The early stress response of jasmonic acid in cell suspension cultures of Catharanthus roseus

Goldhaber Pasillas, G.D.

Chapter 7

General discussion & perspectives

Goldhaber-Pasillas GD[1]

[1]Natural Products Laboratory, Institute of Biology Leiden, Sylvius Laboratory, Leiden University, Sylviusweg 72, 2333BE, Leiden, The Netherlands

7.1 An integrated approach to understand the rapid JA-stress response

During its lifetime, a plant is permanently exposed to a number of environmental conditions which pose a continuous challenge to its survival, and to which it has to adjust accordingly. Environmental cues can include among others: exposure to UV light, suboptimal temperatures, drought, flooding, light quality, osmotic stress, exposure to heavy metals, wounding along with pathogen attack and herbivory, which can lead to decreased growth. Consequently, plants have evolved complex inducible systems that require a coordinated and integrated response that involves the perception of the external stress condition, the translation of such stimuli into an internal signal that will travel from the site of induction to the whole plant to set an "alarm state", and to accumulate enough amounts of messenger signals, which will eventually induce the expression of specific genes involved in the stress response. Such inducible signals are transmitted by small molecule messengers *i.e.* phytohormones like jasmonic acid (JA), that act through signal-transduction pathways to respond to the stress condition. JA along with its precursors and derivatives, *i.e.* jasmonates (JAs), classified as plant oxylipins, are biologically active both in plant development and in defense mechanisms, with roles in both healthy and stressed tissues.

Because in wound-induced experiments in *Arabidopsis thaliana* the JA biosynthetic precursors dinor-12-*oxo*-phytodienoic acid (dnOPDA) and/or 12-*oxo*-phytodienoic acid (OPDA) are rapidly released from galactolipid complexes in thylakoids, combined with rapidly increased endogenous levels of JA and jasmonoyl-L-isoleucine (JA-Ile) (Kourtchenko *et al.,* 2007; Browse, 2009a; 2009b), this suggests that JA can induce its own biosynthesis by a feedback loop mechanism. Further evidence comes from the fact that all JA biosynthetic genes are induced by JAs (Sasaki *et al.,* 2001). Nevertheless, the occurrence of mono- or digalactosyldiacylglycerol (MGDG and DGDG) and the phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylglycerol (PG) containing either dnOPDA, OPDA, linolenic acid (C18:3) and/or (ω5*Z*)-etherolenic acid residues bound to galactolipids, has so far only been reported in members of the Brassicaceae, Convolvulaceae, Asteraceae, Lamiaceae and Linaceae plant families. Additionally, the enzymes 13-lipoxygenase (13-LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) are able to *in situ* transform linoleic acid (C18:2), linolenic acid and roughanic acid (C16:3) esterified in MGDG and DGDG into dnOPDA or OPDA, thus suggesting the constitutive presence of these enzymes, and precluding gene expression of these and/or JA biosynthetic enzymes. However, neither the incorporation of these precursors released from galactolipids nor the *de novo* biosynthesis of JA into the JA-mediated stress response, have been proven yet. This prompts to the question: how do dnOPDA/OPDA esterified in arabidopsides contribute to the stress response, if at all? Originally, our results showed that after JA-treatment, C18:3 was indeed induced after 24 h of treatment suggesting that the *de novo* biosynthesis of JA did not take place. After reevaluating our multivariate data analysis (MVDA) approach and observing the strong effect of the mock-treatment exerted on C18:3, we concluded that under our experimental conditions, *i.e.* JA dissolved in ethanol, the mock treated and JA treated cells cannot be distinguished. In our

