Review of Literature
I. PHYSIOLOGY AND MOLECULAR MECHANISMS OF ADVENTITIOUS ROOTING

AR formation is stimulated as a result of root excision, which possibly reduces the sink size for the basipetally translocating substances, leading to their build up in the hypocotyl base. Root excision could also probably remove the inhibitors of AR being synthesized in roots. Various environmental and endogenous factors, such as temperature, light, hormones (particularly auxin), sugars and mineral salts, act as cues for promoting redifferentiation of predetermined cells resulting in adventitious root induction.

Effect of various pharmacological agents on adventitious rooting

i. Auxins: Regeneration of roots on stem cuttings has long been known to require exogenous auxin in many plant species whereas, in some cases, roots form spontaneously as a result of basipetal transport of endogenous auxin synthesised in the shoot apex (Diaz-sala et al., 1996; Blazkova et al., 1997). Interestingly, application of auxin to these plants strongly enhances the number of roots (Nordström and Eliasson, 1991). IAA, the most abundant endogenous auxin, IBA (subsequently discovered as an endogenous auxin), and NAA (a well-known synthetic auxin), have often been used commercially for AR formation. As a corollary, triiodobenzoic acid (TIBA, an inhibitor of IAA transport), 1-naphthylphthalamic acid (NPA, a phytotropin) and latrunculin B (Lat-B, actin depolymerizing agent) have been reported to inhibit AR formation. Generally, IBA leads to more positive response for AR formation (Diaz-sala et al., 1996; Rout, 2006; Kesari et al., 2009; Dash et al., 2011), followed by NAA and IAA. A difference in the effectiveness of these three auxins could be due to differences in their chemical nature and concentration reaching the target cells (de Klerk et al., 1999). It could also be species-dependent. Auxins are reported to be taken up in cells by pH trapping and by influx carriers, and subsequently, they undergo oxidation and conjugation. IAA, and to a lesser extent IBA, may be irreversibly inactivated by oxidation, whereas NAA does not get oxidized. Auxins may also be reversibly inactivated by conjugation and then released subsequently, thereby limiting the availability of free auxin. Auxins appear to show dose-dependent rooting
response. In *Pongamia pinnata*, 4.92 mM IBA leads to maximum rooting response and concentrations above 7 mM inhibit AR formation (Kesari et al., 2009). An interaction between the auxins (three component mixture) has been reported to be most effective in AR (Kesari et al., 2009).

Associated with the three phases of AR formation i.e. induction, initiation and extension, there occur biochemical changes which modulate AR formation. In *Camellia sinensis* and *Saraka asoka*, IAA oxidase activity is reduced during the initial phases (induction and initiation) and it subsequently increases during the extension phase (Dash et al., 2011; Rout, 2006). This pattern of IAA oxidase activity coincides with the pattern of free IAA available during the three phases, it being high in the initial two phases and decreasing thereafter (Nag et al., 2001), thus supporting the proposition that auxin-sensitive and auxin-insensitive phases exist during AR formation (Hartmann et al., 1993). Accompanying AR formation, there occurs cell wall lignification resulting from peroxidase activity (Sato et al., 1993; Chen et al., 2002). Moreover, IBA treated explants are reported to exhibit enhanced peroxidase activity till the extension phase (Rout, 2006; Dash et al., 2011). Similarly, polyphenol oxidase activity gets reduced during the extension phase and phenol levels are reported to be high during the initiation and extension phase. de Klerk et al. (1999) found that phenolic compounds act as antioxidants, protecting IAA from oxidation, and plant tissues from stress due to wounding.

Auxin-induced gene expression is classified into short-term and long-term responses. Short-term responses upregulate genes within a span of few minutes whereas the long-term responses lead to gene expression in few hours and the response may continue to increase. Members of the AUX/IAA family encode short-lived transcription factors (within 5-60 mins of auxin addition) that serve as activators or repressors for late auxin-inducible genes. The ARF (auxin response factors) family of transcription factors act as activators (as dimers) for the early response genes by binding with auxin response elements. AUX/IAA encoded proteins bind to ARFs, forming inactive hererodimers, thereby blocking either gene activation or suppression. In the presence of high auxin, TRANSPORT RESPONSE 1(TIR1) blocks AUX/IAA proteins, leading to their ubiquitination and degradation and subsequent formation of
active ARF dimmers. Auxin directly binds with TIR1 protein and promotes its binding to AUX/IAA proteins (Li et al., 2009a; Taiz and Zeiger, 2010).

ii. Nitric oxide: Nitric oxide is a bioactive molecule that has emerged as an important plant messenger in the last decade. Exogenous supply of two NO donors- sodium nitroprusside (SNP) and S-nitroso N-acetyl penicillamine (SNAP) to cucumber explants mimicks the effect of IAA, leading to AR formation (Pagnussat et al., 2002). SNP elicits its effect in a dose-dependent manner, with maximal biological response at a particular concentration, which is species-dependent. 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), a NO scavenger, completely inhibits AR formation, emphasizing the involvement of NO in AR. Treatment of explants with IAA in combination with NO donors results in enhanced response, leading to profuse and longer roots as compared to those in explants treated with SNP or IAA alone (Pagnussat et al., 2002; Huang et al., 2007). Explants coincubated in IAA and NO scavenger (PTIO) delays AR emergence and reduces the number and length of roots formed, thus demonstrating the involvement of NO in mediating auxin response leading to AR formation. Employing the inhibitor for guanylate cyclase (GC), it has been demonstrated that NO operates downstream of IAA, promoting adventitious root development through the GC-catalyzed synthesis of cGMP that further induces the Ca^{2+}-dependent protein kinase (CDPK) (Pagnussat, 2003; Lanteri et al., 2006). Alternatively, MAPK signalling cascade is activated by IAA via a NO-mediated but cGMP-independent pathway (Pagnussat et al., 2004).

iii. Hydrogen peroxide: Hydrogen peroxide (H_{2}O_{2}), one of the reactive oxygen species (ROS), has long been considered as a toxic metabolite. However, in the last decade, H_{2}O_{2} has emerged as a signalling molecule in various physiological processes both in plants and animals (Neill et al., 2002; Quan et al., 2008). H_{2}O_{2} is continuously synthesized at basal levels during photosynthesis and respiration in plants. Enzymatically, it is produced by NADPH oxidases, cell wall peroxidases, amine oxidases and other flavin-containing enzymes. Initially, H_{2}O_{2} was known to mediate responses against abiotic and biotic stresses, such as pathogen attack (Bolwell and Daudi, 2009; Vellosillo et al., 2010), wounding (Orozco-Cardenas et al., 2001), water deficit (Jubany-Mari et al., 2010), salinity stress (Miller et al., 2010), alteration in
phytohormone levels, such as ABA (Zhang et al., 2001; Bright et al., 2006) by systemic acquired resistance (SAR) and hypersensitive resistance (HR), stomatal closure and programmed cell death (PCD, Gadjev et al., 2008). Recent reports have shown that H$_2$O$_2$ plays an important role in various plant growth and developmental processes, such as growth of lateral roots (Su et al., 2006), root hair development (Foreman et al., 2003; Rentel et al., 2004; Dunand et al., 2007), root gravitropism (Joo et al., 2001), pollen-stigma interaction and their development (McInnis et al., 2006a,b), and formation and development of adventitious roots (Li et al., 2009b, Liao et al., 2009; She et al., 2011).

