On Plant Responses to D-Amino Acids

Features of Growth, Root Behaviour and Selection for Plant Transformation

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Cover: Dual selection of Arabidopsis with the *DAO1* marker gene: Plants on the left hand side quadrants are wild-type, right hand side quadrants are 35S::*DAO1* plant. Selective agent is D-isoleucine in the top quadrants, and D-alanine in the bottom quadrants.

(photo: O. Forsum)

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Abstract

Amino acids have been regarded as potential plant nitrogen sources for more than a century. Some amino acids have been shown to support growth while others have growth-retarding effects. The D-isomers with notably adverse effects on plants' growth and development include D-serine and D-alanine. Recently, D-serine has been recognised as an endogenous ligand of receptor channels mediating calcium fluxes in plants, but otherwise little is known about endogenous roles of D-amino acids in plants.

In the studies underlying this thesis, the negative responses to D-serine and D-alanine were converted to positive effects in *Arabidopsis thaliana* (Arabidopsis) plants by introducing either of two D-amino acid-metabolising enzymes. Transgenic Arabidopsis lines expressing either the D-serine dehydratase (*dsdA)* gene from *Escherichia coli* or the D-amino acid oxidase (*DAO1)* gene from *Rhodotorula gracilis* grew with otherwise toxic D-amino acids as the sole nitrogen source. I also expressed a transporter specific for D-amino acids, which further increased the transgenic plants' growth with D-serine as sole nitrogen source. Hence, both assimilation and uptake restrictions can limit plant growth on D-amino acids. The growth of transgenic lines on D-serine or D-alanine provides an unambiguous and highly visible phenotype, which is essential for a selectable marker. Thus, expressing of either the *dsdA* or *DAO1* genes generated transformants that are easy to screen. Furthermore, the *DAO1* gene can be readily used for either positive or negative selection, depending on the substrate, thus it provides a unique conditional, substrate-dependent positive/negative selectable marker for plant transformation.

In summary, the presented work demonstrates that introducing the ability to catalyse a single metabolic step can allow plants to exploit an otherwise inaccessible or toxic form of organic nitrogen, and provides a versatile marker based on nitrogen nutrition for selecting transgenic plants. A possible role for D-serine in plants' touch response is also reviewed in the thesis.

Keywords: D-amino acid, metabolism, nitrogen, selectable markers, D-Serine, *GLR* and Arabidopsis

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To Åsa my joy and light, to whom I owe so much and to two dearly loved children, Vidar and Jonathan, all of whom makes life worth living.

"There must be numerous homeostatic adjustments required of cells. The sensing devices and the signals that initiate these adjustments are beyond our present ability to fathom. A goal for the future would be to determine the extent of knowledge the cell has of itself and how it utilizes this knowledge in a thoughtful manner when challenged"

(Barbara McClintock 1984)

Preface

Work towards this thesis was involuntarily interrupted and consequently it is based on three published articles, designated Papers I-III, and unpublished data produced almost a decade ago. These circumstances offer opportunities to apply perspectives that only time can provide. Both subsequent studies that have cited the published work the thesis is based upon and recent advances in understanding of D-amino acids' physiological roles in plants can be considered. Thus, in addition to the usual demarcation in any PhD thesis between background information available before the doctoral studies commenced and insights obtained from the doctoral work, here there are further demarcations between knowledge obtained from these sources and information subsequently presented by other authors. Papers I-III mainly address Arabidopsis plants' ability to use D-amino acids as nitrogen sources, and how introductions of the ability to catalyse single metabolic steps and enhance uptake can alter plants' responses to D-amino acids. The unpublished data presented concerns introduction of a prokaryotic amino acid transporter specific for D-amino acids. A generalised view of D-amino acids as inaccessible nitrogen sources is presented in Paper I. Papers II and III present possible applications of genes conferring the ability to metabolise D-amino acids as markers for selecting transformed plants. However, these articles do not consider possible roles of endogenous metabolism of D-amino acids in plants, which may have been too lightly dismissed at the time. Since I published my articles (in 2004, 2005 and 2008) a number of surprising discoveries have been made indicating that D-amino acids, especially D-serine, have endogenous physiological roles in plants. The recently ascribed role of D-serine can also be related to the emerging, intriguing and controversial field of plant neuroscience. In a section entitled Views and Perspectives *I review the roles of D-serine in plants in the light of recent developments and present preliminary new observations in conjunction with published data. This is intended to close the gap between my old results and the recently recognised endogenous role of D-serine in plants. Consequently, there are two main narratives in the thesis, one regarding D-amino acids as nitrogen sources and another concerning the potential role of D-serine as a signal molecule.*

Contents

List of Publications

This thesis is based on work presented in the following papers, which are referred to by the corresponding Roman numerals in the text:

I O. Forsum, H. Svennerstam, U. Ganeteg, and T. Näsholm. 2008. Capacities and constraints of amino acid utilization in Arabidopsis. *New Phytol*. 179:1058-1069.

II O. Erikson, M. Hertzberg, and T. Näsholm. 2005. The *dsdA* gene from *Escherichia coli* provides a novel selectable marker for plant transformation. *Plant Mol Biol*. 57:425-433.

III O. Erikson, M. Hertzberg, and T. Näsholm. 2004. A conditional marker gene allowing both positive and negative selection in plants. *Nat Biotechnol*. 22:455-458.

The contributions of Oskar Forsum (Erikson) to the papers included in this thesis were as follows:

I Planned the work jointly with the other authors, performed the experiments and analysed the data together with the joint first author. Wrote the paper together with the other authors.

II Planned the work together with the other authors, performed the experiments and analysed the data. Wrote the paper together with the other authors.

III Planned the work together with the other authors, performed the experiments and analysed the data. Wrote the paper together with the other authors.

1 Introduction

1.1 Chirality

In 1848 Louis Pasteur discovered molecular chirality. Objects are described as chiral if their reflected image in a mirror cannot be superimposed on the original. The term originates from the Greek word for hand, and the human hands are good examples of chiral objects. The two mirror images of a chiral object are referred to as optical isomers or enantiomers.

In Pasteur´s pioneering work he discovered spontaneous resolution in the crystallisation of racemic (equal proportions of both enantiomers) tartaric acid, yielding enantio-pure crystals, and that plane-polarised light is rotated either counter-clockwise or clockwise when transmitted through a solution of either of the enantiomers, hence the term optical isomers.

Later in 1857 Pasteur made the first discovery of biological enantioselectivity, when he observed preferential consumption of one optical isomer of tartaric acid by unidentified microorganisms in a fermentation experiment. In 1860 he demonstrated similar enatioselectivity in metabolism of tartaric acid by *Penicillium glaucum* (reviewed in Gal 2008).

1.2 Nomenclature and naming conventions

An enantiomer can be named according to the direction in which it rotates the plane of polarised light. If, as seen by a viewer towards whom the light is traveling, it rotates the light clockwise the enantiomer is labelled (+), while its mirror image that rotates the light counter-clockwise is labelled (-). The (+) and (-) label of enantiomers have also been named *d*- and *l*-(for dextrorotatory and levorotatory) but should not be confused with the D/L labelling commonly used for amino acids and sugars. The D/L system is based on glyceraldehyde, which is chiral itself, as a reference compounds on which amino acids can be overlaid. The D-isomer of glyceraldehyde is a (+) isomer. If a given amino

acid´s backbone can be overlaid upon L-glyceraldehyde it is labelled (L-), regardless of whether it is a (+) or (-) isomer in terms of optical rotation. In fact, nine of the 19 L-amino acids found in natural proteins are dextrorotatory or (+) isomers. The D/L-system and the R/S system, which is generally used to label chiral centres within molecules according to the Cahn Ingold prelog priority rules, have no fixed relation to the (+)/(-) system. Consequently, a D- or R isomer can be either dextrorotatory or levorotatory depending on the molecule (Meierhenrich 2008).

1.3 Homochirality as a signature of life

Many of the key biomolecules associated with life (including proteins, amino acids, DNA, RNA and sugars) are chiral. According to current knowledge all amino acids incorporated into proteins via ribosomal synthesis are L-isomers (Ollivaux *et al.* 2014). Similarly, almost all naturally occurring monosaccharides are D-sugars. Organisms must therefore be able to distinguish enantiomers. Furthermore, while chemical syntheses usually produce both enantiomers in equal proportions (racemic mixtures), most enzymes display stringent enantioselectivity (Cava *et al.* 2011). The correct scaffolding of DNA and proteins into helical chirality, a special case of axial chirality, requires homochirality. DNA and proteins are *P*-helixes (*P* for plus, or clockwise rotation away from the viewer, while helixes with the opposite orientation are denoted *M*- (for minus) helixes (Meierhenrich 2008). It only takes one erroneous residue to break the symmetry required for life´s key molecules. Hence, homochirality has been viewed as a signature of life.

1.4 D-amino acids have functional roles in organisms

Although stringent homochirality is maintained in ribosomal protein synthesis, D-amino acids are found in various forms, including proteins and peptides, in both prokaryotes and eukaryotes. All D-amino acids found in proteins are due to post-translational modification, or are protein or polypeptide products of non-ribosomal protein synthesis (Ollivaux *et al.* 2014). When D-amino acids were first found in organisms it was considered an oddity with little or no physiological relevance. However, since then there have been substantial advances in understanding of their origins, and they have been shown to have diverse structural and physiological functions, especially in microorganisms and animals, as briefly reviewed below.

1.4.1 D-amino acids in prokaryotes

One of the best known examples of D-amino acids' structural functions is as important elements of bacterial cell walls. On the outside of the cytoplasmic membrane of almost all bacteria is a strong and flexible cell wall made of peptidoglycan. This is a net-like polymer of linear glycan strands composed of repeating disaccharide units of *N*-acetyl glucosamine and *N*-acetylmuramic acid cross-linked by short peptides with high proportions of D-glutamic acid and D-alanine (Nagata *et al.* 1998).

D-amino acids are also components of antibiotics produced by bacteria (Urry 1971). They also have several signal functions in bacteria. For example, D-alanine acts as a signal that represses germination of dormant bacteria spores, generated by racemisation of the germinant L-alanine to D-alanine by an alanine racemase (Halvorson and Spiegelman 1952). Moreover, D-amino acids are involved in signalling the transition from a communal to a single cell planktonic life-strategy by controlling biofilm formation and disassembly in some bacteria (Kolodkin-Gal *et al.* 2010). D-amino acids can also serve as carbon and nitrogen sources for bacteria. D-Serine dehydratase (DSDA) (also known as D-serine ammonia lyase) encoded by the *dsdA* gene, enables *Escherichia coli* to use D-serine in this manner (Cosloy and McFall, 1975). Effects of introducing DSDA into plants and the metabolic capacities it confers were major foci of the work underlying this thesis.

1.4.2 D-amino acids in eukaryotes

There has also been substantial progress towards elucidating the physiological roles of D-amino acids and their metabolism in eukaryotes. There are several known human age- and disease-related changes in protein D-amino acid contents, notably increases in crystallin of lenses and dentin in teeth with aging and in fibrillar β-amyloid plaque of Alzheimer's disease brains (reviewed in Fujii 2002 and Hamase *et al.* 2002). Moreover, the toxic properties of some peptide toxins, including frog skin opioids, spider venom toxins, mammal venom toxins and mollusc neuroexcitatory peptides, depend on the D-configuration of specific amino acid residues (Reviewed in Ollivaux *et al.* 2014). Perhaps the most thoroughly studied aspect is that of oxidative deamination by D-Amino acid oxidase (DAAO), D-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3. This is a flavoenzyme, meaning that it requires flavin adenine dinucleotide (FAD) as a prosthetic group that participates in the electron transfer involved in the reaction it catalyses; the stereo-specific oxidative deamination of D-isomers of neutral and polar amino acids into ammonium and corresponding keto-acids with concomitant reduction of molecular oxygen to hydrogen peroxide (Alonso *et al.* 1998).

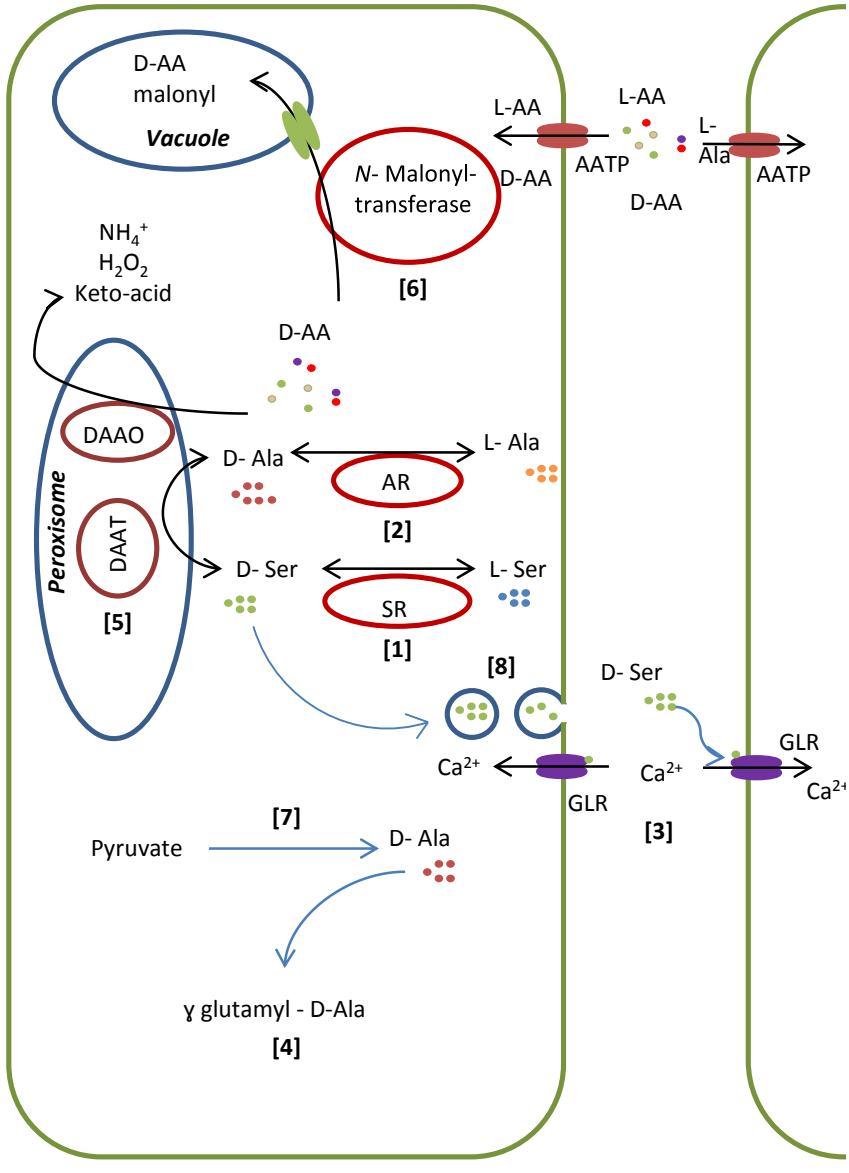

Figure 1. Schematic model of D-amino acid metabolism in plants, based on data gathered from analyses of several species. Key: Amino acid transporter (AATP), D/L-amino acid (D/L-AA), 1) Serine racemase (SR) (Sugimoto *et al.* 2009), 2) alanine racemase (AR) (Ono *et al.* 2006), 3) glutamate receptor-like channels (GLR) (Lam *et al.* 1998), 4) D-alanine (D-Ala) in peptides (Fukuda *et al.* 1973b), 5) D-amino acid aminotransferase (DAAT) (Ogawa & Fukuda 1973, Manabe 1984), 6) malonyl conjugate formation of D-AAand vacuole compartmentalisation (Pokorny *et al.* 1970, Guo *et al.* 1993, Bouzayen *et al.* 1989), 7) *de novo* synthesis of D-alanine from labelled pyruvate (Ogawa 1978), 8) excretion of D-serine from cells by exocytosis, a putative process proposed in this thesis that has not been empirically verified.

