Auxin Biology: Applications and the Mechanisms Behind

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Abstract This chapter describes the state of the contemporary knowledge of auxin action reflected in its applications in agriculture and biotechnology. We summarise the current understanding of the mechanism of action for endogenous and major synthetic auxins highlighting their morphogenic character that modulates numerous aspects of plant development. Various auxins and auxin-like compounds are used in techniques of plant vegetative propagation, in vitro culture and regeneration, and they play also a role as important herbicides. We discuss potential applications of auxins in commercially relevant procedures used in the context of plant generative and fruit development, abscission, apical dominance and tropisms. These technologies are based rather on the phenomenology of auxin applications, and the molecular mechanisms behind are still not fully uncovered.

1 Introduction

The study of the intriguing group of plant hormones known as 'auxins' (from the Greek word *auxein* meaning 'to grow') had its origins in the investigations of Charles and Francis Darwin (Darwin and Darwin 1881) into the bending responses of grass coleoptiles towards a unilateral light source. In an elegant series of experiments, they demonstrated that the directional light stimulus was perceived by the tip of the coleoptile, while the growth response leading to reorientation of coleoptile growth, dependent on differential elongation of either side of the coleoptile, occurred in some distance from the coleoptile tip in response to a 'signal' conveyed from the tip itself. Later work on *Avena* coleoptiles demonstrated that the

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'signal' was a chemical substance transported asymmetrically from the tip (Went 1928). Although still unidentified, the substance involved could be isolated by diffusion into agar blocks which, if placed laterally on *Avena* coleoptiles, would induce growth curvature by stimulating differential elongation. Indole-3-acetic acid (IAA) isolated from human urine (Kögl et al. 1934) was very effective in the *Avena* curvature test and was eventually found to be the predominant endogenous auxin in plants (Davies 2004). Efforts to find other substances with auxin activity led to the discovery of various compounds, both synthetic and natural. The most frequently used synthetic auxins in basic research and applications, 2,4-dichlorophenoxy acetic acid (2,4-D) and naphthalene-1-acetic acid (1-NAA), do not act completely identically as the native IAA. Only some of the compounds identified as auxins, namely, indole-3-butyric acid (IBA) (Zimmerman and Wilcoxon 1935), phenylacetic acid (PAA) (Koepfli et al. 1938) and 4-chloroindole-3-acetic acid (4-Cl-IAA) (Porter and Thimann 1965), are actually synthesised by some plants and could be understood as 'endogenous auxins'. Their roles and mechanisms of action are still not satisfactorily described (Simon and Petrášek 2011).

From the very beginning of auxin research, the definition of the term 'auxin activity' was based on the competence of such substances to promote elongation growth in coleoptiles and to stimulate rooting. Indeed, the ability of auxins to stimulate rooting gave rise to their original name of 'rhizocalines' (Went 1934). On the cellular level, auxin influences both cell division and cell expansion (Skoog and Miller 1957; Perrot-Rechenmann 2010), two major cellular processes that shape the sessile plant body. Together with the effect of auxin on cell differentiation and the determination of cell fate, auxins influence literally all aspects of plant development (Vanneste and Friml 2009) displaying a morphogenic character (Bhalerao and Bennett 2003) modulated by other phytohormones and the environment and defined by dynamic changes in the machinery of auxin signal transduction. Therefore, auxin concentration gradients are important for the coordination of plant growth and development (Leyser 2011) and have had impact also on the evolution of plant body (Finet and Jaillais 2012).

The knowledge on auxin biosynthesis, metabolism, transport and mechanism of action contributes vastly to all fields of plant biology. Following this reasoning, auxin could be understood as a tool for studying many aspects of plant developmental and cell biology and consequently also as a tool for agronomy, horticulture and biotechnology applications. In application, the interpretation of 'auxin activity' is somewhat different and reflects the phenomenology of exogenous additions of auxins to plants or plant organs. This applies to auxins as compounds used in procedures for in vitro cultures, regeneration, organogenesis and vegetative propagation as well as compounds with herbicide effects. In addition to summarising these procedures in the current chapter, we also want to point to the fact that our understanding of the mechanisms that collectively regulate responses to auxin is not widely exploited in its field applications and might therefore represent good opportunity for the future.

2 Auxins and Their Mechanisms of Action

2.1 Auxin Concentration Gradients in Plant Morphogenesis

The habitus of plants is largely set through the complex balance of cell division and expansion in various plant tissues. Auxin is (in addition to other plant hormones, namely, cytokinins) a central substance that influences both of these processes (Skoog and Miller 1957). It is the concentration of auxin that is very often crucial for the resulting response, and therefore auxin concentration gradients are instructive during literally all phases of plant development. This fact is a prerequisite for a plethora of practical applications. Already from the very early phases of embryogenesis, auxin concentration maxima are generated in developing embryos marking sites for future development of cotyledons and root apical meristems (Friml 2003). During postembryonal development, auxin concentration maxima regulate root and shoot apical meristem patterning and lateral organ development (Benková et al. 2003; Sabatini et al. 1999). They are also important for vasculature development (Mattsson et al. 2003; Scarpella et al. 2010), root and shoot bending responses (Friml et al. 2002), flower and fruit development (Sundberg and Ostergaard 2009) and also during senescence (Ellis et al. 2005) and plant-pathogen interaction (Kazan and Manners 2009). For all of these developmental events, auxin concentration gradients are collectively generated by processes of auxin biosynthesis and metabolism as well as by inter- and intracellular auxin transport. Finely tuned auxin gradients determine how much of auxin will be triggering specific downstream responses that might, but not necessarily needs to, include gene expression.

2.2 What We Know About the Mechanism of Action of Endogenous and Synthetic Auxins

As mentioned in the introduction, the most frequent natural auxin is IAA. However, for numerous applications, various endogenous and synthetic auxin-like compounds are used, inadvertently taking advantage of differences in their biosynthesis, transport and mechanism of action.

As reported for *Arabidopsis thaliana*, IAA is synthesised to various levels literally by every cell (Ljung et al. 2001), predominantly in young tissues. Several pathways from the IAA precursor L-tryptophan (L-Trp) have been described, and also aL-Trp-independent biosynthetic pathway has been postulated (for review see Ljung 2013). The physiologically active pool of IAA is balanced by a complex conjugation and degradation enzymatic machinery, where IAA conjugation to sugars and amino acids and oxidative degradation are the most important (Ljung 2013; Ludwig-Müller 2011). However, for IAA, directional cell-to-cell auxin transport through integral plasma membrane carriers seems to be the major

mechanism establishing auxin gradients during plant development (for review see Petrášek and Friml 2009). This machinery includes auxin influx carriers from the AUX1/LIKE AUX1 (AUX1/LAX) family (Bennett et al. 1996), auxin efflux carriers from the PIN-FORMED (PIN) family (Gälweiler et al. 1998) and ATP-binding cassette subfamily B (ABCB) (Noh et al. 2001). Directional influx by the influx carriers acts in concert with non-directional uptake of IAA from the apoplast through an ion-trap mechanism (Rubery and Sheldrake 1974) which is important for auxin canalisation (see Sect. 3.3). Moreover, some PINs (Mravec et al. 2009; Ding et al. 2012; Dal Bosco et al. 2012) and PIN-LIKE (PILS) proteins (Barbez et al. 2012) that are localised on endomembranes are supposed to maintain intracellular IAA homeostasis by transporting IAA or perhaps even IAA conjugates between ER (or its derivatives) and cytoplasm. It is hypothesised that cellular IAA homeostasis includes mechanisms that coordinate IAA transport and metabolism (Rosquete et al. 2012). Depending on its actual concentration, IAA binds to a nuclear receptor belonging to the TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) family of F-box proteins, subunits of the SCF E3-ligase complex (Dharmasiri et al. 2005a, b; Kepinski and Leyser 2005). Upon binding, degradation of transcriptional repressors of the Aux/IAA family releases auxin response factors (ARFs) that activate or suppress specific gene expression. It was shown that various combinations of TIR1/AFBs and Aux/IAAs determine the specificity of this 'co-receptor' system to IAA (Calderón Villalobos et al. 2012). In addition, two additional IAA receptors have been described, AUXIN BINDING PROTEIN 1 (ABP1, actually the first identified auxin receptor) and S-phase kinase-associated protein 2A (SKP2A). ABP1 affects predominantly events at the plasma membrane linked with cell expansion, e.g. the activation of plasma membrane ATPase and K^+ channels, clathrin-mediated endocytosis and cytoskeletal rearrangements (for review see Sauer and Kleine-Vehn 2011). SKP2A, another F-box protein, regulates cell division through the proteolysis of cell cycle transcription factors and is degraded upon binding of IAA (Jurado et al. 2010).

