Discussion
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CHAPTER 5

DISCUSSION

Branch-point genes present favorable targets for metabolic engineering efforts for redirecting metabolic fluxes towards desired secondary metabolite as plants produce these in meager amounts. OSCs positioned at a key metabolic branch point execute the indispensable enzymatic cyclizations of 2, 3-oxidosqualene for varied triterpenoid biosynthesis. However, detailed information regarding the candidate OSCs covering different branches and their regulation is necessary for desired genetic manipulation. The aim of the present study, therefore, was to characterize selected members of OSC gene family from *W. somnifera*, a medicinal plant of immense repute known to synthesize a large array of pharmacologically important triterpenoids called withanolides.

Three members of OSC gene family belonging to both protosteryl and dammarenyl cation group from *W. somnifera* were cloned and characterized. Being juxtapositioned at a pivotal metabolic branch point, WsOSCs lead to the production of sterols, withanolides and an array of triterpenoids. It is evident that OSCs signify an essential cog of a decisive branching point between primary and secondary metabolites. The three OSCs in *Withania* utilize the common substrate pool of 2, 3 oxidosqualene and lead to the formation of different triterpenoids serving both primary and secondary functions for the plant. Co-occurrence of OSCs from both these groups for structuring different triterpenoid skeletons is often found in many plants such as *G. glabra, Kalanchoe daigremontiana, A. thaliana* etc. (Hayashi et al., 2004, Wang et al., 2010, Husselstein–Muller et al., 2001).

WsOSC/BS, WsOSC/LS and WsOSC/CS showed moderate similarity at nucleotide and amino acid levels with each other. This similarity encompasses various conserved domains which were detected in all the three OSCs and play an essential role for the proteins to carry out the cationic cyclization of linear triterpenes into fused ring compounds. Whereas, one of the conserved motif (Motif B) was detected exclusively in WsOSC/BS and WsOSC/LS (Figure 6.1). Interestingly, on the basis of site-directed mutagenesis, tryptophan and leucine positioned at the second place in these motifs have been proved to be characteristic for functional β- amyrin and lupeol synthases (Kushiro et al., 2000). Though a point mutation can radically mutate such specificities, but in many situations numerous other sequence alterations may counterbalance each other without modifying the enzyme specificity. This
implicates the significance of both diversity and similarity for definite functioning of each OSC.

Phylogenetic clustering also grouped WsOSC/BS, WsOSC/LS and WsOSC/CS in three separate sub-groups comprising of β-amyrin, lupeol and cycloartenol syntheses respectively (Figure 8.1). Several factors are supposed to drive this expansion and diversification of OSC family. Recruitment of mutated/duplicated genes for new functions (neo-functionalization) involves one key mechanism for this pathway evolution and extension. And such post-speciation expansion indicates the members to have evolved mostly for utility other than the primary metabolism. This is significantly evident in various OSC sub-families wherein the ones that use the dammarenyl cation intermediate show comparatively more lineage specific diversity (Phillips et al., 2006, Shiu et al., 2004). In contrast, cycloartenol synthase subfamily performing core housekeeping functions have undergone minimum post speciation expansion. Evolutionarily, it accounts for the existence of multiple OSCs and numerous skeletal types of triterpenes found in a single plant (Brendolise et al., 2011).

Previous studies have revealed many members of OSC gene family exhibiting multifunctional properties wherein a single OSC contributes more than one functional triterpene rather than being a single product specific enzyme. Thus, sequence homology is far from being a definitive argument to illustrate the defined enzymatic activity of different OSCs. For instance, C. speciosus CsOSC2 is a multiproduct synthase producing lupeol, germanicol, and β-amyrin (Kawano et al., 2002). Likewise, orthologous mixed-amyrin synthases have been identified in L. japonicas and P. sativum (Iturbe-Ormaetxe et al., 2003, Morita et al., 2000). Functional validation of the three WsOSCs in S. pombe revealed their mono-functionality. LC-MS profile of each WsOSC extract was essentially identical to its respective authentic standard showing a single product whose retention time was coincident with that of the standard (Figure 9.1). MS and fragmentation patterns of WsOSCs products and their respective standards further authenticated their mono-functionality. The combined results clearly demonstrate that WsOSC/BS, WsOSC/LS and WsOSC/CS encode mono-specific proteins committed for biosynthesis of defined single product and thus being a favourable attribute for future pathway engineering endeavors (Figure 10.1, 11.1).