DAAO was one of the first enzymes to be described, and the second flavoprotein discovered (Krebs 1935, Warburg and Christian 1938). It is a model flavoprotein, and very well characterized (Umhau *et al.* 2000). Due to its absolute stereospecificity (L-amino acids are neither substrates nor inhibitors) and broad substrate specificity DAAO is widely used as an enzyme in chemical analysis and as a biocatalyst, notably in production of cephalosporin antibiotics (Pollegioni and Molla 2011).

DAAO activity has been detected in numerous organisms, including prokaryotes, fungi, invertebrates, fish, birds and mammals (Pollegioni *et al.* 2007), but when the work this thesis is based upon started in 2001 no DAAO activity in plants had been reported. The enzyme has been ascribed diverse physiological functions in various organisms. For example, in yeast it allows D-amino acids to be used as nitrogen and carbon sources, while in humans it eliminates D-amino acids in kidney, liver and dietary tracts. DAAO has also been attributed a regulatory role in controlling D-serine levels in the brain, which is as an agonist of N-methyl D-aspartate (NMDA) receptors, involved in regulating nerve synapses and overall brain function (reviewed by Pilone 2000, Pollegoni *et al.* 2007). The capacities conferred by functional expression of DAAO were also major foci of the work underlying this thesis.

1.5 Plant D-Amino acid metabolism

1.5.1 Metabolism of administered D-amino acids

The first studies on D-amino acid metabolism in plants, in the 1960s and 1970s, focused on measuring plants' contents, uptake, metabolism and responses to various D-amino acids. These included a number of experiments in which D-amino acids were exogenously supplied. The results showed (*inter alia*) that *Hordeum vulgare* (barley) plants supplied D- and L-isomers of phenylalanine, valine, leucine, isoleucine, tyrosine, tryptophan, alanine, and glutamic acid formed malonyl derivatives from all D-isomers, but not L-isomers (Rosa and Neish, 1968). Aldag and Young (1970) reported that both *Lolium perenne* (ryegrass) and *Zea mays* (maize) seedlings readily absorb D-valine, D-leucine, D-alanine, D-methionine, and D-lysine. They suggested that at least some of the initial metabolic conversions were analogous to those involving the corresponding L-α-amino acids. The α-keto acid analogue of the supplied D-amino acid was a significant product, and possibly a major intermediate in every case. Further they corroborated the occurrence of decarboxylation, as evidenced by loss of radioactivity when the ^{14}C label was in the carboxyl position. When they supplied ryegrass seedlings with ^{14}C-D-alanine they detected labelled valine in the plants' roots, but labelled isoleucine

in their shoots. They also observed conversion of carboxy ^{14}C-D-alanine to labelled ^{14}C-α-methylserine, and proposed the presence of a α-methylserine hydroxymethyl-transferase.

Pokorny *et al.* (1970) made an extensive comparative study of L- and D-methionine metabolism in different taxa of plants, fungi, algae and bacteria. They found that all plants (but only plants) formed *N*-malonyl conjugates when fed D-methionine, but not when fed L-methionine. Some conifers also formed *N*-acetyl-D-methionine. Subsequent reports included observations by Guo *et al.* (1993) that an *N*-malonyltransfarase active towards 1-aminocyclopropane-1-carboxylate (ACC), which is achiral, also conjugates D-phenylalanine in *Vigna radiata* (mung bean). In addition, a tonoplast membrane transporter for ACC-*N*-malonyl and malonyl-D-amino acids, which mediates vacuole loading, has been characterised (Bouzayen *et al.* 1989).

1.5.2 Naturally occurring D-amino acids

Various conjugated forms of alanine in D-configuration also naturally occur in pea, as demonstrated by Ogawa *et al.* (1973), who detected *N*-malonyl-D-alanine in the ninhydrin-negative fraction of ethanol extracts of decotylised pea seedlings, and Fukuda *et al.* (1973a), who found that the alanine moiety of γ-glutamylalanine in the ninhydrin-positive fraction of such extracts is in D-configuration. Fukuda *et al.* (1973b) also found that D-alanine accounted for ca. 80% of the total alanine in a hydrolysate from 6-day-old *Pisum sativum* (pea) seedlings, and only small amounts were present in free form. Furthermore, Ogawa *et al.* (1976) found that 30-70% of amino acids formed from α-amino-*N*-butyric acid in extracts of pea seedlings disappeared during treatment with DAAO, and Ogawa *et al.* (1978) demonstrated that D-alanine is synthesised *de novo* in germinating pea seedlings via racemisation of L-alanine. Analyses by Kawasaki *et al.* (1982) corroborated that D-alanine is synthesised from L-alanine by a racemase reaction and that several other D-amino acids are synthesised via action of a D-alanine aminotransferase in pea seedlings. An alanine racemase from alfalfa seedlings was subsequently characterised in more detail (Ono *et al.* 2006). The presence of a D-amino acid aminotransferase has also been demonstrated in pea (Ogawa and Fukuda 1973), *Oryza sativa* (rice) (Manabe 1984) and Arabidopsis (Funakoshi *et al.* 2008). Several dipeptides containing D-alanine have been isolated from rice (Manabe 1992). Brückner and Westhauser (2003) quantified free L- and D-amino acids from a large number of gymnosperms and angiosperms, and concluded that D-amino acids in the low percentage are principle constituents of plants.

1.6 Plant responses to D-amino acids

A number of studies describing D-amino acids' influence on plant's growth, development and associated physiological responses have been reported. Some early reports concerned effects of racemic mixtures of D/L-amino acids, probably due to difficulties in obtaining enantiopure amino acids, which makes interpretation of results difficult. For example, inhibitory effects of various exogenous racemic D/L-amino acids on flower and frond production in duckweed have been demonstrated (Nakashima 1964). Valle and Virtanen (1965) report growth performance of barley and peas with different D-, L- and D/L- amino acids given solely or in combination with nitrate. Their study showed that several D- but also L-amino acids (e.g. D-alanine, D-methionine , D-histidine, L-methionine, D-histidine and L-histidine) reduced growth (less than nitrogen free control) of both barley and peas, when providing the sole nitrogen source. Addition of nitrate abolished the negative effect of some of the amino acids (e.g. L-histidine and D-histidine) whereas D-alanine and L-valine allowed less growth than nitrogen free control irrespectively of addition of nitrate.

In Bollard (1966) over 160 organic nitrogen sources, including six D-amino acids, were provided as the sole nitrogen source to the plant *Spirodela oligorhiza* (duckweed). None of the D-isomers supported growth. Among D-amino acids, the strongest inhibitory effect was reported for D-serine (Scheffer *et al.* 1966) and D-alanine (Valle and Virtanen 1965 and Manabe and Ohira 1980). D-Serine also reportedly strongly inhibits root production (Scheffer *et al.* 1966), and suppresses uptake of potassium, nitrate, phosphate and sulphate by slices of *Beta vulgaris* (beetroot) tissue by 40-50% (Ellis *et al.* 1964).

The notion that D-amino acids cannot be used for plant growth is supported by findings presented in Paper I, that no D-amino acid supported growth of Arabidopsis when provided as the sole nitrogen source. Other data show that some metabolism (at least transamination, decarboxylation or racemisation) of D-amino acids occurs (Hill *et al.* 2011, Gördes 2011, Gördes 2013), but there is no evidence that any plants can grow on D-amino acids as sole nitrogen sources, to the best of my knowledge. This is not an exhaustive review of relevant reports, but rather an illustration that different D-amino acids do not induce the same responses in plants.

1.7 An endogenous role for D-Serine in plants

The plant GLUTAMATE RECEPTOR-LIKE genes (*GLR*s) are homologous to the genes for mammalian ionotropic glutamate receptors (iGluRs) (Lam *et al.* 1998). In animals, these ligand gated receptor channels mediate calcium fluxes

in nerve cells at synapses, which play key roles in their functions. D-Serine has been recognised as the main co-agonist of some of these receptors and strongly influences animal synapse and brain functions (Martineau 2014).

In plants, homologous genes to these animal receptor channel genes have been shown to participate in several developmental processes. For example, initiation of lateral root primordia is associated with *GLR*3.3 and 3.4 (Vincill *et al.* 2013), *GLR*3.4 expression is sensitive to touch (Mayerhoff *et al.* 2005), while *GLR*3.3 is involved in geotropic response (Miller *et al.* 2010) and both pathogen defence signalling and resistance responses (Manzoor *et al.* 2013). GLRs are also putative nutrient sensors in roots that influence root architecture (Walch-Liu *et al.* 2006, Forde and Walch-Liu 2009).

A finding of particular interest, in the context of this thesis, is that in both Arabidopsis and *Nicotiana tabacum* (tobacco) *GLR*1.2 and *GLR*3.7 mediate Ca^{2+} fluxes in pollen tubes and are regulated by D-serine produced in the pistil and (particularly) the ovary, by a serine racemase (SR). *GLR*1.2 knock-out and anti-sense plants showed reduced fertility in terms of seeds per silique than wild-type counterparts (Micharde *et al.* 2011). The SR is also expressed in tip regions of primary and lateral roots, developing leaves and meristems (Sugimoto *et al.* 2009) (Fig. 2). Finally, rather intriguingly, a DAAO has been found in maize (Gholizadeh and Kohnehrouz 2008) that is encoded by a gene induced by both D-alanine in the growth medium and drought stress (Gholizadeh and Kohnehrouz 2009). Putative DAAOs, based on sequence similarity, have also been detected in rice and Arabidopsis (Gholizadeh and Kohnehrouz 2008).

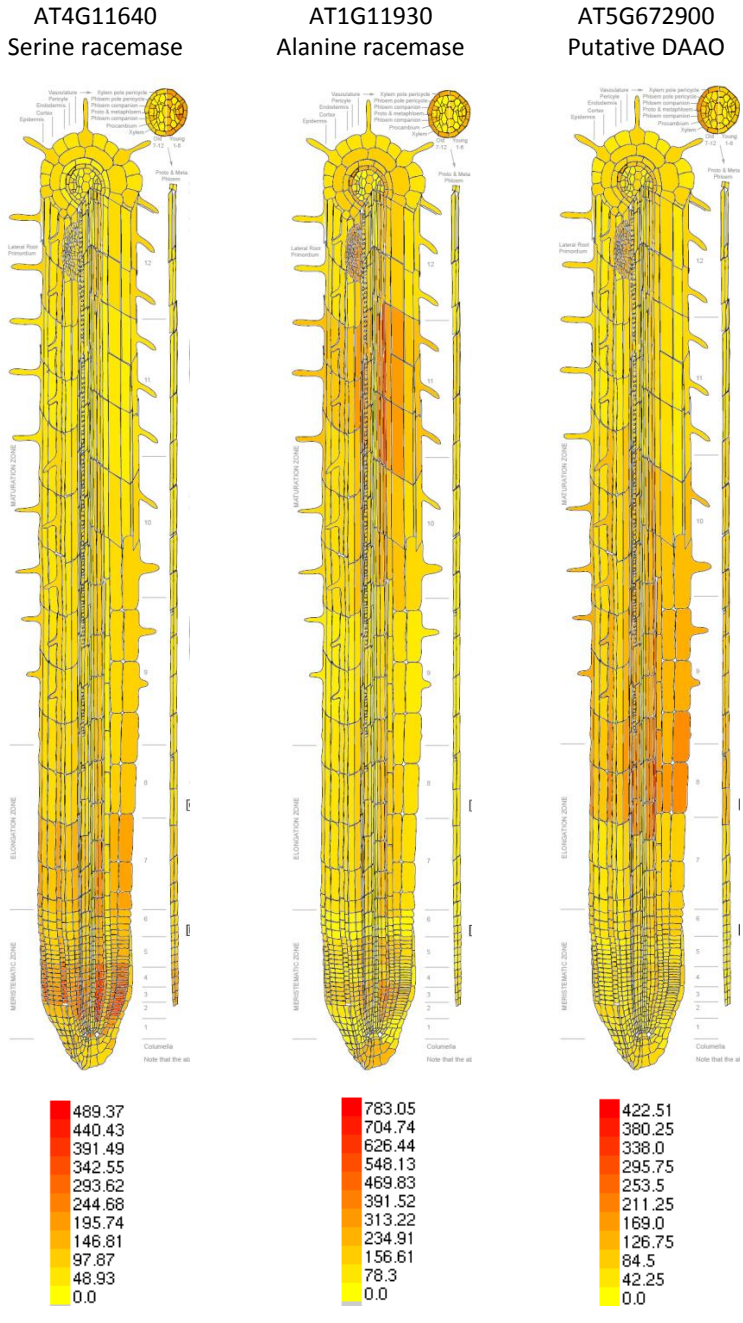

Figure 2. Expression patterns of serine racemase, alanine racemase and putative DAAO genes in Arabidopsis roots. Note the different scales (image generated from Arabidopsis eFP Browser at bar.utoronto.ca, Winter *et al.* 2007).