Over the past few years, role of H$_2$O$_2$ as a signal molecule during adventitious root formation has gained momentum. It has been reported to play an important role downstream to auxin (IBA) during AR formation in mung bean (Li et al., 2009b) and cucumber seedlings (Li et al., 2007). As a wounding response, H$_2$O$_2$ levels reach maximum in mung bean seedlings within 36 hrs of removal of primary root, whereas treatment with 10 µM IBA leads to H$_2$O$_2$ maxima within 3 hrs. This indicates that IBA induces higher production of H$_2$O$_2$ and that high concentration of H$_2$O$_2$ may be an essential component for AR formation. Chou et al. (2010) reported the expression of some anionic peroxidases (POX, pl 4 and pl 5.3) in soybean hypocotyl explants when incubated in auxins (IAA, IBA and NAA). Sequencing of the 5’ end of pl 5.3 POX has revealed the presence of ARF/AuxRE motif in the promoter region which is homologus to the sequences commonly found in auxin response elements. Exogenous supply of H$_2$O$_2$ promotes the formation and growth of AR in cucumber and mung bean seedlings (Li et al., 2007, 2009b), and the promotory effect of H$_2$O$_2$ has been shown to be partly or completely eliminated by the inhibitors or scavengers of H$_2$O$_2$ (diphenylene iodonium (DPI), catalase, ascorbic acid). This indicates the clear involvement of H$_2$O$_2$ in AR formation. H$_2$O$_2$ has been reported to activate the calcium permeable channels in the plasma membrane, which acts as a second messenger linking H$_2$O$_2$ to the activation of processes for AR formation (Huang et al., 2011). The role of both extra- and intracellular calcium pools has been confirmed by the use of calcium channel inhibitors [Ruthenium Red (RR), Lanthanum Chloride (LaCl$_3$)] and calcium chelator-EGTA, that have been reported to inhibit AR formation in H$_2$O$_2$ treated seedlings.
Employing the fluorophore dichlorofluorescein (H$_2$DCF), Huang et al. (2011) have shown spatiotemporal changes during the initiation and development of AR. H$_2$O$_2$ signals have been localized in the pericycle cells between vascular bundles, 24 hrs after the removal of the primary root. Thereafter, H$_2$O$_2$ signal gradually increases with the development of root primordia, and it is primarily confined to the meristem.

The role and relationship of nitric oxide and hydrogen peroxide during AR formation has also been investigated in marigold and chrysanthemum (Liao et al., 2009, 2010). It has been reported that a combined treatment of NO and H$_2$O$_2$ leads to the formation of greater number of roots, and roots are longer than those produced with individual treatments of NO or H$_2$O$_2$. Interestingly, NO treatment leads to enhancement of endogenous H$_2$O$_2$. It is reported that 5'-cyclic guanosine monophosphate (cGMP) is involved in the NO-induced AR formation in marigold whereas it is not involved in H$_2$O$_2$-mediated rooting (Liao et al., 2009). This suggests that NO and H$_2$O$_2$ mediated signalling pathways may share some common targets and they may have some unique components to them. Consequently, NO and H$_2$O$_2$ enhance AR synergistically and independently. Among the biochemical parameters, NO and H$_2$O$_2$ treatments in a proper dosage, increase the activities of polyphenol oxidase (PPO), indole-3-acetic acid oxidase (IAAO) and the content of water soluble carbohydrates and total nitrogen while repressing the total polyphenol content (Liao et al., 2010).

iv. Ethylene: Ethylene, the gaseous plant hormone, is also known to be involved in adventitious rooting. Although enough work has not been done in this context but a few recent reports clearly suggest an ethylene-auxin cross-talk during AR. Employing various mutants of tomato, ones which exhibit a block in ethylene responses and delayed ripening (nr – never ripe, gr – green ripe, rin – ripening inhibitor, nor – non ripening) and those with enhanced ethylene production (epi – epinastic), the role of ethylene has been demonstrated in reduced and enhanced AR response, respectively (Negi et al., 2010a). Interestingly, ethylene exerts a negative effect on lateral root formation as a consequence of drastic reduction of free auxin content in roots but not in hypocotyls. This depicts auxin transport as a pivotal aspect in the ethylene-auxin crosstalk. Employing these tomato mutants, AR formation in response to waterlogging has also been studied (Vidoz et al., 2010). Water logging appears to
stimulate auxin transport, leading to its accumulation in the lower part of the stem. The accumulated auxin initiates rooting in the submerged stem. Ethylene diffusing from the vascular bundles leads to localized accumulation of auxin that also promotes AR formation (Aloni et al., 2006). Ethylene facilitates AR emergence by loosening the cell wall of hypocotyl (by regulating the apoplastic pH and regulation of expansin genes) and by promoting programmed cell death (Vidoz et al., 2010). Ethylene has been demonstrated to have stage-specific effect on AR, it being promotory in the initial stage (thus rendering more cells responsive to auxin) and inhibitory thereafter (de Klerk and Hanecakova, 2008).

v. Phosphatidic acid: Auxin-induced NO triggers a rapid and transient accumulation of phosphatidic acid (PA) in cucumber explants by the activation of phospholipase D (PLD), which hydrolyzes structural phospholipids, such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) (Lanteri et al., 2008). PA has been reported to accumulate within one min of auxin or NO treatment and its level returns back to control within 20 to 30 min. Treatment with IAA or NO donor for 10 min increases AR number by 200% as compared to controls. Interestingly, when PA is supplied exogenously, upto 300% increase in the number of AR has been reported. It is evident that auxin relies on NO to form PLD-derived PA, which is involved in early signalling events during AR formation (Lanteri et al., 2008; Distéfano at al., 2010). But investigations on the role of exogenous phospholipids in the regulation of plant growth are still in their infancy.

NO-auxin crosstalk during adventitious rooting

The polar movement of auxin from the source (meristem) to the sink (base of hypocotyl) leads to auxin accumulation in the basal portion of hypocotyl segments, where adventitious roots are formed by nitric oxide-dependent signalling cascade (Pagnussat et al., 2002, 2003, 2004). Though both auxin and NO are involved in AR, the induction phase is governed by auxin alone, independent of NO. NO becomes operative in this auxin-modulated response (AR) during the initiation and extension phases only (Huang et al., 2007). The spatial distribution of NO during different stages of AR formation has been mainly visualized by DAF-2DA, a fluorescent probe
for NO (Kojima et al., 1998). In the initial stages, both apoplastic and symplastic NO fluorescence has been reported in the actively dividing interfascicular parenchyma cells, which gradually increases and is intense, particularly in the meristem at the later stages of AR development (Huang et al., 2007). Localization of NO to the meristem suggests it to be related to cell division and differentiation (Huang et al., 2007). These spatiotemporal changes in NO levels are accompanied by similar changes in the expression pattern of NO generating system in mung bean hypocotyl cuttings (She and Huang, 2004; Huang et al., 2007). NADPH-diaphorase, a commonly employed marker for NOS activity, co-localizes with NO distribution in hypocotyl explants, specifically in the root primordia. By employing a NOS specific inhibitor-[L-NAME (N⁰-nitro-L-Arg-methyl ester)], significant inhibition of NADPH-diaphorase activity, NO fluorescence and AR formation have been reported, thus suggesting that endogenous NO may be partially catalyzed by putative NOS during adventitious rooting.

Application of exogenous auxin (IAA, IBA) and NO (SNP) has been reported to promote AR formation (Pagnussat et al., 2002, 2003) in hypocotyl cuttings. Tewari et al. (2008 a,b) have reported that in Panax ginseng, exogenous NO activates NADPH oxidase (NOX) activity, which leads to increased generation of superoxide ion (O₂⁻), subsequently inducing adventitious rooting. Thus, O₂⁻ generation is involved in NO-mediated AR formation. Interestingly, H₂O₂, the dismutation product of O₂⁻, has been found to be drastically reduced by SNP supply, as a result of the upregulation of H₂O₂ scavenging antioxidant enzymes (SOD-superoxide dismutase, CAT-catalase, POD-peroxidase, APX-ascorbate peroxidase and GR-glutathione reductase). Thus, NO stimulates both prooxidant (NOX) and antioxidant enzymes during AR formation in Panax ginseng.