Copy number validation of WsOSC/BS, WsOSC/LS and WsOSC/CS was done using southern blot analysis. Both WsOSC/BS and WsOSC/LS exist as single copy number genes whereas WsOSC/CS may have more than one copy in W. somnifera genome. In view of
the fact that cycloartenol synthase participates in both sterol and withanolide biosynthesis, we wanted to know that how cycloartenol synthase confronts the high metabolic demand in terms of gene copy number (Figure 19.1). The results suggest that possibly dual copies of WsOSC/CS might be involved in carrying primary and secondary functions separately in *W. somnifera*. However, it needs further validation.

The spatial expression pattern revealing high levels of WsOSC/CS in leaves supports earlier finding where leaves were reported to be the richest source of withanolides in *W. somnifera* (Figure 14.1) (Lattoo et al., 2007). Previous reports have shown high expression of WsSQS, WsSQE and WsCPR2 in leaves comparatively (Bhat et al., 2012, Rana et al., 2013, Razdan et al., 2013).

In view of the complexity of secondary metabolism, present investigation demonstrated variation in withanolide production at different ontogenetic stages in two morpho-chemo variants of *W. somnifera*. Metabolite flux was found to be related with the transcript abundance of five pathway genes. HPLC based chemoprofiling of WS-Y-08 and WS-R-06 revealed significant differences in the concentration of the three key withanolides in leaf and root tissues at different phenophases. However, the metabolite dynamics through different developmental phases in the two accessions did not present any congruence in the pattern of metabolite accumulation.

Towards the over-maturation stage WS-1, WS-2 and WS-3 withanolides showed a dwindling trend with either absence or accumulation in meager amounts (Figure 21.1). This may be attributed to the allocation and utilization of finite resources towards the reproductive maturation and seed development. In terms of energy budgeting, *W. somnifera* invests considerable resources in reproductive effort as per plant the average flower count that mature into fruits is around 6525.3 ± 231.47 (~49%) and the number of seeds per fruit average 25.24 ± 0.54 (Lattoo et al., 2007). Presumably, the channelization of limited resources results in such qualitative and quantitative metabolite variations which are essential to optimize various putative cost expenses involved in adaptation and growth of a plant.

Present investigation suggests that there is no particular single developmental stage which is suitable for obtaining the maximum amount of withanolides as peak accumulation stages differ with regard to three withanolides. For WS-2 and WS-1, vegetative stage seems to be the optimum time of harvesting while as WS-3 displays its highest accretion at fruit set stage (Figure 21.1). Purview of literature reveals that metabolite accumulation in several plant
species is coincident with different developmental stages and there is not always a direct association between the developmental stage and metabolite accumulation. For instance in *A. annua*, sesquiterpene lactone endoperoxide artemisinin production peaks during flowering (Ferreira et al., 1995). However, induction of flowering well in advance by constitutively expressing flowering promoter factor gene (fpf1) and the early flowering gene CONSTANS from *Arabidopsis*, in *A. Annua*, did not lead to a parallel increase in the artemisinin content (Wang et al., 2004, Wang et al., 2007). This suggests that there is no straight regulatory relationship between flowering and artemisinin synthesis. Hence it may be contended that there are several other factors that concur to regulate the production of secondary metabolites. Among these, physiological demands, weather, condition of plantation and various aboveground and belowground stresses are a few factors leading to variation in secondary metabolite production. As plants are sessile and devoid of immune system, alternative strategies have evolved to guard them against various vagaries by enormous variety of secondary metabolites. Additionally, secondary metabolites play enormous role in surmounting stress constraints and to survive changing environment by showing huge variations both qualitatively and quantitatively (Edreva et al., 2008). This resultant systemic plant defense response can impinge on the outlying tissues making plants the potent intermediaries of exchanges.