2 Aims

Plants are known to contain, absorb and even synthesise D-amino acids. However, there are no reports that plants can grow on D-amino acids. Thus, a primary aim was to determine what prevents plants from growing on D-amino acids as nitrogen sources, particularly whether restrictions lie at acquisition or assimilation steps.

Some other organisms have enzymes that allow growth on D-amino acids as sole nitrogen and carbon sources. Thus, a second aim was to assess the scope for engineering plants to grow on D-amino acids as sole nitrogen sources, through introducing such enzymes.

If plants with the ability to metabolise a new nitrogen source can be engineered, uptake may be a growth-limiting step. Plant amino acid transporters mediate uptake of D-amino acids as well as L-amino acids, but usually at lower rates. Specific D-amino acid transporters with high affinities for D-amino acids have been reported. Thus, a third aim was to determine whether expression of such a transporter can increase plant uptake of D-amino acids and enhance growth on them.

A fourth aim was to address potential applications of plants that can grow on D-amino acids.

Recent advances towards elucidating physiological roles of D-amino acids, especially D-serine, shed new light on some of my old results and observations. D-Serine is now known to be a strong activator of some plant GLRs, and to participate in interactions between pollen tubes and ovaries. However, it may also be involved in other physiological processes of plants, notably I have observed strong effects of exogenously administered D-serine on root growth and behaviour. Thus, the final aim was to elucidate D-serine's mode of action in these phenomena.

3 Methodological considerations

To meet the aims of the work underlying this thesis regarding the capacity of plants to take up and utilise various D-isomers of amino acids, and associated phenomena, growth rates were used as indirect indicators of their acquisition and assimilation rates. The material used comprised wild-type Arabidopsis (ecotype *Columbia*, *Col-0*) plants and transgenic lines with modified capacities to metabolise and/or transport D-amino acids. Growth was considered (and is considered in this thesis) as a crude indicator of nitrogen uptake and assimilation rates, as it demonstrates that all growth requirements were met, including sufficient uptake and subsequent metabolism of nitrogen.

In order to sustain growth, concentrations of amino acids in the growth media had to be much higher than physiologically relevant to natural conditions, especially for the relatively rare D-isomers. In the growth system used there is no growth at all without high concentrations. This work was not intended to reflect natural conditions, but rather to probe the limit of plant´s ability to grow on D-amino acids. However, use of growth in this context can be criticised as a poor measure of metabolism that may grossly underestimate metabolic rates of D-amino acids. Plants can, probably, assimilate several D-amino acids, without being able to use them as a sole nitrogen source.

Arabidopsis was regarded as the most suitable species for the project partly because it is a small plant that germinates and grows rapidly, which greatly facilitates replication of multiple treatments in convenient space and time frames. Arabidopsis is also attractive from a physiological perspective as it does not form mycorrhizae or associations with dinitrogen-fixing organisms (which would have confounded observed effects by extending access to other metabolic capacities, through symbionts, and hence potentially influenced the plants' responses to D-amino acids). Plants associated with dinitrogen-fixing bacteria are likely to be exposed to D-amino acids from the nodules, while plants in mycorrhizal associations are likely to benefit from the metabolic

capacities of their fungal symbionts. Fungi and bacteria are known to metabolise D-amino acids, while the corresponding capacities of plants are less well established. Arabidopsis is a member of the Brassicaceae, which are not normally considered to form mycorrhizal associations, but instead grow extensive hairy root systems. Nevertheless, there are variations among Arabidopsis ecotypes and (for example) *Landsberg erecta* has been shown to have much lower D-amino acid transferase capacity than 17 other ecotypes (Gördes *et al.* 2013). In all experiments reported in this thesis ecotype *Columbia* (Col-0) was used. Other reasons for using Arabidopsis include the enormously extensive information available about optimal methods for growing, manipulating and analysing the species.

4 Results

4.1 Uptake and Growth on D-Amino acids

4.1.1 Plant growth and uptake of D-isomers of amino acids

Solution depletion measurements in Arabidopsis root uptake experiments suggested that all tested D-isomers of amino acids were absorbed. Uptake rates of D- and L-isomers of alanine were similar, while D-serine, D-valine, D-isoleucine and D-arginine were taken up at about ~25%, 60%, 60% and 75% lower rates than the corresponding L-isomers, respectively (Fig. 1, Paper I).

Analysis of the growth of Arabidopsis seedlings supplied with 15 different amino acids (nine L-isomers, five D-isomers and glycine) as the sole nitrogen source revealed that six amino acids (the achiral glycine and five L-isomers: Glutamine, asparagine , aspartic acid, alanine, arginine and glycine) supported stronger growth than nitrogen-free medium. Growth was weaker on seven of the amino acids (three L-isomers and four D-isomers: L-Serine, L-valine, L-isoleucine, D-alanine, D-serine, D-arginine D-valine) than on nitrogen-free medium, and there was no significant difference between growth rates on the other two amino acids (L-glutamic acid and D-isoleucine) and nitrogen-free medium (Fig. 2, Paper I).

When the 15 amino acids were added together with 3 mM nitrate in the growth medium, three (L-glutamine, L-asparagine and L-aspartic acid) supported stronger growth, nine (D-serine, D-alanine, D-arginine, L-isoleucine, L-serine, L-valine, D-valine, L-arginine and L-alanine) reduced growth, and three (L-glutamic acid D-isoleucine and glycine) had no significant effect, relative to nitrate alone. Interestingly, in the context of this thesis, all of the tested D-isomers inhibited growth, as either sole nitrogen sources or together with nitrate, except D-isoleucine, which had no negative effect on growth even together with nitrate. If anything, it had a slightly positive effect (Figs. 2, Paper I, and 1C, Paper III). In contrast, L-isomers of valine and valine strongly

reduced growth when added either solely or together with nitrate. A possible explanation for the toxicity of L-isoleucine and L-valine is discussed in Paper III.

L-lysine toxicity and tolerance of D-lysine have also been observed in studies with tobacco and Arabidopsis (Chen *et al.* 2010), in which lysine racemase was used as a selectable marker for transformation and L-lysine (10 mM) as a selective agent. Selection was slow, it took 18-20 days for unambiguous phenotypes to develop, but 96% escape-free. The cited authors also successfully selected transformants using L-lysine as the sole nitrogen source. They argue, with the support of credible data, that L-lysine toxicity involves the accumulation and feed-back inhibition of aspartate kinase, leading to depletion of precursors required for L-isoleucine and L-methionine synthesis. No tests of the possibility that wild-type Arabidopsis could grow on D- lysine as the sole nitrogen source are mentioned in the report. Expression of lysine racemase increased glutamic acid concentrations in transformed tobacco, which somewhat compromises its applicability as a selectable marker.

Growth-retarding effects of various D- and L-isomers of amino acids on several species have been reported (Bollard 1966, Valle and Virtanen 1965). Clearly, it is not only the optical configuration that determines whether or not an amino acid can sustain growth or exert toxic effects, but there are no reports of D-isomers supporting growth of plants as the sole nitrogen source. When Bollard (1966) screened more than 160 organic nitrogen compounds' ability to serve as a sole source of nitrogen, he tested and found significant differences in the ability of a fungus (*Neurospora crassa*), an alga (*Chlorella vulgaris*) and a plant (duckweed) to grow on various sources. Duckweed, *Chlorella vulgaris* and *Neurospora crassa* grew on six, 14 and 19 of the tested protein amino acids, respectively. Of particular interest in the context of this thesis, Duckweed, *Chlorella vulgaris* and *Neurospora crassa* grew on none, three and six of the tested D-amino acids, respectively.

This dichotomy in ability to use D-amino acids for growth prompted the hypothesis that plants may lack the metabolic capacity to assimilate absorbed D-amino acids sufficiently to support growth. DAAO activity has been detected in *Chlorella vulgaris* (Pistorius and Voss 1977) and various yeasts, including *Rhodotorula gracilis* (Alonso *et al.* 1998), *Candida boidinii* (Yurimoto *et al.* 2000), and *Trigonopsis variabilis* (Ju *et al.* 1998). When the doctoral project was initiated, in 2001, there had been no reports of a DAAO in plants. However, there had been reports of uptake of ^{14}C D-valine followed by its conversion to labelled carbon dioxide and the corresponding keto acid, indicative of DAAO activity, in both maize and *Helianthus annuus* (sunflower) (Aldag *et al.* 1970, Aldag and Young 1970). The presence of a DAAO that was

inducible by D-alanine and drought stress was subsequently confirmed in maize (Gholizadeh and Kohnehrouz 2009, Gholizadeh *et al.* 2009 and Gholizadeh 2011).

To test the hypothesis that a plant´s ability to grow on D-amino acids is limited by a metabolic constraint I constructed two transgenic lines with enhanced D-amino acid catabolic capacity.

4.1.2 Arabidopsis plants with enhanced D-amino acid metabolism capacities grow on D-alanine and D-serine as sole nitrogen sources

I constructed two transgenic Arabidopsis lines, one expressing the *dsdA* gene from *Escherichia coli* encoding D-serine ammonia lyase and the other expressing the *DAO1* gene from *Rhodotorula gracilis* encoding DAAO, both catalysing deamination of D-amino acids into ammonium and keto acids. Both of the transgenic Arabidopsis lines grew well on D-serine, which is toxic to wild-type plants. Plants expressing DAAO also grew well on D-alanine. The transgenic plants showed clear positive growth responses to increases in D-serine or D-alanine concentrations (Fig. 7, Paper I). D-Serine in combination with nitrate promoted growth of the 35S::*dsdA* transgenic plants more than corresponding concentrations of ammonium and nitrate (Fig. 8, Paper I). The introduced D-amino acid metabolism enables selective nitrogen nutrition, available only to the transgenic plant. The transgenic plants can also grow with D-serine or D-alanine as the sole nitrogen source. These findings demonstrate that the ability to exploit an otherwise inaccessible organic nitrogen source can be conferred by the introduction of capacity to catalyse a single metabolic step.

4.1.3 Enhanced uptake can increase growth

The 35S::*dsdA* and 35S::*DAO1* transgenic plants needed 30 mM D-serine or D-alanine to grow at 50% of the rate observed with 20 mM NO_3^-. This raised questions regarding the constraint(s) limiting growth after the transformations had eliminated the metabolic constraint on the plants' ability to grow on D-amino acids. In the study reported in Paper I the hypothesis that uptake may be a bottleneck, in Arabidopsis growth on amino acids, was corroborated by results of overexpressing an endogenous amino acids transporter, lysine histidine transporter 1 (LHT1). The particulars of the uptake experiments and transporters tested in the study, including LHT1, are covered in an earlier thesis by Svennerstam (2008). Briefly, overexpression of LHT1 dramatically increased growth with amino acids as sole nitrogen sources at low concentrations (0.5 mM of L-asparagine, L-glutamic acid and L-glutamine), and this effect of overexpression gradually declined as the concentrations

increased. To test the possibility that a specific D-amino acid transporter may have similar effects I introduced a prokaryotic D-serine/D-alanine/glycine transporter, from *Escherichia coli K-12* (the *cycA* gene) into wild-type, 35S::*dsdA* and 35S::*DAO1* Arabidopsis plants. Unpublished, preliminary, data show that heterologous expression of a D-amino acid transporter can almost double growth rates of 35S::*dsdA* plants on D-serine (Table 1a). Expression of the *cycA* gene in wild-type background also increased growth on glycine (Table 2). In contrast, expression of the *cycA* gene in 35S::*DAO1* Arabidopsis plants had no or negative effects on their growth on D-alanine or D-serine (Table 1b). These preliminary data show that uptake of D-amino acids also limits growth of the 35S::*dsdA* plants, but increasing uptake in the 35S::*DAO1* plants is detrimental for their growth. This may be because uptake then exceeds the metabolic capacity of the DAAO enzyme, and the excess D-amino acids have toxic effects rather than contributing to nitrogen nutrition.

Table 1. Growth (final biomass), in percent, of Arabidopsis 35S::*dsdA* (A) and 35S::*DAO1*(B) lines expressing *cycA* relative to that of 35S::*dsdA* controls, grown on nitrogen-free ½-strength MS (Murashige and Skoog 1962) medium amended with 3 mM of D-serine (D-Ser) or D-alanine (D-Ala), solidified with 0.8% agar, for 20 days after germination.

A) Line	N source	Relative growth	B) Line	N source	Relative growth
dsdA 4:9	D-Ser	100	*DAO1* 10:7	D-Ala	97
dsdA x *cycA* 1:1	D-Ser	143	*DAO1* x *cycA* 4:4	D-Ala	77
dsdA x *cycA* 1:3	D-Ser	133	*DAO1* x *cycA* 9:1	D-Ala	90
dsdA x *cycA* 1:4	D-Ser	132	*DAO1* 10:7	D-Ser	52
dsdA x *cycA* 1:6	D-Ser	141	*DAO1* x *cycA* 4:4	D-Ser	52
dsdA x *cycA* 2:6	D-Ser	184	*DAO1* x *cycA* 9:1	D-Ser	39

Table 2. Growth (final biomass), in percent, of Arabidopsis lines expressing *cycA* relative to that of wild-type (WT) controls, grown on nitrogen-free ½-strength MS medium amended with glycine (Gly) at indicated concentrations, solidified with 0.8% agar, for 20 days after germination.

	Gly		
	1 mM	3 mM	10 mM
WT	100	83	74
Cyc 4:2	180	199	182
Cyc 7:2	122	178	169
Cyc 8:2	143	165	161

Note on methods:
Construction of plants expressing the transporter *cycA* gene listed in Tables 1 and 2.