Non-hormonal metabolic changes

i. Alkamides: Plants use a diverse array of small molecules for extra- and intracellular signalling, along with the classical plant hormones. Of them, plant lipids are of particular interest because they not only act as structural components of membranes but also as signalling molecules which regulate developmental and adaptive responses
of plants to environmental stimuli (Cruz-Ramírez et al., 2004; Wang, 2004). Reports have suggested the involvement of N-Acylethanolamines (NAEs) in diverse physiological processes, including seed germination, pathogenesis, regulation of root architecture and response to herbivory (Blancaflor et al., 2003; Chapman, 2004; Wang et al., 2006). NAEs are a group of lipids produced from the hydrolysis of N-acylphosphatidylethanolamide (NAPE; a minor lipid constituent of cell membranes) by the action of phospholipase D. NAE-related compounds, including alkamides (N-alkyl amides), alter root development and regulate cell division and differentiation processes in Arabidopsis thaliana (Ramírez-Chávez et al., 2004; López-Bucio et al., 2006). Campos-Cuevas et al. (2008) suggested a novel role for alkamides in the regulation of AR development, independent of auxin, and probably operating in the NO signal transduction pathway. The role of auxin in mediating AR proliferating activity of alkamides was tested. The effect of N-isobutyl decanamide (a natural alkamide with saturation grade and medium length of the fatty acid chain) was evaluated in the morphogenetic response of explants obtained from hypocotyl and roots of dark-grown A. thaliana seedlings (Campos-Cuevas et al., 2008). Previously, Ramírez-Chávez et al. (2004) showed that affinin (an alkamide produced in Heliopsis longipes roots) increases the number of lateral roots in A. thaliana seedlings. This effect has been attributed to the dual activity of this compound to induce pericycle cells to divide and form new primordia, and to stimulate the emergence of existing primordia (Ramírez-Chávez et al., 2004). These findings have been further confirmed by Campos-Cuevas et al. (2008) by reporting the effect of alkamides in the regulation of AR formation. Both N-isobutyl decanamide and affinin have been shown to have a promoting effect on the number of adventitious roots. However, opposite effect was observed for AR length. This suggests that alkamides can mimic the effect of an endogenous compound that plays some role in the generation and development of adventitious roots. Both lateral and adventitious roots are formed post-embryonically but lateral roots typically arise from the root pericycle. The molecular mechanisms of AR formation are still poorly understood. Two possible pathways as potential targets of the alkamide signal could be: 1. the classical auxin pathway, wherein physiological and genetic evidence have demonstrated a critical role of auxin, which is supplied by shoot tissues through polar transport system for the initiation of AR. 2. the nitric oxide
response route. But still, the target molecules of both auxin and NO in the shoot response to generate AR are not known. NO accumulation at different stages of development of explants was shown to be induced by N-isobutyl decanamide. NO level at the sites of AR formation increases with alkamide treatment, as has been shown through confocal microscopic analysis (Campos-Cuevas et al., 2008). Whether NO mediates AR response to alkamides, remains to be investigated.

ii. Jasmonic acid: NO is an interesting signalling molecule having homeostatic properties for the coordination and synchronization of cellular metabolism (Lamattina et al., 2003). NO stimulates both prooxidant (NADPH oxidase; NOX) and antioxidant (SOD, CAT, POD, APX, DHAR and GR) enzymes in the AR formation in mountain ginseng (Tewari et al., 2007). NO, thus, acts as an antioxidant, most likely by directly reacting with ROS (particularly with H$_2$O$_2$). NO being a redox active molecule, it can also interact with ascorbate and thiol. SNP elicitation also increases the total ascorbate contents. NO modulates ascorbate and glutathione, most likely by modulating the activities of enzymes (APX, DHAR and GR) involved in ascorbate glutathione cycle. Overall, antioxidant defense system is upregulated upon NO elicitation (Tewari et al., 2007).

The synthesis of secondary metabolites in plants is believed to be a part of their defense response against biotic and abiotic stresses. NO plays a crucial role in the synthesis of secondary metabolites in cell suspension cultures via chemical agents (e.g. methyl jasmonate; Wang and Wu, 2005), physical agents and ultrasound (Wang and Wu, 2004; Wach et al., 2005; Xu et al., 2005). ROS production is an early event in the elicitation-induced synthesis of secondary metabolites in plant cell cultures through biotic and abiotic means (Yuan et al., 2001; Wu and Lin, 2003). Wu et al. (2007) provided first report on the relationship among NO, oxidative burst and secondary metabolite synthesis in the adventitious roots of *Echinacea purpurea*. They also investigated the relationship between NO and other elicitor responses (H$_2$O$_2$ production), the activation of antioxidant defences and production of useful secondary metabolites from *E. purpurea* AR cultures. They showed that exogenous NO supply through SNP is beneficial for the accumulation of secondary metabolites (phenolics, flavonoids and caffeic acid derivatives) as well as for the upregulation of antioxidant
defense in AR of *E. purpurea*. NO modulates the levels of both ascorbate and glutathione in *E. purpurea* AR.

AR formation in leafy stem cuttings is a crucial physiological phenomenon for the propagation of many plant species. However, a lack of knowledge on the molecular physiological basis hampers the development and application of reliable technologies. AR formation is a complex process that requires successive developmental phases involving different hormonal signals and other factors (de Klerk et al., 1999). After detachment of the shoot, basipetal polar transport of auxin contributes to auxin accumulation at the base of stem (Garrido et al., 2002) and the increase in free auxin at the basal region of stem contributes to the early events of AR formation (Blakesley, 1994; de Klerk et al., 1999; Sorin et al., 2005). Wounding in majority of the plants also leads to an increased production of jasmonates (Schilmiller and Howe, 2005). The role of jasmonates in AR formation is almost unknown. They are synthesized via the octadecanoid pathway (Feussner and Wasternack, 2002) and the enzyme allene oxide cyclase (AOC) is considered crucial for JA biosynthesis (Wasternack and Hause, 2002). Wound-induced responses, involving a complex network of signalling cascades, activate one of the best-characterized class of signal molecules, called as jasmonates (Wasternack, 2007). Jasmonic acid (JA) and its precursor-OPDA (12-oxo-phtodienoic acid), accumulate transiently with maximum at 0.5 h post-excision, and this occurs locally within the stem base as well as systemically in other parts of cutting, but at lower levels (Ahkami et al., 2009). Further analysis has revealed constantly high activity of a protein present in vascular tissues which is involved in the increased biosynthesis of JA, as previously reported in tomato (Hause et al., 2003; Stenzel et al., 2003). The wound-induced elevation of jasmonate levels is followed by activation of JA-responsive genes (Wasternack and Hause, 2002). One of the JA biosynthetic enzymes, called *PhAOC*, shows a quick increase following the rise in OPDA and JA, exhibiting a transient maximum of RNA accumulation at 1 and 2 h post-excision. Additionally, cell wall invertase transcript accumulation at the stem base seems to be induced by wound-induced jasmonates, as also previously reported for other cell wall invertases (Godt and Roitsch, 1997; Schaarschmidt et al., 2006).
Role of carbohydrate metabolism in adventitious rooting

Adventitious root (AR) formation, being an energy consuming process, relies on adequate supply of carbohydrates to the region of root regeneration (Veierskov, 1988). A high input of energy and carbon skeletons is required during cell division and enlargement. A major carbon source, like sucrose, formed in photosynthetically active tissues, is translocated towards sink parts of the plant. Sucrose is then either cleaved into hexoses as direct carbon source or converted to storage compounds, like starch. Also, increasing evidence suggest a regulatory role of sugars in AR formation. The speed or intensity of AR formation is limited if the level of carbohydrates in cuttings is low at the beginning of rooting (Veierskov et al., 1982; Druege et al., 2004) whereas application of sugars to the rooting medium increases subsequent root formation (Eliasson, 1978; Li and Leung, 2000; Takahashi et al., 2003). Moreover, application of different sugars suggests distinct functions of glucose, sucrose, and starch during different phases of AR formation (Li and Leung, 2000; Correa et al., 2005), thus pointing to a regulatory role of sugars (Druege et al., 2004; Correa et al., 2005). In the stem base of Petunia cuttings, the levels of soluble and insoluble sugars start to increase at 24 h post excision, although high metabolic activity starts from 12 to 24 h post excision. Sucrose can be translocated to the stem base for further metabolism. Although a regulatory action of sugars cannot be excluded, the data presented here support the view that carbohydrates in Petunia cuttings may play an important role in root growth rather than during root initiation (Ahkami et al., 2009). The basipetally translocated sucrose is, however, not only used to deliver energy for differentiation (cell division and enlargement), but a considerable portion of sucrose is converted to starch, which probably acts as the major carbon source when adventitious roots grow. As shown previously in Pinus radiata, sucrose in the growing medium leads to higher levels of sugars and starch in rooting regions of hypocotyl cuttings and it enhances root formation (Li and Leung, 2000). Starch does not seem to be involved in root initiation since there is only a marginal increase in starch content during early stages. However, analyses indicate that starch can be synthesized and stored in different cell types to meet their demand of increased metabolic activity when AR emerge. Cell wall invertase activity has been found to
increase in the first few hours after excision, which is in accordance with the low sucrose content directly after excision. It has been found to decrease again to the basal level before root formation. In contrast, activities of vacuolar and cytosolic invertases decrease continuously to a low level. Cell wall invertases are not only key enzymes of the apoplastic phloem unloading of transported sucrose but they also link phytohormone action with primary metabolism (Roitsch and González, 2004). Invertases located in the apoplast can also establish a sink function of certain tissues and, thus, provide a mechanism for flexible and appropriate adjustment to a wide range of internal and external stimuli (Roitsch et al., 2003). Further work suggests that at early stages of AR formation, basipetally translocated sucrose is degraded via cell wall invertase, and hexoses produced are transported into the cytosol for further metabolism.