While metabolism and mode of action are relatively well understood for IAA, there is significantly much less information for three other naturally occurring auxins, 4-Cl-IAA, PAA and IBA. 4-Cl-IAA is the auxin predominantly found in developing seeds of legumes, including agronomically important species like pea, alfalfa or lentil. A typical activity of 4-Cl-IAA is the stimulation of pea pericarp growth (Reinecke 1999). This auxin is produced by enzymatic conversions of 4-chlorotryptophan to 4-Cl-IAA (Tivendale et al. 2012) and can also be conjugated with amino acids (Ludwig-Müller 2011). Auxin transport competition assays showed that 4-Cl-IAA might be transported by auxin influx and efflux carriers (Simon et al. 2013). It might also share some mechanisms of action with IAA, as indicated by high competition of 4-Cl-IAA with IAA in binding displacement assays (Zažímalová and Kutáček 1985), by the ability of 4-Cl-IAA to trigger ABP1-mediated events on the plasma membrane in maize (Karcz and Burdach 2002) and, interestingly, also by 4-Cl-IAA-stimulated interaction of TIR1 with the Aux/IAA repressor (Yu et al. 2013). Another endogenous auxin, the phenyl-

derivative PAA, was detected in a number of plant species (Wightman and Lighty 1982; Korasick et al. 2013). It is synthesised by the nitrilase pathway (Ludwig-Muller and Cohen 2002) and rather poorly transported by auxin influx and efflux carriers (Simon et al. 2013). It is not known whether PAA is a good substrate for TIR1/AFB, but it seems likely that the mechanism of action of PAA involves both TIR1 (Simon et al. 2013) and ABP1 (Napier and Venis 1990). The fourth endogenous compound that is often classified as endogenous auxins is IBA. Although recent reports from Novák et al. (2012) using novel analytical approaches in various tissues of *A. thaliana* (liquid chromatography-multiple reaction monitoring-mass spectrometry) failed to detect IBA at all, its presence in a wide range of plants including *A. thaliana* has been documented in multiple previous reports (Korasick et al. 2013). As summarised in Strader and Bartel (2011), IBA represents an important IAA precursor that is formed by β-oxidation, thus regulating the active IAA levels during plant development. IBA conjugates with glucose were reported to increase resistance to water stress (Tognetti et al. 2010). In addition, it seems that IBA is transported by a transport machinery that differs from that for IAA and includes the ABCB36 and ABCB37 auxin efflux carries (Strader and Bartel 2011). So far, it does not seem to be likely that IBA directly triggers TIR1- or ABP1-mediated responses. The finely tuned metabolic conversion of IBA to IAA might actually be the reason why IBA is often better suited for various applications producing a remarkably stable auxin activity that even excels that of IAA or its synthetic analogues.

Since IAA has been reported to be less stable in culture media, where it is photodegraded within several days (Yamakawa et al. 1979; Nissen and Sutter 1990), numerous experiments and applications use more stable synthetic structural and functional auxin analogues. The most frequently used compounds are 1-NAA and 2,4-D. Metabolism, transport and mechanism of action of these molecules share many features with those of IAA, but as described in next few lines, there are also some specific points that need to be considered. 1-NAA and 2,4-D are more stable when compared with IAA in terms of their slower metabolic conversions as documented for 2,4-D (Delbarre et al. 1996; Hošek and Kubeš et al. 2012) and 1-NAA that is reported to be not as readily converted by some IAA auxin-conjugating enzymes (Peat et al. 2012). Radioactively labelled trace amounts of 1-NAA and 2,4-D turned out to be suitable tools for selective measurements of auxin transport, i.e. carrier-driven auxin influx (2,4-D) and carrier-driven auxin efflux (1-NAA) (Delbarre et al. 1996; Petrášek et al. 2006). Interestingly, the evidence accumulated from these assays, and supported by mathematical modelling (Hošek and Kubeš et al. 2012), shows that for auxin efflux, 2,4-D, in contrast to the literature, is transportable to a certain degree, however, less efficiently. Lower cellular efflux of 2,4-D together with 2,4-D-specific auxin influx through the ABCB4 transporter (Kubeš et al. 2012) might thus be responsible for the high herbicidal activity of this synthetic auxin (see later). Both 1-NAA and 2,4-D can activate the TIR1/AFB-Aux/IAA co-receptor system (Calderón Villalobos et al. 2012), but their affinity to TIR1 is somewhat lower in comparison with IAA (Dharmasiri et al. 2005a; Kepinski and Leyser 2005). It is important to mention that

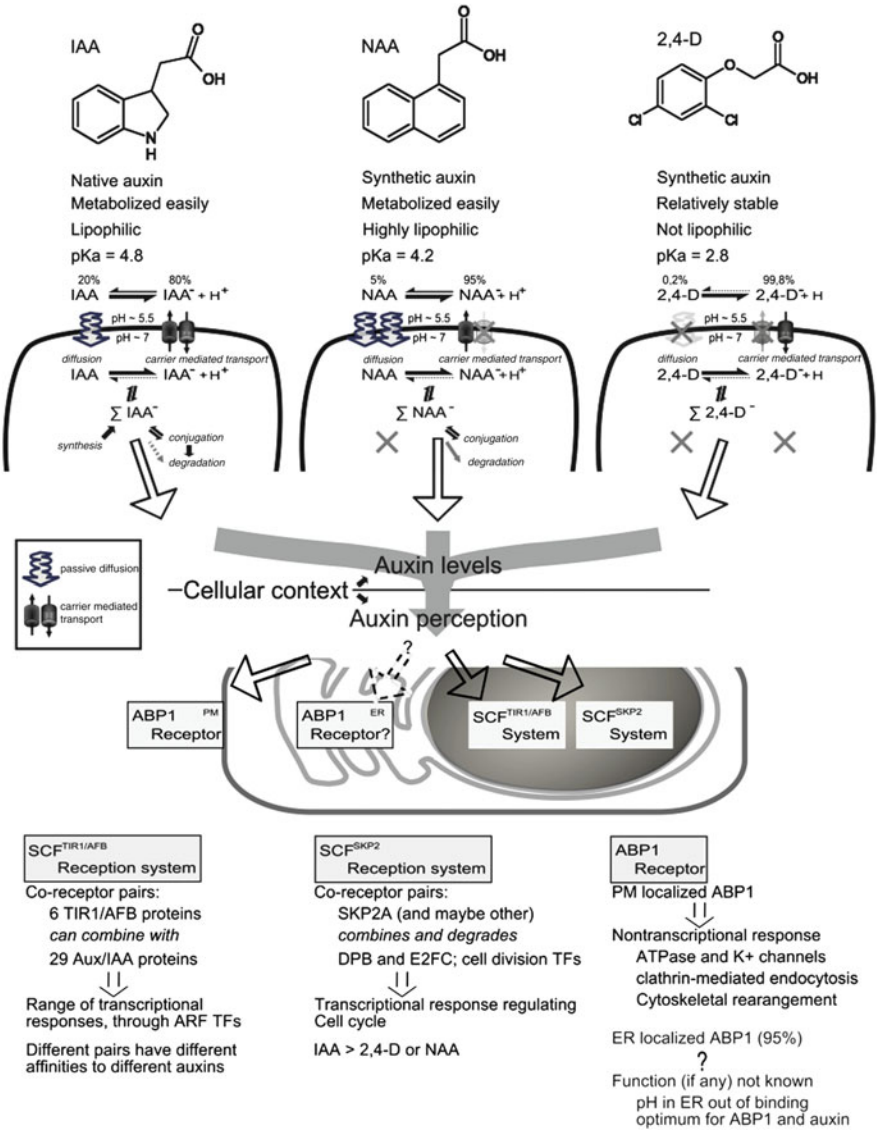

Fig. 1 Schematic depiction of the mechanisms involved in the timely and spatially restricted tuning of endogenous levels of native auxin IAA and the two synthetic auxins, 1-NAA and 2,4-D. Both free diffusion and carrier-mediated transport have been described for auxins. The amount of the dissociated form of the particular auxin depends on the pH of the compartment. As calculated based on the dissociation constant, the amount of the non-dissociated form is 20 % for IAA, 5 % for NAA and 0,2 % for 2,4-D. Based on this dissociation rate and also on the lipophilicity, 2,4-D is uptaken almost exclusively by carriers, IAA by both carriers and diffusion, and NAA almost exclusively by diffusion. Within the cell, the pH is higher and under this situation, for all three auxins, carrier-mediated transport predominates. Here, while IAA and NAA are very good substrates for the described auxin carriers, 2,4-D is much less transported. Moreover, the metabolic conversions that deactivate the pool of auxin are the slowest for 2,4-D, keeping its concentration high for downstream transcriptional control that is based on the proteasome-mediated degradation

picloram, an auxinic compound with herbicide effects, is selectively bound by the AFB5-Aux/IAA co-receptor (Calderón Villalobos et al. 2012). This indicates that individual TIR1/AFBs and their combinations with Aux/IAAs might determine the sensitivity of the particular tissue to auxins or auxin-like compounds. In addition to TIR1/AFBs, 1-NAA and 2,4-D can also trigger responses through the activities of auxin receptors ABP1 (Löbler and Klämbt 1985) and SKP2 (Jurado et al. 2010). It seems that there might exist in general two pathways for the control of cell division and cell elongation that are triggered by 2,4-D (cell division) and 1-NAA and IAA (cell elongation) (Campanoni and Nick 2005; Simon et al. 2013). A schematic summary of the current knowledge on transport and signalling is given in Fig. 1.