The *cycA* gene was transferred to 35S::*dsdA*, 35S::*DAO1* and wild-type Arabidopsis plants, using standard DNA manipulation techniques (generally as describe in paper I and II), as follows. The *Escherichia coli* gene *cycA* was cloned by PCR using primers 5´-GGGGACAAGTTTGTACAAAAAAGCAGGCTCATGGTAGATCAGGTAAAAGTCGTT and 5´-GGGGACCACTTTGTACAAGAAAGCTGGGTGGTTATTTCCGCAGTTCAGC, the PCR fragments were recombinated into the pDONR vector, according to the manufacturer's guidelines, and subsequently recombinated into the CaMV 35S expression cassette of the binary vector pH7WG2D. The vector was analysed by sequencing and enzymatic restriction and then transferred by electroporation into Agrobacterium tumefaciens strain GV3101::pMP90 RK. Arabidopsis plants carrying the *dsdA* gene from *Escherichia coli* (Erikson *et al.* 2005), or the *DAO1* gene from *Rhodotorula gracilis* (Erikson *et al.* 2004) and wild-type plants were transformed with *Agrobacterium tumefaciens* strain GV3101::pMP90 RK through the floral dip method. T1 seeds were selected on hygromycin. All isolated lines were screened by PCR amplification of the *cycA* gene to confirm integration of the transgene.

4.1.4 D-amino acids as nitrogen sources

In plants' natural environments, D-isomers of amino acids are scarcer than L-isomers. Nevertheless, there are several sources of D-amino acids in the rhizosphere. Peptidoglycan from microorganisms is likely to be a major contributor, particularly to pools of D-alanine and D-aspartic acid, which are substantial constituents of bacterial cell-wall peptidoglycan and frequently present at levels of several mg per kg of soil (Brodowski *et al.* 2004). A likely source of D-serine in same soils is a phosphagen named lombricine that contains D-serine. It is present (uniquely) in earthworms and is a high-energy phosphate with similar function to adenosine triphosphate (ATP), (Beatty at al. 1960). Since plant amino acid transporters also transport D-isomers (Paper I), plants are likely to acquire D-amino acids released by decomposition of such compounds from the soil solution. Hill *et al.* (2011) also argue that these relatively low abundance D-isomers are metabolised by plants. Furthermore, Arabidopsis plants have been shown to snare yeast and bacteria in contact with their root surfaces by actively modifying their cell walls and engulfing adjacent microbes (Paungfoo-Lonhienne *et al.* 2010).In the cited study trapped yeast and bacterial cells were labelled with green fluorescent protein (GFP) and could be followed into root hairs and roots. Gradual losses of GFP tracer indicated that the cells disintegrated. Such heterotrophic nutrient acquisitions will inevitably expose plants to D-amino acids.

Peculiar as it may seem to develop a transgenic plant that can grow on D-amino acids as the sole nitrogen source, it answers a couple of questions initially not asked. First, the dogmatic role of inorganic nitrogen for plant nitrogen nutrition has been momentous and persistent to change, despite timeless efforts to challenge that view and to also incorporate organic nitrogen sources (Paungfoo-Lonhienne *et al.* 2012). First, the role of nitrate for Arabidopsis growth can in a way be revisited using data presented in this thesis. Under these laboratory conditions, my results show that neither nitrate nor ammonium is needed for Arabidopsis to grow and these inorganic N sources can even be substituted with a D-amino acid, provided that the metabolic pathway is there (paper I). The notion that inorganic nitrogen is not needed also holds true when some L-amino acids are used as a nitrogen source (Fig. 2, paper I). Second, the inability of Arabidopsis to grow with ammonium as a nitrogen source (Fig. 8, paper I), must involve uptake regulation. Similarly, excessive ammonium uptake is toxic and inhibits growth of barley, despite energetically wasteful ammonium effluxes countering its accumulation (Britto *et al.* 2001). Since D-serine and D-alanine are converted into

ammonium by the transgenic plants they essentially grow on metabolically-generated ammonium.

4.2 Selection of transformed plants

4.2.1 D-Serine and D-alanine inhibit plant growth

After characterizing the 35S::*DAO1* and 35S::*dsdA* transgenic lines, as reported in Paper I, an obvious objective was to test the utility of the *DAO1* and *dsdA* genes as selectable markers for transformation, which requires cultivation conditions in which the transgenic plants are clearly distinct from wild-type counterparts in the presence of appropriate substrates. When I tested different D/L-amino acids as nitrogen sources for wild-type Arabidopsis, D-serine was found to be one of the strongest growth inhibitors (Fig. 2, Paper I). Other authors have also reported that D-serine is toxic to plants of several species, including duckweed (Bollard, 1966), sunflower (Aldag and Young, 1970) and *Gossypium hirsute* (cotton) (Valdovinos and Muir, 1965). In further tests I found that D-serine was toxic to a number of other species (*Solanum lycopersicum* (tomato), barley, maize, *Populus tremula* (poplar), tobacco and Arabidopsis), although their responses varied somewhat.

D-Serine's underlying physiological and biochemical mode of action on plants is largely unknown. Papers II and III propose that D-aminoacylation of tRNA, which is normally specific for L-amino acids, and possible inhibition of L-serine synthesis, may be involved in D-serine toxicity, as shown in *Escherichia coli* (Soutrina *et al.* 1990 and Cosloy and McFall 1973). These mechanisms may be contributory factors, but no corroborating data have been published. However, D-serine has been shown to inhibit serine:glyoxalate aminotransferase (by up to 85%) in isolated *Spinacia oleracea* (spinach) leaf peroxisomes, in the characteristic manner of an allosteric inhibitor (Rehfeld and Tolbert, 1972). In the same study D-serine inhibited alanine:glyoxalate aminotransferase activity by 35%, while D-alanine inhibited serine:glyoxalate aminotransferase and alanine:glyoxalate aminotransferase activity by 16 and 23%, respectively. This could partly account for the slightly lower toxicity of D-alanine towards Arabidopsis (Fig. 1, Paper III).

The 35S::*DAO1* and 35S::*dsdA* Arabidopsis plants share several characteristic growth responses to D-amino acids. Both gene products metabolise D-amino acids and allow plants to grow on media containing otherwise toxic D-amino acids and in both cases their activities provide carbon and nitrogen sources. They can also respectively grow on D-alanine or D-serine as the sole nitrogen source (Paper I). DSDA catalyses deamination of D-

serine, yielding ammonium, pyruvate and water, while DAAO catalyses oxidative deamination of D-amino acids, yielding ammonium, a corresponding keto-acid and hydrogen peroxide. DSDA has a narrow substrate range (Fedreriuk *et al.* 1983), while DAAO recognizes a broad range of neutral and polar D-amino acids (reviewed in Pollegioni *et al.* 2007).

I chose the *Rhodotorula gracilis DAO1* gene as the candidate DAAO gene since its product is one of the most intensively studied DAAO, and almost the model flavoprotein (Umhau *et al.* 2000, Pollegoni and Molla 2011). The gene contains five introns, six exons and five regions conserved in all DAAOs. The first intron divides the consensus FAD-binding sequence, glycine-X-glycin-X-X-glycine, into two exons, while the enzyme's other conserved regions are encoded by the last exon. The last conserved region, serine-lysine-leucine, is a peroxisome-targeting signal peptide. The cDNA encodes a protein of 368 amino acids (Alonso *et al.* 1998).

Two variants of the plant cloning vector were constructed, one with the intact *DAO1* gene (cDNA) and one carrying a truncated variant, 35S::$DAO1_{\Delta 1098}$, encoding $DAAO_{\Delta 366}$ with the peroxisome-targeting signal peptide deleted. The signal peptide was eliminated by omitting the last six nucleotides when amplifying the *DAO1* cDNA by nPCR (data not shown). Both gene constructs were under control of the CaMv 35S promoter in otherwise identical constructs.

Two versions of the vector constructs were constructed to test the possibility that cytosolic expression could be favourable for detoxification of D-amino acids, and effects of the associated cytosolic hydrogen peroxide production on the plants' cells. However, Arabidopsis plants expressing either construct were resistant to D-alanine and D-serine, although the 35S::$DAO1_{\Delta 1098}$ plants were more sensitive to D-alanine and (especially) D-serine (Figs. 3 and 4). The enzymes were not localized and presumably the lower D-alanine and D-serine tolerance of the 35S::$DAO1_{\Delta 1098}$ plants is due to one or more of the following possibilities: hydrogen peroxide release in the cytosol, sub-optimal functionality of the enzyme in the cytosol, and/or impairment of its functionality by the truncation. The construct with the intact gene was selected for further studies, and the 35S::$DAO1_{\Delta 1098}$ plants were not further characterized.

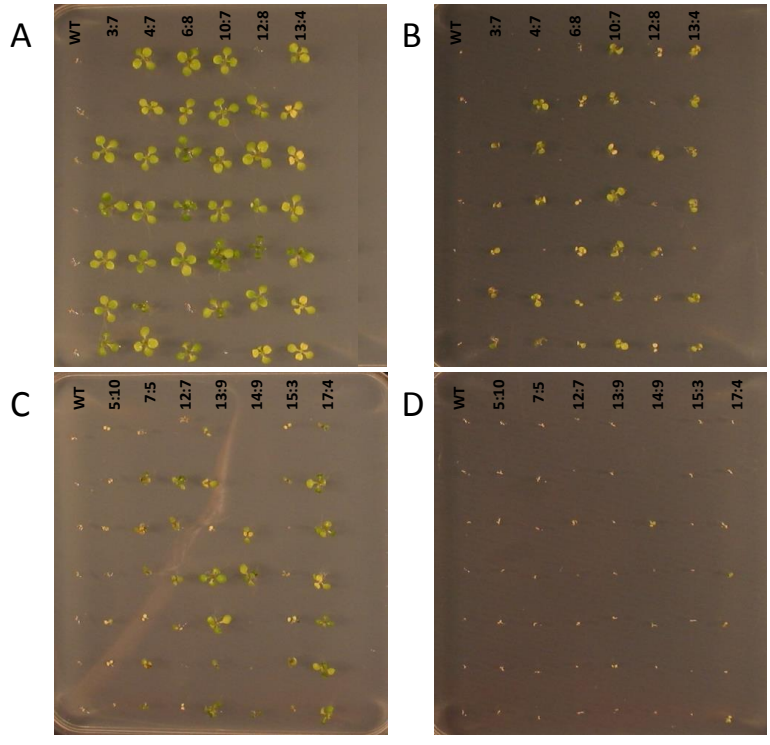

Figure 3. Upper panels: Wild-type (WT) and 35S::*DAO1* Arabidopsis seedlings grown on 3 mM (A) and 30 mM (B) D-serine. Lower panels: wild-type 35S::$DAO1_{\Delta 1098}$ Arabidopsis seedlings grown on 3 mM (C) and 30 mM (D) D-serine.

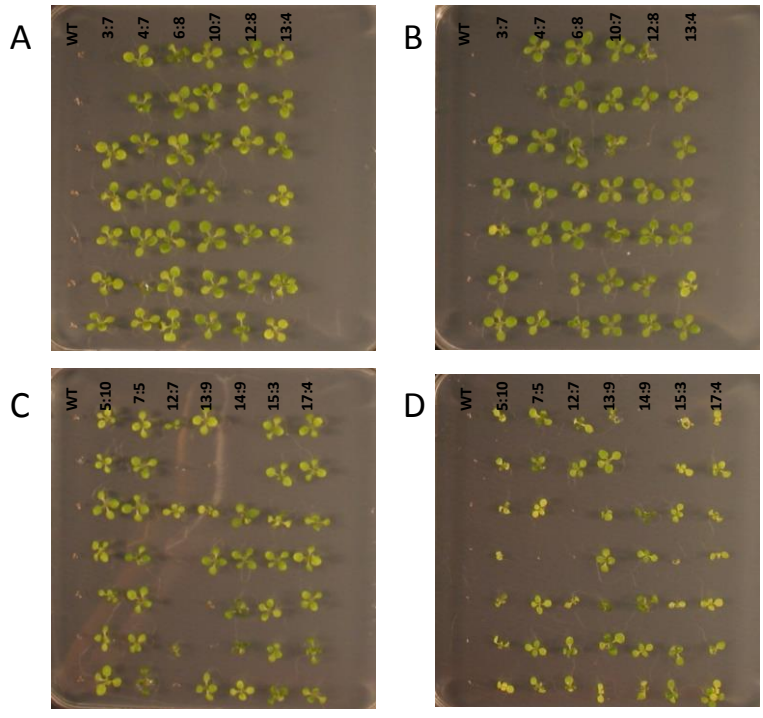

Figure 4. Upper panels: wild-type (WT)and 35S::*DAO1* Arabidopsis seedlings grown on 3 mM (A) and 30 mM (B) D-Alanine. Lower panels: wild-type and 35S::$DAO1_{\Delta 1098}$ Arabidopsis seedlings grown on 3 mM (C) and 30 mM (D) D-alanine.

4.2.2 Transformation of Arabidopsis with the *dsdA* and *DAO1* gene as selectable markers

The transformation vector used to produce the 35S::*dsdA* transgenic plants also contained the pNOS::*nptII* element, which confers resistance to kanamycin, so offspring successfully transformed with the T-DNA from the vector were resistant to both kanamycin and D-serine. Seeds from the same transformation event were split into two batches and selected on either kanamycin or D-serine. All recovered seedlings were confirmed to be transgenic by PCR and progenies were also kanamycin resistant, therefore, there were no escapes. Both selection regiems generated essentially similar transformation rate (Paper II).

An important feature for a selectable marker is generation of an unambiguous phenotype. Transformed seedlings selected on D-serine were distinguishable immediately after germination (Fig. 7), while seedlings selected on kanamycin developed a clear phenotype 5-7 days after germination. Since growth of wild-type plants arrested directly after their radicles emerged when grown on D-serine at 3-30 mM, while 35S::*dsdA* plants grew well on that

concentration, the seeds could be sown at very high densities (Fig. 6, Paper II). I also evaluated the possibility of selecting seeds in liquid culture, by germinating them in an Erlenmeyer flask with MS nutrient solution (Murashige and Skoog 1962) amended with 3 or 30 mM D-serine. As all seeds sank to the bottom and only growing seedlings became buoyant and rose to the surface it was easy to recover the transformants from large numbers of seeds. Most importantly there were no escapes.

