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A genomic approach for studying the biological activity of humic substances

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

February 2nd, 2009 Sara Trevisan

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Riassunto

Le Sostanze Umiche (SU) costituiscono la parte di materia organica maggiormente diffusa in natura. E' stato ampiamente dimostrato come le sostanze umiche, agendo sulla struttura e fertilità del suolo, sono in grado di manifestare numerosi effeti postitivi sulla fisiologia della pianta, modificandone l'architettura dell'apparato radicale e conseguentemente influenzando l'assorbimento dei nutrienti.

Nonostante numerosi studi ne abbiano evidenziato una forte attività biologica, i meccanismi biochimici e molecolari che regolano la risposta della pianta alle SU sono solo parzialmente conosciuti.

La presenza di auxina, accertata in frazioni umiche diverse, supporta l'ipotesi avanzata da diversi autori che le SU possano agire sulla pianta attraverso una via auxino-simile. Tuttavia questo non basta a chiarire il complesso meccanismo dell'interazione SU-pianta.

La prima parte di questa tesi ha avuto come obiettivo quello di verificare l'attività auxinica esercitata dalle SU nella pianta modello *Arabidopsis thaliana* attraverso l'osservazione dello sviluppo delle radici laterali.

I risultati ottenuti grazie anche all'utilizzo di specifici inibitori del trasporto o dell'azione auxinica hanno dimostrato come le SU inducano la formazione di primordi radicali principalmente attraverso una attività auxinica.

Inoltre le SU usate in questo studio hanno sia attivato l'espressione del costrutto DR5::GUS che stimolato la trascrizione di un *early auxin responsive gene*, *IAA19*, con una *time-course* non diversa rispetto a quella osservata in risposta all'auxina. I risultati ottenuti, comunque, non escludono la presenza di fattori addizionali diversi dall'auxina, i quali potrebbero contribuire all'attività biologica riscontrata in presenza di SU.

Nella seconda parte di questa tesi lo studio si è concentrato sui meccanismi molecolari che regolano la risposta della pianta alle SU. Per capire quali geni siano coinvolti in questo processo è stato scelto un approccio trascrittomico ad ampio spettro, in grado di individuare una vasta gamma di trascritti differenzialmente espressi in *Arabidopsis thaliana* in seguito a trattamenti con SU.

La tecnica del cDNA-AFLP (cDNA-amplification fragment length polymorphism) ha permesso di individuare più di 100 geni, i quali sono stati in seguito suddivisi in categorie

funzionali secondo la loro annotazione di *gene ontology*.

Questo studio ha permesso di ottenere il primo ampio profilo trascrizionale in risposta alle sostanze umiche, iniziando a chiarire alcuni degli eventi chiave nell'interazione SU-pianta e evidenziando il ruolo regolativo dell'auxina sin dalle prime fasi di questo processo.

Summary

Humic substances (HS) represent the organic material mainly widespread in nature. HS have positive effects on plant physiology by improving soil structure and fertility and by influencing nutrient uptake and root architecture. The biochemical and molecular mechanisms underlying these events are only partially known. HS have been shown to contain auxin and an "auxin-like" activity of humic substances has been proposed, but support to this hypothesis is fragmentary. In the first part of this thesis the "auxin activity" of HS has been investigated in detail by studying the induction of lateral root development in *Arabidopsis thaliana* with a combination of genetic and molecular evidence. The data obtained, also by using specific inhibitors of auxin transport or action, showed that HS induce lateral root formation mostly through their "auxin activity". These findings were further supported by the fact that the humic substances used in this study activated the auxin synthetic reporter DR5::GUS and enhanced the transcription of the early auxin responsive gene *IAA19* with a time course not different from that of auxin. The presence of additional factors, different from auxin and responsible for part of the measured HS biological activity, was also shown and discussed.

Furthermore in the second part a comprehensive transcriptomic study was used in the model plant *Arabidopsis thaliana* to investigate the global molecular regulation that plant acts in response to HS, providing a broad overview of differentially expressed transcripts involved in this process.

The cDNA-AFLP (amplification fragment length polymorphism) technique was used to identify more than 100 genes, which were sequenced and sorted into functional categories according to their gene ontology annotation.

This study provides the first wide transcriptional profile for the comprehension of key events of the HS-plant interaction and highlights the regulative role for auxin from the early phase of this process.

Chapter 1 - General Introduction

Towards a sustainable agriculture

Agriculture is essential to everybody, everyday, everywhere. It provides us with food and biomaterial, rural employment and even renewable energy. It plays an important role in maintaining the rural landscape and semi-natural habitats. However, it is not clear which are greater, either the successes of modern high-intensity agriculture or its shortcomings.

A deep interest towards agriculture and sustainable agriculture has been coming out in recent time. Not only farmers and soil scientists, but also economists and politicians are redirecting their attention to such a topic. In 2002 the Dalai Lama declared: "*The threat of nuclear weapons and man's ability to destroy the environment are really alarming. And yet there are other almost imperceptible changes - I am thinking of the exhaustion of our natural resources, and especially of soil erosion - and these are perhaps more dangerous still, because once we begin to feel their repercussions it will be too late.*"

The price to pay for an huge increment in agricultural production after the *Green Revolution* has been shown to include contamination of groundwater, release of greenhouse gases, loss of crop genetic diversity and eutrofization of rivers, streams, lakes and coastal marine ecosystems (contamination by organic and inorganic nutrients that cause oxygen depletion, spread of toxic species and changes in the structure of aquatic food webs) (Matson, 1997; Vitousek, 1997).

Beyond the conceptual debate about the substitutability of organic amendments by chemical fertilizers (Smil, 1999; Rigby and Caceres, 2001), societal criticisms concerning the sustainability of intensive farming arose as early as the 1930s, when the hypothesis of a connection between the decline in soil fertility, the quality of the human diet and human health (Balfour, 1944), already expressed in the 18^{th} and 19^{th} centuries (Marald, 2002), began to be reformulated. The potential of chemical fertilization for increasing crop yield was widely recognized at the end of the 19^{th} century, and industrial synthesis of N and processing of P fertilisers were mastered by the early 20^{th} century.

Since 1960 the worldwide rate of application of nitrogen fertilizers has increased by seven times (Vitousek, 1997), and now exceeds 7^{7} tonnes of nitrogen per year. Inputs from humans now equal all natural inputs to the nitrogen cycle and are seriously affecting terrestrial, freshwater and marine ecosystems, because half to two-thirds of nitrogen

fertilizers enter these non-agricultural ecosystems (Tilman, 1998).

After the Second World War the renewed interest in the study of soil properties not only stem from internal scientific dynamics, but was also forced by the onset of the oil crisis, as a consequence of the agriculture motorization (Mazoyer and Roudart, 1997).

Erosion, usually the most spectacular, immediate and irreversible symptom of modern agricultural practices, was the first indication of the drawbacks of intensified practices that left vast areas of soil deprived of the protection of plant cover. Italy and all the Mediterranean regions are particularly prone to erosion processes (Figure 1). It was considered as a natural process, because the Mediterranean regions are subject to long dry periods followed by heavy bursts of erosive rainfall. But inappropriate agricultural practices have forced erosion to a stage of irreversibility and in some places it has practically ceased because there is no more soil left (European Soil Commission, 2000). Although more than 99% of the world's food comes from the soil, experts estimate that each year more than 10 millions hectares (25 millions acres) of crop land are degraded or lost as rain and wind sweep away topsoil. An area big enough to feed Europe - 300 millions hectares- has been so severely degraded and it cannot produce food, according to UN figures (Radford, 2004).

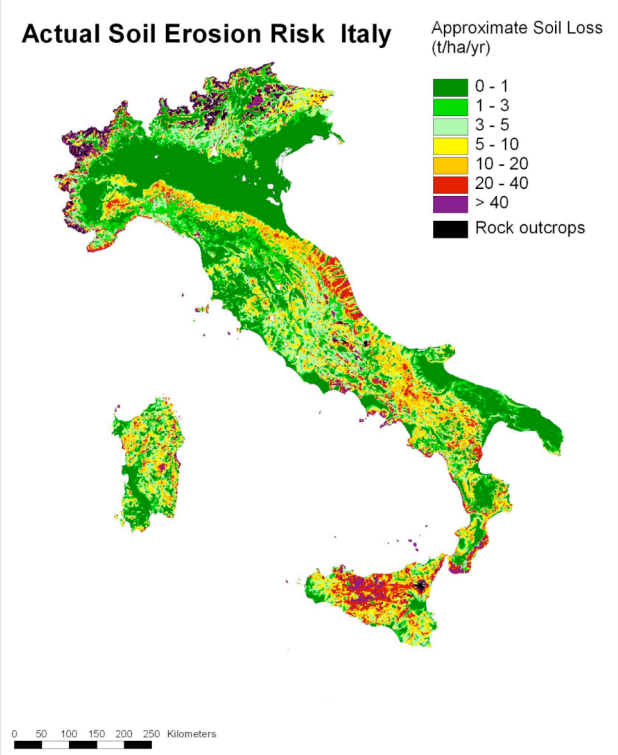


Figure 1 - Actual soil erosion risk in Italy

All these findings turn to the conclusion that no other activity has transformed humanity, and the Earth, as much as agriculture (Diamond, 1997).

Scientific concern and societal demands for sustainable farming practices that would save non-renewable resources such as fossil fuel and nutrients, maintain soil agricultural and environmental functions, and be ecologically and economically relevant when applied to

the whole planet, have led to the reassessment of the real value of biological maintenance of soil quality in agro-ecosystems (Izac and Swift, 1994; Tilman, 1997; Swift, 1999).
Thus, understanding of the nature, properties, dynamics and functions of the soil as part of agricultural landscapes and ecosystems is becoming the main objective of research in soil science.

Soil and Soil Organic Matter

The soil change concept

Scientific recognition of soil and Soil Organic Matter (SOM) and their implications for farming practices have taken a long time to settle into accepted wisdom. Farmers for millennia have recognized many of the soil physical constraints to plant growth and crop cultivation, although unable to describe and quantify them scientifically.
The first occurrence of the term "soil organic matter" in science cannot be dated with any precision. The term "humus" may well be considered as its precursor, although it has encompassed a wide range of meanings, which relate it more or less closely to modern understandings of SOM. The etymology of the word humus is Latin, and has been used to describe three substantially different concepts: humus as a chemical constituent, humus as a horizon and, less frequently, humus as a principle (Feller and Boulaine, 1987; Feller, 1997).
To Roman writers (Virgilio, Plinio il Vecchio and Columella), "humus" meant "soil" or "earth". Thus, Virgil named loamy soil "*pinguis humus*" and used the words "*humus*", "*solum*" or "*terra*" interchangeably to convey the notion of soil or earth rather than restricting the meaning to soil organic matter. At the beginning of the 1st century, after Cicero (106–43 b.c.), "*humus*" in the sense of "soil" progressively died out and was replaced by "*terra*" (Martin, 1941). Humus probably re-entered the European scientific vocabulary in the 18th century. It appeared in Diderot and d'Alembert's Encyclopaedia (1765, vol. 8), with the meaning "mould, garden earth, earth formed by plant decomposition".
Nowadays soil is considered as fundamental natural resource being the substrate of all

renewable productions and the site of all human activities (Giordano, 1999).

Soil accounts many definitions according to which scientific branch considers it.

The Soil Society of America (SSA) reports: *"soil is the unconsolidated mineral or organic material on the immediate surface of the earth that serves as a natural medium for the growth of land plants. (ii) The unconsolidated mineral or organic matter on the surface of the earth that has been subjected to and shows effects of genetic and environmental factors of: climate (including water and temperature effects), and macro and microorganisms, conditioned by relief, acting on parent material over a period of time. A product-soil differs from the material from which it is derived in many physical, chemical, biological, and morphological properties and characteristics"*.

Life, soil, the atmosphere, water and landforms have all evolved together; none would be the same without all of the others. Soil connects, responds to, and shapes the land, the atmosphere and its climates, surface water and groundwater, and ecosystems.

It is reasonable to assume that soil is the "Earth's living skin".

Soil Organic Matter

The SSA definition evidences that soil is the natural substrate for plant growth and highlights how soil is not simply the product of weathered parent material: it is the combined product of climate-driven physical site factors and vegetation, affecting soil properties by adding organic matter to the soil.

The term SOM is generally used to represent the organic constituents in the soil, including undecayed plant and animal tissues, their partial decomposition products, and the soil biomass.

SOM has been adopted as an indicator of soil fertility based on the rationale that it contributes significantly to soil physical, chemical, and biological properties that affect vital ecosystem processes of soil.

Organic matter within the soil serves several functions. From a practical agricultural standpoint, it is important for two main reasons: (i) as a "revolving nutrient fund"; and (ii) as an agent to improve soil structure, maintain tilth and minimize erosion.

Most soil organic matter originates from plant tissue. Plant residues contain 60–90 percent moisture. The remaining dry matter consists of carbon (C), oxygen, hydrogen (H) and small

amounts of sulphur (S), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg). Although present in small amounts, these nutrients are very important from the viewpoint of soil fertility management. SOM includes, in varying proportions and many intermediate stages, an active organic fraction including microorganisms (10–40%), and resistant or stable organic matter (40–60%), also referred to as humus.

Forms and classification of SOM have been described by Tate (1987) and Theng (1987). For practical purposes, organic matter may be divided into two major categories:

- the living fraction which is being actively used and transformed by living plants, animals, and microbes (Figure 2)
- the stabilized organic matter, which is highly decomposed and stable.

The living part of soil (about 5% of the total soil organic matter) includes a wide variety of microorganisms such as bacteria, viruses, fungi, protozoa and algae. It also includes plant roots, insects, earthworms, and larger animals such as moles, mice and rabbits that spend part of their life in the soil. The non-living biomass, which forms up to 98% of the total organic carbon, can be sub-divided in two main categories: macroorganic matter and humus. Macroorganic matter, commonly referred to as the labile one, makes up 10–30% of the total organic carbon (mainly as carbohydrates) and consists largely of plant residues in varying stages of decomposition.

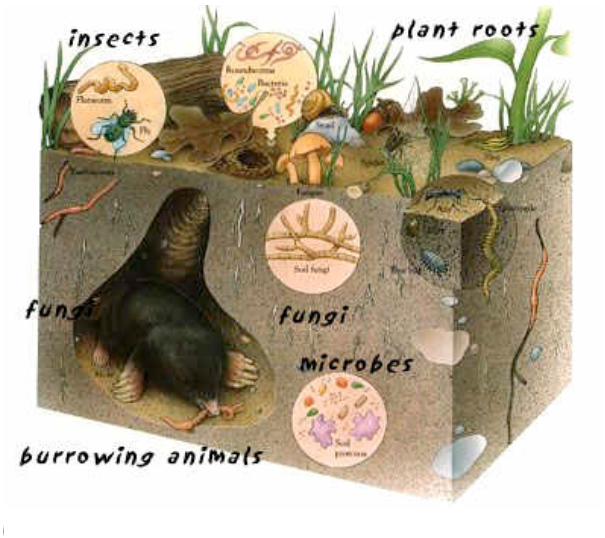


Figure 2 - Living part of the soil.

Carbohydrates occur in the soil in three main forms: free sugars in the soil solution, cellulose and hemicellulose; complex polysaccharides; and polymeric molecules of various sizes and shapes that are attached strongly to clay colloids and humic substances (Stevenson, 1994). The simple sugars, cellulose and hemicellulose, may constitute 5–25% of the organic matter in most soils, but are easily broken down by microorganisms.

Polysaccharides (repeating units of sugar-type molecules connected in longer chains) promote better soil structure through their ability to bind inorganic soil particles into stable

aggregates. Research indicates that the heavier polysaccharide molecules may be more important in promoting aggregate stability and water infiltration than the lighter molecules (Elliot and Lynch, 1984). Some sugars may stimulate seed germination and root elongation (Gibson, 2004). Other soil properties affected by polysaccharides include CEC, anion retention and biological activity.

The soil lipids form a very diverse group of materials, of which fats, waxes and resins make up 2–6% of soil organic matter. The significance of lipids arises from the ability of some compounds to act as growth hormones. Others may have a depressing effect on plant growth.

Soil N occurs mainly (> 90%) in organic forms as amino acids, nucleic acids and amino sugars. Small amounts exist in the form of amines, vitamins, pesticides and their degradation products, etc. The rest is present as ammonium (NH_4^-) and is held by the clay minerals (Gregorich et al., 1994; Franzluebbers et al., 1995). This pool of non-humic substances is important since it serves as a readily decomposable substrate for soil microorganisms, a short-term reservoir of plant nutrients and fragments become the centre for formation of water-stable aggregates (Golchin, 1994).

Table 1 - Functions of Humus in soil

Functions of Humus
Improve fertilizer efficiency
Long life N
Improve nutrient uptake, particularly of Ca and P
Stimulation of beneficial soil life
Provide magnified nutrition for reduced disease insects and frost impact
Salinity management – humates “buffer” plant for excess Sodium
Increasing soil C levels

The dead organic matter, remaining after separation of the labile fraction, is called humus. It is a relatively stable component formed by humic substances, including humic acids, fulvic acids, hymatomelanic acids and humins (Tan, 1994). It is probably the most widely distributed organic carbon-containing material in terrestrial and aquatic environments (Andreux, 1996; Stevenson, 1994). Humus cannot be decomposed readily because of its intimate interactions with soil mineral phases and is chemically too complex to be used by

most organisms (Stevenson, 1994). However when removed from their mineral matrices and exposed to microbial attack, humic substances decompose rapidly (Gramss et al., 1999), revealing the fact that intimate association with soil minerals is key to their stabilization (Schulten and Leinweber, 1996; Zech et al., 1997; Baldock and Skjemstad, 2000; Laird et al., 2001). It has many functions (Tab. 1). One of the most striking characteristics of humic substances is their ability to interact with metal ions, oxides, hydroxides, mineral and organic compounds, including toxic pollutants, to form water-soluble and water-insoluble complexes. Humus consists of different humic substances, according to their solubility under acidic or alkaline conditions: fulvic acids (FA), humic acids (HA), humin. The two fractions of the non-living biomass are strictly related because both of them are involved in storage, development and stabilization of SOM in soil (Piccolo, 1996).