2.3 *Mechanisms of Co-operation Between Auxins and Cytokinins: Setting the Plant Morphogenesis*

The morphogen-like effect of auxin is regulated by crosstalk between numerous signal transduction pathways triggered by external stimuli like gravity, light quality and length, temperature or nutrient availability. With respect to the applications of auxin, crosstalk with other phytohormones is of particular importance. Among them, cytokinins are the most frequently applied phytohormones that act in concert with auxin in basic morphogenic processes, i.e. cell division and cell growth/differentiation. The importance of mutual co-operation between auxin and cytokinins for organogenesis was elegantly shown in the pioneering experiments with regenerating tobacco stem pith explants (Skoog and Miller 1957; see Sect. 3.2 and chapter by Opatrný, this volume). However, it is only recently that the molecular mechanisms of auxin-cytokinin interplay were at least partially understood. Cytokinins (see chapter by Šmechilová and Spíchal, this volume) repress auxin signalling through the activation of the positive (type B) cytokinin response regulator ARR1, thus activating the transcription of the Aux/IAA auxin signalling repressor IAA3/SHY2. This co-operation was reported to regulate root growth and root meristem size, where auxin supports meristem activity and cell division, whereas cytokinin supports differentiation (Dello_Ioio et al. 2008; Moubayidin et al. 2009). In contrast, the establishment and maintenance of the embryonic root pole

Fig. 1 (continued) of ubiquitinated transcriptional regulators upon binding of auxin to F-box proteins from the TIR/AFB family. This mechanism is common for all three IAA, NAA and 2,4-D, albeit with differential affinity of the particular auxin receptor for the particular auxin that is further regulated by the interaction with specific transcriptional repressors from the Aux/IAA family. Other inputs, not shown here, modulate on various levels auxin effects through the regulation the 'cellular competence', i.e. they might be instructive for a plethora of developmental processes. The processes represented in the figure are depicted in a schematic way, for the sake of lucidity. Therefore minor actions (such as some small metabolisation of 2,4-D) were disregarded in the scheme

(hypophysis) is regulated in inverse manner. Here auxin upregulates the negative (type A) cytokinin response regulators ARR7 and ARR15 (Müller and Sheen 2008). As nicely summarised in recent reviews (Bishopp et al. 2011; Su et al. 2011; Bielach et al. 2012; Vanstraelen and Benková 2012), programming of cell division and growth/differentiation through the interaction of auxin and cytokinin is critical for key developmental processes involving meristematic activity. The mechanism includes a number of regulatory loops and signalling components that are under the transcriptional control of both phytohormones, e.g. the genes for auxin efflux carriers, auxin and cytokinin biosynthetic and metabolism enzymes as well as upstream transcription factors. Moreover, non-transcriptional crosstalk has been described recently for the cytokinin-stimulated vacuolar degradation of auxin efflux carrier PIN1 (Marhavý et al. 2011).

3 Auxin Applications

3.1 Auxin as a Tool

Auxin became incorporated as a tool in the hands of plant breeding from very early on. The early applications included already most of applications used nowadays, namely, prevention of fruit and leaf drop, fruit thinning, induction of parthenocarpic fruits, stimulation of rooting of cuttings and auxin herbicidal effects (Preece 2003; Loach 1988).

As mentioned in the introduction, auxin was discovered through its ability to mediate plant responses to the environment. With continuing theoretical research, it became evident that auxin is of fundamental importance for a wide spectrum of vital processes, of which growth responses to environmental stimuli represent only a small, albeit important, subset. In fact, the capacity of this single molecule to cause a very diverse range of seemingly unrelated effects in different body parts, different time or under different conditions is one of the fundamental, yet most puzzling, characteristics of auxin. Each cell in the plant body may respond to auxin differently depending on its position, ontological and/or positional context, with a range of different possible cellular responses (Trewavas 1982; Kieffer et al. 2010; see also Sect. 2.2, and also Opatrný, this volume).

The plant body as an entity is shaped through physiological response of the tissues to particular pattern of auxin gradients distributed over the plant parts. As the auxin gradients stimulate auxin signalling pathways differentially along the gradient, various growth responses and cell differentiation are triggered (see Sect. 2.1). These processes may result in the change of cell specifications based on the positional information provided by the gradient and subsequently in the formation of new cell patterns, tissues and organs and also in responses of existing plant cells, tissues and organs to the environment (Friml 2003; Vanneste and Friml 2009). This morphogen-like character of auxin action in plant tissues contributes to

the control of large array of essential developmental processes (specified in Sect. 2.1 and Opatrný, this volume).

Therefore, auxin may be seen as signalling molecule, as a mobile unit conveying information of either endogenous or exogenous origin. All auxin molecules, irrespective of their origin, are relocated by the plant organism, and informational value of this distribution is translated with regard to the responsiveness (competence) of the receiving local tissue. And in that regard, embryo formation either from the zygote or from various somatic cells (see chapter by Smertenko and Bozhkov in this volume), as well as subsequent morphogenesis of the plant body, represents probably the most common applications of auxin signals in plant life. Alternatively, this informational wealth is employed for numerous regenerative events, as background for either wound healing or plant vegetative propagation (see Sect. 3.3 and the chapter by Opatrný in this volume). Another 'systemic' application of the auxin signal is utilised frequently in the case of auxinic herbicides. Synthetic auxins are applied as potent species-selective herbicides and defoliants (described in Sect. 3.4). The partially related abscission effect is also smartly used to cause fruit drop in apple orchards in order to regulate the number of fruits per tree. Several diverse applications related to fruit development have been conceived, with induction of parthenocarpy being probably the most interesting. Moreover, there exist also some indirect applications, where auxin metabolism, transport or perception is targeted, complementing the exogenous application of auxin per se (Sect. 3.5).

3.2 Auxin and In Vitro Cultures

Propagation of plants using in vitro techniques has become an important commercial technology, where auxin represents a fundamental component for the large-scale propagation of cell mass, organs or whole plants, or, to get rid of innate pathogens, in the generation of virus-free plants (Preece 2003).

The crucial importance of adequate endogenous levels of phytohormones has been demonstrated already during early stages of in vitro techniques. This hormone dependence is considered to be one of the main reasons why the pilot attempts of Gottlieb Haberlandt to cultivate a variety of functionally differentiated cells in vitro failed (Haberlandt 1902, see also Opatrný, this volume). In contrast, root cultures of tomato (White 1934) were the first plant entities growing in vitro without limitations, thanks to the production of IAA and cytokinins in the root apical meristem. Moreover, the first plant tissue cultures were callus cultures supplied with auxin either thanks to their tumour (e.g. auxin producing) origin (White 1939; Smith 1988) or due to the external application of IAA into the culture media (Nobécourt 1939; Gautheret 1939). While some cultured plant cells or tissues retain the capacity to synthesise auxin on their own, in most cases, however, external auxin has to be added into the culture media. The reason may partially be a so-called elution effect that is also known to contribute to losses of other essential

metabolites, typically after using subcritical density of culture inoculum (see also Opatrný et al., this volume). These days, plant tissue or cell cultures are propagated only rarely with the addition of the natural IAA itself, and the dominant auxins for technical applications are 2,4-D or 1-NAA, administered either alone or in combination. This applies also for the most frequently used cell lines/strains in basic research, i.e. Arabidopsis (Menges and Murray 2002), tobacco BY-2 (Nagata et al. 1992) and VBI-0 (Opatrný and Opatrná 1976).

In extreme cases, auxin autonomy can be induced by genetic and epigenetic changes that accompany in vitro propagation of plant tissue and cell cultures. The process of acquired auxin autotrophy, originally noticed by Gautheret (1942) and designated as 'anergy', leads to progressive insensitivity of long-term cultures to auxin even to a level where exogenous auxin can cause growth inhibition (Gautheret 1942, 1955, 1985). For such gradually acquired hormone autotrophy, the term 'habituation' had been coined by F. Meins (Meins 1982, 1989), originally for cytokinins. The mechanism of habituation is still not fully understood, and it is even not clear whether it is of genetic or rather of epigenetic background (Pischke et al. 2006). However, it has been reported to include up-regulation of some auxin ABC-type transporters in tobacco cells (Shimizu et al. 2006). Irrespective of the underlying mechanisms, habituation has significant implications for auxin applications. Hormonal substances are generally indispensable and one of the most expensive components of culture media. So, the possibility of their elimination is appealing and even more in the context of large-scale cultivation technologies for industrial purposes. On the other hand, relevant spontaneous phenotypic changes of production lines (or strains) which are associated with their (sudden or gradual) habituation may have adverse economic consequences.

Both natural (endogenous) and exogenously applied auxins participate in regenerative responses of various types of plant explants. In general, two main alternatives of regenerative procedures are employed to receive new organisms from the somatic cells of the donor plant: organogenesis and somatic embryogenesis (see Opatrný, this volume). For each of these procedures, rather specific regenerative protocols ('cookbooks') exist, both based on the empiric experience of their first users.