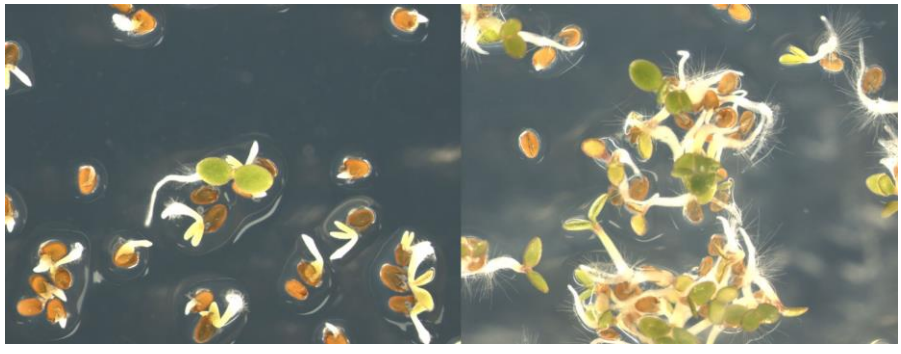

Figure 5. Photographs (taken 3 days after germination) illustrating ease of phenotypic selection for transformants carrying the *dsdA* and *nptII* genes, via growth on 3 mM D-serine (left) and 50 ug/ml kanamycin (right), respectively.'

The transformation vector used to produce the 35S::*DAO1* transgenic plants also contained the pNos::*nptII* element. Like those transformed with the *dsdA* gene, the resulting transformants were distinguishable immediately after germination when grown on D-alanine or D-serine. The phenotypes of the seedlings selected on D-alanine or D-serine were unambiguous, with no intermediate responses, as sometimes found with kanamycin selection. Furthermore, when transgenic seedlings were rescued by D-alanine/D-serine selection and transferred to soil there was no lag period with slow growth, as frequently observed with kanamycin selection (Lindsey and Gallois, 1990). The vigorous growth of the DAAO- and DSDA-expressing plants throughout the selective cultivation on D-alanine or D-serine and transfer to soil was probably due to effective degradation of the selective agent into ammonium and pyruvate (or hydroxypyruvate in the case of D-serine selection with DAAO) that contributed to the plants' nutrition.

Foliar sprays of one-week-old soil-grown 35S::*DAO1* and 35S::*dsdA* seedlings with either D-alanine or D-serine also separated transformed from untransformed seedlings, but they were less effective than spray-based selection for plants carrying genes conferring resistance to Basta or Roundup, since several applications with high concentrations were needed to eliminate escapes. Spray selection rapidly lost effectiveness as the plants grew, possibly due to the leaves' cuticles thickening or some other developmental changes.

When this project was carried out in 2001-2002 plants were generally believed to lack the pathway for oxidative deamination of D-amino acids via DAAO, which is common in other eukaryotes. However, nearly a decade later Gholizadeh and Kohnehrouz (2009) published the first report of a D-alanine-inducible DAAO gene in maize. They also predicted, from amino acid sequence alignment, putative DAAOs in rice and Arabidopsis with 88 and 62% identity, respectively. If Arabidopsis has an inducible DAAO, the spray applications mentioned above may have induced DAAO activity and hence reduced the effect of subsequent sprays.

4.2.3 mRNA and DSDA and DAAO enzyme analysis

Transcript levels and activities of the DAAO/DSDA proteins encoded by the transgenes in six independent lines with single 35S::*DAO1* and 35S::*dsdA* inserts correlated well: all lines with high mRNA levels also had high DAAO/DSDA activities. However, despite differences in mRNA levels and DAAO/DSDA activities there was no visually apparent phenotypic variation among the 35S::*DAO1* and 35S::*dsdA* lines when grown under D-alanine or D-serine selection conditions, suggesting that the DAAO/DSDA enzymes can effectively remove D-alanine and D-serine from plant tissues, even possibly without a strong promoter like CaMV 35S.

Another desired feature of a selectable marker is stringent specificity in only rescuing the transformed seedlings, and not alter any other characteristics. A clear risk when introducing an amino acid-metabolizing enzyme is that it may interfere with endogenous amino acid metabolism. However, the DSD enzyme from *Escherichia coli* is highly specific for D-serine and D-threonine, with K_m values of 0.086 and 0.083 mM, respectively, while the K_m for L-serine is more than two orders of magnitude higher, at 11 mM (Federiuk *et al.* 1983). Furthermore, I found no significant differences between pools of free L-amino acids of 35S::*dsdA* and wild-type plants, in either relative or absolute concentrations, indicating that the transformation did not interfere with endogenous L-amino acid metabolism. Nevertheless, given the recently recognised endogenous role of D-serine, further characterization is needed,

because obviously the markers' applicability would be compromised if possible interference with D- amino acid metabolism is not ruled out.

Measurements of DAAO activity showed that it was very low in wild-type controls, relative to the 35S::*DAO1* plants, but not zero (Fig. 2, Paper III). Although the plants were grown in soil and not under conditions that would intentionally induce a possible DAAO gene, some agent(s) in protein extracts from the wild-type plants acted on D-alanine that increased absorbance of the test solution in a similar manner to increases in pyruvate concentrations. An uncorroborated speculation is that the increase in absorbance was due to oxidative deamination. Similar phenomena were observed when DSDA activities were measured in the 35S::*dsdA* and wild-type plants, although a putative DAAO would generate hydroxypyruvate with D-serine as substrate (Fig. 5, Papers II). In summary, the possibility that endogenous D-amino acid metabolic activities may be present induced during certain developmental stages or by certain soil (or other) factors during cultivation under some conditions cannot be excluded.

4.2.4 Transformation of maize with the *dsdA* marker

Following the first reports of the *dsdA* gene's potential utility as a selectable marker in plant transformation, it has also been tested in maize. Lai *et al.* (2011) evaluated its suitability as a selectable marker for *Agrobacterium*-based transformation of maize embryos, and found that transformation frequencies with *dsdA* were equivalent to those with mutated acetohydroxy acid synthase, a well-established marker for maize transformation. They also found no significant phenotypic differences between wild-type and *dsdA*-expressing maize grown in the field in terms of various agronomically important traits, including seeds' protein, starch, fatty acid, fibre and phytic acid contents. In further safety assessments Lai *et al.* (2011) found that the DSDA protein did not show 35% or more identity over any 80-amino acid segment, or any sequence of eight or more consecutive identical amino acids, with any potential allergen. It is also highly digestible under simulated digestion conditions with typical protease activities of mammalian digestive tracts (in contrast to some food allergens). Furthermore, they found that the DSDA protein showed no significant homology to any toxins listed in 40 CFR part 725.421 (Code of Federal Regulations, US Food and Drug Administration). In addition, no animals died when exposed to DSDA in an acute oral mouse toxicity test, thus the median lethal dose (LD50) could not be calculated, but it must exceed 2 g DSDA/kg for male and female DC-1 mice.

4.2.5 D-Amino acid oxidase is a versatile enzyme that allows positive and negative selection with the same marker gene

When I characterized the 35S::*DAO1* Arabidopsis and wild-type plants on media containing D-isomers of the 19 proteinogenic amino acids there were two distinct types of visual responses. On media containing D-alanine (1-30 mM) and D-serine (1-3 mM) the 35S::*DAO1* plants grew well whereas growth of wild-type plants was inhibited, while on media containing D-isoleucine (5-30 mM), D-valine (10-30 mM) the 35S::*DAO1* plants were inhibited whereas wild-type plants grew relatively well (Fig. 1, Paper III).

The keto acid produced in DAAO catabolism of D-isoleucine and D-valine is the same as when L-isoleucine and L-valine is metabolised by the endogenous branched chain amino acid transaminase [EC: 2.6.1.42], that is: 3-methyl-2-oxopentanoate and 2-oxo-isopentanoate (Kyoto Encyclopedia of Genes and Genomes, KEGG). I suggest that the endogenous transaminase is specific for the L-isomer, so the corresponding D-isomer is not metabolised in wild-type plants, but only in DAAO-expressing plants. The negative effects of L-isoleucine and L-valine observed in wild-type plants resemble those of the corresponding D-form on the transgenic plants. Incubation of cell-free extracts from 35S::*DAO1* transgenic plants with D-isoleucine and D-valine resulted in production of 3-methyl-2-oxopentanoate and 2-oxo-isopentanoate, respectively. Further, addition of 3-methyl-2-oxopentanoate and 2-oxo-isopentanoate to growth media impaired growth of Arabidopsis, supporting the hypothesis that these compounds are responsible for the negative effects of D-isoleucine and D-valine on the transgenic plants. This substrate-dependent cultivation that allows selection for or against a transgene is unique to *DAO1*-expressing lines.

4.2.6 Selection with the *DAO1* gene in other species

The *DAO1* gene proved to be a functional selectable marker gene in Arabidopsis. It generates an unambiguous phenotype at a very early stage, as transformed seedlings grow more vigorously on the nutritionally selective media than counterparts on antibiotic resistance-based selection media. It is the first gene shown to function as a dual positive/negative substrate-dependent selectable marker in plants. In Paper III, I propose that in combination with marker excision techniques this gene could potentially be used to generate marker-free transgenic plants. In an elegant extension of the concept García-Almodóvar *et al.* (2014) combined the site-specific Cre-LoxP recombination system (Zuo *et al.* 2001) and positive/negative selection with the *DAO1* gene to generate marker-free tobacco plants. They developed an efficient protocol for recovering transformed lines generated from leaf disks on regeneration

medium supplemented with D-alanine (6 mM). The selection regime was stringent and allowed no escapes. Leaf disks from transgenic and wild-type plants were also tested on regeneration media for negative selection with D-valine (6 or 8 mM). Regeneration of the transgenic lines was severely reduced or abolished, while the regeneration capacity of wild-type plants remained high, under this treatment. Application of the D-valine selection treatment to tissue cultures or progeny seeds a week after induction of marker removal generated chimeric plants. Regeneration of chimeric leaf disks or progeny seed on D-valine selection medium facilitated stringent selection of only solid marker-free plants.

In parallel with my work in Arabidopsis I also attempted to introduce the *DAO1* and *dsdA* markers into poplar and tobacco. In contrast to García-Almodóvar *et al.* (2014), I never obtained stringent positive selection in tissue cultures of these species. On D-alanine or D-serine (3-6 mM), which should have prevented regeneration of wild-type leaf disks of tobacco and poplar stem segments, there were many escapes among the relatively rare true transformants. Similar, insufficiently robust, results were obtained following transformation with both the *DAO1* and *dsdA* genes (data not shown). Several subsequent transfers of shoots to selective media were necessary to eliminate escapes. However, established transgenic plants, generated through selection on kanamycin, tolerated up to 30 mM D-serine (for 35S::*dsdA* tobacco and poplar), which completely inhibited regeneration of wild-type plants.

Similar results have been reported for transformation of apple, by Hättasch *et al.* (2009). They did not obtain an escape-free protocol but confirmed that *DAO1* transgenic apple tissue, recovered via kanamycin selection, was resistant to 20 mM D-serine. They also found that negative selection with D-isoleucine could efficiently eliminate transgenic plants, but not D-valine since it had too strongly negative effects on wild-type plants. Lim *et al.* (2007) successfully generated transgenic tobacco plants using a DAAO gene from *Trigonopsis variabilis* (*TvDAO1*) and selection on 3 mM D-serine. Interestingly Lim *et al.* (2007) and García-Almodóvar *et al.* (2014) used the same cultivar (Xanthi) of tobacco, and both successfully established an escape-free protocol.

Furthermore, growth of transplastomic cells expressing a DAAO gene from *Schizosaccharomyces pombe* (*SpDAO1*) in chloroplasts was tolerant to D-alanine and inhibited by D-valine (Gisby *et al.* 2012). The cited authors suggest that *SpDAO1* could be used for controlling marker excision or retention in spontaneous recombination events between direct repeats by negative/positive selection. *SpDAO1* was not effective as a primary marker, like some markers conferring herbicide or actinonin resistance, but it can be used for maintaining unstable recombination. Foliar spraying for either positive

selection with D-alanine or negative selection with D-valine was effective for separating transplatomic from wild-type plants.

4.2.7 Can the *dsdA* and *DAO1* markers interfere with endogenous D-amino acid metabolism?

Given the information in the introduction and the views and perspectives on D-serine and its interactions with GLRs, the possible physiological effects of constitutively expressing a D-serine metabolising enzyme e.g. DSDA or DAAO in plants warrant consideration. Michard *et al.* (2011) found that *glr1.2-1* Arabidopsis mutants (lacking the receptor in the pollen tube that is activated by D-serine synthesised in the ovary) produced fewer seeds per silique than wild-type counterparts. I found no difference in total seed production between 35S::*dsdA* and wild-type Arabidopsis plants (Paper II). This is not exactly the same parameter as the one measured by Michard *et al.* (2011), but seed production is often used as a measure of general fitness, and if overexpression of DSDA directly affects pollen tube growth and guidance one would expect it to reduce fertility and seed production.

As mentioned above, Lai *et al.* (2011) found no differences in a number of agronomic traits between wild-type and DSDA-expressing maize plants. The absence of observed differences between 35S::*dsdA* and wild-type Arabidopsis and maize plants may merely reflect shortcoming of the techniques used for comparison, as it is impossible to cover all the conditions in which phenotypic differences may be manifested experimentally. An alternative possibility is that the DSDA enzyme has too low affinity to interfere with the endogenous D-serine metabolism. A third possibility is that D-serine production via SR may be spatially separated from DSDA activity in the transgenic plants. Clearly, further characterisation is needed to exclude any possible interference with endogenous D-amino acid metabolism. Nevertheless, the *DAO1* marker gene is still a valid tool for transformation if subsequent marker removal or negative selection is desired.

5 Summary of major results

Uptake and metabolic restrictions limit plant growth

Metabolic constraints limit wild-type Arabidopsis plants' ability to grow on D-amino acids, but introduction of the capacity to catalyse a single metabolic step can eliminate that constraint. Furthermore, introduction of an amino acid transporter can increase their growth on amino acids.

Novel nitrogen sources for transgenic plants

Arabidopsis plants with introduced capacity to metabolise D-amino acids can grow on D-amino acids as sole nitrogen sources. This was the first demonstration of selective cultivation using a nitrogen source only available to a transgenic plant.

Selectable markers for plant transformation

The *dsdA* and *DAO1* genes that encode D-amino acid-metabolising enzymes are functional as selectable markers for transformed plants, enabling convenient and robust screening of Arabidopsis using a selective agent that provides an additional nitrogen source for transformants. The markers have also been used in several other species.

A conditional positive/negative selectable marker

The *DAO1* gene offers potent advantages for plant transformation, uniquely allowing both negative and positive selection with the same marker gene, and (hence) convenient screening for marker-free transgenic plants after excision of the gene.