Humic substances

Humic substances are the largest constituent of soil organic matter (~60%) and are considered as a key component of the terrestrial ecosystem, being responsible for many complex chemical reactions in soil (Stevenson, 1994).

The America Society of Agronomy classifies the three components of HS based on solubility under acidic or alkaline conditions (Figure 3).

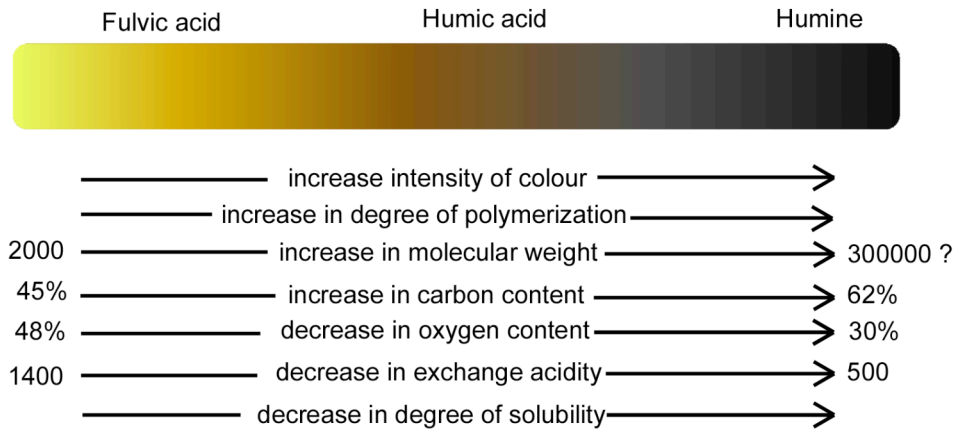


Figure 3 - Humic Substances classification.

Fulvic acids (yellow) are very soluble in water and all pH conditions; humic acid (brown) precipitate when acid (pH<2) is added to humic acids solutions; humins (with a brown-black colour) are insoluble in water at any pH and are believed to consist of humic acid molecules firmly attached to clay (such as kaolinite) or a mineral (such as hematite).

Many investigators now believe that all dark coloured humic substances are part of a system of closely related, but not completely identical, high - molecular - weight polymers. According to this concept, differences between humic acids and fulvic acids, can be explained by variations in molecular weight, numbers of functional groups (carboxyl, phenolic OH) and extent of polymerization.

The postulated relationships are depicted in figure 3, in which it can be seen that carbon and oxygen contents, acidity and degree of polymerization all change systematically with increasing molecular weight.

The low-molecular-weight fulvic acids have higher oxygen but lower carbon contents than the high-molecular-weight humic acids. Fulvic acids contain more functional groups of an acidic nature, particularly carbossilic group. The total acidities of fulvic acids (900-1400 meq/100g) are considerably higher than for humic acids (400-870 meq/100g).

Another important difference is that while the oxygen in fulvic acids can be accounted for largely in known functional groups (COOH, OH, C=O), a high portion of the oxygen in humic acids seems to occur as a structural component of the nucleus.

Electron microscope observations revealed the humic acids of different soils to have polymeric structure, appearing in form of rings, chains, and clusters. The sizes of their macromolecules can range from 60 to 500 A, what is mainly decided of by the occurring humification process, which also exerts an influence on their spatial structure.

From all these evidences humic substances appear as a heterogeneous mixture of compounds for which no single structural formula will suffice.

The molecular structure of humic substances

Unlike any other group of natural products, HS have remained a mystery to structural analysis. The obstacles involved are the result of a complex, virtually chaotic genesis (Ziechmann, 1994). Many authors believe that it is essential to have a basic understanding of the compositions and structures of HS in order to make better predictions of their

interactions in the soil and water environments such as association/dissociation processes, binding and trapping of biological and anthropogenic materials, and many other biological functions (Schulten and Leinweber, 2000).

Three different points of view on the humic substances structural conformation are actually reported in literature. One suggests that HS are macromolecular and assume random coil conformations in solution (Swift, 1999); a second proposes that HS are molecular associations of relatively small molecules held together by weak interaction forces, thus forming a supramolecular structure (Piccolo and Conte, 1999); a third considers that HS are in solution as micelles or "pseudomicellar" structures (Wershaw, 1999). Viewpoints two and three could be broadly summarized in a main theory (Clapp and Hayes, 1999), while the standpoint of Swift (Swift and Anderson, 1989) held assumption that HS are macromolecular polymers and should be considered as the other main macromolecules present in nature, such as proteins, polysaccharides, nucleic acids and lignin. This view is based on the hypothesis that humus synthesis derives either from the lignin or the polyphenolic theories (Flaig et al., 1975). These theory rely on the supposition that progressive polymerization of humic matter takes place via covalent bonding, and that the processes are often mediated by soil enzymes and abiotic catalysts such as primary minerals and layer silicates (Bollag et al., 1995). These conclusions were drawn on the basis of results obtained, among others, through molecular shape and size analyses by ultracentrifugation and computation of frictional ratios of various HS (Cameron et al., 1972).

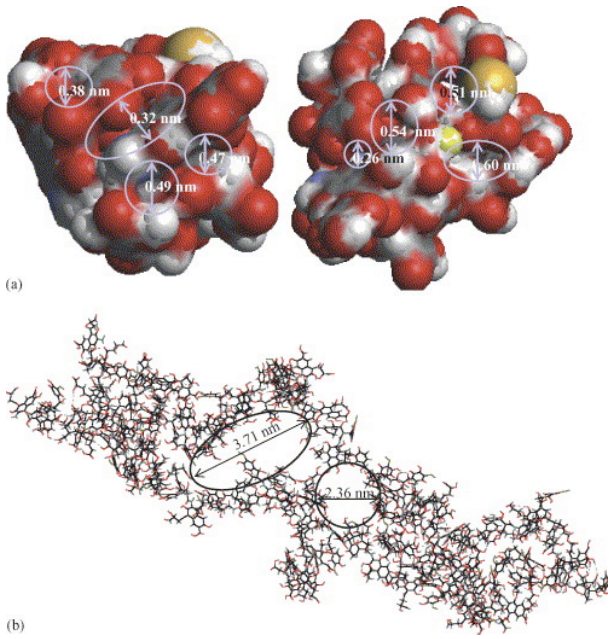


Figure 4 - Two perspectives (van der Waals surfaces) of two aggregated monomer HS. Circles indicate the pores and their size. (b) Results obtained for two polymers of 23 units under the same conditions.

Because of this structural representation, the scientific community have started to consider HS as macromolecular polymers in which simple (though heterogeneous) monomeric units progressively build up into high molecular weight polymers by random condensation and oxidation processes (Alvarez-Puebla et al., 2005). In this model of humus conformation (Figure 4), the randomness of the covalent polymerization of monomers accounts for the observed large polydispersity of humic macromolecules. Moreover, this theory demonstrated as a linear or branched polymeric chain, assuming several conformational folding, would become more resistant to microbial degradation and lengthen their turnover in the soil (Insam, 1996).

During the last years, this idea of HS as a macromolecular polymer, has been developed and a chemical model structures have been proposed combining geochemical, wet-chemical, biochemical, spectroscopic, thermal, agricultural and ecological data with analytical pyrolysis and modern computational chemistry (Schulten and Leinweber, 2000).

The supramolecular model of HS is based on results obtained by low-Pressure Size Exclusion Chromatography (SEC), High Pressure Size Exclusion Chromatography (HPSEC) and spectroscopic techniques. The supporters of this theory reported that the 280 nm absorption of humic acids was reversibly shifted from high to low molecular size

ranges when organic acids (for example acetic acid) were added to lower the pH of a humic solution from 9.2 to 2 before the elution in a 0.02 mol L^{-1} alkaline borate buffer (Nardi et al., 1986; Dell'Agnola and Nardi, 1987; Piccolo et al., 1996). Moreover, recovering the normal pH levels, HA adsorption turned to a high molecular size range.

In vivo organic acids, as phenolic and aliphatic acids, usually released in the rhizosphere from microorganisms and root systems (Nardi et al., 1997) can depolymerize these supramolecular association, creating humic compounds similar to those produced in the laboratory.

The results clearly showed that HS, at neutral or alkaline pH values do not behave as stable polymers, confirming the supramolecular theory. According to this theory, HS can be identify as relatively small and heterogeneous molecules of various origin that self-organize in supramolecular conformations. Humic superstructures of relatively small molecules are stabilized only by weak forces such as dispersive hydrophobic interactions (van der Waals, π- π, and CH- π bonding) and hydrogen bonds, the latter being progressively more important at low pH values. In humic supramolecular organizations, the intermolecular forces determine the conformational structure of HS, and the complexities of the multiple non-covalent interactions control their environmental reactivity (Piccolo, 2001). Even if this theory is still under debate (Swift, 1999; De Nobili and Chen, 1999), it is gaining importance in soil science literature (Burdon, 2001; Sutton and Sposito, 2005) and many authors consider humic substances as both macromolecules and supramolecules.

The genesis of humic substances

In order to properly discuss the chemical composition of HS it is important to investigate the possible contribution of the main precursor of the humic macromolecule.

The genesis of humic substances is complex. A variety of chemical reactions and biological transformation processes are involved in their formation. Therefore, it is reasonable to focus on the process of humification and gain insight into HS structural properties.

In the past, attempts have been made to simulate natural HS formation in restricted systems, especially with a restricted selection of conditions. Among the pioneering studies was Miller's experiment (1955) on chemical evolution, which first produced HS. Contemplating chemical evolution from a different point of view, HS may be regarded as buffers for

entropy that assume a state of lower order to compensate for a state of higher order in living systems (Ziechmann et al., 1994).

During the last years the mechanisms of humic substance genesis have been widely reviewed (Hatcher and Spiker, 1988; Hedges, 1988; Stevenson, 1994; Shevchenko and Bailey, 1996), and there is general consensus that humic substances are mainly originated from the transformation of organic plant residues (humification). This transformation can be the result of two contrasting processes: (1) partial biotic biopolymer degradation, where the integrity of the biopolymer is not completely destroyed, but rather modified such that it forms the humic substance backbone, and (2) abiotic condensation polymerization, in which simple products of biopolymer degradation (phenols, quinones, sugars and aminoacids) repolymerize to form humic substances. These two mechanisms are not mutually exclusive, since biopolymer degradation is a prerequisite for the abiotic condensation pathway (Hedges, 1988).

The term abiotic is used to refer to the later steps in the pathway in which chemical reactions between simple compounds released by biotic processes condense to form the humic substances. In the proposed biotic biopolymer degradation pathway, recalcitrant macromolecules, as lignin, enter the humin fraction in a partially altered state.

In the abiotic condensation-polymerization pathway, molecular fragments released during the decomposition of precursor molecules and/or molecules released as metabolites by products from microorganisms, polymerize via chemical reactions. As the molecular weight of the synthesized polymer increases, a progression from fulvic acid to humic acid to humin occurs. Four abiotic mechanisms have been proposed and schematic representations of the various chemical reactions were presented by Hedges (1988) and Stevenson (1994): (1) the polyphenol theory, (2) the melanoidin model (a sugar/amine condensation reaction), (3) phenol and quinone formation from carbohydrates, and (4) photo-oxidation of polyunsaturated lipids. In the polyphenol theory, monomeric phenolic species (mono-, di-, and tri-hydroxy phenols) are produced by the enzymatic degradation of lignin or synthesized by various soil microorganisms (Martin and Haider, 1971). These monomeric phenolic species are capable of forming a quinone structure in the presence of O_2 or polyphenoloxidase enzymes, which spontaneously polymerize with each other or with amines or ammonia to produce polymeric compounds. In the melanoidin model, sugars and

amines react to form an N-substituted glucosamine which can dehydrate, rearrange, or condense to form simple fragments (e.g., glyceraldehyde) and structurally complex brown nitrogenous polymers.

Spectroscopic studies of Humic Substances

No single analytical tool can provide definitive structural or functional information about HS because of its heterogeneous, ill-defined nature (Chen et al., 2003). Among analytical characterization methods, spectroscopic techniques appear to be most useful, and they are directly applicable to both solid and liquid sample analyses. These methods have a number of attractive advantages including: (a) they are non-destructive; (b) only small amounts of samples are needed; (c) they are experimentally simple and do not require special manipulative sample preparation; and, perhaps most importantly, (d) they provide valuable information on molecular structure and chemical or functional properties of HS (Stevenson, 1994). Spectroscopic techniques such as nuclear magnetic resonance (NMR), Infrared (IR) and Fourier-Transform Infrared (FTIR), Electron Paramagnetic Resonance (EPR), Ultraviolet–Visible (UV/Vis), and fluorescence have been previously applied for both quantitative and qualitative characterization of soil organic matter and humic substances (Martin et al., 2006; Sciacovelli et al., 1977; Montecchio et al., 2006; Golonka et al., 2005; Hur et al., 2006; Senesi et al., 1989, 1991). In the present work Nuclear Magnetic Resonance (NMR) and DRIFT spectroscopic techniques have been applied and described.

Biological Activity

The addition of organic matter to the soil not only maintains but even improves soil fertility and, from earliest time, SOM has been recognized as one of the major natural resources for agricultural purpose (Nardi et al., 1996). Related to this soil improvement, from the beginning of the 20th century the positive interaction between humic substances and the plant growth has been studied (Bottomly, 1917, 1920).

It is not easy to distinguish between direct and indirect effects of HS on plant physiology. In fact, some of their positive effects may be the consequence of an improvement of soil fertility, leading to a higher nutrient availability for plants. Whilst, in other cases, HS seem to positively influence metabolic and signalling pathways involved in the plant

development, by acting directly on specific physiological targets.

Since ’80 different authors hypothesized that HS may be adsorbed by plant roots, even if HMW (High Molecular Weight) and LMW (Low Molecular Weight) fractions seem to behave differently (Vaughan and Ord, 1981; Vaughan et al., 1974, 1986).

Up to today it has not been yet completely clarified the mechanisms through which HS interact with the root cells and may subsequently influence plant physiology and growth.

Among the modifications induced by HS on treated plants, changes in size and development were the first to be studied analytically. Under particular conditions, HS can stimulate plant growth in terms of increase in plant length and dry or fresh weight (Blanchet, 1958; Gumiński, 1968). These effects depend on the concentration (Elgala et al., 1978) and source of the substance (Hernando et al., 1977), on the plant species (Blanchet 1958; Gumiński, 1968) and age, as well as on the culture conditions of the trial. Recently many studies have confirmed the hypothesis of a direct effect of humic substances on plant physiology, in particular concerning root hair formation (Schmidt et al., 2005) and lateral root development (Canellas et al., 2002; Zandonadi et al., 2007).

The effects of HS on plant metabolic processes have been extensively reviewed (Nardi et al 1996; Nardi et al., 2002). For instance there are many reports which show that humic substances influence respiration, protein synthesis and enzyme activity in higher plants (Sladký, 1959; Vaughan, 1967; Nardi et al., 2000, 2007). As far as the photosynthesis process is concerned, few reports, focusing on the chlorophyll content and electrons transport, are available (Thomas et al., 1978; Oettmeier et al., 1988; Jezierski et al., 2000; Pflugmache et al., 2006).

The effects of HS on ion uptake represent one of the topics which received more attention by scientists. They appear to be variable and selective, depending on the HS typology and concentration, on plant species, and on composition and pH of the medium (Chen and Aviad, 1990; Varanini and Pinton, 1995; Varanini and Pinton, 2001; Clapp et al., 2001; Nardi et al., 2002; Tan 2003; Chen et al., 2004). A positive effect of HS on nutrient uptake has been reported for the major inorganic elements, such as nitrogen, phosphorus, potassium (Mylonas and McCants, 1980) and sulphur (Gumiński 1968), but different HS fractions seem to differently affect their uptake (Vaughan and Linehan, 1976).

In particular, as a consequence of the environmental impact that the nitrogen fertilization

has assumed during the last century, several studies were conduced to figure out how the presence of HS may interfere with the nitrate uptake and assimilation by plants (Vaughan et al., 1985; Chen and Aviad, 1990; Varanini and Pinton, 2001; Clapp et al., 2001; Pinton et al., 1999; Sessi et al, 2000; Quaggiotti et al., 2004). Results obtained demonstrated a strong positive effect of the LMW fractions on nitrate uptake and assimilation, whereas HMW fractions induced only weakly the same pathways (reviewed in Nardi et al., 2002), in agreement with previouos data (Vaughan, 1986).

More recently, three independent studies aimed to better clarify the mechanisms through which HS stimulate the nitrate uptake in maize (Pinton et al., 1999; Canellas et al., 2002, Quaggiotti et al., 2004), demonstrated that the increment of nitrate influx measured in response to HS depends on a transcriptional activation of a gene encoding a major H^+-ATPase of maize (*Mha2*), leading to the generation of a more favourable proton motive force. In fact, nitrate influx across the plasmalemma of root cells is coupled to the favourable H^+ electrochemical gradient created by the plasma membrane H^+-ATPases (Thibaud and Grignon, 1981; Ruiz-Cristin and Briskin, 1991; Meharg and Blatt, 1995; Miller and Smith, 1996).

Even if the exact mechanisms through which HS exert their effects on plant physiology are still partially unclear, many evidences suggest that they may involve at least in part a hormone-like activity.