publication of 2014 in the *Molecules* journal (Goldhaber-Pasillas *et al*., 2014, retracted), we described the analysis of the fatty acids (FA), but further experiments showed that a calculation error was made, affecting only the quantitative values but not the qualitative ones. Moreover, a thorough analysis confirmed our conclusions that the FA C18-series has a time-dependent uptrend. Changes in FA profiles also occurred in the mock treatment (40 % v/v ethanol) (**Chapter 3**), thus suggesting the possibility of the post-translational event of SUMOylation (small ubiquitin-like modifier), that occurs during drought stress which is extensively described in *A. thaliana* (Benlloch and Lois, 2018). Interestingly, the JA biosynthetic genes *AOC3, AOS, DEFECTIVE ANTHER DEHISCENCE1* (*DAD1*)*, JASMONATE RESISTANT1* (*JAR1*)*, JASMONIC ACID CARBOXYL METHYLTRANSFERASE1* (*JMTI*) and *LOX3* are drought-inducible (Catala *et al.,* 2007). Therefore, we conclude that the use of JA without dissolution into an organic solvent is definitely encouraged, to bypass the strong effect observed with ethanol. In previous JA feeding and elicitation studies in plants and plant cell cultures, dimethyl sulfoxide (DMSO) has been often used as a solvent for JA and other substances. Although it has been published that DMSO does not induce the expression of the transcriptional factor (TF) OCTADECANOID-DERIVATIVE RESPONSIVE CATHARANTHUS AP2-DOMAIN3 (ORCA3) in *in vitro* cultures of *C. roseus* (Vom Endt *et al.,* 2007) and *A. thaliana* plants (Montiel *et al.,* 2011), there is also evidence that DMSO induces the expression of *TRYPTOPHAN DECARBOXYLASE* (*TDC*) and *STRICTOSIDINE SYNTHASE* (*STR*)*,* two TIA biosynthetic genes (Menke *et al.,* 1999), and ROS production (Pauw *et al.,* 2004), both cases in *C. roseus* cell suspension cultures. Consequently, the use of DMSO is also discouraged. Even if our results are inconclusive towards the undivided effect of JA without the mock on FA C18-series, MVDA clearly showed that the largest variation in FA profiles occurred at 24 h, thus partially answering the question that feeding cells with JA does not affect its precursor C18:3 and most especially, that these events do *not* fit into the fast JA-mediated early stress response, that can be better understood as occurring without JA biosynthetic genes expression. We attempted to understand the link between primary metabolism and JA in regard to possible changes in FA profiles, in a wider context. As our work shows that this possibility cannot be excluded, therefore it needs further research.

The lack of knowledge concerning the role of dnOPDA in the JA-mediated signaling pathway and the domination of knowledge generated about the early steps in the JA biosynthesis in plant models like *A. thaliana*, motivated us to explore the early stress response in a plant cell culture system like the cell suspension cultures of *Catharanthus roseus*. Short time-framed events intended to explore the uptake and biosynthetic fate of JA and its precursor dnOPDA, as well as their potential effect on the production of endogenous JA and unlabeled JAs, deuterium-labeled dnOPDA (*d*5-dnOPDA) and deuterium-labeled JA (*d*5-JA) were used. The deuterium-labeled JAs allowed us to monitor their incorporation in cells and to discriminate between endogenous JA-Ile and other JAs. Feeding with labeled dnOPDA resulted in the immediate accumulation of *labeled* JA and *labeled* JA-Ile in cells and

labeled JA in the growth medium, thus showing that the enzymes needed to convert (labeled) dnOPDA into (labeled) JA and JA-Ile, are constitutively present and active. On the other hand, feeding labeled JA did *not* result in the accumulation of neither endogenous unlabeled JA nor JAs, therefore disproving the putative feedback loop mechanism of exogenous JA fed to cell suspension cultures of *C. roseus* (**Chapter 5**). The fact that a multi-analytical platform with different ultra-high resolution abilities was needed to clearly distinguish each JA isotopologue from endogenous JA to avoid false positives, shows how dedicated extraction protocols for each plant material and analytical methods should be carefully validated. The preanalytical steps are of utmost importance, as the use of deuterium labeled phytohormones, proved to contribute to false positives. Consequently, the fact that under our experimental approaches using ^{1}H-NMR, GC-MS, GC-FID, TLC and UHPLC-HRMS/MS we could not confirm yet, the presence of arabidopside-like complexes in *C. roseus* when applying reported methods (**Chapter 5**), does not mean that such complexes do not occur in *C. roseus.* After all, questions like: do all plants have arabidopside-like complexes? Are the dnOPDA/OPDA esterified in galactolipids, entering the stress response? How does the rapid JA stress response work in non-*Arabidopsis* plants? are just some of the most important and intriguing questions in the field awaiting to be answered.