6 Views and perspectives

6.1 D-Serine influences root development

An interesting observation I repeatedly made during the growth experiments with both Arabidopsis and other plant species was that roots were more affected than shoots during cultivation on media containing D-serine. Strikingly, root growth of several plant species is hampered even at low D-serine concentrations, while shoots are only affected at much higher concentrations (Fig. 8, Paper II). Aldag and Young (1970) observed similar patterns in sunflower seedlings grown on D-serine. D-Serine has pronounced effects on Arabidopsis root development before there is any detectable difference in whole plant biomass (Fig. 1, Paper II), and the effects are even more visually apparent when plants are grown on vertical plates (Fig. 6).

Interestingly, as shown in Fig. 6, root architecture is affected even at very low D-serine concentrations (0.5-2.0 µM). In control plants, not exposed to D-serine, the angle the primary root diverges from vertical (often referred to as the slant or skew), is approximately 35°. In the presence of D-serine, the primary root grows almost straight down. Furthermore, the characteristic waving pattern of the primary and lateral roots is almost absent, resulting in a straight growth pattern. At the same D-serine concentrations, and even more strongly at 5 µM, lateral roots bend, develop a shoulder and shift aberrantly from lateral downward to vertical downward growth, and fail to proliferate outward from the primary root. Although these are only preliminary observations, D-serine clearly influences root development even at very low concentrations, like the hormone auxin (Sánchez-Parra *et al.* 2014) (Fig. 7). These observations raise two intriguing questions: What precise effects does D-serine have on root development, and by what mechanisms.

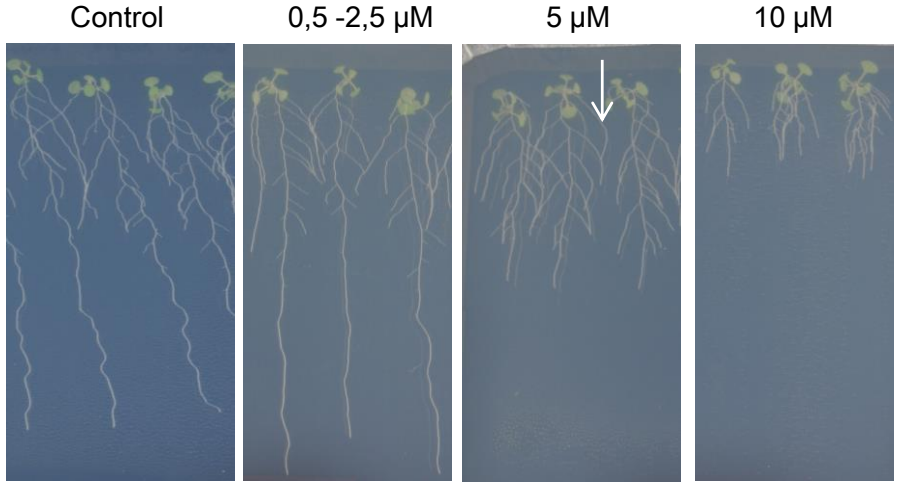

Figure 6. Wild-type Arabidopsis (15 days old) grown on vertical plates with indicated concentrations of D-serine. Concentrations in the range 0.5- 2.5 µM all induced very similar phenotypes. The arrow indicates where the lateral roots bend and shift from lateral to downward growth. Photographs courtesy of Cambui C.A).

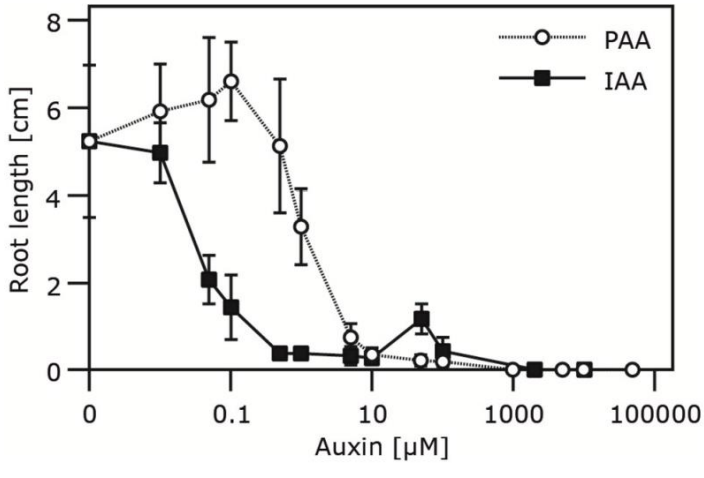

Figure 7. Root lengths of two-week-old Arabidopsis seedlings grown on medium containing indicated concentrations of the hormones indole-3-acetic acid (IAA) or phenyl-2-acetic acid (PAA): Means and standard errors of sets of at least 10 seedlings removed from petri dishes and measured. (Reproduced from Sánchez-Parra *et al.* (2014) with permission of the publisher).

6.2 Root skewing and waving

Root skewing and waving is frequently seen in Arabidopsis grown on semi-solid medium in inclined or vertical orientations. They are surface-dependent phenomena, and do not appear in roots growing embedded in agar (Olivia and Dunad, 2007). When plants are grown on vertical agar plates they actually grow along the surface, rather than within the agar. The skewing and waving processes are influenced by many factors, (*inter alia*) ecotype, nutrient salts, presence/absence of sucrose in the growth medium, type of solidifying agent, gas permeability of petri dish sealing, and ethylene concentration (Buer *et al.* 2000, 2003, Migliaccio *et al.* 2013). Skewing and waving are generally considered to be results of circumnutation, and possibly thigmotropism (response to touch) and torsion, a kind of twisting that causes cell file rotation (reviewed in Migliaccio *et al.* 2013). Circumnutation is a helical organ movement widespread among plants. Buer *et al.* (2003) present data suggesting that root waving and skewing result from two processes driven by partly separate mechanisms: Twisting and differential flank growth. Furthermore, they suggest the hypothesis that endogenous circadian rhythms are involved in root waving, especially the differential flank growth component. Gravity is not required to stimulate skewing and waving since both phenomena have been observed in roots of Arabidopsis plants grown on orbit at the International Space Station (Paul *et al.* 2012). D-Serine may speculatively affect any permutation of the processes apparently involved in root skewing and waving, e.g. circadian rhythms, circumnutation, thigmotropism, differential flank growth and/or root torsion.

6.3 Endogenous production of D-serine in plants

D-Serine is a known endogenous ligand of the NMDA type of iGluR and is involved in neuronal signal transmission in vertebrates (Mothet *et al.* 2000). In animals, a SR is responsible for D-serine production and subsequent activation of NMDA receptors (Wolsker *et al.* 1999a, Wolsker *et al.* 1999b). Plants possess iGluR-like receptors, referred to as GLRs (Lam *et al.* 1998), and a genealogical analysis of iGluRs, including animal NMDA type receptors and plant GLRs, by Chi *et al.* (1999) suggests that the receptors' evolution predates the divergence of plants and animals. However, they found no similar receptor among sequenced unicellular organisms, suggesting that these receptors involved in cell-cell signalling are unique to multicellular organisms and have a common ancestry. Other authors argue that the receptors may date all the way back to the last common universal ancestor (Price *et al.* 2012).

Fujitani *et al.* (2006) detected a homolog to mammalian SR in Arabidopsis, which specifically catalyses production of D-serine from L-serine. SR has also been described in barley and *O. sativa* (Fujitani *et al.* 2007). Sugimoto *et al.* (2009) showed that this racemase is expressed in developing cells at sites such as tip regions of primary and lateral roots, developing leaves and meristems of Arabidopsis. They also conclude, based on microarray data from GENEVESTIGATOR, that SR expression is not significantly influenced by light irradiation or quality, anatomical or developmental stage, plant hormones, or environmental factors such as cold, drought, heat, osmotic, oxidative, or salt stresses.

6.4 Are GLRs involved in processes that result in root skewing and waving?

Michard *et al.* (2011) showed that D-serine production via SR in pistils and (particularly) the ovule affects pollen tube morphogenesis in Arabidopsis. GLR knock-out (*Atglr*1.2-1 and *Atglr*3.7-1) pollen tubes grew abnormally and slowly, with deformations, in wild-type pistils (Fig. 8). Similarly, wild-type pollen tubes grown in SR knock-out (Atsr1-1) pistils were deformed and branched. The cited authors concluded that GLR activity participates in Ca^{2+} signalling in pollen tubes by controlling cytosolic Ca^{2+} influxes (and hence cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_{cyt}$), and that D-serine is the most active agonist of GLR activity in both Arabidopsis and tobacco pollen tubes. In addition to changes in Ca^{2+} flux intensity, D-serine reproducibly triggered strong sustained oscillations in $[Ca^{2+}]_{cyt}$. This is intriguing as circadian oscillations in $[Ca^{2+}]_{cyt}$ are thought to control many calcium-dependent enzymes and processes involved in circadian outputs (Johnson *et al.* 1995).

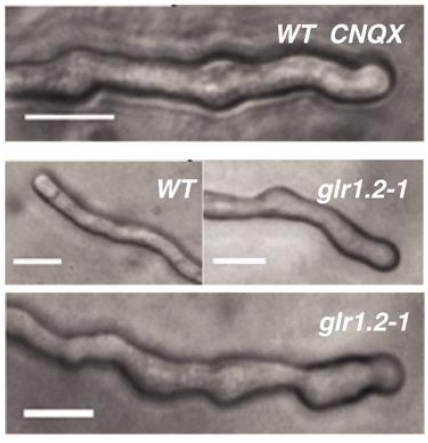

Figure 8. Arabidopsis wild-type (WT) pollen tube grown in the presence of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 172 µM) (upper panel). Wild-type and *Atglr*1.2-1 Arabidopsis pollen tubes grown in control conditions (lower panels). From Michard *et al.* 2011, reproduced with the permission of the publisher.

Other *GLR*s have been associated with functions in roots. *GLR3*.3 is involved in root tip bending in gravitropism-related responses (Miller *et al.* 2010). Interestingly, several amino acids stimulate Ca^{2+} influxes mediated by *GLR*3.3 (including L-glutamic acid, glycine, L-serine , L-alanine, L-asparagine and L-Cys), but not D-serine, according to Nicholas *et al.* (2006). Two other *GLR*s (*GLR*3.2 and *GLR*3.4) are involved in control of lateral root initiation in Arabidopsis, as knock-out plants have increased numbers of lateral root primordia with aberrant distribution patterns (Vincill *et al.* 2013). Photographs published in the same article show an absence of skewing and reduced waving in *Atglr*3.4 (and *Atglr*3.4 and *Atglr*3.2 double mutant) plants grown on vertical plates, in contrast to wild-type and *Atglr*3.2 plants (Fig. 9). These differences are not highlighted in the text; therefore it is a risk that the phenotypes in the photographs are not representative for the particular mutants when it comes to waving and skewing. The cited authors present Förster resonance energy transfer (FRET) data supporting interactions between *GLR*3.4 and 3.2, which complicates interpretation of the results. However, the inference that *GLR*3.3 and 3.4 interact is contradicted by other FRET studies (Price *et al.* 2013). Relevant observations in this context are that *GLR*3.4 expression is sensitive to touch (Mayerhoff *et al.* 2005), and cytoplasmic free Ca^{2+} levels in Arabidopsis roots change in response to touch (Legue *et al.* 1997). Root tip touch sensing and thigmotropism are proposed mechanisms underlying root skewing and

waving (Migliaccio *et al.* 2013). Thus, *GLR*3.4 may be involved in touch responses that result in root skewing and waving.

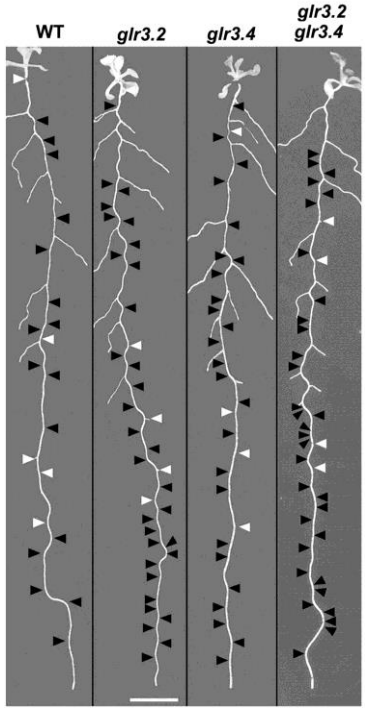

Figure 9. Spatial distributions of lateral root primordia and emerged lateral roots in wild-type (WT) and *GLR* mutant plants. Black arrowheads mark positions of primordia detected by microscopic inspection and white arrowheads indicate emerged roots not visible in the image. Note the degree of skewing of the wild-type and *glr3.4* mutant (from Vincilli *et al.* 2013, the material is copyrighted by the American Society of Plant Biologists and is reprinted with permission

Lam *et al.* (1998) grew Arabidopsis seedlings in the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX), a known antagonist of animal iGluRs, to investigate the possible in vivo function of putative GLRs in plants. They found that DNQX partly blocked the ability of light to inhibit hypocotyl elongation and induce chlorophyll synthesis. An effect of DNQX that was not reported in the cited paper, but is clearly visible in presented photographs, is that it increased root waving of seedlings compared to controls.

6.5 Can exogenous D-serine disrupt roots' ability to sense touch?

A plausible mechanism whereby D-serine in the growth media may reduce root waving and skewing is that it may act as a GLR agonist and disrupt normal Ca^{2+}-mediated touch responses. If exogenously administered D-serine activates GLRs in membranes in the root tip, and depolarises the membrane, any endogenous signalling mediated by D-serine may be outweighed. Normally root tips probe surfaces, a process enabling the detection of suitable routes to circumnavigate obstacles in their growth paths. However, when actually growing on a vertical agar surface, and not embedded in the substrate, that probing motion, together with other processes like circadian rhythms and torsion (which results in cell file rotation), is believed to contribute to skewing and waving. Thus, when deprived of the ability to respond to physical touch roots grow with less waving and skewing. Presumably gravity and light predominantly guide the direction of root growth, through gravitropism and negative phototropism, respectively, in the absence of the proprioceptive sense of touch. Nevertheless, the possibility cannot be excluded that the absence of root skewing and waving patterns in plants grown with D-serine may be due to interference with some of the other mechanisms proposed to underlie root waving and skewing, e.g. circadian rhythms, circumnutation, root torsion or differential growth. An alternative explanation to the possibility that D-serine disrupts touch responses (thigmotropism), is that it may perturb differential growth in the elongation zone.