All recent protocols for the induction of organogenesis are variations of the original procedure of Skoog and Miller (1957), performed on the in vitro cultures of tobacco Wisconsin 38 stem pith primary explants modulated by various combinations of IAA and the cytokinin kinetin (See also Sect. 2.3). These parenchymatic tissue cultures sensitively responded to exogenously applied kinetin and IAA by rapid cell division and massive formation of callus. The addition of IAA alone or the massive predominance of IAA over kinetin stimulated pronounced root formation. Inversed ratios with higher kinetin concentration promoted the generation of shoot meristems that developed into shoot buds on medium with decreasing concentration of exogenous IAA. Our recent understanding of auxin-cytokinin interaction during de novo organogenesis has significantly profited from studies of the 'stem cell niches' in *A. thaliana* and the regenerative capacity of primary explants from roots, leafs, hypocotyls or cotyledons (Opatrný, this volume). These primary

explants are regenerated on media containing optimised ratios of auxin and cytokinin for callus induction (callus induction medium, CIM), shoot induction (shoot induction medium, SIM) or root induction (root induction medium, RIM). According to Valvekens et al. (1988), induction of callus with CIM needs the establishment of tissue-specific auxin concentration maxima by addition of higher concentrations of 2,4-D, the auxin that is very stable, because it is less exported from the cell and less metabolised (see Sect. 2.2). Subsequent shoot induction with SIM is then initiated by lowering the concentration of external auxin by use of the transportable (but instable) IAA. Depending on genotype and type of primary explant, the length of cultivation on SIM or RIM (medium with IAA only) has to be adjusted to the particular experimental system and the respective goal of regeneration. Based on this knowledge, a huge number of combinations of auxins, cytokinins and other phytohormones have been used in the propagation of broad spectrum of plant species (George and Sherrington 1984; Vasil 1986). However, organogenesis in some species has remained unsuccessful to variable degrees, a phenomenon known as 'recalcitrance'. To explain the reasons for these failures is, of course, a problem of both high theoretical and applied impact (see Opatrný, this volume).

As shown by gene expression profiling and the analysis of cell-type-specific protein markers in Arabidopsis, increased concentration of auxin in CIM applied to primary explants either in the form of less transportable 2,4-D or in the form of higher concentrations of IAA or 1-NAA triggers the ectopic activation of the developmental programme for lateral root initiation, one of the processes induced *in planta* by local auxin maxima (Sugimoto et al. 2010). This programme is initiated in the population of pluripotent, pericycle-like cells that might be derived not only from roots but also from cotyledons and petals. Therefore, a common mechanism characterised by the activity of specific sets of transcription factors and signalling components has been proposed (Atta et al. 2009; Sugimoto et al. 2010). During the initiation of pluripotent cells, auxin-stimulated expression of negative cytokinin response regulators ARR7 and ARR15 (see Sect. 2.3) prevents shoot formation (Buechel et al. 2010). Moreover, as shown on hypocotyl explants, auxin-induced root organogenesis involves also tissue-specific activation of cytokinin signalling that subsequently negatively regulates auxin transport through the inhibition of PIN auxin efflux carriers (Pernisová et al. 2009). Shoots are formed after transfer to SIM from shoot progenitor cells that appear upon a transcriptional switch triggered by combination of cytokinin and decreased concentrations of auxin (Gordon et al. 2007).

In addition to de novo organogenesis, auxin is also involved in the regeneration via the process of somatic embryogenesis (see chapters by Smertenko and Bozhkov, and Opatrný, this volume). This process can be initiated in vitro, either directly from primary explants or from callus derived from these explants. Typically, high concentrations of 2,4-D (Raghavan 2004) are applied to initiate embryogenic callus on immature zygotic embryos during early phases (globular and torpedo phases), while 2,4-D applied to immature embryos in the late cotyledonary stage initiates direct embryogenesis (for a protocol in Arabidopsis, see Gaj 2011).

Subsequent transfer of somatic embryos to auxin-free media initiates the development of plantlets. The technique of in vitro plant regeneration and propagation via somatic embryogenesis has been successfully used in a broad range of plant species (Raghavan 2004). In some systems, like cereals or conifers, it has even became the method of choice since for these species micropropagation via organogenesis has not been very successful, due to unknown genetic or developmental factors. As a rule, some hardwood trees, in particular conifers, are recently preferentially propagated only by means of either zygotic or somatic 'seeds'. Here, 2,4-D is the preferentially used auxin in the induction and proliferative media. The main reason is perhaps similar to the case of de novo organogenesis: 2,4-D generates stable and effective auxin concentration maxima that are not disrupted by excessive auxin transport and metabolism.

3.3 Auxins and Vegetative Propagation

In agriculture, horticulture and forestry (plant-propagating industry), vegetative propagation is used to produce large numbers of plants of equal genotype, and it is extensively used for multiplication of elite plant clones (Hartmann et al. 1990). Vegetative propagation is often the only option for plant growers, because only by this strategy, they are able to preserve all desirable traits of the parent plant (Salaš et al. 2012). Vegetative reproduction as a method of choice is exploiting natural competences of plants, both their innate ability to reproduce clonally and the general phenomena of plants to regenerate. A key step and essential part of most protocols for vegetative propagation is the formation of adventitious roots at some stage on the plant propagule. That is notably true for plant propagation by cuttings. In contrast to other forms of vegetative plant propagation, propagation by cuttings is frequently used as particularly efficient and attractive technique for industrial propagation (Ford et al. 2002; Salaš et al. 2012), and is making use of a long history of rooting stimulants based on auxins, and therefore will be our main focus here.

Development of the main root and the formation of lateral roots from the main axis of the root are meanwhile relatively well understood; the biology of adventitious root formation has received less attention, however. Adventitious roots are formed postembryonically in a de novo process either on stems or leaves or on already lateralised root axis or on any other plant organ (Chriqui 2008; Barlow 1994; Taiz and Zeiger 2002). Many plants, as part of their strategy for reproduction and survival, possess the ability to form adventitious roots naturally or under specific circumstances such as environmental stress or after mechanical damage. While some species, such as willow, might be grown simply by placing their cutting into a moist soil, the majority of species requires special attention, and there are many plant species on the other side of the spectrum, which are, for various reasons, very difficult to propagate even with the help of auxin-containing 'rooting substances'. The ability to root (without the help of rooting stimulants) is severely limited both with regard to the range of species amenable to spontaneous rooting

and also in the yield of those species that root easily (Ford et al. 2002). With the help of auxin regulators, plant growers can now go far beyond the original capacity of plant parts to produce new plants. With the proper use of these agents, cuttings will form better and more uniform roots in a shorter length of time. Cutting is a technique in which a piece of stem (rhizome, root or leaf) is excised from the parent plant and encouraged to grow into a plant that is independent of the parent. While narrowed to only certain species, conditions and with various efficacies, the natural disposition of plant parts to form roots has been exploited by gardeners for centuries.

Before describing the whole process of adventitious root formation (ADRF), it is necessary to differentiate between adventitious root primordia that are induced de novo and those that are already preformed on the stem (see also the classification by Němec recapitulated in the chapter by Opatrný in this volume). Preformed root initials are produced during the normal development of some plants; they are present mostly in woody plants and tend to develop slowly. If root primordia have yet to develop de novo in response to a triggering event, they are most conveniently formed in response to wounding (Blakesley et al. 1991). Actually, cuttings may be understood as a wounding event. Based on experiments with apple microcuttings, a sequence of successive phases has been defined by De Klerk et al. (1999) for formation of adventitious roots, namely:

(i) Callus induction(0–24 h)
(ii) Root induction (24–96 h)
(iii) Root outgrowth from the stem (96–120 h)

Endogenous IAA plays a central role for ADRF in vivo. Auxin is predominantly produced in shoot apical meristems or, more specifically, in the youngest leaves nearest to the meristem and, from there, is transported down through the adjacent stem parts (Ljung et al. 2001). Since auxin moves downwards, it is progressively loaded into the phloem (Berleth et al. 2000), its main transport route on longer distances (Baker 2000). When parts of the stem vasculature are wounded (severed), auxin would accumulate, in response to such wounding, in high concentration at the upper border of the wound (Sachs 1991). In concert with wound response factors, accumulation of auxin triggers leads to initial reprogramming of the cells into callus cells. When auxin is transported through this callus further downwards, to the lower parts of the plant, this leads to differentiation of new vascular tissue along the route of transported auxin, as described by canalisation hypothesis proposed by Sachs (1969). In a case of a cutting wound, endogenous auxin may move naturally downwards (rootwards) until it arrives at the position of incision, and it may be safely assumed that, after auxin has hence accumulated there, its rising levels help to induce a first wound response. Accumulated auxin leads then to transdifferentiation of several cell layers near this cut (see also Opatrný, this volume). This event would correspond to the first phase of the rooting as proposed by De Klerk et al. (1999).

Different roles for auxin during those phases of root initiation were shown by Ludwig-Müller et al. (2005). In the first phase, callus is formed preferentially from

cambium cells. According to Sugimoto et al. (2010) and also other authors (see Opatrný, this volume), these primary callus cells exhibit molecular markers of pericycle-like pluripotent stem cells. Under in vivo conditions, under long-term effect of exogenously applied IAA or NAA, they further differentiate into root apical meristems. The team of Ludwig-Müller has shown on *Arabidopsis* segments that both IAA and synthetic 2,4-D are comparable in their 'auxin' potency during this phase. Considering that neither 2,4-D nor PAA are ever used as rooting stimulants, it is interesting that both 2,4-D and even PAA were shown to be effective in this stage (Sugimoto et al. 2010), where the role of auxin is to induce the cells to proliferate into callus, from which roots may arise during the following phase. The shift from the first to the second phases of ADRF seems to be related to different gene expression during the two phases. The chromatin-remodelling component PROPORZ1 (PRZ1) mediates auxin effects on gene expression through chromatin remodelling. Noteworthy for the effect of PRZ1 is the differential auxin response of the mutant to auxin: whereas application of auxin promotes formation of lateral roots in wild type, it only triggers formation of tumorous callus-like tissue on *prz1-1* roots (Sieberer et al. 2003).