The emerging topic of JA's catabolism is giving a broader standpoint into the "switch-off" events of the JA-mediated stress response. Not only can JA be metabolized into a number of derivatives, but also JA-Ile and OPDA can undergo metabolic conversions into inactive derivatives, although the presence of OPDA-Ile in *A. thaliana*, raises the question regarding their biological role in the JA-mediated signaling pathway, and if JAR1 is responsible for the conjugation of OPDA with isoleucine. Sixteen conversion pathways have been described so far for JA and JA-Ile, and our results show that oxidation, conjugation, hydroxylation, reduction and glycosylation of both JAs were the major turnover metabolites, and it appears that these are the start of the major catabolic pathways in cell suspension cultures of *C. roseus* (**Chapter 6**). CYP enzymes, among others, were recently described to catalyze these reactions *e.g.* CYP94B3, CYP94B1, CYP94C1, IAA-ALANINE RESISTANT3 (IAR3) and JASMONATE-INDUCED OXYGENASE 1-4 (JOX), suggesting their presence in our cell suspension cultures. Given that a larger percentage of JAs was observed in the growth medium than in cells, one may ask: what is the minimum concentration of JA that can elicit the stress response in an *in vitro* model system like ours? How does the cell perceive an excess of JA? Why do some metabolites of JA occur more than others and do they have any biological activity? Apparently, the potential overdose of JA is quickly taken care of by irreversible events such as the hydroxylation, oxidation and reduction of JA, JA-Ile and 12-hydroxyjasmonic acid (12-HOJA) in cells and their excretion to the growth medium (**Chapters 5** and **6**).

The main goal of the work described in this PhD thesis was to get a better understanding of the basis of the rapid JA-mediated stress response in cell suspension cultures of *C. roseus*. Even if our

research did not deliver new information about a putative self-induction mechanism of JA, it disproved this hypothesis and instead, showed that fed dnOPDA is immediately converted to JA-Ile. Moreover, it contributed to expand the knowledge of the metabolic conversions that attenuate the signal propagation of JA *in vitro.* Especially, because it demonstrated the need to keep devising new, more specific methods for the analysis of other oxylipins present in *C. roseus*, and it raised the question about the possibility of other mechanisms involved in the fast stress response. Additionally, our work undertook the task to investigate the link between primary and secondary metabolism occurring during the JA-stress response (**Chapters 3, 5** and **6**). Due to the highly complex JA-mediated stress response, a holistic approach to evaluate and map all events taking place at the metabolite level is mandatory. Metabolic profiling of plant systems is a prerequisite for an integrated approach to study the effects of JA and its derivatives in cells, tissues and organs, especially outside plant model organisms. In this way, the field has greatly benefited from the development of more robust *omics* technologies as well as from analytical platforms such as hybrid quadrupole-orbitrap mass spectrometers with higher sensitivity and selectivity for phytohormone profiling, identification, confirmation and quantification. Particularly dose response studies seem of interest, as is the feeding with labeled JA and labeled dnOPDA which resulted in very different observations. It remains to be proven if feeding with dnOPDA induces the same set of genes in the JA-signaling pathway as JA or OPDA, as it is known that a differential gene expression is triggered by these two JAs in wounding and elicitation experiments. Both OPDA and JA are able to initiate the stress signal-related transduction pathways, although OPDA in a CORONATINE INSENSITIVE1 (COI1)-independent way. Further questions such as: what is the specific biological activity of each JAs, how many more are there, and which genes do they regulate? Are there other components in the JA signaling pathway? Why are some stress responses COI-JAZ-(in)dependent? Why does the extent of JA's effectiveness depend greatly on the plant species or its concentration? Why does the same JAs have different biological activities in different plants? All questions which are still unanswered.