**Contribution of tricarboxylic acid cycle and energy metabolism**

Among the amino acids, citrate has been shown to increase dramatically whereas all other amino acids remain unchanged and at low levels. During AR formation, citrate accumulates within the tissue and is probably stored within the vacuole. Considering the dramatic increase concomitant with the formation of meristem, citrate level may be used as a biochemical marker for entering later stages of AR formation in Petunia. Analysis of Petunia stem indicates that mainly glutamine and asparagine are synthesized in source tissue and are transported downward to sink tissue. Since glutamine biosynthesis is the key step for nitrate assimilation in plants and supplies nitrogen for amino acid synthesis, a basipetal translocation of glutamine is an important process to meet the demand of the cells during root formation. Levels of glutamine and asparagine remain low at later stages of AR formation, thus indicating a high turnover of the translocated amino acids into other forms that appear to be essential for the accelerated protein synthesis during AR formation. Ahkami et al. (2009) proposed a three-phase mechanism for the metabolic response involved in AR formation in Petunia: 1. Sink establishment phase: wounding leading to JA accumulation and then to induction of genes coding for enzymes that degrade sucrose within the apoplast to hexoses. These hexoses are then taken up by the monosaccharide transporter and used for the production of energy necessary for
wound healing and cell division. Thus, wounding initiates the establishment of a sink tissue in which all the resources are depleted. 2. Recovery phase: characterized by replenishment of resources and ends with the formation of meristemoids. 3. Maintenance phase: characterized by symplastic transport of sugars translocated from source leaves to stem base. These are converted either directly to intermediates flowing into root development or to the intermediate storage compounds, such as starch and citrate, which are then used for root formation.

AR formation thus appears to be modulated by a combination of different pathways, including hormone biosynthesis and primary metabolism, whose individual steps might exert a control over AR formation. Future work might help us to understand the causal interactions between hormones and specific genes and also how these interactions regulate the process of AR formation.

**Lipid signalling during AR formation**

Considering the signal transduction pathway leading to AR formation in cucumber explants, it has been proposed that NO induces cGMP production by regulating the activity of the enzyme guanylate cyclase (GC) (Pagnussat et al., 2003). Subsequently, Pagnussat et al. (2004) have demonstrated that NO activates a MAPK cascade in a cGMP-independent pathway. Recently, Ca\(^{2+}\) and CDPK have been established in mediating the auxin response leading to AR formation (Lanteri et al., 2006). Lanteri et al. (2008) further investigated phospholipid (PL) signalling during auxin- and NO-induced AR formation in cucumber explants. The signalling phospholipids, phosphatidylinositol phosphate (PIP) or phosphatidylinositol 4,5-biphosphate (PIP\(_2\)), are hydrolyzed to generate the second messengers-diacylglycerol (DAG) and inositol 1,4-biphosphate (IP\(_2\)) or inositol 1,4,5-triphosphate (IP\(_3\)), through the enzyme phospholipase C (PLC). IP\(_2\) can then be further phosphorylated to IP\(_3\), and calcium is then released from intracellular compartments into the cytosol by IP\(_3\) action (Bootman et al., 2001). It has been pharmacologically proved that IP\(_3\)-regulated Ca\(^{2+}\) channel inhibitors, like lithium chloride and neomycin sulphate, suppress AR formation induced by auxin or NO in cucumber explants (Lanteri et al., 2006). It has earlier been demonstrated that auxin generates a rapid and transient increase in both PIP and PIP\(_2\).
levels concomitant with an increase in IP$_3$ levels in the suspension cultures of *Catharanthus roseus* cells (Ettlinger and Lehle, 1988; Grabowski et al., 1991). Similarly, a rise in IP$_3$ level has been shown to be triggered by auxin, followed by the activation of Ca$^{2+}$ channels located in the intracellular compartments of isolated membranes of carrot (Zbell and Walter-Back, 1988; Zbell et al., 1989). Still, there are no data regarding the involvement of PLC/DGK pathway in auxin signalling although evidence regarding the auxin stimulation of PLC activity has been monitored. Still another enzymatic source of PA in plants is phospholipase D (PLD), which hydrolyzes the structural PLs, such as PC or PE, at the terminal phosphodiester bond, to produce PA and a free head group (Wang, 2000). Earlier studies have shown that that auxin has no effect on PLD activity in membranes isolated from *Cucurbita pepo* hypocotyls or in suspension-cultured parsley and soybean cells (André and Scherer, 1991; Paul et al., 1998). Whether PLD participates in the auxin signal transduction pathway in plants, still remains to be elucidated. Work from Lanteri et al. (2008) has provided evidence that the signalling PLs, namely-PA, PIP and PIP$_2$, accumulate in cucumber explants treated with auxin or NO and that this accumulation is transient and dependent on the nature of NO donor as well. Auxin induces a NO-dependent PIP and PIP$_2$ accumulation in cucumber hypocotyls. The existence of different isomers of PIP and PIP$_2$ creates specificity, determining different downstream signalling events, and it has also been shown that the relative abundance of PIP and PIP$_2$ isomers is quite variable, depending on the cell types and /or growth conditions of the plants (Westergren et al., 2001). However, which isomer is induced upon auxin and NO treatments, is still unknown. Previous reports from Lanteri et al. (2006) have suggested the involvement of PLC activity during auxin and NO-induced ARF in cucumber by regulating cytosolic Ca$^{2+}$ concentration via increase in IP$_3$. Evidence suggesting the involvement of PLD activation to trigger PA formation upon induction by auxin and NO in cucumber explants, has been provided by Lanteri et al. (2008). This PA accumulation is due to increased PLD activity. A similar recent report by Im et al. (2007) has also suggested the expression of a human PIP kinase in suspension-cultured tobacco cells, leading to PIP$_2$ accumulation and subsequent production of IP$_3$ via PLC. Additionally, pharmacological data from *Arabidopsis* seedlings have suggested that PLC signalling upon salt stress involves IP$_3$-regulated Ca$^{2+}$ release
from intracellular compartments but not from DAG (Parre et al., 2007). PIP$_2$ has been shown to be a stronger activator than PIP to induce in vitro activities of PLDs (Chung et al., 1997; Pappan et al., 1997; Qin et al., 1997). Most of the members of *Arabidopsis* PLD family are PIP$_2$-dependent and require Ca$^{2+}$ for their in vitro activities (Bargmann and Munnik, 2006). Considering this evidence and the observation from Lanteri et al. (2008) that the accumulation of PIP and PIP$_2$ precedes PLD-derived PA, it can be proposed that PIP and/or PIP$_2$ may directly activate cucumber PLD and/or fulfil its requirement of Ca$^{2+}$ via PLC activity. However, the effect of PIP and/or PIP$_2$ has not yet been studied on cucumber PLD activity. Further work has to be carried out on the characterization of molecular events that link NO with PLD activation. Activation of PLD in vivo during auxin and NO treatments leading to PA generation, has also been shown by Lanteri et al. (2008). The evidence that PLA$_2$ is stimulated by auxin, has been provided by Scherer and André (1989). PLD-derived PA has been shown to be involved in auxin- and NO-induced AR formation (Lanteri et al., 2008). Although exogenously applied PA significantly stimulates AR formation, PA does not exert the same magnitude of response compared with auxin and NO treatments. Auxin is known to induce several signal transduction events other than PLD activity, like cGMP-dependent and cGMP-independent pathways (Pagnussat et al., 2003, 2004). Therefore, exogenous supply of PA should not be expected to fully mimic auxin action. The blockage of PLD activity with 1-butanol results in reduced NO induction of vacoular H$^+$-ATPase and H$^+$-PPase (key enzymes in salt stress responses. Also, Zhang et al. (2006) have shown that exogenously applied PA stimulates these enzyme activities. However, an understanding of the effect of exogenous PL on endogenous PL homeostasis and coupling to endogenous lipid signalling networks, need further investigations (Cowan, 2006). Further research is required to establish the identity of PLD involved in auxin and NO signalling leading to AR formation. Till date, PLD gene from cucumber has not been cloned, making it impossible to correlate the enzymatic activities to a specific gene product (Lanteri et al., 2008). It has been established that protein kinases belonging to diverse families act downstream of PA. A CDPK localized in the membrane has been found to be activated by PA in carrot, consistent with a role in signal transduction pathways involving PLD (Farmer and Choi, 1999). PA has been
shown to induce a wound-activated MAPK in soybean (an important mediator in stress and ethylene signalling; Lee et al., 2001). These observations suggest that PA could possibly activate the CDPK and MAPK pathways, which have been proposed to be involved in the auxin- and NO-induced AR formation in cucumber explants (Pagnussat et al., 2004; Lanteri et al., 2006). Findings from Lanteri et al. (2008) contribute to the identification of new components in the auxin signal transduction pathway and highlight the remarkable complexity of the mechanisms controlling root growth and developmental processes. However, further progress in unravelling the precise crosstalk between various signalling components will certainly improve the pathway of auxin, NO and lipid signalling in plants.