Hormone-like activity

A putative HS hormone-like activity is not surprising as it is known that soils vary in their native auxin content (Hamence, 1946) and fertile soils contain greater amounts of auxin then less fertile ones (Stewart and Anderson, 1942; Dahm et al., 1977). Auxin and gibberellin levels are usually higher in the rhizosphere than in the bulk soil, probably as a consequence of increased microbial populations or of an accelerated metabolism owing to the presence of root exudates. Although numerous soil and rhizosphere microorganisms, as well as the root systems of higher plants, have been reported as producing auxin (Lebuhn and Hartmann, 1993) and gibberellins (Rademacher, 1992), there is little information about their stability and only indirect conclusions have been made about their presence in amounts high enough to be biologically active (Frankenberger and Arshad, 1995).

At the beginning of the 19th century Bottomley hypothesized that the growth promoting activity of the humic substances reflects a hormone-like activity. Such hypothesis was then corroborated by results demonstrating the immunological or spectrometric identification of indol acetic acid (IAA) inside several humic substances (Muscolo et al., 1998, 1999; Canellas et al., 2002; Quaggiotti et al., 2004). In addition, the auxin-like activity of HS is supported by reports showing a positive effect of such substances on specific targets of auxin action. *Mha2*, a major maize isoform of H^+-ATPase that is preferentially expressed in guard cells, phloem and root epidermal cells and that appears to be strongly stimulated at the transcriptional level in response to auxin (Frias et al., 1996), evidenced also a significant up-regulation of its mRNA abundance in roots of maize seedlings treated for 48 hours with earthworm low molecular size humic substances (Quaggiotti et al., 2004). Furthermore, Russel and collaborators (2006), by studying the effects of two different molecular weight fractions of humic substances extracted from earthworm faeces on pea, evidenced an auxin-like effect of both fractions on stomatal opening as influenced by phospholipase A2, that is considered to be involved in auxin-mediated signalling (reviewed by Macdonald, 1997; Scherer, 2002).

Moreover, Muscolo et al., (1993, 1996) have shown that humic substances induce morphogenetic and biological changes in leaf explants of Nicotiana plumbaginifolia. They affect the patterns of peroxidase and esterase, enzymes that are involved in organogenesis, and may be indicators of somatic embryogenesis. These effects, peculiar to humic fraction with a low relative molecular mass (<3,500 Da), were similar to those produced by IAA. A study on homogeneous carrot (*Daucus carota*) cell cultures compared the effects of the low relative molecular mass humic fraction to different auxins (Muscolo et al., 1999). This humic fraction caused an increase in carrot cell growth similar to that induced by 2,4-dichlorophenoxyacetic acid (2,4-D) and promoted morphological changes similar to those induced by IAA. In addition, Muscolo et al., (2007) demonstrated that IAA and LMW fractions richer in carboxylic groups bind in the same way with carrot cell membranes. Zandonadi et al., (2007) comparatively evaluate the effects of indole-3-acetic acid and humic acids isolated from different soils substances on root development and on activities of plasmalemma and tonoplast H^+ pumps from maize roots. They observed that HA as well as low IAA concentrations (10^{-10} and 10^{-15}M) improved root growth through a markedly

proliferation of lateral roots along with a differential activation not only of the plasmalemma but also of vacuolar H^+-ATPases and H^+-pyro-phosphatase.

A different theory about the auxin-like activity of the humic substances has been assumed by Schimdt et al., (2007). To further investigate a possible hormone-like effect of water-soluble humic molecules (WEHS), they grown *Arabidopsis* plants in sterile medium containing WEHS in concentrations ranging from 1 to 20 mg C org l^{-1}. Application of WEHS were found to significantly increase the number and length of root hairs. Further experiments reported that the phenotypes of *Arabidopsis* auxin-related mutants, all exhibiting a reduced number of root hairs, were not rescued by the application of WEHS. In addition, mutants defective in root hair initiation such as *rhd6*, known to develop normal hairs in the presence of ethylene or auxin, were not affected by a wide range of applied concentration of WEHS. The authors concluded that HS cannot substitute for these hormones in promoting root hair growth, and suggested that HS can alter root development without significantly affecting the plant's auxin homeostasis. This assumption was also supported by the lack of responsiveness of the auxin-responsive GH3 gene. Transcripts of genes from the GH3 family accumulate following auxin exposure, probably to dampen auxin signalling by inactivating IAA via conjugation to amino acids (Hagen and Guilfoyle, 1984). Application of high concentration of HS (more than 5 mg C l^{-1}) for two hours evidenced only a slight increase in transcript abundance in roots and caused no significant change in message levels in leaves, further confirming the hypothesis that the changes in root morphology are not mediated via an auxin-signalling pathway.

It is important to note that this theory is not complemented with a detailed characterization of the humic substances analyzed. Because of the different features and the complex chemical composition of humic substances, an efficient and complete characterization, from both a chemical and a spectroscopic point of view, is an essential requirement to match data obtained from different studies. In this case the extraction procedure and the lacking in characterization of the humic substances analyzed make the results not completely suitable for further comparison with any other information present in literature. Moreover the authors expressed the theory that WEHS do not exert their effects in an auxin-like manner without investigate its inner content of indole-3acetic acid. According to all these lacking in description, the conclusion of the authors could be speculative rather

than theoretical and it would need a more detailed investigation to be considered.

On the contrary, recently Dobbss and collaborators (2007), using *Arabidopsis* and tomato, demonstrated that various characterized humic acids need the auxin-signalling transduction pathway to be active. The increase in number of lateral root exhibited in *Arabidopsis* and tomato wild-type seedlings treated with different humic substances lead authors to hypothesize the presence of auxin-like compounds in the organic matter. Nevertheless the same substances did not induce lateral root formation in a tomato mutant (*dgt*) characterized by a defective gene for auxin response. They concluded that probably humic substances may act as a "buffer", either absorbing or liberating signalling molecules, according to modifications in the rizosphere, such as the acidification brought about by the activity of plasma membrane H^+-ATPase (Canellas et al., 2002; Zandonadi et al., 2007) or exudation of organic acids (Façanha et al., 2002; Canellas et al., 2008), thus behaving as a regulator of hormonal balance with respect to lateral root emergence.

Because of their complicated and changeable nature, a debate on HS auxin-like activity is still open. However, the observed hormonal effects did not always correlate to the amount of IAA detected in the humic acids. For this reason, the presence of different compounds of the auxin family or of molecules that might either mimic the action or stimulate the plant endogenous metabolism of auxin cannot be ruled out. Functional genomics, transcriptomics or proteomics may represent a good strategy in spreading light on HS biological activity.

Humic substances and environment

HS have been seen in the upper sections to constitute a physically and chemically heterogeneous mixture of macromolecular organic compounds of mixed aliphatic an aromatic nature. They are rich in chemically reactive functional groups, and formed by different processes originating during the microbial and chemical transformation of fresh organic matter in soil. As part of the soil organic matter HS contribute substantially in improving the global soil fertility status by exerting, besides several general fertility functions that they posses in common with other soil components, a number of functions which are specific and typical of humified SOM (Mackowiak et al., 2001). These include, among the others, slow release of nutrients (N, S, P), high cation exchange capacity, pH buffer capacity, and extended interactions with micronutritive and/or microtoxic metals ion

and xenobiotic organic molecules such as pesticides.

Bioavailability/phytotoxicity, mobilization/transport and immobilization/accumulation in soil are thus greatly influenced. Plants can exploit these soil-improved characteristics to develop a more specific response system to external stress, such as salt (Oliver et al., 2006). Moreover, Turan and Angin (2004) found HS effective in enhancing heavy metals desorbtion from soil and increasing their accumulation in crop species. The lower stability constant of HS, makes HS an ideal soil amendment for phytoextraction and prevents the possible movement of heavy metal-humic acid complexes across the soil profile (Chen and Aviad, 1990; Wood, 1996; Arslan and Pehlivan, 2008).

These features together with a major demand of safe food and sustainable agricultural have contributed to enlarge the environmental significance of HS.

Fertilizer factories are now redirecting their production to biostimulants, based on humic substances and other organic compounds and recently, in Italy, biostimulants were inserted in the Legislative Decree n. 217/2006 ("New regulation about fertilizer" MiPAF).

This is an important result which supports the fundamental project of recycling partially humified organic wastes, derived from plant, wood, food and other human activities, as beneficial soil amendments.

For this reason, understanding HS biological activity and the molecular mechanisms through which they exert their functions is becoming an important ecological task and a valid tool in facing environmental problems.

The model system: Arabidopsis thaliana

In 1964, a small band of pioneering plant scientists made a bold predicted that *Arabidopsis thaliana*, a weed commonly known as mouse cress, would become the botanical equivalent of Drosophila, a model species to rival the famous fruit fly in its ability to illuminate the biochemical basis of genetic mutation. To a large extent, history has borne out this prediction and *Arabidopsis thaliana* is the most well researched plant species nowadays.

It is a small dicotyledonous species, a member of the Brassicaceae or mustard family. Although closely related to such economically important crop plants as turnip, cabbage, broccoli, and canola, *Arabidopsis* is not an economically important plant. Despite this, it has been the focus of intense genetic, biochemical and physiological study for over 40

years because of several traits that make it very desirable for laboratory study. As a photosynthetic organism, *Arabidopsis* requires only light, air, water and a few minerals to complete its life cycle. It has a fast life cycle, produces numerous self progeny, has very limited space requirements, and is easily grown in a greenhouse or indoor growth chamber. It possesses a relatively small, genetically tractable genome that can be manipulated through genetic engineering more easily and rapidly than any other plant genome.

Arabidopsis, like all flowering plants, dehydrates and stores its progeny at ambient temperature for long periods of time. This fact, together with a newly developed means of creating gene knockout lines, has made many basic biologists realize that *Arabidopsis* may be the best model system for basic research in the biology of all multicellular eukaryotes. A complete knockout collection of *Arabidopsis* seeds can be housed in a room no larger than a closet; to create and store a similar library of knockouts for mouse, flies and worms would be much more labour and space intensive. All together, these traits make *Arabidopsis* an ideal model organism for biological research and the species of choice for a large and growing community of scientists studying complex, advanced multicellular organisms.

Root system in *Arabidopsis thaliana*

Root system is the plant organ closely related to the soil. It is delegated to the uptake of water and nutrients and exerts its function in anchoring the plant body to the ground.

The plant body has a bipolar growth axis with a Root Apical Meristem (RAM) at one end and a shoot apical meristem at the other. One traditional idea is that the axial organs have an indeterminated growth (Sinnott, 1935). This generalization can be interpreted to mean that the root apical meristem will remain active indefinitely in suitable conditions. But there are several studies demonstrating that the roots of many plants do not grow indefinitely and that they are in fact determinate; *Arabidopsis* is one of them (Reinhardt and Rost, 1995). A typical growth curve for *Arabidopsis* primary roots has three phases: an initial phase of accelerating elongation soon after emergence, a steady phase of the seedling primary root, and a decelerating phase leading to termination of elongation. The time it takes to reach the determinate length was variable depending on species and environmental factors (Chapman et al., 2003).

From a developmental perspective, the *Arabidopsis* root is a paragon of simplicity.

RAM is a dynamic structure, that changes with the growth, developmental age, and environmental conditions of the root (Armstrong and Heimsch, 1976; Seago and Heimsch, 1969).

A small number of stem cells at the RAM generate the all cell types through stereotyped divisions followed by cell differentiation and regulated cell expansion. Because root growth is indeterminate, these processes are continual, resulting in all developmental stages being present at all times. The radial symmetry of the root combined with a lack of cell movement means that clonally related cells are frequently found in cell files. These cell files can be traced back to their origins, which are four types of stem cells (or initial cells) at the root tip (Dolan et al., 1993). The epidermal/lateral root cap initials give rise to the epidermis and the outer portion of the root cap known as the lateral root cap (Figure 5).

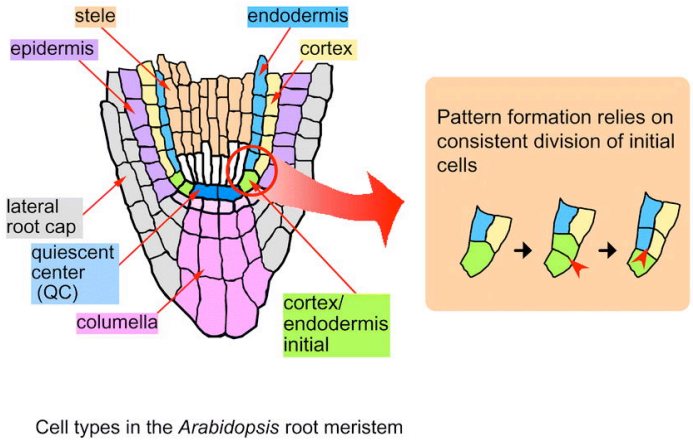


Figure 5 - Longitudinal section of the *Arabidopsis* root tip that has been colour-coded to show the different cell types and a scheme showing the two asymmetric cell divisions that the cortex/endodermis initial and its daughter undergo.

The central portion of the root cap, the columella, has its own set of initials. The ground tissue cells, the cortex and endodermis, are generated by division of the cortex/endodermal initials. Finally, the vascular tissue and pericycle have their own initials.

Internal to and contacting all the initials is a small number of central cells that are mitotically inactive and are known as the Quiescent Center (QC).

Division of initials can be either solely anticlinal (orthogonal to the axis of growth) resulting in a single file of cells or first anticlinal then periclinal (parallel to the axis of growth) resulting in two or more cell layers. The columella initials generally divide only

anticlinally and their progeny undergo rapid cell expansion and then differentiate, producing starch-containing amyloplasts that play a role in gravity sensing. The other three types of initials generally undergo both anticlinal and periclinal divisions, resulting in cell lineages that acquire different identities (Dolan et al., 1993). For example, the cortex/endodermal initials divide first anticlinally to regenerate the initial cell and a basal daughter cell. The basal daughter then undergoes a periclinal division to form the first cells of the cortex and endodermal lineages. These cells will undergo a small number of anticlinal divisions during which time they will acquire their specific fates. Subsequently they will undergo rapid polar expansion.

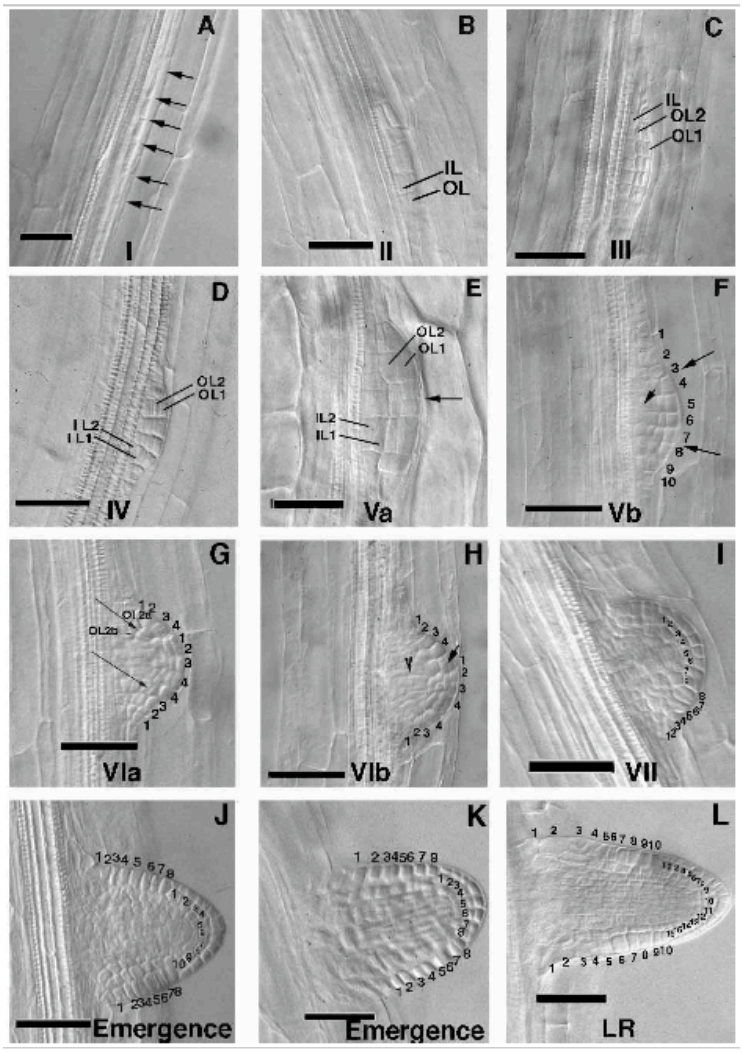


Figure 6 - Stages of lateral root primordium development. Shown are Nomarski images of cleared whole mounts of 2- to 6-week old. (OL= outer layer; IL= inner layer). Bar, 50 µm. (Malamy and Benfey, 1997).

Lateral root formation

Lateral root primordia arise from pericycle cells opposite xylem poles at some distance from the primary root meristem (Figure 6) (Dolan et al., 1993). The initial cell division patterns that give rise to new primordia are very different from those occurring during primary root formation (Casimiro et al., 2001). At later stages of lateral root formation the cellular organization becomes very similar to that of the primary root, although lateral roots display more variability in cell numbers and precise cellular organization. Analysis of cell division patterns aided by enhancer traps that mark various root tissues have been used to stage lateral root development (Malamy and Benfey, 1997). Eight stages were defined based on specific anatomical characteristics and cell divisions.