The process of adventitious root formation proceeds after some 24 h to the next phase, rhizogenesis, lasting from the second to approximately the fourth day. This process of root initiation might be roughly compared to the root initiation on RIM during in vitro rhizogenesis (see Sect. 3.2) and requires the continuous presence of a strong auxin signal (De Klerk 1999 and literature therein, Ludwig-Müller et al. 2005). During this stage, only NAA, IAA or IBA are effective, but not 2,4-D or PAA. This functional partitioning of the auxins is quite noteworthy (see also Sect. 2.2): 2,4-D and PAA (which cease to be effective just after callogenesis) are not well transported polarly, because they are rather poor substrates for auxin efflux carriers (Simon and Petrášek 2011). In contrast, IAA, NAA and, in a way, IBA (see later) are effective during both the first and second phases of ADRF and are substrates for cellular efflux such that they can participate in the organisation of auxin flow. Several studies confirm the importance of endogenous, basipetal IAA transport for adventitious rooting (for review see Ford et al. 2002). For instance, Jarvis and Shaheed (1986), by application of the polar auxin transport inhibitors TIBA and a morphactin on cuttings of *Phaseolus aureus*, also inhibited ADRF, even when a basal level of IAA was supplied exogenously. This finding insinuates the importance of organised auxin transport for this phase of root primordia establishment. If so, this would draw analogies to general non-restorative organogenesis, where auxin gradients, created by coordinated auxin transport, are crucial (Benková et al. 2003; Friml 2003, see also Sect. 2.1). Proper redistribution of the auxin signal over the neighbouring cells in space and time is most readily achieved by directional transport. Intercellular auxin transport and mainly auxin cellular efflux mediated by PIN proteins are driving this coordination, and it is well documented that not all auxins are equally good substrates for PIN proteins (see Sect. 2.2). The third and last phase of ADRF is marked by the outgrowth of roots from their primordia in the stem, and during this last part of the process, auxin can actually act already even inhibitory. Thus, the promoting role of auxin for ADRF is

mainly confined to the first 4 days (i.e. the first two of three distinct stages) of the process.

The process of rooting is dependent on the condition of the regenerating organ, on the condition of the maternal plant, on the season of the year, on the position of the tissue within the maternal plant and on the technique used (Bojarczuk and Jankiewicz 1975). Each of these parameters has impact on the available endogenous IAA or its transport routes, for example, from outgrowing apical buds, as well as on the responsiveness of the tissue to auxin. Most cuttings used lack their own shoot tips and so the original major source for natural auxin. Therefore, in many of these cases, application of exogenous auxin is required to achieve rooting (Salaš et al. 2012; Diaz-Sala et al. 1996; Blazkova et al. 1997). The practical application of auxin became amenable, when it was discovered that auxin can also induce root formation when applied on the surface of the basal cut (Hitchcock and Zimmerman 1936). Some plants regenerate roots on cuttings spontaneously as described above, but these are plants, where endogenous auxin is produced in the apex (or outgrowing lateral buds in spring) and transported basipetally to the cut surface in sufficient amounts to act as trigger: removal of the apex reduces both the level of endogenous auxin in the basal portion of a cutting and, consequently, the number of regenerated roots (Nordström and Eliasson 1993). Moreover, even in these plants, application of exogenous auxin strongly increases the number of roots (Salaš et al. 2012; Nordström et al. 1991; Liu and Reid 1992a).

Application of rooting stimulants is performed by several but similar methods. In spite of efforts to develop new rooting treatments for commercial operations, new methods have not emerged (Salaš et al. 2012; Hartmann et al. 1990): nowadays almost all auxin applications are conducted through a short dip (for a few seconds) of the cutting base into gel, solution or powder containing active auxin. Thus, the available auxin has to be absorbed via the base of plant cuttings before ADRF is initiated, and the auxin taken up during that period has to suffice for the whole process of rooting. The choice of suitable auxins became eventually narrowed to 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA), with IBA being strongly preferred over NAA (Salaš et al. 2012). The limited spectrum of auxins suitable for rooting provokes the question why? Form and circumstances under which auxin must be applied onto the plant probably predetermine, to the highest degree, the potency and choice of different auxin analogues for the task. The benefits of IBA and NAA in this regard had been discovered soon after the identification of auxin (Preece 2003 and references therein), and even today, IBA is still the predominant auxin of choice for rooting stimulators. IBA is by far the most used, active component of rooting substances on the market. The suitability of NAA (as second most used compound in rooting stimulants) in comparison to IBA or even IAA has been summarised as follows (Salaš et al. 2012): ‘NAA is not that effective as IAA, and its main advantage on the market was just its lower price’. The dominance of IBA on the market might be further illustrated by the decision of the biggest European producer of rooting stimulants – the Rhizopon Company: as of 2012, the company informed their customers that product lines based on any other active compound than IBA would be withdrawn and that, based on their

rooting guide, this actually will not bring any harm to the plant growers, because well-adjusted treatment with product lines based on IBA will replace well all non-IBA-based stimulant lines (recommended before for about 5 % plant-species-dependent treatments by Rhizopon). This decision of Rhizopon relates to the future commercial unavailability of IAA and NAA on the European market, since NAA and IAA will be most probably taken from Annex (Annex 1) of the European Crop Protection Directive list. In Europe, the responsibility of collecting information on properties and safety aspects of substances manufactured or imported at or above one tonne per year lies with the companies. Since the industrial demand for NAA- and IAA-based products was so low, the economic interest was not sufficient to make up for the costs of the evaluation process under the European legislation, such that the company decided not to have them relisted in the Annex 1 of the Directive.

We will try to sum up here by which qualities IBA has been able to outclass all the other auxins for commercial applications of rooting. As written above, auxin stimulators may only be applied through a pulse treatment at a beginning. This time frame is located just at the beginning of the process, but actually auxin is required during the two subsequent phases of root initiation (see above). Unlike the situation for in vitro rhizogenesis, the plant material in commercial rooting will not experience prolonged contact with auxin-containing media, which would continuously supply the plant tissue with the necessary auxin throughout the whole length of the rhizogenetic process. Moreover, a higher concentration (activity) of auxins is needed during the second as compared to the first phase. Thus, if the plant tissue is treated with auxin at the beginning of the process running for several days, chemical and biological instability of different auxins will be a limiting factor. Some auxins, among others 2,4-D and IBA, are stable, with a low rate of metabolism. However, while IBA is valued highly, 2,4-D seems to be the least used and ineffective auxin in root stimulation. This seems to be a further key to determine the requirements posed on an active root stimulator. As Ludwig-Müller et al. (2005) have shown, both IAA and 2,4-D are comparable in their potency during the first phase, whereas the second phase required high concentrations of polarly transported NAA and IAA or (in higher concentration) of IBA. Based on the substrate preference of efflux carriers alone (giving polarity to auxin flow; see Sect. 2.2), possibly the best major auxins for the rhizogenetic stage of ADRF would be IAA and NAA, along with the rare 4-Cl-IAA (Simon et al. 2013). 4-Cl-IAA has not been used as rooting substance for commercial applications so far. Interestingly, this auxin was found by Katayama et al. (2010) to be exceptionally strong as root stimulator, at least on cuttings of *Vigna mungo*. Both IAA and NAA have been successfully employed as rooting substances as soon as root stimulants were commercialised. However, the native auxin IAA is very vulnerable and chemically instable if applied externally. In addition, it is metabolised rapidly. If applied on the root cuttings at the beginning of the process, supply with IAA will run out, presumably already during the first stage of ADRF. So we might expect that even a strong auxin signal delivered by IAA to the cutting would be relatively short lived and with high probability the remaining auxin levels on the beginning of the second stage would be not sufficient for efficient rhizogenesis. This is highlighted by the

differences of rooting potential of cuttings with growing tip from the apical part of shoot compared to those from the basal shoot lacking a tip. Cuttings without shoot tip are much more dependent on the external application of auxin in comparison to the upper shoot parts, as the growing tip in the cutting is able to provide stable, continuous supply of natural auxin necessary for both callogenesis and rhizogenesis underlying ADRF. Most of the cuttings created lack a growing tip, such that the continuous supply of auxin becomes the limiting factor. As found by Correa et al. (2012), the efficacy of different auxins administered during 2–4 days via solid media to sustain ADRF was comparable. Thus, the superior suitability of IBA as rooting substance must be linked to the mode of its application in practice, i.e. the pulse manner, at the beginning of the rooting on the cutting basis: IBA is slowly and steadily converted into polarly transportable IAA that is steadily released into the plant tissue. (Ludwig-Müller et al. 2005). It would be interesting to test if new rooting stimulators might be developed based on forms of actively transported but stable forms of auxin (including 4-Cl-IAA), which might provide the qualities of IBA described above.