7.2 Terpenoid indole alkaloids, the JA-late stress response in *C. roseus*

The JA-signaling pathway in *A. thaliana, Solanum lycopersicum, Nicotiana benthamiana* and *C. roseus* has been thoroughly studied for decades with major breakthroughs such as the inter- and intra-cellular localization of most enzymatic steps of terpenoid indole alkaloids (TIA) biosynthesis in *C. roseus* and the more complete elucidation of the seco-iridoid pathway, all published between 2013 and 2018 (Simkin *et al.,* 2013; Miettinen *et al.,* 2014; Dugé de Bernonville *et al.,* 2015; Qu *et al.,* 2018; Caputi *et al.,* 2018), which also means that involved genes are now publicly available in the GenBank, Medicinal Plant Genomics Resource and PhytoMetaSyn databases. In *C. roseus*, a plethora of information is available on the more than 130 different TIA present in plants, like the antitumor drugs vinblastine and vincristine, as well as the presence of some TIA in *in vitro* cell and tissue cultures. Also,

the biosynthesis is well defined to the enzyme and gene level. Based on this knowledge, the regulation of the biosynthesis has been studied in detail, resulting in the identification of the TF induced by JA like ORCA2 and ORCA3. For these studies, analytical platforms have been developed and designed to extract, isolate and identify the chemically different TIA, where some of them occur in very low amounts. The great clinical and industrial importance of *C. roseus* made it into one of the best studied medicinal plants and thus a suitable model system for JA-mediated plant stress response, including the effect on secondary metabolism. Concerning the late JA-mediated stress response in *C. roseus*, it depends on the signaling pathway that conveys the expression of several genes involved in TIA biosynthesis, taking place after 2 h of induction, where after a significant accumulation of TIA occurs after 24 h. Therefore, the early steps in the JA-mediated stress response include the fast and large accumulation of JAs as early as 30 sec after wounding in *A. thaliana* whereas the late stress response in *C. roseus* involves the increased accumulation of TIA like tabersonine, serpentine, catharanthine and α-TIA-3 after 24 of JA treatment (**Chapter 4**). The presence of 7 different α-methylene-indoline alkaloids (α-TIA 1-7) not previously reported to occur in cell suspension cultures of *C. roseus*, which were JA-inducible, just shows the richness and diversity of TIA in *C. roseus* cells. Likely candidates for some of the α-TIA are 19-hydroxytabersonine, akuammigine and lochnericine. Therefore, our work contributed to broaden the knowledge of JA-inducible minor TIA present in cell suspension cultures of *C. roseus* (**Chapter 4**). Their full identification could not be achieved by means of a HRMS-based platform, therefore reinforcing the undeniable need to develop further analytical platforms sensitive enough to elucidate the structures of the minor compounds of both the JA on TIA biosynthetic pathways.

7.3 Concluding remarks & perspectives

As an ideal plant model system, *C. roseus* served us to explore and understand more about the JA-mediated fast stress response. Our data clearly demonstrated that there are no negative results, just unexpected ones that challenged our scientific minds, redirected our focus, our analytical approaches and made us reformulate our research hypotheses. Moreover, this PhD thesis proved that this topic can be undertaken in a non-*Arabidopsis* plant model system and *in vitro*. Although we still need to test if one can expect the same or similar results *in planta* and *in vitro*, thus adding another layer of complexity to the already complex JA-stress response.