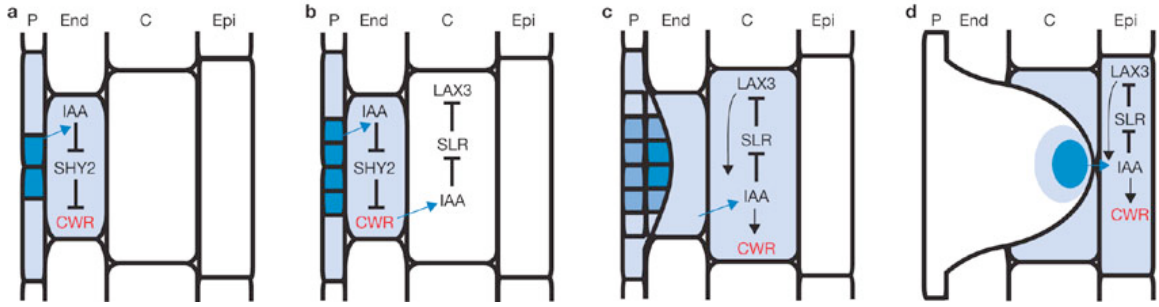


Figure 7 - (a) Auxin (IAA) originating from dividing pericycle (P) cells induces cell-wall-remodelling (CWR) gene expression in adjacent endodermal (End) cells by targeting the degradation of the SHY2/IAA3 repressor protein. **(b)** Auxin derived from the lateral root primordium also induces expression of the auxin influx carrier LAX3 in adjacent cortical cells (C) by targeting the degradation of the SLR/IAA14 repressor protein. **(c)** LAX3 expression increases cell permeability to auxin, creating a positive-feedback loop. Increased auxin accumulation induces CWR expression. **(d)** At a later stage of primordium development, auxin induces LAX3 expression in adjacent epidermal (Epi) cells. The expression of CWR in a few cells of the different layers facilitates the emergence of lateral root primordium. Cellular auxin responses are represented as a blue colour gradient (Swarup et al., 2008).

Analysis of the expression patterns and cell division patterns revealed that a remarkable amount of organization and cell differentiation occur at very early stages of lateral root primordium development, with differential gene expression apparent after the first set of divisions of the pericycle. In contrast, the lateral root meristem does not become active until after the primordium emerges from the parent root, and therefore does not appear to play a role in early pattern formation and organization (Malamy and Benfey, 1997).

Although the nature of the patterning mechanism that determines the longitudinal spacing of root primordia in *Arabidopsis* is unclear at present, auxin obviously represents an important promotive factor (Casimiro et al, 2001, Hirota et al., 2007, Lucas et al., 2008). It

has been proposed as a key signal during lateral root development (Casimiro et al., 2003, Ivanchenko et al., 2008, Dubrovsky et al., 2008), triggering the initial mitotic division of lateral root founder cells in the pericycle tissue and patterning lateral root primordia (Benkova et al., 2003). A model for this process is reported in figure 7.

A recent study has hypothesized that auxin derived from lateral root primordia re-programme overlaying cells in adjacent tissues (Swarup et al., 2008). The authors have suggested that cells in the parent root overlaying new lateral root primordia actively participate in organ emergence. Therefore, roots seem to use a transcellular auxin signalling network designed to synchronize lateral root development and emergence processes.

Auxin

Auxin refers to an important group of phytohormones that has been implicated in most of the quantitative growth changes that occur during a plant's life cycle.

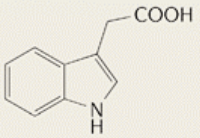
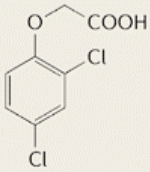
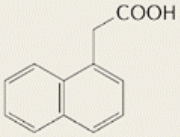
Properties	Natural	Synthetic	
Chemical structure			
	IAA	2,4-D	NAA
Affinity to receptors			
TIR1 binding (K_d)	High	Low	Middle
ABP1 binding (K_d)	Middle	Low	High
Transport capacity			
Influx carriers	Yes	Yes	No (by diffusion)
Efflux carriers	Yes	No	Yes

Figure 8 - Indole-3-acetic acid (IAA) is considered the most important natural auxin, 1-Naphthaleneacetic acid (NAA) is an horticulturally important auxin, and 2,4 dichlorophenoxy-acetic acid (2,4-D) is a common selective herbicide.

Phytohormones are chemicals that have specific effects on plant growth, and are active at low concentrations. Plants use a wide variety of hormones, including steroids and peptides, as well as the five classical classes of phytohormones (auxins, abscisic acid, cytokinins, ethylene and gibberellins), which are all relatively small molecules. The extent and significance of phytohormone transport is not well understood for all of these classes, but is

particularly significant to the action of auxin and to the story of its discovery (Teale et al., 2006). Indole-3-acetic-acid (IAA) is the predominant auxin in plants (Figure 8), and is an indispensable phytohormone with a well-documented ability to regulate many aspects of plant development: cell division, cell elongation and cell differentiation, although the way in which this phytohormone acts remains still unknown.

Auxin is synthesized from indole through tryptophan-dependent and tryptophan-independent pathways, and this topic has been recently reviewed (Woodward et al., 2005). Consistent with its definition as a hormone, IAA can be transported the length of the plant from the shoot to the root; this transport is necessary for normal development, and more localized transport is needed for tropic responses.

Auxin transport

Although virtually all plant tissues appear to be capable of synthesizing auxin, most is normally produced in young developing parts of plant such as the shoot apex, emerging leaves and developing seeds (Ljung et al., 2001; reviewed by Ljung et al., 2002), transported in the root apical meristem and reallocated to the other parts of the plant.

Auxin distribution through the plant is based on two spatially separated transport pathways: a fast, non-polar transport in the phloem (Morris and Thomas, 1978) and a slower, cell-to-cell polar transport in various tissues. Even though most of the physiological and molecular data evidence the relevance of the Polar Auxin Transport (PAT) system, the importance of auxin translocation via phloem should not be underestimated. In fact the majority of long distance auxin redistribution occurs via phloema, which is especially significant in larger plant species.

Auxin polar distribution involves many proteins, among them, the most investigated belong to a family of polarly localized plasma membrane protein, PIN proteins (Figure 9a). PINs are found throughout the plant kingdom, and mediate auxin efflux. There are eight PIN proteins in *Arabidopsis thaliana*, and they are expressed at distinct times and locations (Papanov et al, 2005). Five have been well characterized and show a distinctive polar subcellular localization. The mechanism that underlies the polar localization of PIN proteins and their ability to relocalize rapidly is the recycling of PIN-containing endocytotic vesicles to and from the plasma membrane.

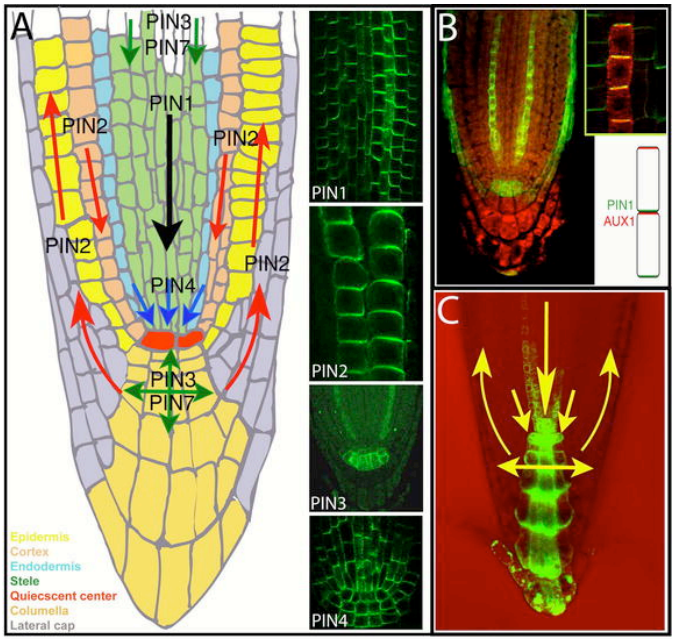


Figure 9 - Polar localization of auxin efflux and influx carriers, and the direction of auxin flow in the *Arabidopsis* root. (A) A diagram representing the *Arabidopsis* root tip with arrows indicating the direction of auxin transport as mediated by PIN1, PIN2, PIN3, PIN4 and PIN7. Inset images show the subcellular polar localization of PIN1, PIN2, PIN3 and PIN4 (by immunolocalization). (B) AUX1 subcellular localization (by immunolocalization); inset: a close-up and scheme illustrating opposing PIN1 and AUX1 polar localization in protophloem cells. (C) A diagram showing the predominant directions of auxin flow and auxin accumulation in the root tip as visualized by DR5rev::GFP (Swarup et al., 2001)

The auxin influx through the cell is known to be mediated by auxin cellular influx carrier, AUX1 (Fig 9b). The first evidence favouring AUX1 as a protein involved in auxin influx came from the *aux1* mutant. The *aux1* mutant was originally found in a screen for seedlings resistant to 2,4-D (Maher and Martindale, 1980). The auxin resistant and agravitropic root growth phenotype of the mutant suggested a defect in auxin transport. Additional auxin uptake assay performed in *aux1* and wild type roots revealed the role of AUX1 in the influx of auxin (Marchant et al., 1999). Its auxin transport is direct, and this capacity was demonstrated in *Xenopus laevis* oocytes at physiologically significant concentrations (Yang et al., 2006).

AUX1 is member of a small gene family in *Arabidopsis* consisting of three other LIKE AUX1 (LAX) genes (Swarup et al., 2001, 2008), but their functional characterization has not yet been reported. In *Arabidopsis* root tips, AUX1 is strongly expressed with cell membrane protein localization in the protophloem, columella, lateral root cap and epidermal cells. Although in most of these tissue AUX1 appears to be uniformly distributed around all side of the cell, in the protophloem it was found to be enriched in the upper side

of cells, that is opposite to PIN localization in the same cells (Swarup et al., 2001, 2004). The characterization of other members of the LAX gene family should improve the understanding of auxin influx.

Gene expression regulation

IAA biosynthesis, metabolism, and transport together ensure that appropriate auxin levels that are in place to orchestrate plant development. The signalling between auxin and downstream effectors has been the subject of many researches. Auxin rapidly and transiently induces accumulation of at least three families of transcripts: SMALL AUXIN-UP RNAs (SAURs), GH3-related transcripts and AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) family members.

SAUR, GH3 and Aux/IAA transcripts accumulate rapidly after auxin exposure in many species, including *Arabidopsis* (Gil et al., 1994; Hagen et al., 1984; Abel et al., 1994; Abel and Theologis, 1996).

Aux/IAA genes encode proteins that generally have nuclear localization signals and four conserved domains (I–IV). Domain III has a predicted ribbon–helix–helix DNA-binding domain that is found in bacterial transcriptional regulators (although it is not thought that Aux/IAAs bind DNA in plants) (Ulmasov et al., 1997). Aux/IAAs have indeed been found in the nucleus. Several Aux/IAA genes are transcribed within minutes of plants or cells being exposed to exogenous auxin or protein synthesis inhibitors. Most strikingly, they were shown to form homo- and heterodimers not only with one another, but also with members of the ARF family.

ARFs are transcription factors that contain an amino-terminal B3-like DNA-binding domain, which binds to an auxin-responsive element (ARE; TGTCTC) in the promoter of auxin-responsive genes (Ballas et al., 1993; Ulmasov et al., 1999). The carboxy-terminal domain is similar to the carboxy-terminal region of the Aux/IAA proteins and is likely to promote direct interaction between both groups of proteins while bound to the ARE (Ulmasov et al., 1997). This interaction blocks ARE-mediated transcription.

With the potential involvement of many proteins, auxin signalling is unexpectedly complicated. This auxin-receptor-binding signal is relayed to the subsequent phase of auxin-responsive gene expression, which is anticipated to be an intricately intertwined set

of processes. The effects of auxin are thought to depend on its concentration, with high and low doses eliciting different responses.

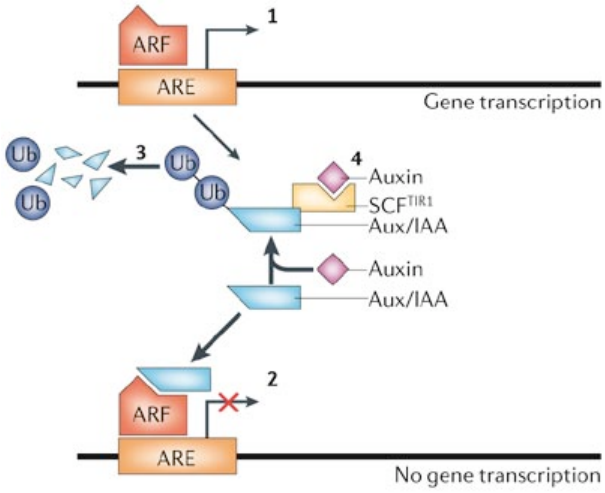


Figure 10 - Auxin signalling (Teale at al., 2006).

A framework for understanding how auxin can have such different roles in plant development is now in place (Figure 10). At basal auxin levels, Aux/IAAs are relatively stable, homodimerize and heterodimerize with ARFs that can bind to AREs in the promoters of auxin-responsive genes (Kim et al., 1997). The ARF-bound Aux/IAA proteins block transcription from auxin-responsive promoters by controlling the amount of free ARF transcription factors to the promoters (Ulmasov et al., 1997).

An increase in auxin levels causes the proteasome-mediated degradation of Aux/IAAs (Bernard et al., 2001), which in turn allows for a gradually increasing number of functionally active ARF proteins and the transcriptional activation of auxin regulons.

The regulation of auxin transport, and specifically the role of auxin itself in its own transport is going to explain much about how plant growth is controlled.

Technical approaches

Humic Substances characterization

Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance, or NMR as it is abbreviated by scientists, is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin. Nuclear magnetic resonance spectroscopy is the use of the NMR phenomenon to study physical, chemical, and biological properties of matter. As a consequence, NMR spectroscopy finds applications in several areas of science.

Many of the molecules studied by NMR contain carbon. Unfortunately, the carbon-12 nucleus does not have a nuclear spin, but the carbon-13 (C-13) nucleus does, due to the presence of an unpaired neutron (Hornak, 1999). Recently there has been a great deal of interest in obtaining cross-polarization magic angle spinning (CPMAS) ^{13}C NMR spectra of humic substances. This interest has rapidly developed because humic substances are not readily soluble in organic solvents and the CPMAS ^{13}C NMR spectra provide a quantitative measurement of the aromatic, carboxylic acid and other groups in humic substances. Of all the spectroscopic methods, it is the only one for which a complete analysis and interpretation of the entire spectrum is normally expected. Although larger amounts of sample are needed than for mass spectroscopy, NMR is non-destructive, and with modern instruments good data may be obtained from samples weighing less than a milligram.

Diffuse Reflectance Infra Red Spectroscopy (DRIFT)

Transmission infrared spectroscopy, either dispersive (IR) or Fourier transform (FTIR) has been widely used for the characterization of complex organic molecules such as humic substances (Schnitzer, 1971; Stevenson and Goh, 1971, 1974; Stevenson, 1982; Inbar et al., 1989). Another analytical technique for IR spectroscopy of powders is diffuse reflectance using infrared Fourier transform (DRIFT). According to Painter et al., (1985) and Niemeyer et al., (1992), this technique offers several advantages over transmission infrared

spectroscopy: (i) a simple sample preparation procedure; (ii) insensitivity to water associated with the sample and enhanced resolution; (iii) high resolution of the spectra because of reduction in the sensitivity towards light scattering; and (iv) a more reliable method for quantitative estimations of groups.

The technique and theory of DRIFT are well documented and reviewed (Griffiths and Fuller, 1982; Painter et al., 1985; Childers and Palmer, 1986). In practice diffuse reflectance measurement involves the collection of radiation reflected from the sample by mirrors in an integrating sphere with a definitive fraction of the reflected radiation directed through an exit slit of the detector. Powders and rough surface solids reflect light diffusely (in addition to specular reflectance) (Baes and Bloom, 1989). The diffuse reflection spectra are strongly dependent upon the conditions under which they are recorded. These spectra can exhibit both absorbance and reflectance features due to contributions from transmission, internal and specular reflectance components as well as scattering phenomena in the collected radiation (Griffiths and Olinger, 2002). Diffuse reflectance infrared Fourier transform detects molecular vibrations and is useful for functional group analysis and for identification of molecular structures of SOM (Stevenson, 1994). DRIFT spectroscopy is nowadays considered one of the most sensitive IR techniques for analysis of humic substances from different origins (Inbar et al., 1989; Haberhauver and Gerzabek, 1999; Francioso et al., 2001).

Auxin action investigation

Auxin Inhibitors

In this PhD thesis, three different auxin inhibitors, NOA, TIBA and PCIB were used to prove the auxin-like activity of humic substances. They belong to two distinct classes of anti-auxin, a first one affecting the auxin transport and a second one competing for the specific auxin-binding site.

As it was mentioned before, many aspects of plant development, including patterning and development, are largely dependent on the asymmetric distribution of the plant signalling molecule auxin. Auxin transport inhibitors (ATIs), which interfere with directional auxin transport, have been essential tools in formulating this concept. However, despite the use of ATIs in plant research for many decades, the mechanism of ATI action has remained

largely elusive. According to their target, ATI can be divides in auxin influx inhibitors (1-Napthoxyacetic acid, 1-NOA) and auxin efflux inhibitors (Triiodobenzoic acid, TIBA).
TIBA has been recently demonstrated to dramatically interfere with actin dynamics in plants, providing a mechanism by which these drugs disrupt vesicle subcellular trafficking, including that of PIN auxin efflux carrier (Dhonukshea et al., 2008).
Despite auxin transport inhibitors, the auxin action can be blocked exploiting the competition for its binding site. p-Chlorophenoxyisobutyric acid (PCIB) is one of the most often used inhibitors of auxin and is assumed to inhibit auxin action by competing with auxin for the binding site of the auxin receptor (Oono et al., 2003). Although PCIB and auxin are structurally similar, the mode of action for PCIB has not been completely defined.