6.6 Roots can grow differentially in presence of exogenous D-serine, but not respond to touch

To further probe processes that may be disrupted by D-serine, I performed a number of pilot experiments. The experiments are not fully replicated studies and should not be regarded as such, hence the results should be considered cautiously. To test if D-serine inhibits differential growth in the elongation zone, I grew Arabidopsis plants with 0.5 and 2.5 µM D-serine in vertically oriented growth media for a period and then rotated the plates through 90 degrees. After rotation, root growth responded to the new gravitational vector, turned through 90 degrees, and continued to grow towards the new centre of gravity (Fig. 10). These findings indicate that the plants can grow differentially in the elongation zone even with 0.5 or 2.5 µM D-serine in their growth medium.

Figure 10. Wild-type plant grown in the presence of 0.5 µM D-serine on a vertical plate that was rotated 90 degrees counter-clockwise mid-growth. After rotation, roots quickly adjusted their direction of growth towards the new gravitational vector.

Normally coordinated touch and gravitropic responses modulate growth of primary roots. Touch stimulation, by an obstacle, causes step-like growth with bends forming in the central and distal elongation zone that cause the main root axis to grow parallel to (but not touching) the obstacle, while the root cap is in touch with the barrier (Massa and Gilroy 2003). To test how roots behave upon contact with an impenetrable obstacle with and without D-serine in the media, a glass barrier was placed in their growth-paths. Preliminary results show that Arabidopsis roots exposed to D-serine behave aberrantly upon contact with an impenetrable obstacle. The normal responses with bending in the distal elongation zone upon touching a barrier and growth parallel to the obstacle are disrupted, and the root tip grows as if it does not perceive or respond to touch, resulting in uncoordinated growth (Fig. 11).

a) b)

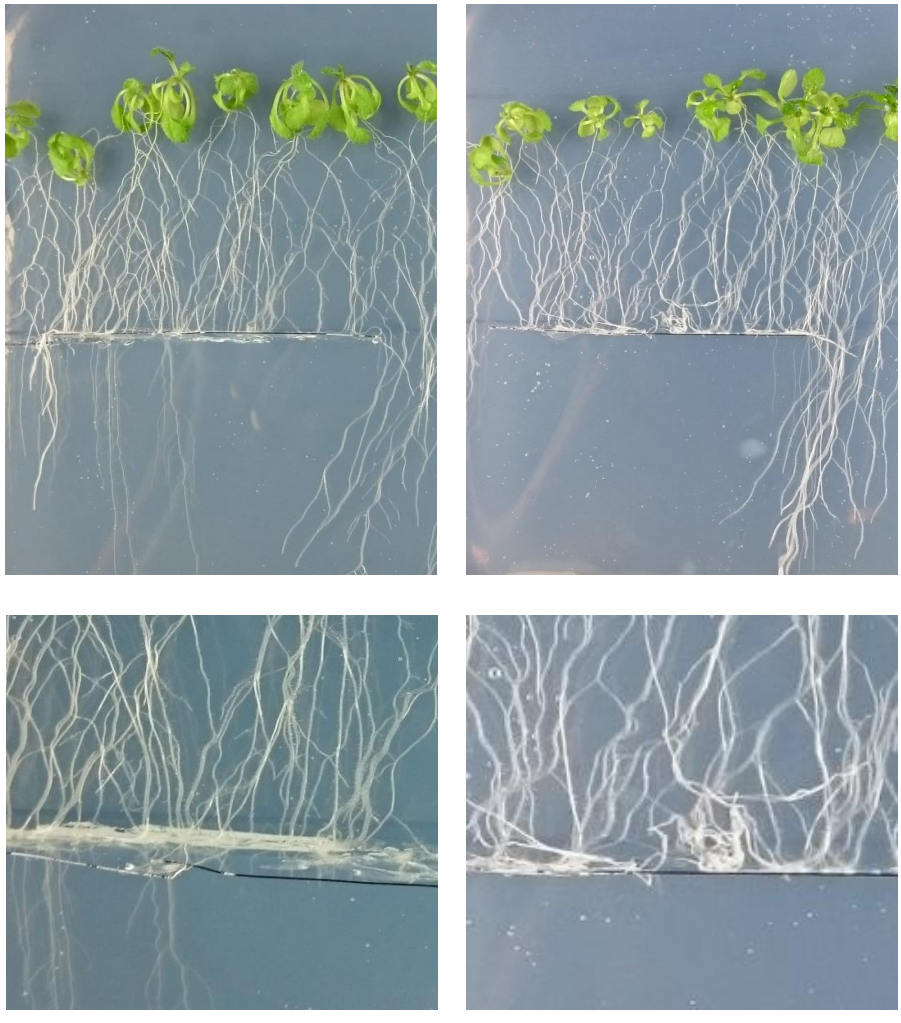

Figure 11. Upper panels: Wild-type Arabidopsis seedlings grown on vertical plates without (a) or with (b) D-serine (2 µM) in their growth media, showing their roots' responses to a glass barrier. Lower panels: Close ups of the roots. Plants were grown on ½-strength MS, solidified by 1.0 % agar (with pH buffered by 0.9 % MES to 5.8) in vertical position for approximately 20 days. Photographs courtesy of Holmlund M.

6.7 Defects in vesicular transport reduce root skewing and waving

There are also mutants that produce roots that do not skew or wave, e.g. mutants carrying loss of function alleles of Arabidopsis *ROOT HAIR DEFECTIVE3* (*RHD3*), which putatively grow without skewing due to defects in trafficking of cell wall- or plasma membrane-associated determinants of anisotropic cell expansion (Yuen *et al.* 2005) (Fig. 12).The same gene is also implicated in the control of vesicle trafficking from the endoplasmatic reticulum (ER) and Golgi compartment (Galway *et al.* 1997 and Zheng *et al.* 2004).

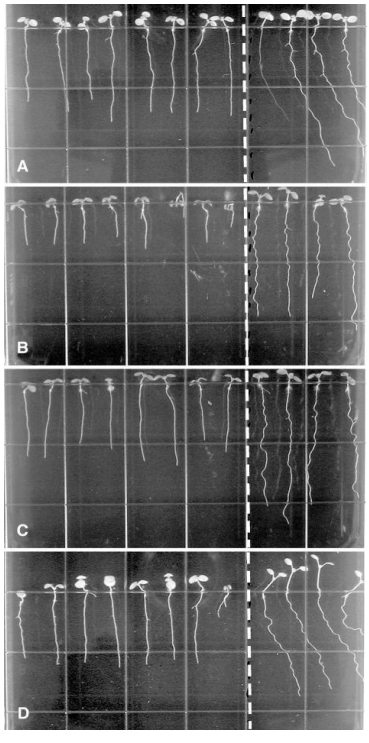

Figure12. Root-waving phenotypes of wave dampened6 (*wvd6)* and *rhd3* Arabidopsis seedlings. A, *wvd6* (Ws ecotype); B, *rhd3*-1 (Col ecotype); C, *rhd3-2* (Col ecotype); and D, *rhd3-3* (No-0 ecotype) seedlings, plated left of the dotted line, with wild-type seedlings of the corresponding ecotypes plated on the right. Seedlings were grown for 8 to 9 d on 1.5% (w/v) agar-solidified medium, tilted backward 30° from the vertical. Images were taken from the bottom of the plates through the medium. The *wvd6* mutant is a result of translocation of the locus harbouring the *rhd3* gene. (from Yuen *et al.* 2005, the material is copyrighted by the American Society of Plant Biologists and is reprinted with permission).

In animal astrocytes (glial cells of a type that closely interact and regulate functions of synapses) vesicles facilitate the transport and release of D-serine from the cellular interior to the synaptic cleft via exocytosis. Specific vesicular transporters enable D-serine loading. Furthermore, SR is also anchored on the surface of these vesicles (reviewed in Martineau *et al.* 2014) (Fig. 13).

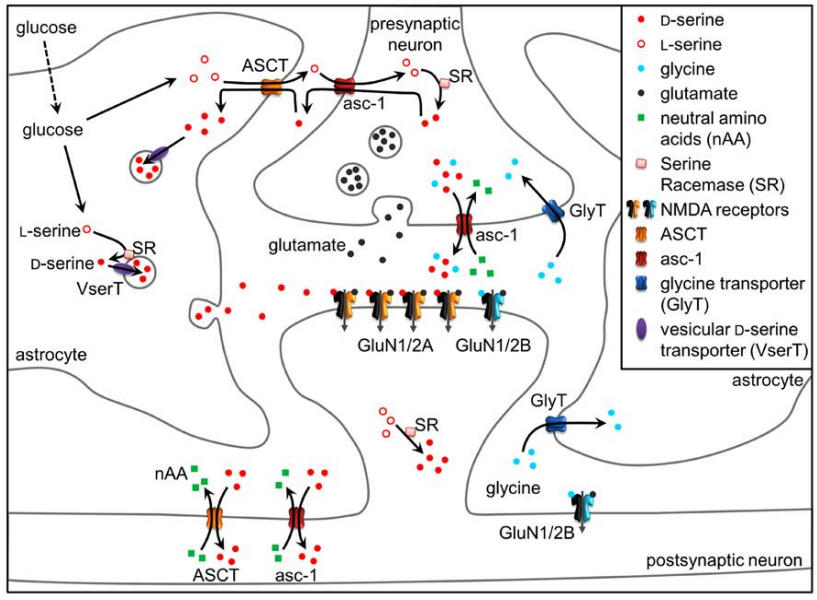

Figure 13. D-Serine at the tripartite synapse. L-serine produced in astrocytes shuttles to neurons through alanine–serine–cysteine transporter 1 (asc-1) and fuels the synthesis of D-serine by SR. D-Serine in turn shuttles from neurons to astrocytes, where it accumulates in glial vesicles. Neuronal D-serine release may occur following depolarisation through asc-1, while astroglial D-serine release may occur through exocytosis following the activation of receptors at the astrocyte plasma membrane. Once in the synaptic cleft, D-serine binds to synaptic NMDA-containing GluN2A subunits. Conversely, extra-synaptic receptors containing GluN2B preferentially bind glycine, diffusion of which towards the cleft is prevented by GlyT1 transporters. Glycine released by neurons through asc-1 could, however, activate synaptic NMDAs to a lesser degree than D-serine. D-Serine is finally removed from the synaptic cleft via ASCT and asc-1 transporters. Reproduced from Martineau *et al.* 2014, with the kind permission from the publisher.

A speculative but tempting parallel, based on circumstantial evidence, can be drawn between vesicle transport of D-serine in animal astrocytes and the absence of skewing and waving of Arabidopsis *rhd3* mutant roots, which have vesicle trafficking defects. If D-serine in plant cells is also transported in vesicles and delivered to the extracellular space by exocytosis, and it is important for roots' touch responses, *rhd3* plants may grow without root skewing and waving due to defects in vesicle transport of D-serine, rather than solely defects in trafficking of cell wall- or plasma membrane-associated determinants of anisotropic cell expansion as proposed by Yuen *et al.* (2005).

Exocytosis of L-glutamate has been proposed in tobacco in response to Cryptogein. Cryptogein is a protein secreted by the pathogenic oomycete *Phytophthora Cryptogea*, and is part of an pathogen-associated molecular pattern (so called PAMP). The L-glutamic acid delivered, as a response to Cryptogein, to the extracellular space trigger GLR mediated Ca^{+2} fluxes across the plasma membrane. Brafeldin A (BFA), which is a lactone antibiotic produced by fungal organisms, depolymerise actin filaments and inhibit vesicle trafficking. BFA inhibits the Cryptogein induced L-glutamic acid release, therefore exocytosis is the expected delivery mechanism, as shown by Vatsa *et al.* (2011). Similar experiment with BFA, in an expected D-serine secretion event, should indicate whether or not D-serine is also transported in vesicles. To further elaborate on the similarities between neurones and plant cells; L-glutamic acid is also delivered by exocytosis by the presynaptic neuron (Maritineau *et al.* 2014).

6.8 Involvment of D-serine in tip growth

In addition to reduced skewing and waving, *rhd3* Arabidopsis mutants also develop abnormal short and wavy root hairs (Galway 1997) (Fig. 14). The defects resemble in some ways the short and wavy (or dented) deformations of GLR knock-out (*Atglr*1.2-1 and *Atglr*3.7-1) pollen tubes grown in wild-type pistils (Fig. 8). Similar effects are also observed when wild-type pollen tubes are grown in SR knock-out pistils (Michard *et al.* 2011).

Root hairs, and pollen tubes, are examples of tip-growing cells (Pierson *et al.* 1996, Schiefelbein *et al.* 1992). Tip growth is a process allowing growth to be focused at a particular point of the cell membrane, which mediates directional growth. Several cellular processes are involved, including modification of the microtubules, microfilaments, ER and Golgi subcellular arrangement towards the tip-growing region, but leaving the tip-most zone clear and devoid of larger organelles (reviewed in Hepler *et al.* 2001 and

Konrad *et al.* 2011). Normally vesicular transport from ER and Golgi stacks is concentrated in the tip region of growing root hairs and pollen tubes, but in root hairs of *rhd3* mutants the number of vesicles is reduced at the very tip (Galway *et al.* 1997).