3.4 Auxinic Herbicides

Almost 70 years ago, when auxins started to be used as herbicides (Hamner and Tukey 1944), a new era of modern weed control in agriculture started, due to the advantages those herbicides offered for the first time. Even now, 2,4-D remains to be the most widely used herbicide in the world (Szmedra 1997; Monaco 2002; Industry Task Force II on 2,4-D Research Data). 2,4-D and other auxinic herbicides offer the advantage of having well-defined crop selectivity, i.e. susceptibility of dicot plants and substantial resistance of monocot crops, with low cost and high efficacy of application, systemic effect of the herbicides on plants (Monaco 2002; Kelley and Riechers 2007) and minimal development of herbicide-resistant weeds over time (Heap 1997; Mithila et al. 2011). To the date 2006 more than 1,500 products were registered declaring 2,4-D as their active ingredient (Industry Task Force II on 2,4-D Research Data).

Auxinic herbicides mimic both effects of auxin application and its phytotoxicity at superoptimal concentration (Monaco 2002). In lower concentration, they stimulate cell divisions, elongation and the growth responses typical for auxins. With increasing dose, they develop symptoms of phytotoxicity as observed by overdoses of IAA, such as disturbed growth and signs of herbicidal injury. Typically, a dicot weed will get at least 100 μg of 2,4-D in a typical field application, which is exceeding the level of endogenous auxin in a plant by at least 1,000 times (Cobb and Reade 2010). Monocots and in particular grasses respond differently to these high levels of exogenously applied auxins. Lack of phytotoxicity in monocots is attributed to a spectrum of different reasons, such as processes involved in auxin management and auxin response (Kelley and Riechers 2007; Grossmann 2007, 2010), but also to the fact that the apical meristem before heading is hidden in the

interior of the plant, where it is protected by the leaf sheaths. Within minutes of auxin application, profound changes in membrane permeability to cations can be discerned. The first phase, over the first hours after application, is stimulatory in susceptible plants, gene expression patterns are changed, and growth and metabolic activity are abnormally stimulated. Some typical growth responses can be seen within hours after auxin herbicide application, such as leaf epinasty (cuplike downward bending of leaves), stem curling and tissue swelling. Later, growth of malformed leaves, often with ectopic, parallel veins, leaf abscission and abnormal elongation of roots can be observed. Meristematic tissues in the stem tend to undergo excessive cell proliferation, with cambium, endodermis, pericycle and phloem parenchyma being most sensitive (Leopold 1955; Dnyansagar and Khosla 1969; Monaco 2002). Often, depending on the species, adventitious roots are formed (see also Sect. 3.3) at the basal parts of stems. Those phenomena are later followed by chloroplast damage, by foliar senescence with progressing chlorosis, and by the destruction of membrane and vascular system integrity, plant withering and, eventually, tissue necrosis and decay (Cobb 2010; Grossmann 2000), accompanied by many secondary symptoms (Monaco 2002).

While the phenotypic response to auxin-like herbicides is well described and decades of research have been dedicated to the development of new auxinic herbicides, many aspects of their mode of action and the basis for crop selectivity of auxinic herbicides have remained unclear (Kelley and Riechers 2007). Countless results from the rapidly growing body of knowledge on the role of auxin in plant development need to be connected with application to resolve the puzzle of selective toxicity of applied auxins and the primary mechanism of their herbicidal action. Over the years, several theories, all connected with the concepts and findings on auxin action, have emerged (Leopold 1955; Kelley and Riechers 2007). While several phytotoxic mechanisms in susceptible plant species have been proposed, the question of the ultimate reason for plant death has remained open; so far, no single mechanism was identified as the exclusively deciding factor in herbicide activity. Probably several effects in concert attribute to the herbicidal impact, and their relative contribution to the global effect of herbicidal treatment remains to be properly resolved.

The actual amplitude and character of an auxin response at a particular point depends on the integration of two major factors in any target cells: first, the pool of the signalling molecules present in the cell and, second, the tuning of the signal perception in the cell. There are two possible basic processes which both contribute to the establishment and modulation of the pool of the signalling molecule: metabolism and transport. While the concentration of natural auxins is a matter of tight regulation as described above in Sect. 2.2, synthetic compounds selected as auxins for herbicidal formulations significantly differ in their stability and persistence in plant tissues and evade normal homeostatic control (see Sect. 2.2). As a consequence, they may more easily trigger overinduction of auxin response in susceptible plants. Differential potential to metabolise 2,4-D and other active auxin herbicides was often proposed as one potential reason for monocot resistance towards these herbicides (Gauvrit and Gaillardon 1991; Monaco et al. 2002). IAA conjugation

with sugars and amino acids and oxidative degradation are the most frequent form of auxin deactivation (see also Sect. 2.2). IAA-glucose conjugates participate significantly in the deactivation of IAA. However 2,4-D is not a substrate for IAA-glucosyl transferase (Jackson et al. 2001), and, consequently, overexpression of this enzyme failed to cause 2,4-D tolerance (Jackson et al. 2002). Six IAA-amino acid-conjugating enzymes from the GH3 gene family have been isolated from *Arabidopsis*, and all six conjugate IAA to multiple amino acids in vitro (Staswick et al. 2005). Interestingly, the expression of these enzymes is induced in response to exogenous auxins, including 2,4-D or dicamba (Staswick et al. 2005) suggesting they may play an important role in inactivating excess auxin to support auxin homeostasis (Kelley and Riechers 2007). However, as auxin substrates for these GH3 enzymes, in addition to IAA, indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPA), phenylacetic acid (PAA) and α-naphthaleneacetic acid (NAA) are metabolised as well. In contrast, Trp, the active halogenated natural auxin 4-chloroindole-3-acetic acid (4-Cl-IAA) and the herbicides 2,4-D and dicamba are not substrates for GH3 enzymes (Staswick et al. 2005). Hormonal imbalance due to application of persistent auxinic herbicides inducing strong conjugation (and consequently inactivation) of native auxins is also suggested as possible mechanism of their phytotoxicity. All those factors may explain the particular choice of auxinic compounds based on their chemical structures. While at the beginning of the research NAA was in 1944 also successfully used as selective agent with herbicidal action (Gauvrit and Gaillardon 1991; Cobb 2010), its effects were much weaker, much higher doses had to be applied, and it was abandoned as soon as the potency of 2,4-D was discovered.

All current auxin herbicides are weak acids with pK values ranging from 2 to 4 (Monaco 2002). Structurally, their dissociated molecules share weaker positive charges on the planar aromatic ring in about 0.5 nm distance from the strong negative charge of the carboxyl group (Grossmann 2003) and can be further divided into four classes based on their particular chemical structures with slightly differing crop selectivity: phenoxycarboxylic acids (e.g. 2,4-D), benzoic acids (e.g. dicamba), pyridines (e.g. picloram) and the newer group of quinoline-carboxylic acids (e.g. quinclorac). Crop selectivity and maybe partly mode of action of the last group differ more from the other groups. Some quinolone carboxylic acids are able to control monocot weeds in the background of a monocot crop, and others target some dicotyledonous weeds in resistant dicot crops (Grossmann and Kwiatkowski 2000). The fact that 2,4-D is less toxic to monocots is often explained by differences in susceptibility for metabolism and degradation. Gauvrit and Gaillardon (1991) actually proposed that selectivity of auxin herbicides might be based on differences in auxin homeostasis, as 2,4-D is rapidly degraded in maize (Gauvrit and Gaillardon 1991; Monaco et al. 2002). Different induction of GH3 enzymes in response to 2,4-D application is also debated as possible factor contributing to the 2,4-D resistance of monocots (Kelley and Riechers 2007). The principal routes for the metabolism of phenoxyalkanoic acids are conjugation, hydroxylation and side-chain cleavage (Cobb 2010), depending on the respective species. A strategy of metabolic deactivation was utilised recently by Dow

AgroScience Company, which successfully developed new transgenic line of corn (DAS-40278-9) resistant to 2,4-D and other phenoxy-auxin herbicides. Resistance was based on expression of aryloxy-alkanoate dioxygenase (AAD-1) from *Sphingobium herbicidovorans*, a gram-negative soil bacterium (Tagliani 2011; Wright et al. 2010). AAD-1 is able to cleave phenoxy acids, and AAD-12 acts on pyridyl-oxyacetate auxin herbicides such as triclopyr and fluroxypyr (pyridines) (Wright et al. 2010).

The type of herbicidal formulation modulates the effects of auxinic compounds significantly. The choice whether auxins will be applied as free auxins in their salt (mostly the amino salts) or in their ester form will impact the permeability of auxinic herbicides into the plant through the cuticular layer and also affects transportability through the plant and between different tissue layers. Esterised auxins enter into the plants more easily, yet they are less readily transported through the plant body (Leopold 1955). The choice of the respective auxinic chemical should also consider their stability *in planta* and the above-mentioned systemic effects. Transportability of herbicide through the plant is a precondition for a systemic action, which allows to kill the plant as a whole including its underground part. For instance, 2,4-dichloropropionic acid has a strong local effect, yet it does not enter the vascular system and therefore is not transported across plant body (Leopold 1955). As a result, it fails to kill regrowing weeds. While endogenous auxin is transported through the plant by combination of polar auxin transport and passive flow in the phloem, auxinic herbicides mostly do not participate in polar auxin transport and therefore must at least enter the vascular system to be then carried by the passive flow through the plant body. This has been demonstrated for 2,4-D and other auxinic herbicides that are transported well both downwards to the root in the phloem and up from the roots to the stem and leaves in the xylem, with speed being in both cases concentration dependent (Leopold 1955; Monaco 2002).