Studies have shown artificial damage of root or shoot to be an inducer for enhanced terpenoid concentrations (Bardgett and Wardle, 2003, Bezemer et al., 2004, Rasmann et al., 2005). As, withanolides are presumed to behave like growth regulators (Sangwan et al., 2008), thus withanolides probably intercede through many innate interactions. Predominant leaf concentration of withanolides has been reported earlier also and presumed to result in import of withanolides from leaves to roots. Likewise, leaves of both WS-R-06 and WS-Y-08 exhibit maximum concentrations of all the three withanolides as compared to roots. However, a relative analysis between root and leaf chemoprofiles of the present study refutes the idea of import by showing no subsequent increase in root withanolides with the declining leaf withanolide concentrations. This implicates *de novo* biosynthesis of withanolides in roots that has also been argued by providing experimental evidence for the biosynthesis of withanolide A in cultured roots as well as native roots of *W. Somnifera* (Sangwan et al., 2008).
The spatial, temporal, and inducible biogenesis of secondary metabolites and the transcripts of related biosynthetic genes are under strict transcriptional regulation. Transcriptional control involving mRNA helps in integrating both developmental and environmental signals. WsSQS, WsSQE, WsOSC/CS, WsCPR1 and WsCPR2 genes comprise vital genic components of biosynthetic pathways leading to production of phytosterols and a large array of triterpenoids. These are the upstream genes involved in the withanolide biosynthesis which regulate the flux by channelizing the downstream precursors. Expression level of all the five genes was significantly higher in leaves of both the varieties as compared to the roots. WsSQS showed slightest digression in expression in leaf and root tissues of both the varieties. Increase in WsSQE transcript level displayed an up-regulation. WsOSC/CS transcripts followed an increasing trend along progressing ontogenetic stages (Figure 20.1). Cycloartenol synthase performs the important function of breaking 11 bonds and forming 11 new ones to transform 2, 3-epoxysqualene to the plant sterol precursor cycloartenol (Rees et al., 1968, Rees et al., 1969). It is presumed that cycloartenol bifurcation takes place for the biogenesis of sterol and withanolides in W.somnifera (Figure 1.1). Probably because of this division of cycloartenol, WsOSC/CS expression was the maximum and on the rise with each advancing phenophase to generate a reservoir of cycloartenol which may get channelized towards the two routes leading to phytosterols and withanolides. WsOSC/CS also revealed a copy number of two possibly indicating the separate role of each copy of WsOSC/CS in sterol and withanolide biosynthesis. Duplicate copy number of WsOSC/CS is plausibly a trigger for higher expression. CPR shuttles electrons derived from NADPH through FAD and FMN domains into the heme iron-centre of the various P450 enzymes and thus confront the high demand of electron supply during biotic and abiotic stress or differential expression at various stages of plant development (Simmons et al., 1985). WsCPR1 transcript level demonstrated no change in comparison to WsCPR2 that showed a slight increase along the developmental phases possibly implicating its role in biosynthesis of withanolides. Thus by integrating and understanding the various interactions between genomes, metabolomes or molecular networks in their entirety, changes in secondary metabolite composition during ontogenesis that are characteristic for each developmental stage can be inferred. All such ontogenetically determined changes are the product of interaction between genotype and environment. To get an insight into the regulatory mechanism of the three OSCs, promoter regions of WsOSC were isolated and presence of various cis-acting elements were confirmed using in
silico tools (Figure 15.1). Cis-acting regulatory elements and their corresponding transcription factors constitute one of the transcriptional regulatory mechanisms induced by different environmental and extracellular conditions to help the plants in adaptive strategies (Walther et al., 2007). Therefore, presence of numerous putative cis-regulatory motifs in the three OSC promoters suggests control over their transcriptional activity being mediated in response to various signals. Elicitations mediated by MeJA, GA3 and YE altered OSC transcript profiles and demonstrated change in withanolide content.

Transcription, RNA processing, translation and post-translational modification constitute different levels at which regulation of cellular processes takes place. Nevertheless, numerous studies have revealed transcriptional modulation of genes as a frequent response to elicitations (Zhao et al., 2005). Generally, mRNA concentrations are broadly employed as a surrogate for protein expression. However, various studies evaluating mRNA and protein expression on a global scale, point towards their partial correspondence (Brockmann et al., 2007). Approximately, it has been assessed that only 20%–40% of protein expression is determined by their analogous mRNA concentrations (Tian et al., 2004, Nie et al., 2006). Consequently, examination of translational differences along with mRNA measurements is imperative for a better interpretation of obtained results (Kolkman et al., 2006).

In present study the three elicitors acted as both positive and negative regulators for the three OSCs. Differential transcript and translational profiles were clearly reflected in relation to elicitor treatments with discernible changes in withanolide concentrations. MeJA elicitation significantly increased the WS-3 accumulation over a period of 48 h. These results are in conformity with our earlier studies where MeJA mediated induction of WsSQS, WsSQE and WsCPR2 mRNA also led to enhanced withanolide accumulation. It may be attributed to increased synthesis of 2, 3-oxidosqualene produced by induced upstream genes. As a consequence, WsOSC/CS is able to utilize an increased precursor pool for withanolide biosynthesis. Although the OSC mRNA expression model in case of GA3 coincided with MeJA treatment, the total withanolide accumulation demonstrated a regular drop with increasing time-course. This may be attributed mainly to the decrease in WsOSC/CS protein concentration as evident from the western blot study. Nevertheless, transcript abundance of WsOSC/BS showed a rise which hinted towards the decrease in the total substrate availability for WsOSC/CS but at protein level, WsOSC/BS expression declined with increasing time intervals. Thus possibly substantiating the drop in WS-3 concentration due to decreased WsOSC/CS protein availability (Figures 16.1, 17.1, 18.1).
Discussion