Visualization of auxin distribution *in vivo*

DR5 synthetic element

The recent availability of the DR5::GUS reporter construct to visualize regions of auxin accumulation allows plant biologists opportunities to test old and new questions about the roles of auxin in developmental processes.
DR5 is a synthetic auxin-responsive promoter that consists of a 7 tandem repeats of an auxin-responsive TGTCTC element from the soybean *GH3* promoter and a 35S minimal promoter fused to the GUS-encoding reporter gene (Liu et al., 1994; Ulmasov et al., 1995).
In *Arabidopsis*, DR5::GUS is sensitive to auxin in a dosage dependent manner, and its activity reflects endogenous auxin levels in plant parts under the staining conditions typically used (Sabatini et al., 1999; Casimiro et al., 2001, Nakamura et al., 2003).
In this thesis the expression pattern of the DR5::GUS gene is used to investigate the auxinic activity of HS during lateral root development in *Arabidopsis* seedlings treated with HS and IAA.

aux1 mutant

The original *aux1* mutant was isolated in a screen for auxin resistance (Hobbie et al., 2004). Additional mutant alleles have been recovered in screens for altered growth orientation and resistance to the plant hormone ethylene. All of the alleles have a similar phenotype. Using an *aux1* mutant tagged with the transferred Agrobacterium T-DNA, Bennett et al., (1996)

were able to clone the gene and show that it encodes a protein with sequence similarity to a family of auxin amino acid permeases (AAAP) family of proton-driven transporters identified in plants and fungi.

During the last several years, the characterization of AUX1 has proceeded to the molecular level. The morphological effects of the *aux1* mutations are restricted to the root. The number of lateral roots is reduced (Marchant et al, 2002), and root gravitropism abolished (Marchant et al., 1999), in *aux1* mutant plants (Hobbie et al., 1995). The *aux1* mutant tissues also display a modest reduction in auxin-induced gene expression (Timpte, 1995).

Identification of pathways involved in HS response

Functional Genomics

Functional genomics is a rapidly developing technology that allows the identification of large sets of genes that influence a particular biological process. Because most plant processes are mediated by large numbers of genes, the technology must necessarily be focused on large scale profiling of genes, mRNAs, proteins and metabolites that participate in cellular processes.

cDNA-AFLP

The cDNA-AFLP technique is based on the same molecular principles of the AFLP (Amplified Fragment Length Polymorphism; Vos et al., 1995; Bachem et al., 1996, 1998), but is applied on double-strand cDNA instead of the genomic DNA (Figure 11). This technology is well known as a tool for transcriptome profiling and gene cloning, and has proved to be a very popular procedure because of its ability to display several transcripts simultaneously and to detect differentially expressed genes. Vuylsteke et al., (2006) have recently demonstrated that cDNA-AFLP is also a powerful genome-scale transcript profiling technique to generate quantitative gene expression profiles for eQTL mapping.

Transcriptome differential profiling of genotypes with antagonistic phenotypes (i.e. mutant vs. wild type) is theoretically one of the most useful strategies for identifying and isolating genes underlying the related metabolic pathway. In the area of plant research, such a method is broadly diffused and relies on synthesizing cDNA subsets from roots, stems, leaves, flowers and/or fruits at specific developmental stages of individuals sharing the

same genetic background but arbitrarily selected for interesting morphological traits or experimentally subjected to different treatments and then screening for differentially expressed transcripts (between selected or treated samples and controls) (Albertini et al., 2004; Albertini et al., 2005; Citterio et al., 2005 2006; Craciun et al., 2006; Quaggiotti et al., 2007; Huang et al., 2008, Polesani et al., 2008). This approach makes the assaying of a large set of mRNA-derived fragments possible and increases the reliability of amplification-based transcriptome analysis.

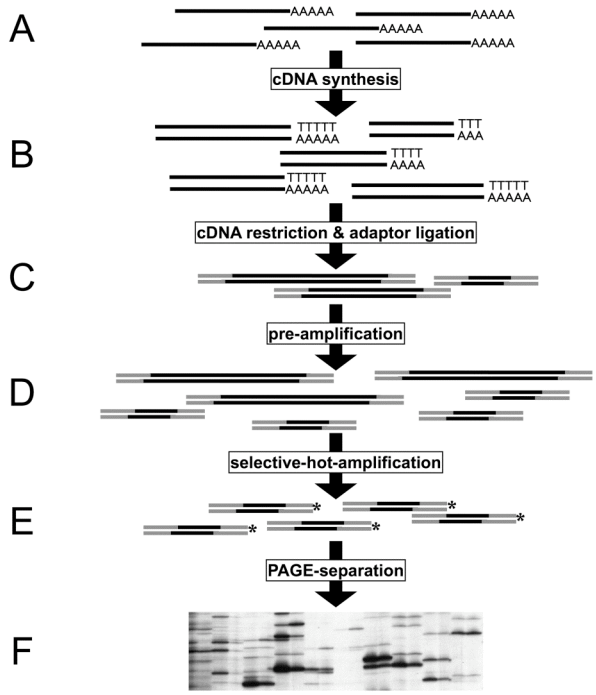


Figure 11 - Schematic representation of the cDNA-AFLP technique. The messenger RNAs (A) are used to synthesize double-strand cDNAs (B). The cDNAs containing the specific restriction sites are digested with two different endonucleases and ligated to specific adaptors (C), to be successively amplified with PCR (D). In the following step, primer pairs with selective nucleotides are used to selectively amplify the fragment pool in D. Such primers are conjugated to radioactive phosphorus (usually ^{33}P), so that the amplicons (E) can be visualized after separation by polyacrylamide gel electrophoresis (PAGE) and autoradiography (F).

Real-time PCR

Among all the methods available for the quantification of specific gene transcripts, PCR is the most sensitive method that can discriminate closely related mRNAs, simply by using opportunely designed specific primers. It is technically simple and sensitive but it is difficult to get reliable quantitative results using conventional PCR. Methods such as competitive PCR were set up to make the method more quantitative but they are very cumbersome and time-consuming to perform. These problems were overcome with the

development of real-time PCR (or reverse transcriptase real-time PCR). In contrast to traditional reverse transcriptase-PCR and analysis by agarose gels, real-time PCR gives quantitative results. Real Time PCR is a kinetic approach in which you look at the reaction in the early stages while it is still linear and when it would not be detectable with ethidium bromide staining.

The technique is based on the detection of the fluorescence produced by a reporter molecule, which increases as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (SYBR Green) or sequence specific probes (TaqMan Probes). In real time PCR the fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase. If a graph is drawn between the log of the starting amount of template and the corresponding increase the fluorescence of the reporter dye fluorescence during real time PCR, a linear relationship is observed.

Several practical advices have to be taken into account when approaching a real-time PCR-based quantification, both concerning the experiment itself and the elaboration of the data acquired during the PCR run (Udvardi, 2008). In order to avoid the presence of aspecific amplicons, a melting point analysis of the PCR products has to be performed since there may be aspecific amplicons invalidating the target quantification. After real time PCR amplification, the PCR machine can be programmed to do a dissociation curve, in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases. The software plots the derivative of fluorescence on the Y-axis versus the temperature on the X-axis, showing a peak at the melting temperature (Fig 12a). According to the melting curve analysis, a further step for fluorescence data collection has to be added in the real-time PCR program. The temperature of this quantification step should be chosen in correspondence of the beginning (just before) of the denaturation of the specific amplicons, when the fluorescent signal starts to decrease in the dissociation curve, and all the non-specific amplicons and eventual primer dimers are completely denatured. Ideally, no aspecific signal should be detected at higher temperature. When all the

previously described parameters have been checked and the PCR program and the mixture have been set-up, the experiments can start.

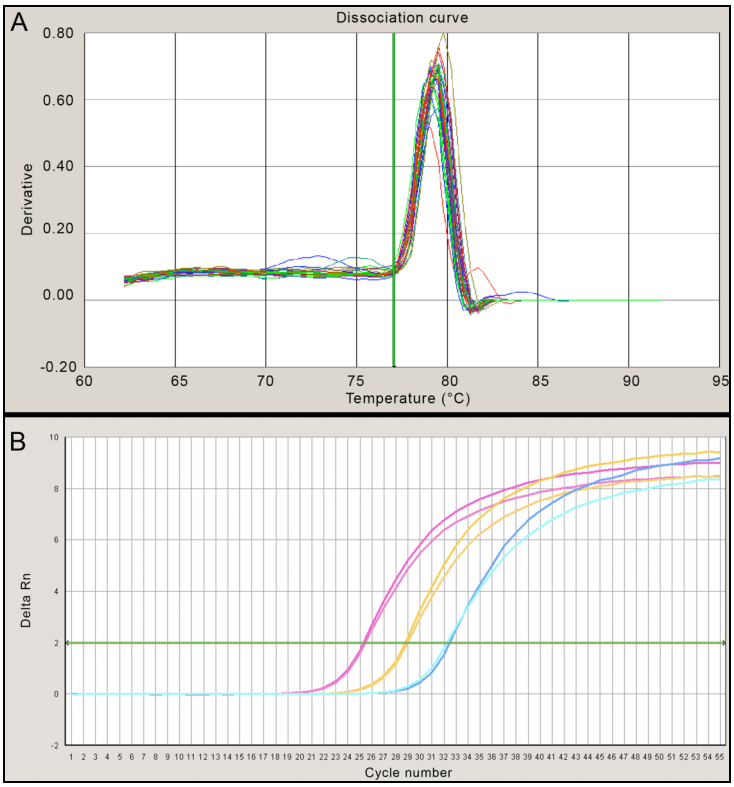


Figure 12 – (**a**) Dissociation curve of real-time PCR products, reported as a derivative of fluorescence. The green vertical line marks the temperature at which the double strand amplicons start to denature with a consequent decrease of fluorescence. A peak is reached when the PCR products are completely denatured. **(b)** Serial dilutions of template cDNA for the calculation of efficiency according to Pfaffl (2001). The green horizontal line indicates the arbitrary threshold, in correspondence of the exponential phase, at which Ct are collected.

According to Pfaffl (2001) method, the first experimental phase includes the assessment of the PCR efficiency for each primer combination used in the quantification assays, so that the final Mean Expression values can be adjusted according to the this parameter and normalized according to the expression of a housekeeping gene. In this way, the expression values of different genes can be compared each other. To do the efficiency curve, serial dilutions of cDNA (Figure 12b) should be used as templates to calculate the PCR yield at different concentrations and the Ct values plotted versus the cDNA amounts. According to the slope of the linear plot, the efficiency of amplification is calculated. The following phase consists in the experimental quantification of the sample templates, for which at least three technical replicates have to be performed. After each PCR run, a dissociation step is performed, to check the specificity of the reaction. Finally, the Ct values are adjusted

according to the PCR efficiency and normalized according to the housekeeping gene (Pfaffl, 2004).

As pointed out above, the main advantage of the Pfaffl's method is that it allows to compare the expression of different genes each other (Gutierrez 2008). Finally, the main advantages of using Real-Time PCR could be summarized as follows:

- Traditional PCR is measured at End-Point (plateau), while Real-Time PCR collects data in the exponential growth phase
- An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated
- Increased dynamic range of detection
- No-post PCR processing
- Detection is capable down to a 2-fold change

The experimental approach

In the present PhD dissertation the biological activity of Humic Substances was studied, focusing at first on the auxinic activity of these substances and then on the global plant response to HS.

In chapter 2 a combination of genetic and molecular approaches was utilized to unravel the HS auxinic activity during initiation of lateral roots in *Arabidopsis thaliana*. To this aim HS were firstly characterized by means of DRIFT and ^{13}C CP/MAS NMR spectroscopy, and their endogenous content of indoleacetic acid was measured. DR5::GUS construct was used to detect the auxin activity induced *in vivo* during the lateral root primordia (LRP) development in response to IAA and HS, in both wild type (Col-0) and *aux1* mutant background. Moreover the action of three different auxin inhibitors on the LRP development were tested. Real time PCR was used to quantify the expression of two *AUX/IAA* genes in seedlings treated with IAA, HS and auxin inhibitors at two time points.

In chapter 3, a transcriptomic approach was adopted to study the global plant-HS interaction. The cDNA-AFLP technique allowed the identification of more than 100 transcripts derived fragments (TDFs) differentially expressed, which were sequenced and then sorted into functional categories according to their gene ontology annotation. The expression profiles of the identify HS-related transcripts were validated by means of Real-

Time PCR.

Finally, in chapter 4 some general conclusions are reported along with the possible future developments and implications of the information herein acquired.

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Chapter 2 – Humic substances induce lateral root formation and the expression of the auxin-responsive *IAA19* gene and *DR5* synthetic element in *Arabidopsis*

Abstract

Humic substances (HS) have positive effects on plant physiology, but the molecular mechanisms underlying these events are only partially known. HS exert an auxin-like activity, but data supporting this hypothesis is under debate. In this paper, to investigate the auxin-like activity of HS, we studied their biological effect on lateral root initiation in *Arabidopsis thaliana*. To this aim we have firstly characterized HS by means of DRIFT and ^{13}C CP/MAS NMR spectroscopy, and measured their endogenous content of indoleacetic acid. Then we have utilized a combination of genetic and molecular approaches to unravel their auxin activity in the initiation of lateral roots. The data obtained by using specific inhibitors of auxin transport or action showed that HS induce lateral root formation mostly through their "auxin activity". These findings were further supported by the fact that the HS used in this study activated the auxin synthetic reporter *DR5::GUS* and enhanced the transcription of the early auxin responsive gene *IAA19*. The presence of functional groups (COOH) are responsible for part of the measured HS biological activity.

Introduction

Humic substances (HS) are recognised as a key component of soil fertility properties since they control the chemical and biological properties of the rhizosphere (Nardi et al., 2005). According to some authors, HS present a random polymeric, amorphous structure formed by poly-aromatic building blocks bridged to each other by ester, ether and C links and carrying variable proportions of carboxyl, hydroxyl, amino and other hydrophilic groups (Andreux 1996). Other authors support a new theory which consider HS to be a supra-molecular association of heterogeneous molecules held together by hydrophobic interactions (van der Waals, π-π, ion-dipole) and hydrogen bonds (Piccolo 2001; Sutton and Sposito 2005).

Besides the controversy about the structure of HS there is also a debate on their functional properties on plant development and physiology. Since the 1980s it has been hypothesized that the positive effects of HS on plant metabolism may depend on the uptake of some macro and micronutrients (Vaughan and Ord 1981; Clapp et al., 2001; Chen et al., 2004; Pinton et al., 2007). However, the hypothesis that they may interact with the root cells by inducing endogenous activities, independently on their uptake, could not be excluded. Furthermore, several studies have hypothesized that physiological mechanisms through which the HS exert their effects may depend on hormones and, in particular, on the presence of auxin or auxin-like components in their structure (Nardi et al., 2002 and references therein). Such a hypothesis is partially based on results that allowed the immunological or spectrometric identification of indoleacetic acid (IAA) in a number of HS isolated from different sources: earthworm faeces (Muscolo et al., 1998), forest soils (Pizzeghello et al., 2001) and compost (Canellas et al., 2002), and on data obtained in *Nicotiana plumbaginifolia* by using two inhibitors of auxin (TIBA=2,3,5-triiodobenzoic acid and PCIB=4-chlorophenoxy-isobutyric acid) (Nardi et al., 1994).

This hypothesis is also supported by other reports showing a positive effect of HS on specific targets of auxin action. This is the case of the Mha2 maize isoform of H^+-ATPase that appears to be strongly stimulated at the transcriptional level in response to auxin (Frias et al., 1996) and that showed a significant up-regulation of its mRNA abundance in roots of maize seedlings treated for 48 h with earthworm low molecular size HS (Quaggiotti et al.,

2004). Furthermore, another study (Russel et al., 2006) conducted on pea by using two different molecular weight fractions of earthworm faeces HS, highlighted an auxin-like effect of both fractions on stomatal opening as influenced by phospholipase A2, that is considered to be involved in auxin-mediated signalling (reviewed by Macdonald 1997; Scherer 2002). However, the measured effects did not always correlate with the amount of IAA detected in the humic acids, but seemed to be also connected with the structural complexity of the HS utilized (Muscolo et al., 2007). Although there has been a noteworthy interest towards different auxin compounds or molecules that might either mimic the action or stimulate the *in planta* endogenous metabolism of auxin very little has been done on how the HS induce lateral root formation.

In order to shed light on the auxin-like activity of HS, we studied their biological effect on lateral root initiation and showed that the HS utilized induce lateral root formation in *Arabidopsis thaliana*. Root development is a complex process under the regulatory control of a number of both exogenous (i.e. nutrient availability, soil characteristics) and/or endogenous (developmental and hormonal) factors. However, lateral root formation has been shown to rely on auxins as a primary dominant signal in promoting the mitotic activity of pericycle cells in the process of *primordia* initiation (reviewed by De Smet et al., 2006; Casimiro et al., 2003). In fact, mutations in genes involved in auxin polar transport or action, such as *AUX1* (Marchant et al., 2002) and *TIR1* (Ruegger et al., 1998), or treatments with chemicals inhibiting the same processes, such as 1-NOA (Parry et al., 2001) and PCIB (Oono et al., 2003) respectively, result in a reduced number of lateral root *primordia* (LRP). HS, extracted from worm coprolytes, were analysed by means of Diffuse reflectance infrared Fourier transforms (DRIFT) spectroscopy, ^{13}C CP/MAS NMR spectroscopy, and their endogenous content of indoleacetic acid was quantified by using immunoassay method.

To further unravel the auxin activity of humic substances in the initiation of lateral roots we have employed the widely used auxin reporter DR5::GUS (Ulmasov et al., 1997), to visualize auxin responses in roots (Sabatini et al., 1999; Benkova et al., 2003) and to characterize the distribution of lateral root *primordia* stages, in both wild type (Col-0) and *aux1* mutant background. In addition we have evaluated the transcription of the known early auxin responsive genes *IAA5* and *IAA19* (Oono et al., 2003; Goda et al., 2002;

Nakamura et al., 2003; Yamazoe et al., 2005) in parallel treatments with humic substances and comparable IAA concentrations.