**Involvement of Haem oxygenase-1 in hydrogen sulfide-induced adventitious root formation**

The process of AR formation has, in the recent past, been observed to be regulated by hydrogen sulfide (H$_2$S), along with nitric oxide and carbon monoxide. H$_2$S is known to regulate many aspects of plant growth and development, including seed germination, responses to abiotic stresses, such as salinity, osmotic stress and drought. The molecular mechanism and signal transduction underlying the physiological roles of H$_2$S are, however, still poorly understood. Recently, Lin et al. (2012) have reported the role of Haem oxygenase-1 as a downstream signalling system in the auxin-induced adventitious rooting in *Cucumis sativus*. Haem oxygenase (HO; EC 1.14.99.3) catalyzes the conversion of the haem to biliverdin, a well known antioxidant, leading to the release of CO and iron (Fe$^{2+}$) in animals, plants and other organisms (Shekawat and Verma, 2010; Shekhawat et al., 2011). So far, three isozymes of HO have been reported in mammals. These are: 1. the inducible form HO-1, 2. the constitutively expressed HO-2 and 3. HO-3 isozymes with very low activity. In plants, HO-1 is known to be involved in phytochrome chromophore biosynthesis, and it has also been observed to play a role in adaptive plant responses against UV-B radiation, salinity, osmotic stress, lateral root formation and programmed cell death. The function of HO has been observed to vary according to the need of individual species (Shekhawat et al., 2011). HO-1 has also been observed to be induced by hydrogen peroxide, nitric oxide, auxin and abscisic acid (Li et al.,
2011). It is likely that the upregulation of HO-1 in plants could act as an antioxidant barrier against stress-induced oxidative damage.

In conformity with the above facts, HO-1 donor (sodium hydrosulfide; NaHS), HO-1 inducer (haemin) and HO-1 inhibitor (zinc protoporphyrin) have been observed to alter naphthylnphaltamic acid-triggered inhibition of AR formation in cucumber (Lin et al., 2012). Thus, NaHS leads to a concentration-dependent increase in root formation in conditions which are otherwise inhibitory (presence of NPA). Likewise, HO-1 inducer (haemin) shows maximum inducible effect on AR development at 10 µM. The fact that these effects are due to H$_2$S or HS$^-$ rather than sulphur, is confirmed by the use of Na$_2$SO$_4$, NaHSO$_4$, Na$_2$SO$_3$ and NaHSO$_3$, which remain ineffective in evoking rooting response. Addition of haemin not only leads to induction of adventitious roots, but it also leads to the enhancement of $CsHO-1$ gene expression in cucumber, which is responsible for the synthesis of HO-1.

All these recent observations point to a confluence of signalling pathways involving auxin, H$_2$S and (HO-1/CO) system during adventitious rooting.

**Signal transduction mechanisms operative during adventitious root formation**

Although many components of auxin transport and signal transduction have been identified, the molecular mechanisms and intermediates underlying the signal transduction of auxin-promoted root formation still need further investigations. Direct evidence indicating the involvement of NO as a second messenger in IAA-mediated AR pathway through the activation of GC-catalysed synthesis of cGMP has been put forward by Pagnussat et al. (2003). GC (one of the most studied targets of NO in mammalian systems) along with phosphodiesterase (PDE) has been found to regulate the endogenous level of the cellular messenger cGMP. cGMP is an important signalling molecule present in both prokaryotes and eukaryotes. Several evidence suggest the presence of a NO-inducible GC in plants (Pfeiffer et al., 1994; Durner et al., 1998). The results provided by Pagnussat et al. (2003) clearly strengthen the hypothesis that cGMP synthesis is required for adventitious rooting. cGMP activates the rooting process in IAA-depleted explants to a similar extent as IAA and SNP. A scheme depicting the suggested pathway involving IAA, NO and cGMP has been
shown by Pagnussat et al. (2003) using different treatments, like GC inhibitor LY83583, cell permeable cGMP derivative (8-Br-cGMP) and PDE inhibitor-sildenafil citrate. IAA synthesized in the apical part of the seedling is basipetally transported and accumulated at the base of the hypocotyls where NO is produced in a yet unknown way. Possible sources of NO release could be nitric oxide synthase (NOS) or nitrate reductase (NR). This local and transient NO production might then activate a cGMP-dependent transduction pathway where nitrosylated active GC might govern cGMP formation (Durner et al., 1998; Pagnussat et al., 2003). However, Ludidi and Gehring (2003) reported the presence of a NO-insensitive GC in *Arabidopsis*. cGMP either through cGMP-dependent protein kinase or cADP ribose (cADPR) could further increase cytosolic free calcium in plants, which might be involved in a cascade to activate the cell division and elongation (Kiegle et al., 2000).