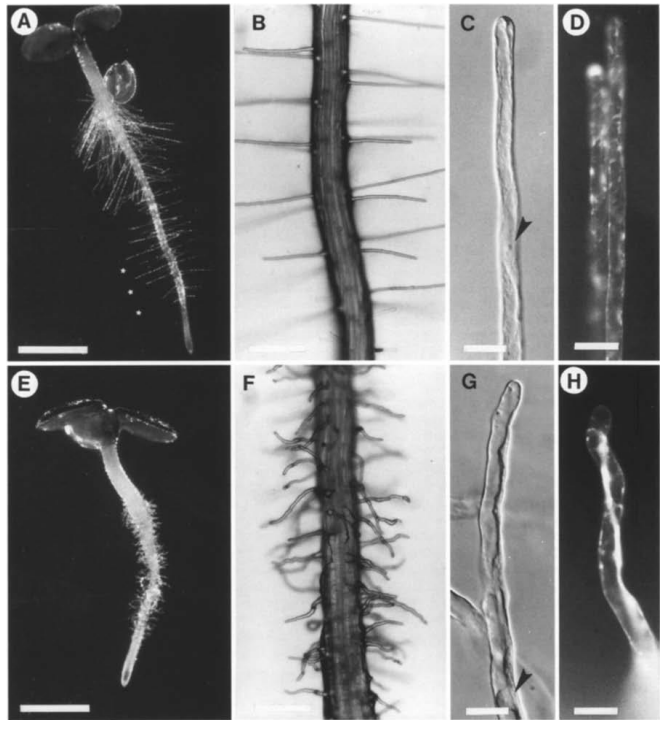

Figure 14. Root hairs of Arabidopsis thaliana. A-D wild-type (Columbia); E-H *rhd3* mutant. A, E Five-day-old seedlings, with the *rhd3* seedlings (E) bearing shorter root hairs compared to the wild-type (A). The file of asterisks in A indicates the zone of growing and elongating hairs. x 8; bars = 2 mm. B, F Root hairs along a wild-type (B) and *rhd3* (F) root; note the short and wavy *rhd3* hairs, x 65; bars = 200 gm. C, G Mature root hairs of wild-type (C) and *rhd3* (G) plants visualized by differential interference contrast optics. Nuclei (arrowheads) are visible in both hairs, x 500; bars = 20 µm. D, H Fluorescein diacetate stains the cortical cytoplasm in a mature wild-type hair (D), and a large, axially-oriented cytoplasmic strand in a mature *rhd3* mutant hair (H). x 500; bars = 20 µm (from Galway *et al.* 1997, reproduced with the kind permission of the publisher).

In attempts to understand the mechanism responsible for the deformation of *rhd3* mutants' root hairs, the similarities in Ca^{2+} fluxes and gradients at the membrane of the growing tips of pollen tubes and root hairs may provide

useful clues. Steep Ca^{2+} gradients outside pollen tubes and root hair tips are significant features for tip growth (Pierson *et al.* 1996, Schiefelbein *et al.* 1992). These gradients are to some extent due to consumption of Ca^{2+} in cell wall synthesis (in demethoxylation catalysed by pectin methyl esterase), and partly to influxes of Ca^{2+} across the plasma membrane (Hepler *et al.* 2011). Since D-serine has been shown to be a strong activator of ligand-gated Ca^{2+} influxes in pollen tubes and vital for normal pollen tube development (Michard *et al.* 2011) the deformations of root hairs in *rhd3* mutants may be related to defects in the suggested vesicular transport of D-serine. Therefore, if D-serine is important for generation of the Ca^{2+} gradients at root hairs' tips too, a defect in the proposed vesicle transport of D-serine could explain why reductions in vesicle densities at the tip generate deformed root hairs. In such cases, adequate Ca^{2+} fluxes across the membrane at root hairs' tips would not be initiated due to D-serine deficiency.

Interestingly, a mutation in a homologous gene to *RHD3* in humans (*Atlastin*) is associated with a neurodegenerative disorder called hereditary spastic paraplegia (HSP). In HSP, axons of the longest neurons that extend from the brain along the spinal cord down to the legs degenerate and impair leg control. Both Atlastin and *RHD3* are implicated in membrane tabulation in Golgi development and vesiculation (Baluška 2010).

6.9 A possible plant synapse, neurotransmission, and a cognitive unit in the root apex

Recently, similarities between plant cells and neurons have been proposed (Baluška 2010) that have possible implications for the role of D-serine in plants. Tip-growing plant cells, like root hairs and (especially) pollen tubes, share similarities with neurons extending axons (Ravishankar and Daphne 2000, Lev-Yadum 2001). The intrusive directed growth of axons is guided by attractive and repulsive cues provided by netrins and semaphorins, respectively, towards their target (Mueller 1999), where a synaptic connection is formed. In plants, growth of pollen tubes is guided by ɣ-amino butyric acid (Ravishankar *et al.* 2003) and D-serine (Michard *et al.* 2011) in their intrusive growth towards the ovary. Further, in neurons, most Golgi apparatus and microtubules are motile organelles that are independent of the perinuclear centrosome and facilitate transport of Golgi outposts towards the neuronal synapse. Plant cells lack centrosomes, and form motile Golgi stacks and trans-Golgi networks (TGN) extending throughout the whole cell. In plant cells, like neurons, TGN are independent organelles that are not connected to the Golgi apparatus and participate in endosomal and vesicular transport (Baluška *et al.*

2004). Adhesion between plant cells, with polar actin- and myosin-enriched domains, has been proposed to function like synapses in plants (Baluška *et al.* 2005).

In root tips, above the apical meristem but below the elongation zone, is a distinct section called the oscillatory transition zone, in which cells have very high rates of vesicle recycling (surpassed only by tip-growing root hairs and pollen tube cells). Further, polar vesicular transport of auxin to the plant cell synapse domain is proposed to act like a neurotransmitter (Baluška *et al.* 2005). I propose a similar function of D-serine, although proof of vesicular transport remains to be seen.

The transition zone has been proposed to harbour cells responsible for computational abilities (reviewed in Baluška and Mancuso 2013). Demand for oxygen is higher in that zone than in any other sites in plants, although the cells do not grow or divide. Electric currents also peak there and (uniquely) synchronously oscillate in the transition zone. Thus, the authors of the cited review argue that cells in the zone have unique properties and resemble neurons in some ways. Interestingly, SR is also highly expressed in that zone, whereas putative DAAO and alanine racemase are expressed more strongly further back in the root (Fig. 2). The three D-amino acid-related genes are distinctly expressed during embryo and seed development (Figs. 15 and 16), suggesting that D-amino acids may be involved in these processes.

Some kinds of plants' behaviour patterns have been described as cognitive or intelligent, extending beyond reactive responses (Trewavas 2005, Calvo-Garzón 2007). Furthermore, there is an emerging field of plant neuroscience, that (*inter alia*) seeks to unify neuroscience of plants and animals (Brenner *et al.* 2006), although the claims are disputed (Alpi *et al.* 2007, Brenner 2007, Trewavas 2007). However, perhaps, in this context D-serine should be viewed as a possible plant neurotransmitter, analogous to its roles in animals.

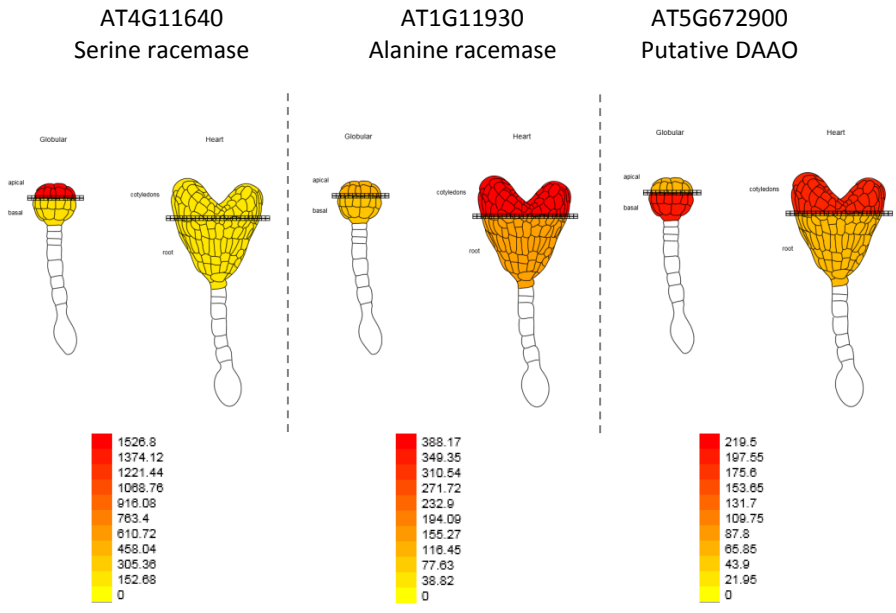

Figure 15. Expression patterns of serine racemase, alanine racemase and putative *DAAO* genes in Arabidopsis embryos. Note the different scales (image generated from Arabidopsis eFP Browser at bar.utoronto.ca, Winter *et al*. 2007)

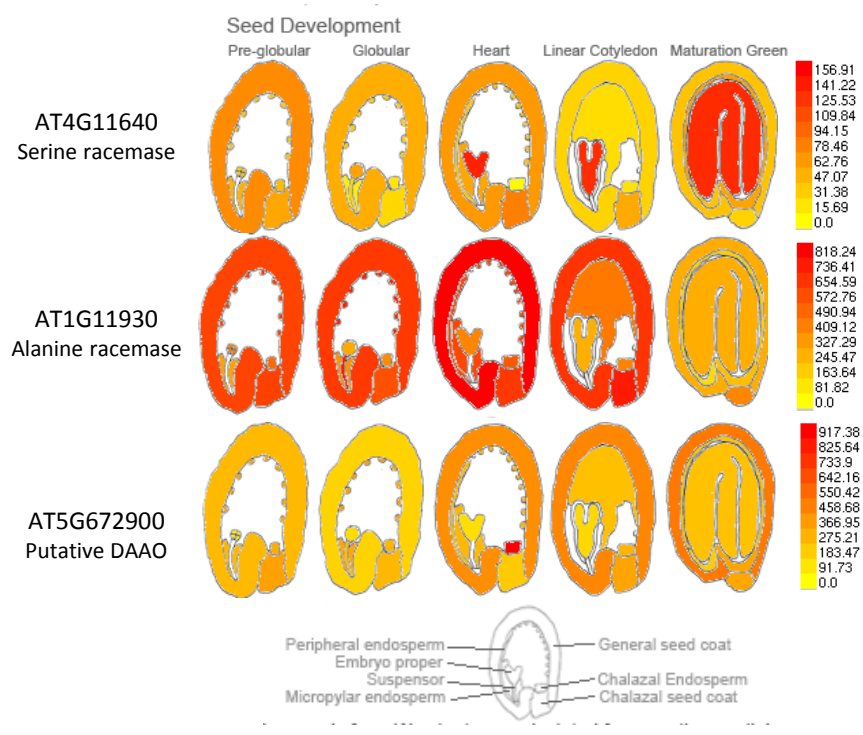

Figure 16. Expression patterns of serine racemase, alanine racemase and putative *DAAO* genes during Arabidopsis seed development. Note the different scales (image generated from Arabidopsis eFP Browser at bar.utoronto.ca, Winter *et al*. 2007)

7 Concluding remarks

7.1 Selective nitrogen nutrition and its exploitation

The concept of selective nitrogen nutrition presented in this thesis is based on introduction of the capacity to catalyse a single metabolic step, which enables conversion of otherwise toxic D-amino acids to ammonium, which can sustain plant growth. It describes (*inter alia*) the first demonstration of the possibility of making an inaccessible nitrogen source available for plant growth, in a selective manner. The approach has clear applicability for selecting transgenic plants in the laboratory. It also has potential utility in field-scale selective nutrition of arable plants, which will (probably) never be realised due to ethical or ecological concerns, although the concept itself holds that potential.

Selection with D-amino acids instead of antibiotics is an attractive prospect. The use of antibiotic, or herbicide, resistance genes in production of genetically modified crops results in vast numbers of gene copies in the environment. The potential risks for horizontal gene transfer has lengthy been debated and the bottom line seems that the risk is low (Keese 2008). However, marker excision techniques circumvent the whole issue. There are also numerous alternatives to antibiotic resistance markers nowadays (Rosellini 2012). However, selecting transformants of recalcitrant species can be a daunting task and the choice of marker can make the difference between success and failure. The presented studies provided an additional two markers to a growing toolbox. Due to the endogenous roles of D-serine in plants, their use in biotechnical applications has uncertain ramifications. Hence, they should be probably be removed after transformation, especially if they are used with commercial crops, in which case a dual function marker like *DAO1* may be an attractive option.

7.2 Patient zero slips out of vegetative state

The presented experiments and reviewed literature renders at best a working hypothesis of D-serine´s role in plants. Simply disrupting a process with an exogenously supplied compound does not prove that the compound has an endogenous role. However, since I have freely speculated about possible roles of D-serine in plants, I may as well go the whole nine yards. A quotation frequently used in articles on plant neuroscience and plant cognition is from Charles and Francis Darwin´s book *The power of movements in plants*, "The course pursued by the radicle in penetrating the ground must be determined by the tip; hence it has acquired such diverse kinds of sensitiveness. It is hardly an exaggeration to say that the tip of the radicle thus endowed, and having the power of directing the movements of the adjoining parts, acts like the brain of one of the lower animals; the brain being seated within the anterior end of the body, receiving impressions from the sense-organs, and directing the several movements".

The discovery of GLR receptors in plants and the fact that some plant GLRs are regulated by D-serine, which is an important regulator of neurotransmission in animals, is tremendous. These ancient receptors, which may predate the plant-animal divergence, are involved in cell-cell communication involving D-serine as a signal transmitter, as shown by their participation in pollen tube-ovary interactions (Michard *et al.* (2011). Perhaps D-serine as a signal transmitter also predates the animal plant divergence. The suggested similarities between neurons and plant cells, especially in the root transition zone, are arguably in line with Darwin's proposal of a root brain.

The discovery of a DAAO in plants has received little attention, in terms of citations, and its role in regulating D-serine is unknown. A tempting parallel with animal neurons, where D-serine concentrations is maintained via production mediated by SR and catabolism mediated by DAAO (Verrall *et al.* 2007), emerges as both of these enzymes have now been found in plants. Of course, more research is required to validate any of the hypothetical roles of D-serine. Nevertheless, the hypothesis that D-serine may be an endogenous signal molecule that relays proprioception information generated from touch from one cell to another can be tested. Root traits, especially responses to touch or other stimuli, in *GLR* and *SR* knock-out plants should provide valuable information in such tests.

I am not predicting the discovery of a hidden brain at the tip of the root, but the recent recognition of features like synapses in plants and the special qualities of cells in the transition zone indicate that plants should be viewed as organisms that can compute information and relay responses that goes further

than simple reflective response. Perhaps, root tips can be regarded as parts of a cluster of connected decentralised cognitive units, as proposed by Calov-Garzon (2007). Most importantly, if we are to pursue cognition in plants we need to recognise what it is, to be able to find it.

In medicine a vegetative state refers to the loss of cognition and perception, leaving a patient with no ability to react to external stimuli. For such patients, any movement or response is purely spontaneous, unpredictable and unconnected to events outside of physical impulse (Brainandspine.org). Simply, plants are far from being in a vegetative state and perhaps D-serine will prove to be a neurotransmitter acting at plant synapses that mediate perception and relay touch sensation.

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