Materials and methods

Earthworm culture conditions

The faeces of Nicodrilus [=*Allolobophora* (Eisen)=*Aporrectodea* (Oerley)] *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) were collected from the surface of the uncultivated couchgrass (*Agropyron repens* L.) fields at the College of Agriculture farm (University of Padua) as already described in Muscolo et al., (1999). The soil was classified as Calcaric Cambisol (FAO-ISSDS, 1999).

Extraction of the HS humic substances

Humic compounds were extracted from the faeces of Nicodrilus (=*Allolobophora* (Eisen)=*Aporrectodea* (Oerley) *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) using 0.1 N KOH as already described in Muscolo et al., (1999). The extract was dialyzed using a 14 kDa cut-off Visking membrane tubing (Medicell, UK) against distilled water. Subsequently, the extract was desalted on ion exchange Amberlite IR-120 (H+ form) (Stevenson, 1994) and then freeze-dried. The nominal molecular size of HS corresponded to high molecular fraction. The C and N contents were measured by using an elemental analyzer (Thermo Electron mod. EA 1110, Waltham, MA, USA).

The concentration of COOH groups was determined by dissolving 12 mg of the freeze-dried HS in 20 mL of Milli-Q Millipore water containing 0.05 M NaCl to keep the ionic strength constant. The pH was adjusted to 3 adding about 1 mL of 0.05 M HCl. The solutions were titrated to pH 10.5 with 0.05 M NaOH using a VIT 90 Titrator Radiometer (Radiometer, Copenhagen, DK). The potentiometric titrations were carried out in triplicate at 25 °C, under N_2 flow and the delivery range was 10 μL min^{-1} (± 0.01). The first derivative method was performed to determine the concentration of COOH groups.

Diffuse reflectance infrared Fourier transforms (DRIFT) Spectroscopy

The spectrum was recorded with a Bruker TENSOR series FT-IR Spectrophotometer (Ettlingen, Germany) equipped with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT). Spectrum was collected as Kubelka-Munk units using KBr (Aldrich Chemical Co. Milwaukee, WI) as the background reference. Spectrum was collected from 4000 to 400 cm^{-1} and averaged over 100 scans (resolution ± 4 cm^{-1}). Analysis of spectral data were performed with Grams/386 spectral software (Galactic Industries, Salem, NH). Spectral sections from 1850 to 500 cm^{-1} were baseline-corrected to an absorbance value of 0.00 at 1850 cm^{-1}.

CP-MAS ^{13}C NMR spectroscopy

Solid state ^{13}C NMR spectrum was obtained using a Bruker AC200 spectrometer equipped for solid state analysis and operating at 50.26 MHz. Samples were spun in the range 3000-7000 Hz in 7 mm diameter zirconia rotors with Kel-F caps. The ^{13}C SPE MAS NMR (SPE=single pulse experiment) spectrum was obtained with high power proton decoupling during acquisition, 30 seconds Relaxing Delay, and processed with a 10 Hz exponential line broadening. ^{13}C chemical shifts were externally referenced to solid sodium 3-(trimethyl-silyl)-1-propane sulfonate at 0 ppm. Magic angle conditions were adjusted by observing ^{79}Br spinning side bands pattern in a rotor containing 5% of KBr (Frye and Maciel 1982).

The following resonance intervals are generally attributed to different carbons (Conte et al., 2007): 220–162 ppm (carbonyls of ketones, quinones, aldehydes and carboxyls), 162–108 ppm (aromatic carbons), 108–80 ppm (anomeric carbons), 80–50 ppm (C–O systems, such as alcohols and ethers), 50–35 ppm (C–N groups and complex aliphatic carbons), 35–0 ppm (alkyl cfrbons).

Indolacetic acid content of HS determination

The quantitative determination of IAA in HS was made by an enzyme linked immuno-sorbent assay (ELISA) (Phytodetek-IAA, Sigma, St. Louis, MO). For ELISA test an anti-IAA monoclonal antibody was utilised that allowed sensitive detection of IAA in the range of 0.05-100 picomoles. The competitive antibody binding method was adopted to measure

concentrations of IAA in HS extracts. IAA labelled with alkaline phosphatase (tracer) was added to antibody coated microwells along with the sample, which had been pretreated with diazomethane to convert the acid to its methyl ester form competing with the tracer for antibody binding sites.

Operationally, tracer and standard solutions were prepared following the manufacturer's instructions (Phytodetek-IAA, Sigma, St. Louis, MO). To each well 100 μL of standard IAA concentration or of serial dilutions of HS and 100 μL of diluted tracer were added. For the standard curve a progression of 500, 100, 50, 20, 5, 1, 0.1, and 0.02 pmol methylated IAA 100 μL^{-1} was used whereas for the HS the progression was 15, 25, 35, 50 and 75 μgC 100 μL^{-1}. After an incubation step at 4°C for 3 h, the wells were decanted and unbound tracer was washed away by adding 200 μL of wash solution before adding 200 μL of substrate solution for colorimetric detection. After 60-mins at 37°C, 50 μL of stop reagent were added to each well and the colour absorbance was read at 405 nm using a 450 Biorad microplater reader (Biorad, Hercules, CA). Both the standard curve and the progression of HS dilutions have been made twice.

Plant growth and treatments

Col-0 and aux1 transgenic lines carrying DR5::GUS construct were provided by Prof. Klaus Palme (Institute for Biology II, Albert-Ludwigs-University Freiburg, Germany). Seeds of *Arabidopsis thaliana* Columbia ecotype (Col-0) and DR5::GUS transgenic plants (Ulmasov et al., 1997) were surface sterilised, sown on solid AM medium and germinated as described by Müller et al., (1998). Usually, between 30 and 50 seedlings were used per sample in each experiment that was repeated three times.

The number of lateral roots per mm of the main root length was scored in all experiments on one- week-old DR5::GUS transgenic plants, for easier visualisation of *primordia* at all developmental stages, after the following treatments. For the HS dose-response curve, seedlings were treated for 24 h with 0.1, 0.5 and 1.0 mg C l^{-1} of HS. For time-course experiments, plants were incubated for 16, 24 or 48 h, with 1.0 mg C l^{-1} of HS or with 34 nM IAA, corresponding to the content of IAA measured in this humic fraction, or with sterile mQ water as a control. For inhibitor treatments, plants were kept for 24 h in 1.0 mg C l^{-1} HS, 34 nM IAA or mQ water (control) in the presence or absence of 50 μM 1-NOA,

50 μM TIBA or 50 μM PCIB. Treatment of *AUX1*-DR5::GUS one week old seedlings was performed with 1.0 mg C l^{-1} HS or 34 nM IAA for 24 h. Histochemical GUS staining was performed as described by Jefferson et al., (1987).

Lateral roots *primordia* at different stages of development (Casimiro et al., 2003; Malamy and Benfey, 1997) were observed at 4.5 x on a SZM-2 Optika microscopy (Optika Microscopes, Italy) and the number of lateral roots in relation to the main root length counted at 10x on an optical microscope (Olympus BX60, Japan) equipped with a C-3040 Olympus digital Camera (Olympus, Japan). Differences among means were calculated by the Kruskal-Wallis H-test (Gibbons, 1976) and a *P* value of less than 0.05 was considered statistically significant. Regression analysis was used to analyze the HS dose-response curve. All statistical analyses were conducted using SPSS for Windows software version 11.0 (SPSS, Chicago, IL).

RNA extraction and cDNA synthesis

Total RNA was isolated using the "NucleoSpin RNA Plant" kit (Macherey-Nagel, Dure, Germany) following the protocol provided by the manufacturer. The concentration of RNA isolated was calculated from the A_{260} in distilled water and the quality assayed by electrophoresis of a 1 μg aliquot on Tris-acetate/agarose gel. First-strand cDNA was synthesised from 1 mg of total RNA, after Dnase I treatment (Promega, Milano, Italy), using 200 u of MMLV Reverse Transcriptase (Promega, Milano, Italy) and oligodT as a primer, in 20 μl reactions, as described in Sambrook et al., (1989). Following the same procedure RNA samples were also processed on which the RT step was omitted to rule out amplification from contaminating genomic DNA.

Real-time PCR

Real-time PCR relative quantification was performed in a total volume of 10 μL using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA) with 3 pmol of every primer and 2 μL of a 1:10 dilution of cDNA. The gene-specific primers for *IAA5* (At1g15580) and *IAA19* (At3g15540), listed in Table 1, were designed with Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the instructions reported in the SYBR® Green PCR Master Mix protocol

(Applied Biosystems, Branchburg, NJ, USA). The specificity of amplification was assessed by subsequent subcloning and sequencing of the PCR products obtained under the same conditions adopted in the real-time experiments. The reaction mixture was amplified in a 7500 Real-Time PCR System (Applied Biosystems, Branchburg, NJ, USA) under the conditions described in Botton et al., (2008). After every PCR cycle, a data acquisition step was introduced to record the fluorescent signals at the optimum temperature, previously determined by melting point analysis of every specific amplification product. Data were acquired, elaborated and exported with the software SDS Sequence Detection System v1.2 (Applied Biosystems, Branchburg, NJ, USA), whereas all the final calculations were carried out with the automated spreadsheet Q-Gene designed by Simon (2003), using the modifications of the delta Ct method suggested by Pfaffl (2001). Two genes (*ACT2,* At3g18780 and *18S*, At2g01010*,*), giving similar expression values, were selected as housekeeping. Gene expression values were normalized to the *18S* gene and reported as arbitrary units (A.U.) of Mean Normalized Expression (Pfaffl, 2001), using the equation 2 of Q-Gene. The correct size of the amplification products was checked by running each reaction in a 1.5% agarose gel stained with ethidium bromide and viewed under UV light.

Table 1 - Target genes for analysis of expression profile. For each couple of primers the annealing temperature (Ta), the number of cycles of the PCR reaction and the size of the corresponding amplification products are also reported.

Gene ID	Primer	Sequence (5'-3')	Ta (°C)	cycles	bp
At1g15580	IAA5	for AGATATCGTCGTCTCCGGTG rev GCCGAAGCAAGATCTTGGTA	61	27	251
At3g15540	IAA19	for GAGCATGGATGGTGTGCCTTAT rev TTCGCAGTTGTCACCATCTTTC	61	27	141
At3g18780	ACT2	for AACATTGTGCTCAGTGGTGG rev TCATCATACTCGGCCTTGG	58	25	206

Results

Characterization of humic substances

Elemental composition of HS showed the following values for total carbon (55.97% ± 0.10) and nitrogen (4.40% ± 0.16), and for total acidity (424 ± 10 mmol/100g).

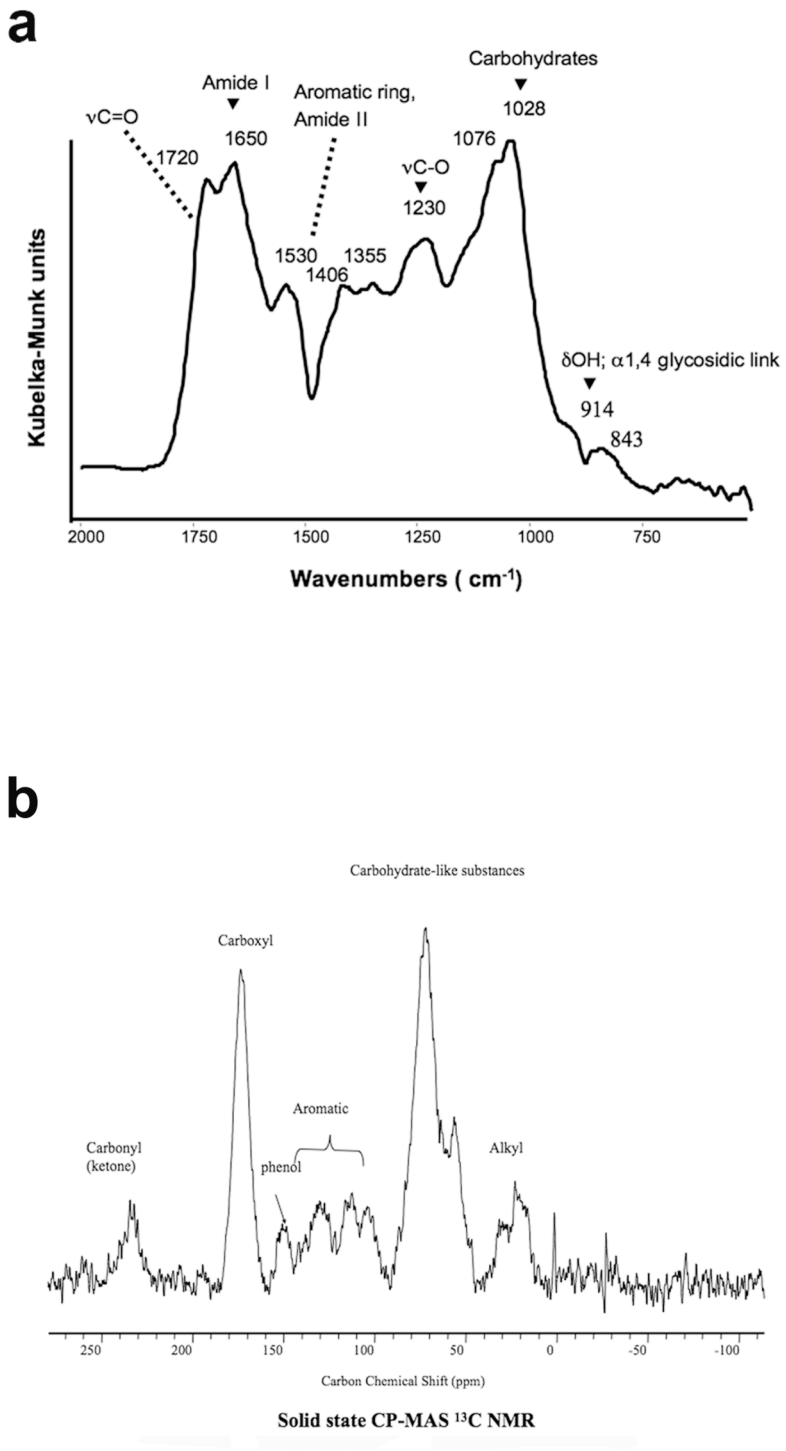

Figure 1 - DRIFT spectrum of HS fraction extracted from earthworm faeces **(a)**. The bands correspond to undissociated carboxylic groups (1720 cm^{-1}); stretching vibration in amide I (1650 cm^{-1}); amide II and C=C vibrations in aromatic rings (1514 cm^{-1}); C-O stretching vibrations of alcohol and carboxyl groups (1230 cm^{-1}); and C-O stretching in carbohydrates (1100-800 cm^{-1}) In CP-MAS NMR spectrum of HS **(b)** the main resonance intervals are attributed to different carbons: 220–162 ppm (carbonyls of ketones, quinones, aldehydes and carboxyls), 162–108 ppm (aromatic carbons), 108–80 ppm (anomeric carbons), 80–50 ppm (C–O systems, such as alcohols and ethers), 50–35 ppm (C–N groups and complex aliphatic carbons), 35–0 ppm (alkyl carbons) (Conte et al., 2007).

DRIFT spectrum (Figure 1a) was dominated by two strong and sharp bands around 1720 cm^{-1} and 1650 cm^{-1} which are characteristic of undissociated carboxyl groups stretching vibrations (Rao 1963; Niemeyer et al., 1992; Francioso et al., 1998, 2002) and to stretching vibration (C=O) in amide I, respectively. Moreover a band at 1530 cm^{-1} indicated the presence of C=C in aromatic rings vibrations from a lignin derivative. The intense band at 1230 cm^{-1} confirmed the presence of C-O stretching vibrations in phenols and carboxyl groups. Moreover, a strong signal in the region between 1100-800 cm^{-1} was unequivocally assigned to C-O stretching in carbohydrates (Bellamy 1975; Stevenson 1994).

CP-MAS NMR spectrum of HS (Figure 1b) showed features very similar to that extracted from earthworm compost (Canellas et al., 2002). Similarly to Canellas et al., (2002) the region between 0-40 ppm assigned to CH_2 in alkyl chains and terminal methyl groups was not well resolved indicating a low content in aliphatic substances. In our sample the resonance between 40 -60 ppm, assigned to C bound to N in amino acids appeared higher that to that of Canellas et al., (2002). The presence of C-N groups was also visible in the vibration of amide I (Figure 1b). The prominent signal around 70 ppm can be mainly attributed to C-O in polysaccharides while the weak resonance around 100 ppm suggested the presence of anomeric C. The aromatic C resonanting between -130 -160 ppm was not well resolved. A prominent signal at around 170 ppm was assigned to quaternary carbon in carboxylic groups. The potentiometric titration supported the high amount in COOH groups found in this sample. Finally, a broad and weak resonance at 240 ppm also suggested the presence of C in ketone.

Humic substances IAA content determination

The quantitative determination of IAA in the HS was made by an enzyme linked immuno-sorbent assay (ELISA). An IAA standard curve obtained by plotting the % Binding ($\%B/B_0$) versus the nmol methyl-Indole-3-acetate concentration was used for quantitation and is shown in Figure 2a. The HS response curve for IAA content determination was made in a range from 15 to 75 μgC HS (Figure 2b). Fitting the % Binding of each point of the HS curve (5 points, n=2 replicates) into the standard's equation an IAA content of 34 ± 0.31 nM was calculated to be present in 1 mg C HS L^{-1}. As far as auxin and conjugated forms of auxin are concerned, molecules such as Indole-3-acetylglycine, Indole-3-acetone, Indole-3-

acetylalanine, Indole-3-butyric acid, Indole-3-acetonitrile, Indole-3-acetamide, Indole-3-propionic acid, Indole-3-aldehyde, Indole-3-acetaldehyde display a low affinity and do not cross-react significantly with the monoclonal IAA antibody used in this study (Phytodetek-IAA, Sigma, St. Louis).