Auxins and auxin-like compounds affect different plant tissues and organs in different manner. Thus, auxin phytotoxicity and symptoms of herbicidal injury differ between stems, roots and leaves. According to McCarthy-Suárez (2011), stems of pea plants did not show elevated levels of reactive oxygen species (ROS), whereas ROS do accumulate in both foliage and roots of auxin-treated pea plants in harmful levels with relevant herbicidic injury, as described later in this section in more detail. Nevertheless, stems of affected plants display also injury, but of a different type. The stems undergo morphological changes depending on the concentration of auxins in different stem tissues (Leopold 1955 and references therein) and different sensitivity of different tissue layers. While the phloem is generally one of the least sensitive tissues in plants towards auxin in general (Leopold 1955), the cells of phloem and its companion cells are the most affected stem tissue after application of auxinic herbicides, which is explained by their close contact with the high concentrations of active auxinic compounds accumulated in the phloem stream (Eames 1950; Dnyansagar and Khosla 1969). As a result, the continuity of the vascular system is affected leading to withering of dicotyledonous plants. Distorted cell division and expansion leads to deregulated growth with collapse of the correlating plant growth structure (Cobb 2010). Except for the phloem, the

strongest radial proliferation was reported in auxin-sensitive tissues with high division rates – especially cambium – followed by endodermis and pericycle (listed in Leopold 1955; Dnyansagar and Khosla 1969) that tend to proliferate and further degrade (Leopold 1955 and references therein). Conversely, the more the cells are differentiated, the stronger their resistance towards auxin-like compounds. It was actually proposed by Struckmeyer (1951) that the selective action of auxinic herbicides against dicotyledonous plants might be based on the different morphology of the stem vasculature in monocotyledonous and dicotyledonous plants. For the grasses being unaffected by herbicide action do not possess neither cambial layers nor weakly differentiated cells adjacent to phloem. Auxin preferentially acts on cells with high division rate, and therefore, meristematic cells are most vulnerable. It is known that severe distortion of patterns of auxin gradients, especially in the apical meristems, may be lethal for the plant (Weijers and Jürgens 2005).

Despite the statement by F.A. Gilbert (1946) that auxin herbicides cause susceptible plants 'to grow themselves to death' and the fact that auxinic herbicides are not good substrates for polar auxin transport, the possibility that the severe detrimental effect may be due to disrupted patterns of auxin transport in apical meristems, with subsequent failure of meristem organisation, has been neglected in the recent literature. Yet, the synergistic effect of the auxinic herbicide dicamba and the auxin transport blocker diflufenzopyr was successfully utilised in field application (Wehtje 2008) and thought to result from elevated concentration of dicamba in meristematic tissues.

Further insight into herbicidal auxin action and auxin herbicidal injury was provided by the observation that strong oxidative damage by excessive levels of reactive oxygen species (ROS) to leaves and roots of affected plants (Schopfer and Liszkay 2006; Pazmiño et al. 2012) is caused by auxin overdose. ROS are particularly responsible for the toxic effects of 2,4-D and other auxinic herbicides, at least in foliage and roots (McCarthy-Suárez et al. 2011). 2,4-D exerts failure of the detoxifying cell mechanisms, causing oxidative damage, fragmentation of nuclear DNA and cell death (Pazmiño et al. 2012). Auxins are also known to induce a programmed and cell-specific generation of ROS and to regulate the level of antioxidants. Onset of 2,4-D-induced leaf senescence is marked by overproduction of H_2O_2 and O_2^- and stimulation of enzymatic and nonenzymatic antioxidative systems (Karuppanapandian et al. 2011). This study also showed that changes produced in ROS metabolism by 2,4-D treatment can cause chlorophyll degradation and lipid peroxidation as typical for leaf senescence. Auxin-related ROS induction is probably conditioned by the activation of phosphatidylinositol 3-kinase activity, and ROS production is considered necessary for some auxin-regulated processes such as gravitropism (Joo et al. 2005).

Induction of ROS species in high doses by 2,4-D is probably mediated by high levels of abscisic acid (ABA Grossmann et al. 1996; Hansen and Grossmann 2000; Zhang et al. 2009) and related stress reactions (Hansen and Grossmann 2000). ABA contributes to the mode of action underlying the late auxin herbicide effects in sensitive dicots, especially the induction of tissue decay and cell death (Grossmann 2000). It was shown that ABA levels are profusely increasing in treated plants due

to cleavage of xanthophyll to xanthoxal, a critical step in ABA biosynthesis (Taylor et al. 2000) catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED; Hansen and Grossmann 2000). The abundance of NCED enzymes is strongly and rapidly upregulated by high levels of several auxinic compounds as observed in several plant models (*AtNCED1*, Raghavan et al. 2006; *AtNCED3* Raghavan et al. 2005; Gleason 2011; *AtNCED5* Gleason 2011; *GaNCED1* Hansen and Grossmann 2000). NCED overexpression in plants is associated with ABA accumulation in the leaves (Taylor et al. 2000). Induction of NCED in the shoots will subsequently lead to ABA transport through the plant to the leaves, where it causes stomatal closure, impinging on carbon assimilation and, consequently, biomass production and growth (Scheltrup and Grossmann 1995; Grossmann et al. 1996; Grossmann 2000; Grossmann and Kwiatkowski 2000; Hansen and Grossmann 2000). During this process, high ABA levels are linked with high levels of ROS (Grossmann 2000; Zhang et al. 2009). Grossmann (2000) has proposed that both auxin and auxinic herbicides primarily induce ethylene, which is then the trigger of an increase in the biosynthesis of abscisic acid (Hansen and Grossmann 2000; Grossmann 2003, 2007). Importantly, strong ethylene biosynthesis starts very early after application of IAA or synthetic auxins through induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase – the rate-limiting factor in ethylene biosynthesis (Woeste et al. 1999). Hansen and Grossmann observed that it was the auxin-induced ethylene that stimulated xanthophyll cleavage. Quinclorac-induced phytotoxicity in several susceptible grasses has been considered to be due to the induction of the ethylene precursor ACC-dependent cyanide (Grossmann and Kwiatkowski 1995). However, the role of ethylene in general 2,4-D toxicity is disputed (Pazmiño et al. 2012), and several authors have demonstrated that while ethylene may participate in the 2,4-D-induced plant senescence (Karuppanapandian et al. 2011), it does not participate in other 2,4-D-dependent symptoms as does leaf epinasty (Keller and Van Volkenburgh 1997; Pazmiño et al. 2012).

It is generally accepted that 2,4-D and IAA share a common signalling pathway (e.g. Taiz and Zeiger 2002), yet there are some specific differences with respect to auxin-triggered cell division and signalling between 2,4-D from 1-NAA and IAA (Campanoni and Nick 2005; Simon et al. 2013; Rahman et al. 2006) that might be potentially relevant to herbicidal action. A detailed analysis of the transcriptome revealed that IAA and NAA induce mainly similar genes, clustered in one group, whereas 2,4-D, in addition to the gene induction shared with IAA and NAA, also induces a subset of genes that cluster in a unique group (Pufky et al. 2003). Some differences between 2,4-D and IAA with respect to regulation and signalling of homeostasis have already been described in Sect. 2.2 of this chapter. Walsh et al. (2006) suggest that some degree of synthetic auxin selectivity and potency may also be based on differences within the auxin reception machinery through the SCF/AFBs complex. The TIR1 homolog AFB5 has been found to confer resistance particularly to auxin herbicides from pyridinecarboxylic acid -type (Picloram; Walsh et al. 2006) and benzoic acid-class auxin herbicides (dicamba; Gleason 2011), with only minimal cross-resistance to 2,4-D or IAA (Walsh et al. 2006). According to Calderon-Villalobos et al. (2012), picloram is selectively

bound by AFB5-Aux/IAA co-receptor pairs. While most attention has now shifted towards the reception through the TIR1/AFBs signalling system, decreased sensitivity of ABP1 to auxinoids is also considered as a potential source of resistance to auxinic herbicides (Mithila and Hall 2005). In the evaluation of auxinic herbicide effects, it seems that several mechanisms contribute to herbicidal injury in parallel, and their contribution and overall importance has to be clarified yet. Further elucidation of the separate pathways triggered by 2,4-D or other classes of auxin herbicides is expected to promote new strategies for the future development of new herbicidal solutions.

3.5 Auxin in Other Biotechnological Applications

Aside from the three major uses of auxinic compounds described in the sections above, auxin is and has been used from the beginning in wide variety of different scenarios to reach a range of diverse practical objectives. Many different practical applications are described in available literature, whereas other practical objectives seem to be, at least theoretically, possible. Nevertheless, many of such applications described in the older literature or the Internet were abandoned later in practice, due to economic or environmental limitations of the technology. Due to legislative constraints, many prospective compounds had to be withdrawn from the market. Therefore, this short review does not intend to encompass full range of applicable solutions in practical use. This section merely intends to review currently often used practices of auxin application in a variety of biological and practical settings and interpret the auxin biology behind them. The choice of a given auxinic substance for a particular application is often a compromise between the desired maximal physiological effect and the avoidance of unwelcome side effects, which present naturally a high threat, symptoms of herbicidal injury after spraying being the most perilous (see also Sect. 3.4).