Interestingly, microbe-derived exogenous YE elicitor played a role of negative regulator for the two competitive OSCs of WsOSC/CS (WsOSC/BS and WsOSC/LS) at both protein and mRNA level. While as WsOSC/CS showed no change in its transcript or protein expression in response to YE. However, there was significant increase in withanolide concentration with YE in comparison to MeJA treatment. The down regulation of WsOSC/BS and WsOSC/LS is possibly indicative of differential channeling of common substrate among the three branch OSCs (Figures 16.1, 17.1). Plausibly, this leads to rearrangement of metabolic fluxes wherein bulk of 2, 3- oxidosqualene substrate pool shifts towards WsOSC/CS leading to much improved withanolide yields.

Further, for homologous intensification of withanolides, these results in totality could be useful to reveal various underlying signal transduction pathways as indicated by elicitations in corroboration with cis-regulatory motifs. Specific transcription factors along with the biosynthetic genes can become prospective targets for pathway engineering. Plausibly, the characterization and validation of WsOSCs seems important for strategizing the enhanced production of withanolides.

Conclusion

Detailed understanding of both biosynthetic and regulatory gears provide potential means to improve the specificity and effectiveness of genetic modifications. And such metabolic engineering efforts augment well for the manipulation of metabolic flux towards efficient biosynthesis of desired secondary metabolites. With this viewpoint, three members of OSC super-family that are juxtapositioned at a critical metabolic branch-point leading to the production of sterols, withanolides and an array of various triterpenoids were cloned and characterized in conjunction with effect of elicitations and dynamics of withanolide biosynthesis.

The main inferences of the present study are as follows:

- Three full length OSC genes WsOSC/BS, WsOSC/LS and WsOSC/CS positioned at a key metabolic branch point were successfully amplified from W. somnifera.
- Presence of conserved catalytic aspartic acid (D) (Motif C) significant for converting squalene into a carbocation necessary for initiating ring cyclization was confirmed in all the three WsOSCs. Further, terpene synthase signature pattern of OSCs in all the three ORFs rich in aromatic residues, substantiated the oxidosqualene cyclase identity of the three WsOSCs.
Copy number validation of WsOSC/BS, WsOSC/LS and WsOSC/CS indicated the presence of dual copies of WsOSC/CS in Withania genome, suggestive of its role involved in carrying primary and secondary functions separately in W. somnifera.

LC-MS profile and fragmentation patterns of WsOSC/BS, WsOSC/LS and WsOSC/CS and their respective standards authenticated their mono-functionality. The combined results clearly demonstrate that WsOSCs encode mono-specific proteins committed for biosynthesis of defined single product and thus being a favourable attribute for future pathway engineering endeavors.

Evaluation of kinetic parameters of purified WsOSC/BS, WsOSC/LS and WsOSC/CS proteins showed WsOSC/BS having the highest affinity towards 2, 3-oxidosqualene along with a higher specific activity of 2.9 µM/min/ml as compared to WsOSC/LS and WsOSC/CS.

Higher accumulation of withanolides in leaves corroborated well with the higher expression profile of WsOSC/CS in leaves, pointing towards the involvement of WsOSC/CS in withanolide biosynthesis.

Study of withanolide accumulation and expression study of withanolide biosynthetic genes at different phenophases revealed that the dynamics of accumulation of withanolides and the transcriptomic abundance of various key pathway genes during ontogenesis are synchronized.

Comparative quantitative and qualitative chemoprofile of leaf and root tissues revealed no correspondence between the patterns of accumulation in the two tissues, thus apparently pointing towards de novo tissue-specific synthesis of withanolides in W. somnifera.

Presence of numerous putative cis-regulatory motifs in the three OSC promoters suggested control over their transcriptional and translational activity being mediated in response to various signals. This was substantiated by the elicitations mediated by MeJA, GA₃ and YE that altered OSC transcript profiles and demonstrated change in withanolide content.

MeJA and YE treatments led to increase in WS-1 and WS-3 withanolide content. However, the negative regulation brought about by YE over the two competitive branch OSCs (WsOSC/BS and WsOSC/LS) led to a pronounced increased in withanolide biosynthesis in comparison to MeJA.
The present investigation has provided detailed knowledge about three OSCs covering three branches of an important metabolic branch point. For further homologous accentuation of withanolides, these results may help to reveal various underlying signal transduction pathways and specific transcription factors which along with the biosynthetic genes can become prospective targets for pathway engineering. Plausibly, the characterization of \textit{W. Somnifera} OSCs seems essential for complementing withanolide biosynthetic route.