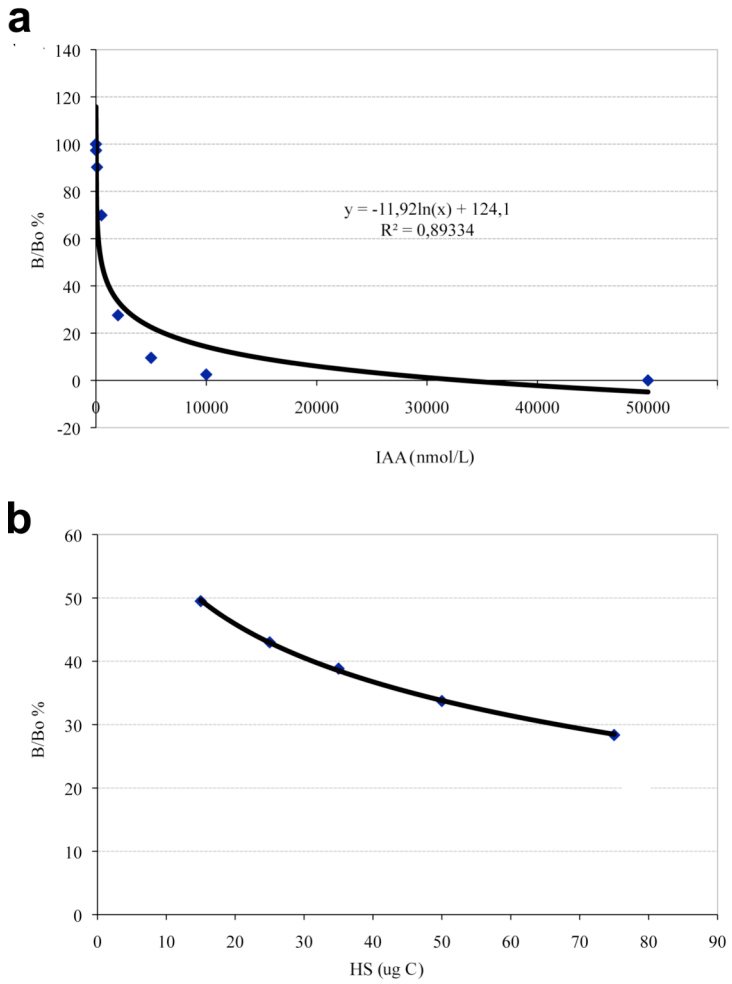

Figure 2 - Results of the quantitative determination of IAA in the HS by enzyme linked immuno-sorbent assay (ELISA). For the standard curve **(a)** a progression of 500, 100, 50, 20, 5, 1, 0.1 and 0.02 pmol methylated IAA 100µL^{-1} was used whereas for the HS dose response curve **(b)** the progression was 15, 25, 50 and 75µgC 100µL^{-1}. The intensity of the colour produced was related to the sample IAA concentration by means of standard curve.

With the exception of the Indole-3-acetylglycine which posses a cross reactivity of 57%, the cross reaction for other molecules is ranging from 5 to <0.01%. Therefore the IAA content determined on HS reflects mainly their content in free IAA.

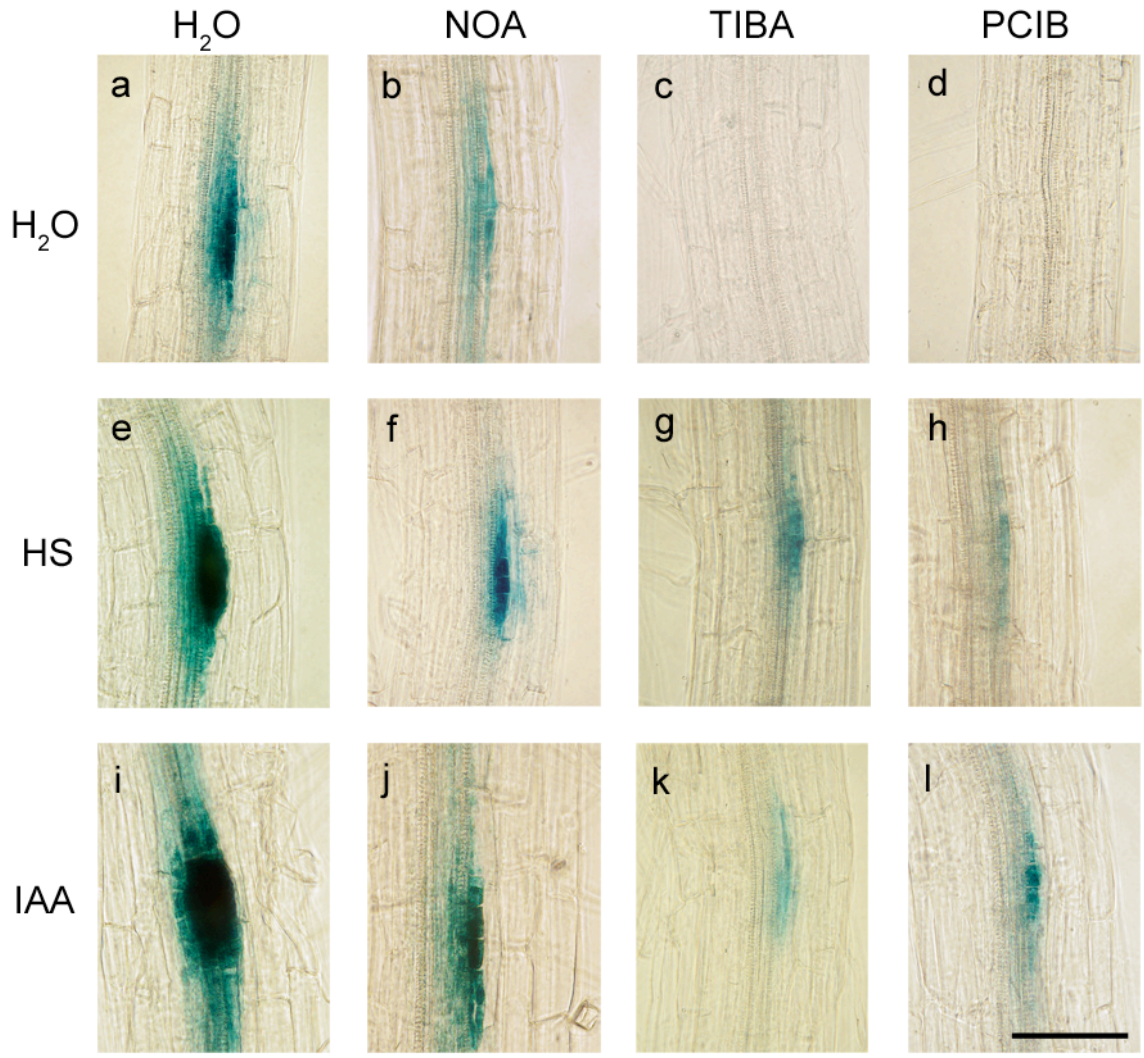

Figure 3 - Visualization of Gus activity in root of DR5::GUS transgenic plants treated with different auxin inhibitors. Plants were grown for 4 days in MS medium plates and then transferred for 24 hours in: water (**a**), 50µM NOA (**b**), 50µM TIBA (**c**), 50µM PCIB (**d**), 1.0 mg C l^{-1} HS (**e**), 50µM NOA+ 1.0 mg C l^{-1} HS (**f**), 50µM TIBA+ 1.0 mg C l^{-1} HS (**g**), 50µM PCIB + 1.0 mg C l^{-1} HS (**h**), 34 nM IAA (**i**), 50µM NOA + 34 nM IAA (**j**), 50µM TIBA+ 34 nM IAA (**k**), 50µM PCIB + 34 nM IAA (**l**). Histochemical GUS staining was performed as described by Jefferson (1987). Scale bar is 50µM.

Humic substances induce expression of the synthetic auxin reporter DR5 and lateral root development in *Arabidopsis* and rescue the lateral root phenotype of the *aux1* mutant

The effect of different concentrations of HS was estimated on the development of lateral roots in wild type plants of *Arabidopsis thaliana* and on the induction of the auxin responsive synthetic reporter DR5::GUS (Ulmasov et al., 1997). In particular the

DR5::GUS has been used as a tool to visualise auxin responses in tissues and mark auxin signalling in lateral roots *primordia* at all developmental stages (Sabatini et al., 1999; Benkova et al., 2003).

It has been also reported to be activated by auxins in a dose dependent manner (Ulmasov et al., 1997). Treatment with HS caused the activation of DR5::GUS expression in lateral roots in a comparable way to exogenously applied auxins as shown in Figure 3e and i, respectively. Consistently, this induction was sensitive to treatment with inihibitors such as PCIB (Oono et al., 2003) (Figure 3d, h, l), or of auxin uptake or transport obtained by treating plants with NOA (Parry et al., 2001)(Figure 3b, f, j) or TIBA, respectively (Figure 3c, g, k). In fact, the auxin inhibitors, even though not fully preventing DR5::GUS expression, delayed it significantly and determined a reduction of both number and intensity of positive signals in roots in response to HS or IAA treatment, as highlighted by representative pictures shown in Figure 3. Pictures shown in Figure 3 evidence also different stages of development of lateral root *primordia* for different treatments, with HS and IAA inducing an earlier lateral root initiation in comparison to the control and the inhibitor treatments. The DR5::GUS staining was also detected at the root apex level, but no significant differences were seen among samples, with the exception of the TIBA treatment which inhibited the DR5::GUS induction in most but not in all apices (Figure 4).

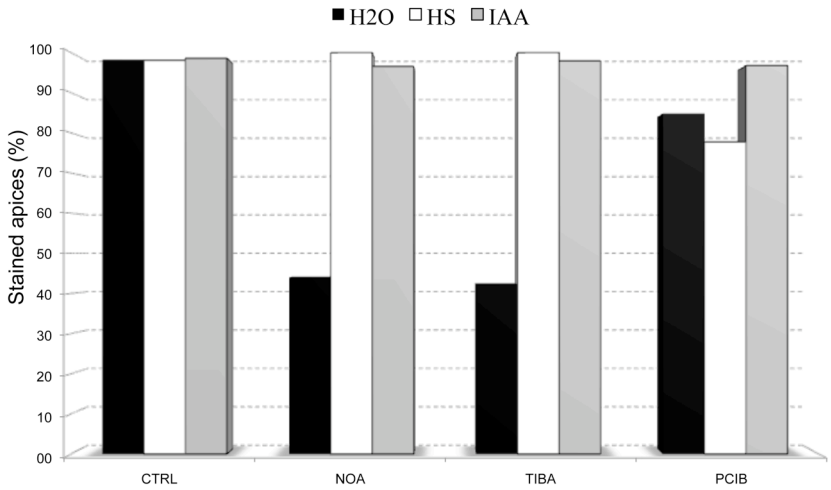

Figure 4 - Frequency of DR5::GUS staining in *Arabidopsis* apices. DR5::GUS *Arabidopsis* seedlings were grown for 4 days in MS medium plates and then treated for 24 h with water (Ctrl), 1.0 mg C l^{-1} (HS), 34 nM IAA (IAA), 50µM NOA, 50µM NOA + 1.0 mg C l^{-1} HS, 50µM NOA + 34 nM IAA, 50µM TIBA, 50µM TIBA + 1.0 mg C l^{-1} HS, 50µMTIBA + 34nM IAA, 50µM PCIB, 50µM PCIB + 1.0 mg C l^{-1} HS, 50µM PCIB + 34 nM IAA. Density was calculated as the total number of stained apices divided by the total number of seedlings analized.

Scoring of the overall DR5::GUS positive lateral root *primordia* showed a significant effect of HS on the number of lateral roots formed in seven-day-old *Arabidopis* plants. A strong and positive linear relationship (Y = a + b*X) was found between number of lateral roots and HS concentration (r= 0.97, $P<0.05$). The high value of R-squared indicated that the model as fitted explains the 93.51 per cent of variability in the dependent variable (Y) (Figure 5).

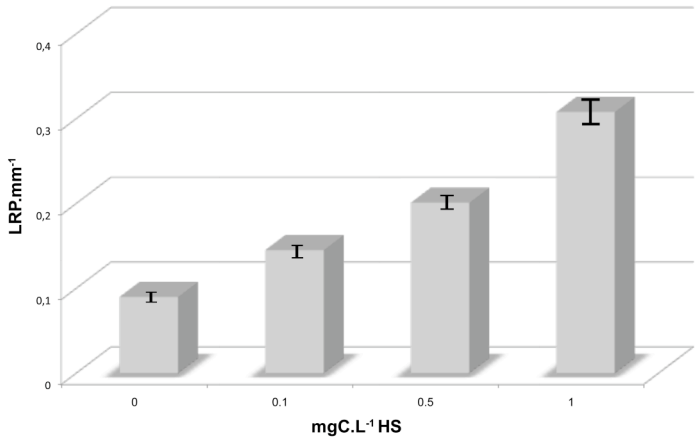

Figure 5 - Relationship between LRP (lateral root *primordia*) formation per mm of primary root in one-week-old DR5::GUS *Arabidopsis* seedlings after treatment for 48 hours with increasing concentrations of HS. Bars indicate standard error of means. A significant linear model Y= 0.094 + 0.197 X ($P<0.05$) described the relationship between variables (r = 0.97).

1mgC L^{-1} was chosen as the concentration for later experiments, since it corresponds to a relatively low IAA content (34 nM). In addition, this concentration for both treatments with HS and IAA caused no significant inhibition of primary root elongation in comparison to control plants. On the contrary, auxin inhibitors induced a limited but statistically significant reduction of root elongation in both the presence or absence of treatments with HS and IAA (Figure 6).

In order to evaluate the dynamics of lateral root induction by HS in comparison to equal concentrations of IAA (34nM) present in 1mgC L^{-1} humic substances, a time-course experiment was carried out. Results reported in Figure 7 showed a statistically ($P<0.05$) higher and faster induction of lateral root development in HS and IAA-treated plants already after 16 h incomparison to control plants, respectively. The positive effect of HS appeared to be slightly higher than that of IAA after 24 h, while after 48 h no significant differences between treatments was shown (Figure 7).

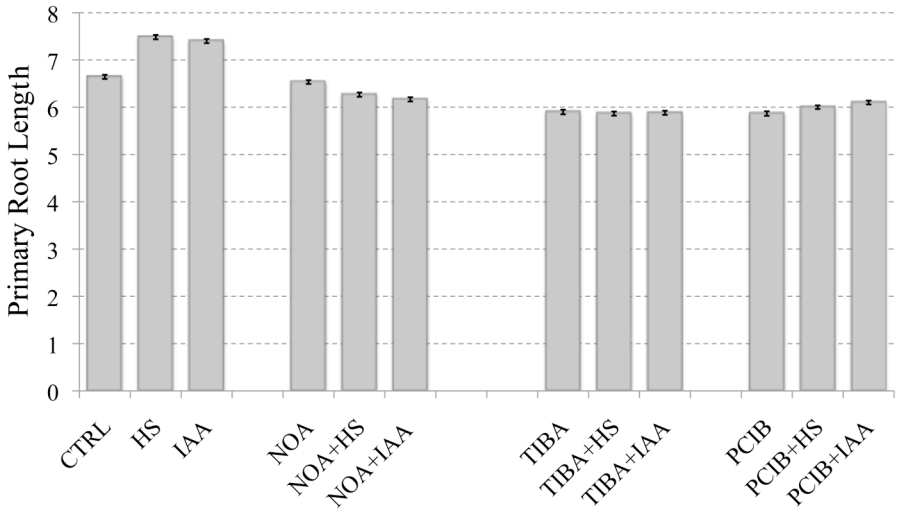

Figure 6 - *Arabidopsis* prymary root elongation in response to applied water (Ctrl), 1.0 mg C l^{-1} (HS), 34 nM IAA (IAA), 50µM NOA, 50µM NOA + 1.0 mg C l^{-1} HS, 50µM NOA + 34 nM IAA, 50µM TIBA, 50µM TIBA + 1.0 mg C l^{-1} HS, 50µMTIBA + 34nM IAA, 50µM PCIB, 50µM PCIB + 1.0 mg C l^{-1} HS, 50µM PCIB + 34 nM IAA. Data are the means ± standard error obtained from more then 30 seedlings pre treatment.

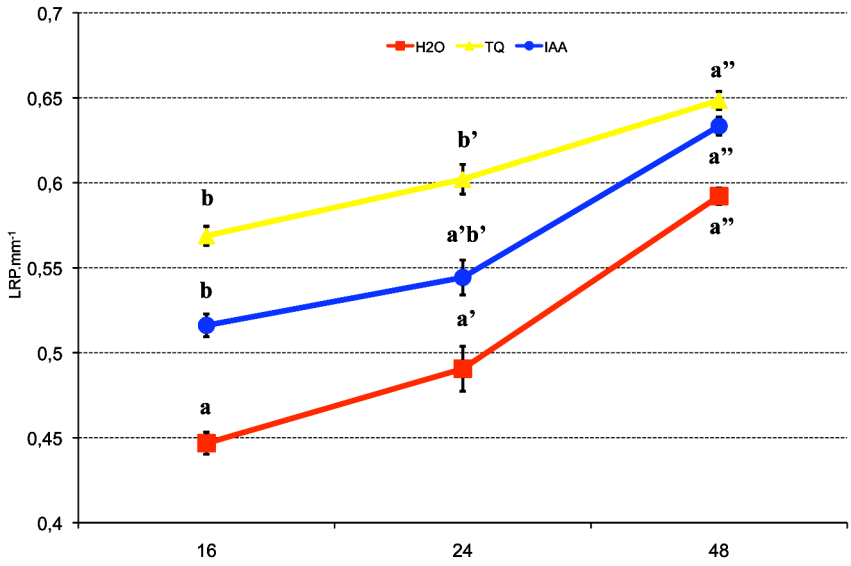

Figure 7 - Time course of LRP formation in response to IAA (34nM) or HS (1.0 mg C l^{-1}) treatments. DR5::GUS seedlings were grown for four days in MS medium plates and then treated for different time lengths (16 h, 24 h and 48 h) with HS or IAA. The number of lateral root *primordia* was counted and then divided by the total root length. The letters on the bars represent the non-significant ranges according to the SNK test ($P \leq 0.05$). Bars indicate s.e.