Auxin had been used to initialise flowering. In 1942, Clark and Kerns found that flowering could be induced in pineapple by auxins (NAA), through evolution of ethylene within 1 day after application (Burg and Burg 1966). Polar auxin transport, driven by PIN1 into subapical tissues, is necessary for correct floral development (Kuhlemeier and Reinhardt 2001). On the other hand, some of the polar auxin transport inhibitors had been used to decrease the degree of apical dominance in pot flowers in order to increase the number of lateral branches bearing flowers. Transgenic increase of auxin synthesis in the ovule epidermis of cotton plants had been the key in developing plants producing higher yield of quality cotton lint fibres – highly elongated cells derived from the ovule epidermis (Zhang et al. 2011).

It was shown that auxin administered to the ovary may cause development of flowers into fruits even in the absence of pollination. Successful pollination initiates ovule growth – known as fruit set. Auxin is normally produced in vicinity of developing seeds and, along with gibberellins, may act primarily to induce fruit set, which trigger endogenous auxin in some fruit tissues (reviewed in Ruan

et al. 2012). This was the base for strategies, where transgenic overproduction of auxin in ovules using the tissue-specific promotor *DefH9* driving the auxin-synthesis gene *iaaM* in tobacco and tomato plants produced parthenocarpic fruits (Ficcadenti et al. 1999). Alternatively, tomatoes with SlPIN4 being silenced in their ovaries produced the same outcome (Mounet et al. 2012). Redistribution of auxin by PIN4 within the ovary is important as also demonstrated by parthenocarpic effects of NPA on tomatoes (Serrani et al. 2010). Application of auxin as sprays can be used in some instances to start parthenocarpic development of fruits in field routine (a common practice for tomatoes and strawberries, figs, watermelons and spiny gourd; Leopold 1955; Rasul et al. 2008; Maroto et al. 2005). Also fruit ripening can be regulated by impairing ripening-related ethylene and auxin metabolism and signalling as used in young developing peach fruits (Torrigiani et al. 2012).

Fruit growers also benefit from auxin applications to regulate different forms of abscission on fruit trees. Application of auxins may influence abscission of leaves, flowers or fruits from plants in a dual manner. In some cases, application of auxins is meant to cause the abscission and shedding of the organs (application of defoliants or fruit-thinning agents), whereas in others it prevents abscission (e.g. premature drop of flowers or maturing fruits). Detachment of abscising organs from the plant body is taking place in a zone of specialised cells – the abscission zone (AZ), which is usually preformed at the base of the petiole or fruit stalk. In some cases, more than one AZ may be present. When cell walls within the abscission layer of the AZ are digested, they become weak, and the connected organ breaks free and falls to the ground. Ethylene is considered to play a crucial role in fruit abscission, by activating new gene sets competent to digest those cell walls, and presence of ethylene receptors in the AZ might define sensitivity of the AZ to the ethylene. The currently accepted model is reviewed in Estornell et al. (2013) and Xie et al. (2013).

In some applications, auxin can prevent abscission in cases where abscission is normally senescence-triggered. As long as endogenous or applied auxin is transported from the organ through the AZ of the plant organ, differentiation of an abscission layer and, consequently, abscission of the organ are prevented. Senescent fruits, leaves or flowers export only small amounts of auxin into stem through the AZ, probably because senescence-born ethylene decreases auxin flow from the organ. The reduced supply of auxin to the AZ together with a loss of its transport polarity enhance the sensitivity of the AZ to ethylene and promote the activation of cell wall-degrading enzymes there (reviewed in detail by Xie et al. 2013). While this process is mainly controlled by environmental factors, auxin, sprayed on plants, can, despite such conditions, prevent undesirable flower or preharvest fruit drop. For example, in potted plants like *bougainvillea*, a spray with 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; from the class of phenoxycarboxylic acids) precludes abscission of bracts and keeps plants in a marketable shape (Meir et al. 2007). Interestingly, the use of picloram application was derived by Goldschmidt and Leshem (1971) to prevent the abscission of floral parts from etrog fruits (*Citrus medica*) on Israeli markets. The shape and condition of *etrogs*

have cultural significance for the Jewish holiday of *Sukkot*. Styles and stigmas still intact on top of the citrus fruits are called *pitam* in Jewish practice, and *etrogs* with an intact *pitam* is considered especially valuable. Spraying with 3 ppm (i.e. 12 μM) solution of picloram on trees at anthesis resulted in almost complete prevention of pistil abscission. It seems that one of the most apparent differences to the abscission process described above is the absence of a priori developed abscission layer tissue capable to separate the organ. Consistently, application of ethylene cannot stimulate abscission under those circumstances (Goldschmidt and Leshem 1971).

Different mechanisms have to be invoked to explain the abscission of young growing fruits which do not show symptoms of senescence yet, and the difference in mechanism also requires a different practice of auxin application. Angiosperms usually produce much less fruits than the initial flower number (Klein et al. 2007), and autoregulation of developing fruits in orchards is a naturally occurring phenomenon relevant to many fruit trees with heavy flowering (apples, citruses, avocado) and is distinct from the later shedding of older fruits, regulated by the mechanism described above. External application of regulators, so-called fruit thinning, is meant to enhance this mechanism in commercial orchards in order to decrease the number of developing fruits, which leads to increase fruit size and improved colour of the remaining fruits, improved plant vigour and annual bearing. Bangerth (2000) offered an explanation in the conceptual framework of correlative dominance, where shoots and fruits and fruits in clusters compete for assimilate relocation. The fruits in a cluster show a clear ranking, and their relative position within the cluster has a direct causal effect on their potential for precocious abscission. The more fruits, the stronger the mutual competition, such that the fruits of the lowest rank are doomed to abscission and precocious shed (Bangerth 2000). The rank of a fruitlet depends on auxin flow: the stronger its auxin export to the phloem of the fruit stalk, the more dominating its position in the competition. Auxin application on leaves and fruitlets leads to amplification of these differences in correlative hierarchy. In the best-studied model, during the so-called June drop of apple trees occurring 3–4 weeks after flowering (Losada and Herrero 2013), NAA and naphthalene acetamide (NAD) as common thinning agents act promotive when sprayed at full bloom or before flower drop. Some non-auxinic compounds such as 6-benzyladenine (BA) can be applied successfully as thinners as well, as they target correlation of plant organs based on different mechanisms (Botton et al. 2011). This mutual competition of auxin flow resembles the mechanisms of apical dominance as described by Balla et al. (2011) for lateral buds dominated by basipetal auxin flow from the apical tissue. In stems of pea, lateral buds are prevented from exporting their own auxin into the stem, due to impaired directional auxin efflux. To overcome the dominance by the apical bud, the axillary bud has first to establish directional auxin export by subcellular polarisation of PIN auxin transporters (Balla et al. 2011). Only after auxin flow has been connected with the main axis, this auxin transport route may start to differentiate into vascular bundles, securing nutrition to the lateral bud. Conversely, if external auxin is applied to and absorbed by the growing tip of the shoot, the conservation of basipetal PIN polarisation in the stem is stronger, and a smaller number of lateral buds are able to escape from apical

dominance. This control of apical dominance by exogenous auxin is utilised in practical applications as well. Tre-Hold, an NAA-containing product, is marketed as tree sprout inhibitor and used to control sprouts and sucker growth on apples, pears, olives and ornamental woody plants and trees. Apical bud dominance is maintained even after pruning, such that lateral bud outgrowth is minimised. This can be used to control branch growth in orchards, residential areas and areas where tree branch growth may pose a hazard, such as power lines.

4 Future Prospects for Auxin Biotechnology

Auxins have been famous and popular chemicals in plant production circles for already extended period of time. A vast amount of practical applications have been attempted, and numerous real-life applications have been developed based on the successful strategies, whereas a multitude of them had to be omitted later, not due to methodological failure but in response to economic or environmental challenges connected to them. The practical use of auxins succeeded to a degree that it is often very challenging for fundamental biology to decipher the biological mechanism behind this application. Under such circumstances, it is rather daring to propose prospective application of auxins.

However, advanced and more detailed understanding of the biological mechanisms can still provide new improvements and chances for fine-tuning of existing practices. However, to successfully transfer this scientific knowledge from the laboratory to the field, sufficient economic payoff is required, allowing to fund the environmental and toxicological tests necessary for the registration of the compounds into the databases of chemicals which are meanwhile strictly regulated in all developed markets.

Other yet unexploited opportunities may come from the field of genetically modified plants, provided that they will be allowed to enter the market. Considering our growing knowledge of auxin-related biology, future plants may be able to utilise many of the mechanisms described above, such as parthenocarpic fruiting, resistance to auxinic herbicides or better regulation of fruit set. More visionary prospects would be to direct artificial shaping of plant architecture through transgenic tuning of auxin-related mechanism. Theoretically, altered plant architecture (see also the chapter by Nick in the current volume), such as changes in branching in the root system or shape and habitus of canopy or change of morphological features such as shape of leaves, flowers and other body parts, should be possible in the future.

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