Whereas JA is considered an important element in plant stress signaling pathways and defense responses, most research efforts are focused on its role on plant growth, development and other physiological activities like fertility, sex determination, reproductive processes, fruit ripening and senescence, to name a few. With the challenge to improve plant resilience to environmental stresses and diseases, it is striking that there is not enough information available about the link of primary and secondary metabolism in plants in regard to JA. In connection to our results, there is an obvious need

to distinguish between a possible SUMOylation effect and JA induction on FA profiles. Increased levels of C18:3 were expected to occur in a shorter timeframe and instead, it was increased at the end of our observation points *along* with C18:3 in ethanol-treated cells. The expression of JA-biosynthetic genes should be tested, and JA *has* to be used undiluted in feeding experiments, together with the comprehensive analysis of oxylipins and metabolomics of unhydrolyzed extracts of *C. roseus*, this will give a better overview of the possible role of C18:3 during the early stress response.

The choice to use deuterium-labeled phytohormones justified our goals, it helped us to confirm that JA does not induce its own biosynthesis and that dnOPDA is converted to JA-Ile but it also questioned our analytical platforms and thinking. The overlap between *d*1-JA and JA accompanied by the lack of access to a higher-resolution mass spectrometer, could have falsely led us to claim that JA induces its own biosynthesis. Thus, collaboration in science, especially in the JA field, is a must. Moreover, the role of dnOPDA in the early and late JA-mediated stress responses in *C. roseus* awaits further research action like testing the differential gene expression triggered by JA and dnOPDA, and the effect of dnOPDA on TIA production, which raises the question whether dnOPDA, like OPDA, act through different signaling pathways; if fed dnOPDA later converted to JA-Ile is responsible for changes in TIA profiles, and if dnOPDA induces the expression of TIA biosynthetic genes. These questions could be answered with the monitoring of dnOPDA-induced cDNA macroarray analysis of *C. roseus* cells, to understand its effects at a deeper systems level. An interesting question is if priming with JA can induce epigenetic changes in plants of *C. roseus*, and if so, which defense-related genes are upregulated. Furthermore, the presence of available enzymes and/or precursors possibly involved in the rapid response, could be spatially and temporally localized with imaging techniques such as imaging and single-cell mass spectrometry, as it was through this technique that catharanthine and serpentine were found to occur in idioblasts and laticifers of *C. roseus.* Concerning the metabolic conversions of JA, JA-Ile and 11/12-HOJA observed in cells of *C. roseus,* our results point to the presence of CYP enzymes among others reported to catalyze these reactions. Further gene exploration for these enzymes in *C. roseus,* the exploration of new JAs along with their exact biological activity and role in signaling pathway, will greatly advance the current understanding of this often neglected topic in the JA-mediated stress response. Possibly, some of these JAs might play a role in mammalian inflammation or as antidepressants such as JA and its methyl ester and will provide insights into their pharmacological potential as plant-based drugs.

In conclusion, in this PhD thesis we established a different perspective for further studies of JA effects on FA, TIA and JAs in *in vitro* systems by showing which experiments, analytical standards and platforms should be used to avoid false positives. In addition, exploring the effects of JA with an *omics* approach will lead to a more comprehensive and integrated understanding of the profound effect that JA exerts in a plant cell, and should be part of a systems biology approach taking into account the total of metabolism in cells or plants. Thus, the research field of JA makes progress day by day, enabled

by the development and access to newer technologies. One finding after the next one will take us to more exciting questions that will challenge our imagination until we have fully dissected the intricate and fascinating world of the JA-stress response.

