). Together with methods which have been developed for extraction and enrichment of plant hormones (Du et al. 2012), this bioanalytical procedure might even enable the robust and comprehensive quantification of trace amounts in pollen grains and tubes.

Finally, one of the central challenges in metabolomics, playing also a crucial role for pollen analytics, is the resolution towards the single-cell level. Recent approaches have indicated the difficulties in these approaches, which, for example, are due to the enormous dynamic range comprising only a few molecules up to millions of molecules per cell. Yet, these approaches have also clearly shown that difficulties in interpretation, e.g. due to dilution effects from tissue-based sampling, might be by-passed by single-cell techniques (Misra et al. 2014).

12.6 Conclusion and Perspective

The resolution of the pollen metabolome and the interpretation of its dynamics in the context of developmental and stress-related molecular processes represent a challenge in the research field of metabolomics. This challenge arises not only from limitations in chromatography, detection and quantification but also from sampling, extraction and biochemical validation. Integrative research platforms merging microscopy and imaging techniques with hyphenated omics techniques

and physiological assays represent a promising constellation which can overcome such experimental limitations. Conclusively, integrative approaches will promote our current understanding of how stress conditions affect pollen morphology, physiology and biochemistry and how this impacts the reproductive success of plant species.

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