In order to provide further insights into the signal transduction mechanisms that are operative during IAA-and NO-induced AR formation, involvement of mitogen-activated protein kinase (MAPK) was tested in cucumber (Pagnussat et al., 2004). MAPKs are known to be involved in signalling various biotic and abiotic stress responses and have also been known to regulate cell cycle and developmental processes. Morris (2001) discussed the role of MAPK cascades in auxin signalling where the extracts from auxin-treated tobacco cells activated MAPKs. Although the above findings represent advancement in the knowledge on auxin signal transduction pathway, but the cell components that act as signal molecules from IAA to MAPK activation and the mitotic initiation, still remain unidentifiable. Activation of MAPK activity during plant defense responses has been shown in tobacco (Kumar and Klessig, 2000) and in *Arabidopsis* (Clarke et al., 2000). Pagnussat et al. (2004) analyzed the effect of MAPK activity during AR formation where protein extracts from hypocotyls of explants treated with water or NO donor SNP or with SNP plus NO scavenger (cPTIO), were assayed for PK activity by in vitro and in gel analyses, using myelin basic protein (MBP) as substrate. An increase in in vitro MBP-kinase activity in cucumber explants after exposure to IAA or SNP was reported with maximal activation being obtained after 3 d of treatments. Also, treatment with MAPKK inhibitor (P098059) showed only a basal MBP-kinase activity in cucumber
explants treated with IAA or SNP. The increase in MAPK activity could even be related to changes observed at the cellular level in hypocotyl sections where maximal MAPK activity was detected simultaneously with cell proliferation and AR primordia formation in IAA and NO treated explants. Treatment of cucumber explants with SNP or IAA plus MAPKK inhibitor (PD098059) produced a dose-dependent reduction in AR formation, indicating that a MAPK cascade could be involved in this auxin-induced process. Anatomical studies have also shown the presence of binucleate cells in transverse sections of IAA and NO-treated explants, including those subjected to MAPKK inhibitor, thus indicating the involvement of MAPK activity in the regulation of cell division rather than during AR primordia formation. Thus, a MAPK signalling cascade is activated during adventitious rooting process induced by IAA in a NO-mediated and cGMP independent pathway. These results are in accordance with the previous findings by Mizoguchi et al. (1994) and by Mockaitis and Howell (2000), who also demonstrated the activation of MAPK cascade in response to auxins. This could mediate AR development by the activation of mitotic process and also by regulating the expression of auxin-responsive genes (Mockaitis and Howell, 2000). However, the observation that MBP-kinase activity was not affected by GC inhibitor and that it is able to prevent AR formation induced by NO, suggested that MAPK pathway does not alone assure AR formation. Thus, at least two different pathways seem to be activated by NO (cGMP-dependent and cGMP-independent) which involve a MAPK signalling cascade during the induction of AR formation. Also, the activation of both pathways is utmost essential for AR development since blockage of anyone of them leads to inhibition of AR. These findings are in accordance with the previous report suggesting an NO action via a cGMP-independent pathway, activating phosphatases and protein kinases, including MAPKs (Lamattina et al., 2003; Neill et al., 2003). AR formation seems to be regulated by a complex set of cellular messengers whereby IAA and NO appear upstream in the signalling cascade and activate MAPK and cGMP. Findings from Li and Xue (2010) have also shown adventitious rooting to be regulated by a complex interplay of various cellular messengers, among which MAPK and cGMP are activated by upstream components that involve hydrogen peroxide, NO and calcium.
The involvement of Ca\(^{2+}\) and the regulation of Ca\(^{2+}\)-dependent protein kinases (CDPKs) activity during IAA-and NO-induced AR formation in cucumber explants has been evaluated by Lanteri et al. (2006). A connection among NO, cGMP, Ca\(^{2+}\), and calmodulin (CaM) pathways has been suggested in previous investigations showing the participation of NO in light-mediated processes in plants (Beligni and Lamattina, 2000). An increase in cytosolic Ca\(^{2+}\) concentration has been triggered by NO by promoting Ca\(^{2+}\) release from intracellular stores through the activation of cADPR/ryanodine-sensitive Ca\(^{2+}\) channels in the fava bean and tobacco cells (Garcia-Mata et al., 2003; Lamotte et al., 2004). Reports suggest a rise in IP\(_3\) concentration following auxin signalling and activation of Ca\(^{2+}\) channels located in the intracellular compartments, such as vacoules (Zbell and Walter-Back, 1988; Ettlinger and Lehle, 1998). In plants, the elevation in cytosolic calcium ions induced by a particular stimulus is perceived and transduced by specific effectors involving Ca\(^{2+}\)-binding proteins and protein kinases which initiate downstream events leading to changes in gene expression, metabolism, cell division and cell elongation (Reddy and Reddy, 2004). These proteins include CaM, calcium-dependent protein kinases (CDPKs), calcium-regulated phosphatases, annexins and integrins. Lanteri et al. (2006) evaluated the involvement of Ca\(^{2+}\) and the regulation of CDPK activity during IAA and NO-induced AR formation in cucumber explants. Whether NO-induced and cGMP-mediated AR formation involve a cADPR/Ca\(^{2+}\)-dependent pathway, was investigated by Lanteri et al. (2006) by treating cucumber explants with compounds that are known to block cADPR/ryanodine-sensitive Ca\(^{2+}\) channels or cADPR synthesis. These inhibitors provoke a significant reduction in both IAA-and NO-induced AR formation. cGMP has also been shown to activate PM-associated Ca\(^{2+}\) channels, called as cyclic nucleotide-gated ion channels (CNGCs), besides their involvement in mediating a cADPR/Ca\(^{2+}\)-dependent pathway. Inhibitors of IP\(_3\)-regulated channels (for the release of Ca\(^{2+}\) from intracellular stores) result in a significant reduction in AR formation in both IAA and NO treated cucumber explants (Lanteri et al., 2006). Earlier reports also provide evidence for auxin-induced increase in IP\(_3\) concentration (Zbell and Walter-Back, 1988). It was, thus, proposed that IP\(_3\)-regulated Ca\(^{2+}\) channels at the tonoplast might be involved in auxin-triggered increase in cytosolic calcium ions. Even the Ca\(^{2+}\) channel blocker (lanthanum chloride) significantly reduces IAA and NO-induced AR formation. These results collectively
indicate that both intracellular and extracellular Ca\(^{2+}\) pools are required for the involvement of Ca\(^{2+}\) as a second messenger, linking both auxin and NO to the activation of processes leading to AR formation. CDPKs utilize changes in cytosolic Ca\(^{2+}\) to couple cellular responses to extracellular stimuli and are, thus, known to be involved in signalling pathways. An increase in 50 kDa CDPK activity has been reported 1 d after exposure of cucumber explants to either IAA or SNP (Lanteri et al., 2006). Involvement of CDPK at earlier stage of AR formation suggests that this protein kinase could be associated with cell differentiation and division. Kobayashi and Fukuda (1994) and Komatsu et al. (1996) have also shown the involvement of Ca\(^{2+}\)-binding proteins and protein kinases in plant differentiation processes. Thus, auxin and NO have been put together in the Ca\(^{2+}\)-mediated signalling pathway that regulates CDPK activity leading to AR formation. The induction of CDPK gene expression in response to auxins has earlier been studied in Medicago sativa (Davletova et al., 2001). The activation of all pathways triggered by NO seems to be required for AR formation since the stimulatory effect of auxins and NO is abolished even when one of the pathways is missing. However, it still needs to be determined whether synchronization between MAPK- and Ca\(^{2+}\)/CDPK-dependent signalling pathways occurs during AR formation. The two signalling events (spatially and temporally specific elevations in cytosolic Ca\(^{2+}\) and reversible protein phosphorylation, including protein kinases and phosphatases) seem to be activated during NO-induced AR formation and they may represent key mechanism of NO responses in plants.

Recent investigations by Liao et al. (2012) provide evidence that Ca\(^{2+}\) and CaM signalling cascade are activated during NO and H\(_2\)O\(_2\)-induced adventitious rooting in marigold. The Ca\(^{2+}\) signal in AR development is still poorly unknown. The effect of exogenous Ca\(^{2+}\) on AR has been found to be dose-dependent. Exogenous Ca\(^{2+}\), NO, and H\(_2\)O\(_2\) evoke similar response on AR development in marigold. However, no synergistic effect has been observed between Ca\(^{2+}\) and NO/H\(_2\)O\(_2\) on mediating rooting. Involvement of endogenous Ca\(^{2+}\) in adventitious rooting has been further confirmed by the use of EGTA and BAPTA/AM since these compounds significantly block NO and H\(_2\)O\(_2\)-induced AR.
Summary of the possible molecular events known to co-ordinate during adventitious root formation. Abbreviations: NO- nitric oxide; PIP-Phosphatidylinositol phosphate; PIP$_2$- Phosphatidylinositol 4,5-biphosphate; PM- Plasma membrane; PLC- Phospholipase C; MAPK- Mitogen-activated protein kinase; GC- Guanylate cyclase; PLD- Phospholipase D; IP$_3$- Inositol 1,4,5-triphosphate; cGMP- cyclic guanosine monophosphate; cADPR- cyclic adenosine diphosphate ribose; PC- Phosphatidylcholine; PE-Phosphatidylethanolamine; PA- Phosphatidic acid; CDPK- Calcium-dependent protein kinase.