7.4 REFERENCES

- **Benlloch R**, Lois LM (2018) Sumoylation in plants: mechanistic insights and its role in drought stress. *Journal of Experimental Botany* 69: 4539-4554.
- **Browse J** (2009a) Jasmonate passes muster: a receptor and targets for the defense hormone. *Annual Review of Plant Biology* 60: 183-205.
- **Browse J** (2009b) The power of mutant for investigating jasmonate biosynthesis and signaling. *Phytochemistry* 70: 1539-1546.
- **Caputi L**, Franke J, Farrow SC, Chung K, Payne RME, Nguyen TD, Dang TTT, Soares Teto Carqueijeiro I, Koudounas K, de Bernonville TD, Ameyaw B, Jones DM, Curcino Vieira IJ, Courdavault V, O'Connor SE (2018) Missing enzymes in the biosynthesis of the anticancer drug vinblastine in Madagascar periwinkle. *Science* 360: 1235-1239.
- **Catala R**, Ouyang J, Abreu IA, Hu Y, Seo H, Zhang X, Chua NH (2007) The *Arabidopsis* E3 SUMO ligase ISZ1 regulates plant growth and drought stress. *The Plant Cell* 19: 2952-2966.
- **Dugé de Bernonville T**, Clastre M, Besseau S, Oudin A, Burlat V, Glévarec G, Lanoue A, Papon N, Giglioli-Guivarch'h N, St-Pierre B, Courdavault V (2015) Phytochemical genomics of the Madagascar periwinkle: unravelling the last twists of the alkaloid engine. *Phytochemistry* 113: 9-23.
- **Goldhaber-Pasillas GD**, Mustafa NR, Verpoorte R (2014) Jasmonic acid effect on the fatty acid and terpenoid indole alkaloid accumulation in cell suspension cultures of *Catharanthus roseus. Molecules* 19(7): 10242-10260 **-Retracted-**
- **Kourtchenko O**, Andersson MX, Hamberg M, Brunnström A, Göbel C, McPhail KL, Gerwick WH, Feussner I and Ellerström M (2007) Oxo-phytodienoic acid-containing galactolipids in *Arabidopsis*: jasmonate signaling dependence. *Plant Physiology* 145: 1658-1669.
- **Menke FLH**, Parchmann S, Mueller MJ, Kijne JW, Memelink J (1999) Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus. Plant Physiology* 119: 1289-1296.
- **Miettinen K**, Navrot N, Schneider T, Burlat V, Pollier J, Woittiez L, van der Krol S, Lugan R, Ilc T, Verpoorte R, Oksman-Caldentey KM, Martinoia E, Bouwmeester H, Goossens A, Memelink J, Werck-Reichhart D (2014) The seco-iridoid pathway from *Catharanthus roseus. Nature Communications* 5: 3606.
- **Montiel G**, Zarei A, Körbes AP, Memelink J (2011) The jasmonate-responsive element from the *ORCA3* promoter from *Catharanthus roseus* is active in *Arabidopsis* and is controlled by the transcription factor AtMYC2. *Plant & Cell Physiology* 52: 578-587.
- **Pauw B**, van Duijn B, Kijne JW, Memelink J (2004) Activation of the oxidative burst by yeast elicitor in *Catharanthus roseus* cells occurs independently of the activation of genes involves in alkaloid biosynthesis. *Plant Molecular Biology* 55: 797-805.
- **Qu Y**, Thamm AMK, Czerwinski M, Masada S, Kim KH, Jones G, Liang P, De Luca V (2018) Geissoschizine synthase controls flux in the formation of monoterpenoid indole alkaloids in a *Catharanthus roseus* mutant. *Planta* 247: 625-634.
- **Sasaki Y**, Asamizu E, Shibata D, Nakamura Y, Kaneko T, Awai K, Amagai M, Kuwata C, Tsugane T, Masuda T, Shimada H, Takamiya KI, Ohta H, Tabata S (2001) Monitor of methyl jasmonate-responsive genes in *Arabidopsis* by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. *DNA Research*. 8: 153-161.

- **Simkin AJ**, Miettinen K, Claudel P, Burlat V, Guirimand G, Courdavault V, Papon N, Meyer S, Godet S, St-Pierre B, Giglioli-Guivarc'h N, Fischer MJ, Memelink J, Clastre M (2013) Characterization of the plastidial geraniol synthase from Madagascar periwinkle which initiates the monoterpenoid branch of the alkaloid pathway in internal phloem associated parenchyma. *Phytochemistry* 85: 36-43.
- **Vom Endt D**, Soares e Silva M, Kijne JW, Pasquali G, Memelink J (2007) Identification of a bipartite jasmonate-responsive promoter element in the *Catharanthus roseus ORCA3* transcription factor gene that interacts specifically with AT-hook DNA-binding proteins. *plant Physiology* 144: 1680-1689.