Earlier, Bellamine et al. (1998) have also reported inhibition of adventitious rooting in poplar cuttings upon application of both EGTA (Ca$^{2+}$ chelator) and lanthanum
chloride (Ca\(^{2+}\) channel blocker). Also, the promotive effects of NO and H\(_2\)O\(_2\) on AR are largely suppressed by simultaneous presence of CaM antagonists. This is the first report on the involvement of Ca\(^{2+}\) and endogenous CaM in H\(_2\)O\(_2\)-induced AR development. Thus, Ca\(^{2+}\), CDPK, MAPK and CaM have been shown to be involved in AR. Still, an understanding of the role and order of action of CaM as a critical mediator in AR development is unknown. Further studies have shown that exogenous calcium could enhance levels of both NO and H\(_2\)O\(_2\) in marigold explants. Thus, a complex interplay between Ca\(^{2+}\)/CaM and NO/H\(_2\)O\(_2\) in AR development has been elucidated. Although some components of NO signalling during adventitious rooting have been revealed but still the entire network responsible for AR development seems to be very complex.

**Proteomic analysis in relation to adventitious rooting**

The physiological and developmental state of any particular cell is best depicted by the array of proteins that it contains at any point of time. Several kinds of proteins from maize correlated with rooting have been identified by 2-DE (Konishi et al., 2005). Enhanced expression of one of the proteins, identified as fructose-biphosphate aldolase, has been found to coincide with increased root development. Also, Sorin et al. (2006) found 11 proteins related to the contents of endogenous hormones and the number of adventitious roots. Proteomics, thus, not only provides information on rooting-related proteins but it also acts as a means of molecular marker to identify rooting ability of plant.

Recently, investigations on gene expression during AR formation in *Pinus contorta* hypocotyls have reported more than 200 differentially expressed genes during different phases. These proteins are related to auxin transport, photosynthesis, cell replication or cell wall synthesis (Brinker et al., 2004). However, the first characterization of two-dimensional (2-D) protein patterns of Arabidopsis mutants has been done by Sorin et al. (2006). Their work has enabled identification of gene markers regulating adventitious rooting in *Arabidopsis*. 2-D patterns of soluble proteins of hypocotyls from four *Arabidopsis* mutants (differing in their auxin content and/or their adventitious rooting ability) have been analyzed with particular reference
to the early developmental events associated with AR initiation. Seventeen proteins are related to metabolism, out of which ten are putatively involved in energy/carbon metabolism and five are associated with nitrogen metabolism. Nine proteins are putatively involved in stress responses, four are related to oxidative stress and the other four to glucosinolate metabolism and biosynthesis. Four proteins have been shown to be associated with hormone metabolism. Also, other proteins involved in protein folding, degradation, protein glycosylation, cell wall biosynthesis and few of unknown function. Proteins linked to energy and carbon metabolism, photosynthesis and TCA cycle, have been found to be up-regulated. Proteins involved in the control of auxin homeostasis are also identified, including three GH-3 proteins, which are significantly positively correlated with AR number. Several other proteins, either positively or negatively correlated, are putative chaperones or stress-related proteins. Eleven proteins have been identified and their content either positively or negatively correlated with early and/or late phases of AR development. Although these proteins are not correlated to adventitious rooting, some overlap among the proteins identified here, and genes potentially described to be associated with AR formation in Pinus (Brinker et al., 2004), could be found, like for 20S proteasome subunits, putative β-tubulin and tubulin α2/α4 chain, and the caffeoyl-CoA methyltransferase-like protein. These results strongly suggest that the identified proteins could be valuable markers for further quantitative genetic analysis of AR development.
II. SEROTONIN BIOSYNTHESIS IN PLANTS AND ITS REGULATION

Occurrence and concentration

Serotonin is a monoamine neurotransmitter that is primarily found in the gastrointestinal (GI) tract and central nervous system (CNS) among mammals. The chemical name for serotonin is 5-hydroxytryptamine, which is often abbreviated as 5-HT. Serotonin is a physiologically active amine and a well-known neurotransmitter that regulates mood, sleep and anxiety in mammals (Veenstra-VanderWeele et al., 2000). Its action as a hallucinogenic drug is well known from the biochemical, electrophysiological and behavioural investigations. It was initially identified as a vasoconstrictor substance in blood serum – hence "serotonin", a serum agent affecting vascular tone. This agent was later chemically identified as 5-hydroxytryptamine (5-HT) by Rapport and coworkers in 1948, and a broad range of its physiological roles were elucidated. 5-HT became its preferred name in the pharmacological field (Rapport et al., 1948). In plants, it was first identified in the legume *Mucuna pruriens* (Bowden et al., 1954), and since then it has been detected in more than 42 species (Roshchina, 2001).

Though serotonin is found in different parts of plants, including leaves, stem, roots, fruits and seeds, the quantity of serotonin within the plant tissues varies widely. Fruits, vegetables and seeds are the major tissues in which serotonin occurs abundantly (Kema et al., 2000). Its quantities in plants vary greatly among species and tissues, ranging from 0.007 µg.g\(^{-1}\) in fresh leaves to 2000 µg.g\(^{-1}\) in seeds of *Griffonia simplicifolia* (Fellows and Bell, 1970). The highest values of 25-400 mg.kg\(^{-1}\) of serotonin have been found in walnuts (*Juglans regia*) and hickory (*Carya* sps.). Serotonin also occurs in fruits (e.g., pineapple, banana and tomato) avocado, eggplant and seeds (walnuts), and also in the sting of the nettle *Urtica dioica* (Collier and Chesher, 1956) and in the pods of cowhage (Bowden et al., 1954), in which serotonin is suggested to play a protective role against predators.

Serotonin has been implicated in diverse physiological functions in plants viz. growth regulation (Murch et al., 2001), flowering, xylem sap exudation (Kang et al., 2007a), ion permeability (Ojakova and Hadjiivanova, 1997) and adaptation to environmental
changes. Serotonin may act as a growth regulator stimulating the growth of roots (Csaba and Pal, 1982) and the hook of oat coleoptiles (Niaussat et al., 1958). It also stimulates the germination of radish seeds (Roshchina, 2001) and the pollen of *Hippeastrum hybridum* (Roshchina and Melnikova, 1998). In addition, serotonin levels are known to increase as fruits ripen in many species, including tomato, although inverse is true of the fruit of pineapple (Udenfriend et al., 1959; Foy and Parrat, 1961).

Serotonin has recently been shown to act as a plant hormone in view of its auxin-like activity. Immunolocalization analysis has revealed that serotonin is abundant in the vascular parenchyma cells, including companion and xylem cells of rice, suggesting its involvement in maintaining the cellular integrity for facilitating efficient nutrient recycling from senescing leaves to sink tissues during senescence (Kang et al., 2009).

**Serotonin biosynthesis**

Serotonin is synthesized from tryptophan, which contains an indole ring and a carboxyl-amide side-chain, similar to all amino acids. Using isotope tracer experiments, it has been reported from in vitro regenerated *Hypericum perforatum* plants that tryptophan is the precursor of melatonin and serotonin biosynthesis (Murch et al., 2001). In animals, serotonin is synthesized from tryptophan in two successive steps involving tryptophan hydroxylase (TPH; EC 1.14.16.4) and aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28), in which tryptophan hydroxylase acts as the rate-limiting enzyme (Veenstra-VanderWeele et al., 2000). In plants, however, serotonin is synthesized in a different manner whereby tryptophan is first catalyzed into tryptamine by tryptophan decarboxylase (TDC; EC 4.1.1.28), followed by the catalysis of tryptamine by tryptamine 5-hydroxylase (T5H) to form serotonin (Schroder et al., 1999). In St. John’s wort (*Hypericum perforatum*), however, serotonin synthesis has been reported to occur via 5-hydroxytryptophan, as in mammals (Murch et al., 2001). Tryptophan decarboxylase (TDC) catalyzes the conversion of tryptophan into tryptamine, followed by tryptamine 5-hydroxylase (T5H), which hydroxylates the C-5 position of tryptamine to form serotonin (Kang et al., 2007a,b; Kang et al., 2008; Kang et al., 2009). TDC serves as a rate-limiting step
because of its high $K_m$ in relation to the substrate tryptophan (690 µM). However, T5H and the downstream enzymes, such as serotonin N-hydroxycinnamoyl transferase (SHT), have low $K_m$ values with corresponding substrates. This suggests that the biosynthesis of serotonin or serotonin-derived secondary metabolites is restricted to cellular stages when high tryptophan levels are present.

**Tryptophan decarboxylase (TDC)**

TDC belongs to the family of aromatic amino acid-decarboxylases (AADCs) and it decarboxylates tryptophan into tryptamine in presence of the coenzyme pyridoxal-5-phosphate. TDC expresses at an undetectable level in rice leaves and seeds. It is the first committed enzyme in serotonin biosynthetic pathway and serves as a bottleneck point regulating serotonin biosynthesis since TDC expression is very low or negligible. The first known TDC gene was isolated from *Catharanthus roseus* (De Luca et al., 1989). Since then, only two other TDC genes have been functionally characterized. These genes encode proteins from *Camptotheca acuminata* and *Ophiorrhiza pumila* that have 67% and 71% amino acid identity to the *C. roseus* TDC, respectively (Lopez-meyse and Nessler, 1997; Yamazaki et al., 2003).

TDC from *Catharanthus roseus* has a low $K_m$ for tryptophan (0.072 mM), but the $K_m$ of other TDC enzymes isolated from tomato (Gibson et al., 1972), *Ophiorrhiza pumila* (Yamazaki et al., 2003) and rice (Kang et al., 2007b) is at least 10 fold higher than that of *C. roseus*. Unlike TDC, all downstream enzymes after TDC have low $K_m$ values for the corresponding substrates. The high $K_m$ values of TDC enzymes suggest that TDC functions actively in cells with high tryptophan accumulations. Furthermore, recombinant rice TDC is slightly tolerant of high temperatures, with maximum TDC activity at 45°C, and 50% enzyme activity at 55°C. In addition, TDC activity is greatest at high pH levels, with peak activity at pH 7.5–8.5, but the activity rapidly decreases to 50% at pH 6.5 (Kang et al., 2008). Rice TDC has high substrate specificity for tryptophan, and aromatic amino acids (like phenylalanine and tyrosine) have no effect on its activity (Kang et al., 2007b).
Due to the low expression of TDC in rice plants, serotonin levels in leaves and seeds have been found to be around 0.3 mg·g\(^{-1}\) fresh weight and 0.12 mg·g\(^{-1}\) seeds, respectively, whereas transgenic rice plants overexpressing TDC produce 25- and 11-fold higher serotonin in leaves and seeds, respectively and show delayed senescence than the wild type (Kang et al., 2007b). However, levels of serotonin in wild type plants increase greatly in response to senescence signals, such as nutrient deprivation or leaf detachment, and serotonin biosynthesis is closely coupled with transcriptional and enzymatic induction of tryptophan biosynthetic genes as well as TDC in senescing leaves (Kang et al., 2009). The lines in which expression of TDC was suppressed through an RNA interference (RNAi) system, produced less serotonin and senesced faster than the wild type line, suggesting that serotonin is involved in attenuating leaf senescence (Kang et al., 2009).

Results of TDC overexpression and TDC RNAi in plants suggest that TDC plays a rate-limiting role for serotonin accumulation, but the synthesis of serotonin depends on an absolute amount of tryptophan accumulation by the coordinate induction of tryptophan biosynthetic genes (Kang et al., 2009). Serotonin has also been reported to accumulate in rice leaves infected by the fungal pathogen, *Bipolaris oryzae* (Ishihara et al., 2008). Ueno et al. (2003) have reported that TDC activity is induced upon infection of Sekiguchi lesion mutant rice with *Magnaporthe grisea*. Serotonin accumulation is preceded by a transient increase in tryptamine content and marked activation of TDC. Serotonin treatment suppresses the growth of fungal hyphae, indicating that the activation of the tryptophan pathway is involved in the establishment of effective defense against the pathogen through serotonin production in rice plants. Collectively, this information indicates that serotonin levels in plant tissues may increase by demand, under particular developmental transitions or when challenged by pathogens (Kang et al., 2009).

Rice plants are known to harbor at least two functional TDC genes, of which TDC1 is functionally implicated in synthesizing serotonin (Kang et al., 2007b). The induction of serotonin accumulation is co-ordinately regulated with the induction of the entire set of tryptophan biosynthetic mRNAs, and is proportional to the induction of TDC protein. It has been suggested that TDC1 plays a major role in serotonin biosynthesis
when rice plants are challenged with senescence. TDC expression is abundant in the senesced rice leaves, and it is strongly induced in parallel with the high production of serotonin as well as tryptophan, as the rice leaves undergo senescence. Upon investigating the effects of plant hormones (such as zeatin and abscisic acid) on serotonin biosynthesis in response to senescence, it has been found that abscisic acid treatment accelerates serotonin synthesis while zeatin treatment delays it (Kang et al., 2007b).

**Tryptamine 5-hydroxylase (T5H)**

T5H is the terminal enzyme responsible for serotonin synthesis using tryptamine as a substrate. It occurs predominantly as a soluble protein and exhibits broad substrate specificity toward aromatic amino acids and their derived amine compounds, such as tyramine and octopamine. However, dopamine derived from phenylalanine has no effect on T5H enzyme catalysis, suggesting that T5H harbors a certain level of substrate specificity among aromatic amino acid derivatives. It is constitutively expressed in healthy rice plants (Kang et al., 2007a) and belongs to the family of amino acid hydroxylases, such as phenylalanine hydroxylases, tyrosine hydroxylase, and tryptophan hydroxylase, because the amino acid hydroxylases require tetrahydrobiopterin as a co-substrate for their enzymatic reactions (Fitzpatrick, 1999).

Serotonin levels have been reported to be greatly influenced by tryptamine and little by tryptophan (Kang et al., 2007a). Rice seedlings grown in presence of tryptamine exhibit a dose-dependent increase in serotonin in parallel with enhanced T5H enzyme activity, suggesting that T5H enzyme is highly expressed in roots, and that tryptamine absorbed through the roots is able to move to the stem, where it is catalyzed into serotonin by T5H expressed in the stem. This indicates that exogenously applied tryptamine can be readily used by T5H in the cells. However, no significant increase in serotonin content was observed in rice seedlings grown in presence of tryptophan, suggesting that tryptamine is a bottleneck intermediate substrate for serotonin synthesis (Kang et al., 2007a). This shows that serotonin synthesis occurs actively in rice seedlings and requires tryptamine as a direct substrate for T5H. It is, therefore, highly probable that the tryptamine content is tightly coupled with that of serotonin.
Regulation of serotonin biosynthesis

Two major rate-limiting steps that seem to regulate serotonin biosynthesis in plants are:

- Level of tryptophan/tryptophan pool
- Induction of TDC

High level of tryptophan induces TDC, which then acts upon tryptophan to form tryptamine. This is further hydroxylated by T5H into serotonin. Thus, high level of serotonin is closely implicated in an increased pool of tryptophan in plants (Kang et al., 2008). TDC activity is prone to regulation. It has been reported in Catharanthus roseus that auxin rapidly downregulates the transcription of TDC gene. By performing run-off transcription experiments, this downregulation has been observed to take place at the transcriptional level within 15 min, and is independent of de novo
protein synthesis (Goddijn et al., 1992). Using isotope tracer experiments, it was found that under low light conditions more radiolabel from $^{14}$C tryptophan was recovered as $^{14}$C serotonin relative to melatonin. However, under increased light conditions, the ratio was found to be reversed with more amount of radiolabel being recovered as melatonin. This indicates that the production of serotonin and melatonin from tryptophan is regulated, at least in part, by light (Murch et al., 2001).

**Feedback regulation of tryptophan and serotonin biosynthesis**

Anthranilate synthase (AS) is the first enzyme in the tryptophan biosynthetic pathway. It has two α subunits and two β subunits and plays an important role in controlling the level of tryptophan in plants. ASα exists in 2 isoforms – ASα1 and ASα2. While ASα1 is insensitive to tryptophan, ASα2 is strongly feedback inhibited by very low concentrations of tryptophan. Thus, it is thought that serotonin synthesis occurs via the tryptophan-insensitive ASα1 pathway since high concentration of tryptophan is required for the induction of TDC (Kang et al., 2008).

![Schematic diagram of the serotonin biosynthetic pathway depicting feedback regulation of tryptophan and serotonin biosynthesis (Modified from Kang et al., 2008)](image_url)