Interestingly, while HS treatment induced reproducibly a higher number of lateral root *primordia* than both control and IAA treated plants after 24 h, the addition of NOA at

saturating concentrations (50 µM) lead to a reduction of the number of LRPs to comparable levels in both treated and control plants (Figure 8a).

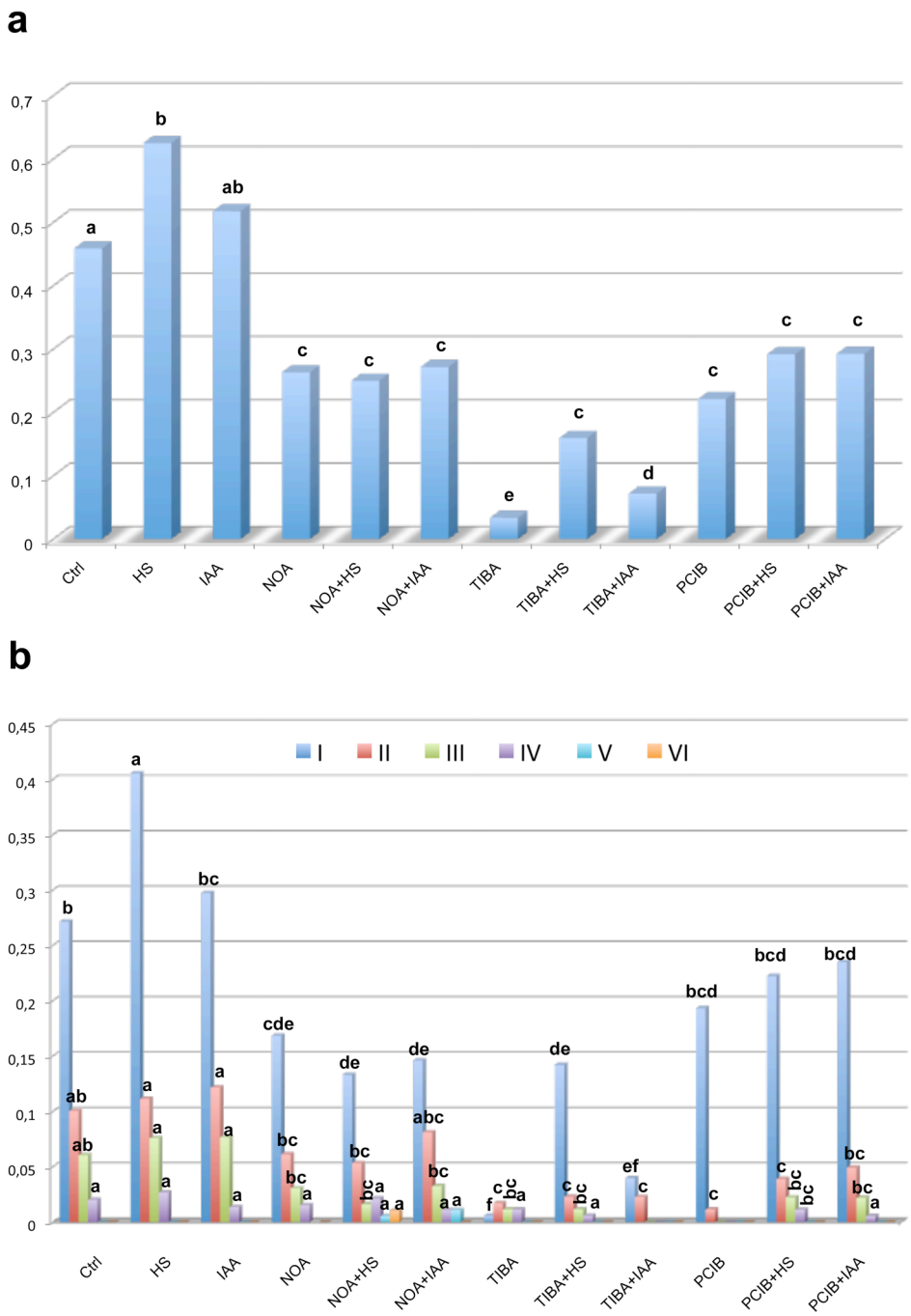

Figure 8 - Changes in the density of lateral root *primordia* in four-day-old DR5::GUS *Arabidopsis* seedlings after 24 hours of treatments with IAA or HS (1.0 mg C l^{-1}) and in response to different auxin inhibitors. Density was calculated as the total number of LRP divided by the total root length (**a**) and as the number of LRP/mm per stage of development (**b**). Developmental stages during lateral root development were identified according to the numbers and the orientations of cell divisions as described by Casimiro et al., (2003). The letters on the bars represent the non-significant ranges according to the SNK test ($P \leq 0.05$).

A similar inhibitory effect was exerted by PCIB, with no significant differences between treatments, on both HS and IAA treated plants and in comparison to untreated plants (Figure 8a). The use of the auxin transport inhibitor TIBA appeared to be the most effective in preventing the formation of lateral root *primordia*. Its action lead to a significantly different reduction of the number of LRP among treatments, resulting in a 92% decrease in control plants, 86 % in IAA treated plants, and only 74 % in HS treated plants (Figure 8a).

The mode of HS enhancement of lateral root formation was further investigated by analysing the number of the different stages (from I to VIII) (Casimiro et al., 2003; Malamy and Benfey, 1997) of lateral root *primordia* in the DR5::GUS reporter line in the presence of the different inhibitors (Figure 8b). Treatment with IAA and HS lead to a general enhancement of the number of root *primordia* ranging from stage I to stage III, in comparison to control plants. No significant differences for later stages of development appeared between treatments. On the contrary a strong induction of overall LRP formation was caused by HS, relatively to higher number of stage I *primordia* ($P<0.05$) (Figure 8b). In addition, the stimulation of stage I LRP formation triggered by HS appeared to be less sensitive to TIBA inhibition (65% reduction in HS as opposed to 85% in IAA treated plants, respectively), while the inhibition of IAA loading by treatment with NOA or inhibition of IAA action by application of PCIB lead to a comparable number of stage I *primordia*.

The auxin activity of humic substances was further investigated by analysing lateral root formation in the *aux1* mutant background. Mutation in the *AUX1* gene, encoding an auxin influx carrier, results in agravitropic roots, reduced ethylene sensitivity and in a 50% reduction of lateral root *primordia* formation (Hobbie and Estelle 1995; Marchant et al., 2002). The *aux1* lateral root initiation phenotype has been shown to be due to a suboptimal loading of auxin into the founder cells of *primordia* that can be rescued by exogenous application of nM concentrations of auxin to the plants (Marchant et al., 2002).

In our study a number of lateral roots in the *AUX1*-DR5::GUS plants treated with HS and IAA (34 nM) showed differences statistically significant with respect to wild type for the same length of time (24 h) (Figure 9a). Interestingly, the higher number of stage I LRP was induced by IAA and HS treatments in the *aux1* mutant background than control (Figure 9b).

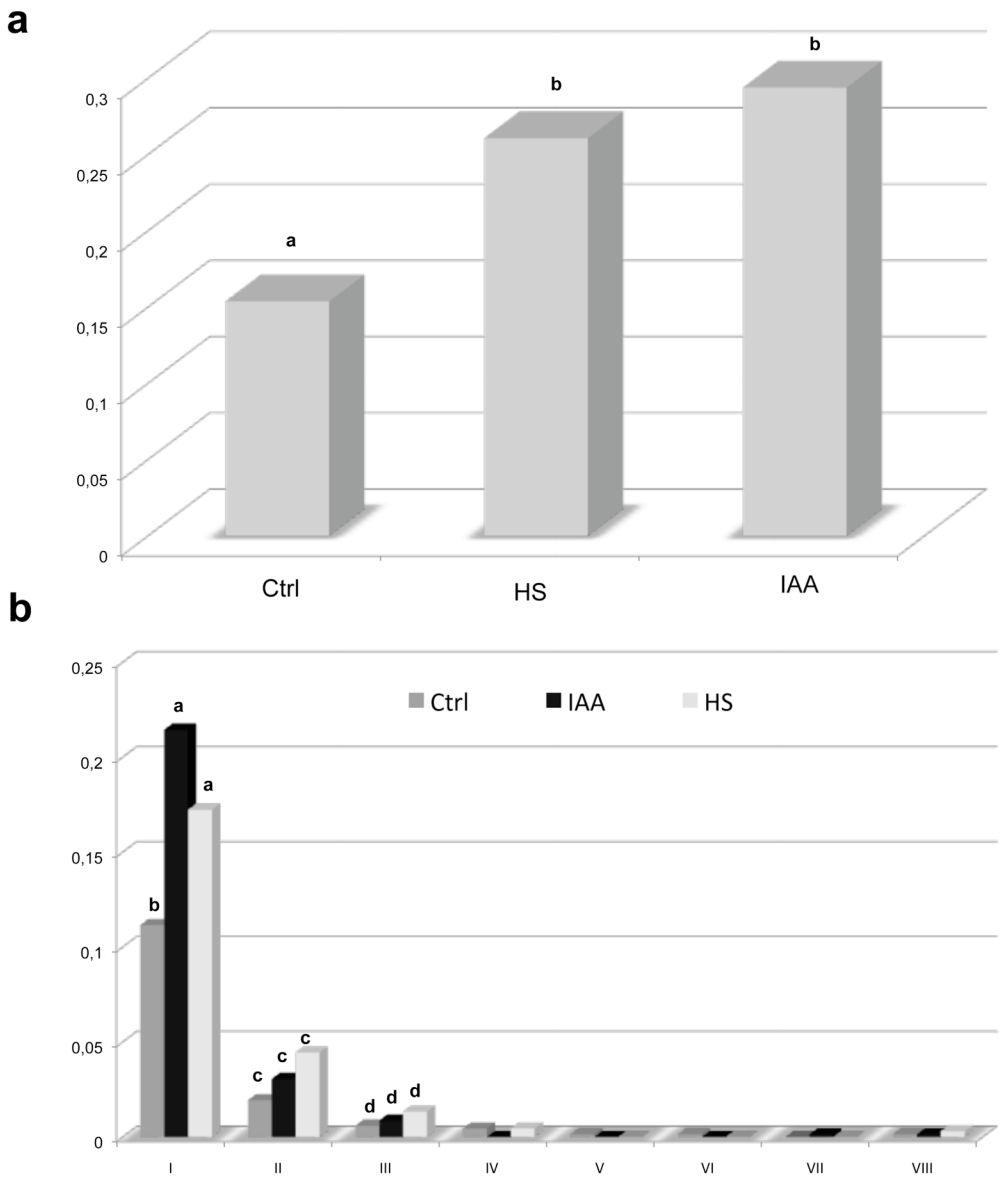

Figure 9 - Statistical analysis of lateral root *primordia* density in 4-day-old *AUX1*-DR5::GUS seedlings after 24 hours of treatments (Ctrl, 34nM IAA, 1.0 mg C l^{-1} HS). Density was calculated as the number of LRP divided by the total root length (**a**) and as LRP/mm per stage (**b**). Developmental stages during lateral root formation were identified according to the numbers and the orientation of cell division (Casimiro et al., 2003). The letters on the bars represent the non-significant ranges according to the LSD test ($P \leq 0.05$).

Humic substances induce transcription of the auxin inducible gene *IAA19*

The AUX/IAA transcription factors IAA5 and IAA19 have been previously shown to be early transcriptionally induced by auxin and brassinosteroids (Nakamura et al., 2003; Oono et al., 2003). The detailed analysis of their time course of expression in response to brassinolide or IAA demonstrated their faster down-regulation already after two hours of auxin treatments as opposed to a longer and steady up-regulation (up to 12 h) in the presence of brassinolide, allowing to diagnose divergent molecular mode of action of these hormones (Nakamura et al., 2003). In an attempt to separate IAA-dependent effects from effects mediated by factors others than auxin present in HS and to further characterise their molecular mode of action, we investigated the time course of expression of *IAA5* and *IAA19* following short parallel treatments with either humic substances or 34 nM auxin in the absence or presence of auxin inhibitors. Real time quantitative RT-PCR showed that the *IAA1*9 gene was up-regulated already after 30' treatments of *Arabidopsis* Col-0 plants with either HS or 34 nM auxin in a similar fashion, and subsequently displayed a decline of its transcription after two hours of treatment with IAA while a steady increase was detected in the presence of HS and, to a lesser extent, in control plants (Figure 10). *IAA5* transcript accumulation did not show a significant stimulation by either IAA or HS (Figure 10). The use of auxin inhibitors resulted in a complex expression pattern pointing to both overlapping and divergent regulatory mechanisms exerted by HS and IAA on auxin responsive genes expression. The auxin influx inhibitor NOA enhanced both *IAA5* and *IAA19* transcription when used in combination with IAA, with a peak after 30' of treatment and a slight decline after two hours while did not induce remarkable changes in gene expression in control and HS treated plants. A significant enhancement of *IAA5* transcription appeared to take place in the presence of TIBA or PCIB with a peak of expression after 30' and a decline to basal levels after two hours of treatment This effect was counteracted partially by IAA and to a larger extent by HS, while the stimulation exerted by PCIB was nearly abolished by IAA and sustained by HS. *IAA19* transcription appeared to be not substantially inluenced by TIBA or PCIB in relation to control plants but showed a significant upregulation when the inhibitors were used in conjunction with IAA or HS (for TIBA) or HS only (for PCIB).

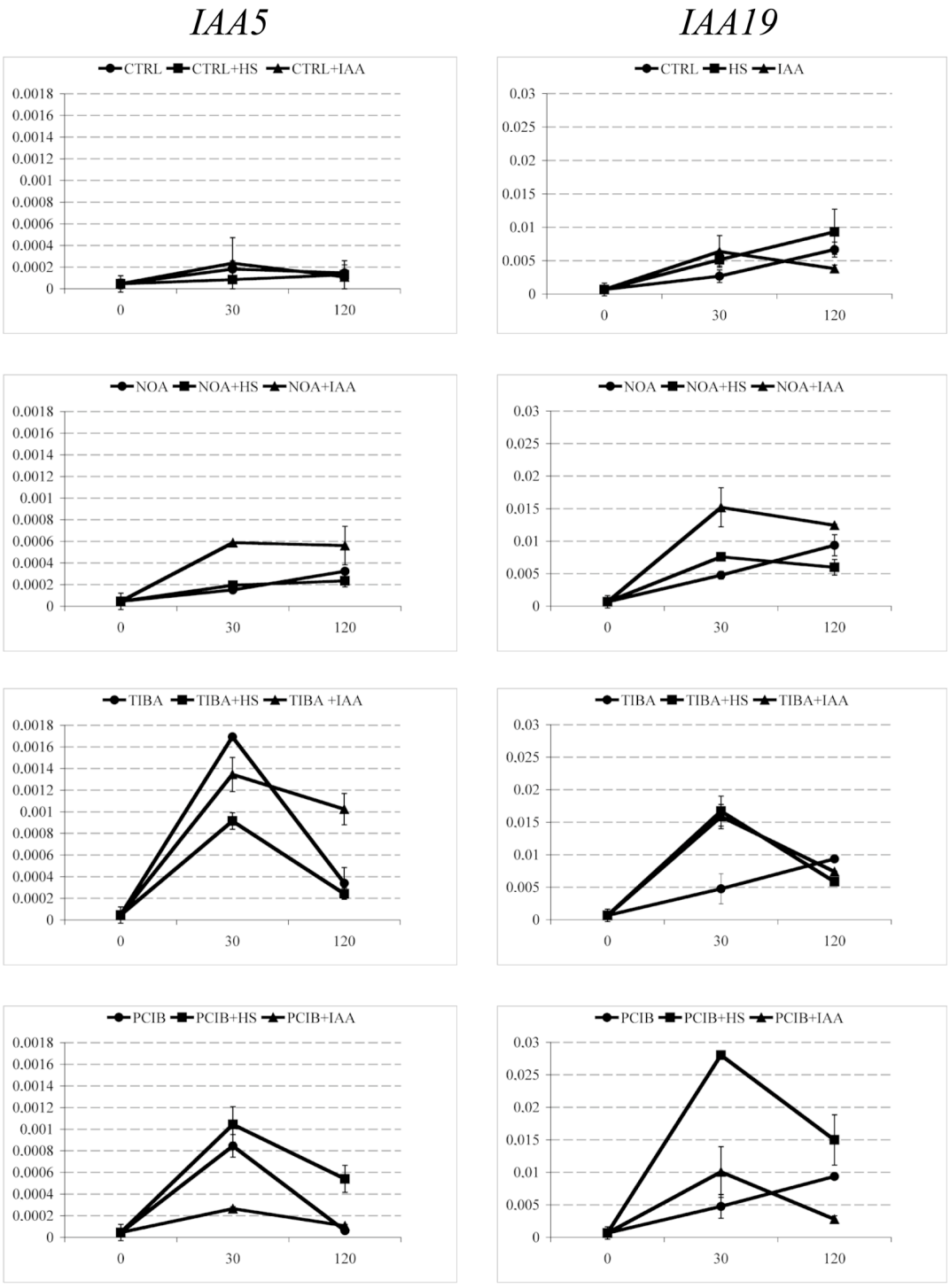

Figure 10 - Expression of IAA19 (At3g15540) and IAA5 (At1g15580) measured by Real Time-PCR using the *18S* gene (At2g01010) as a reference. *Arabidopsis* seedlings were grown for four days in MS plates and then transferred for 30 minutes or two hours in: water (Ctrl), 1.0 mg C l^{-1} (HS), 34 nM IAA (IAA), 50µM NOA, 50µM NOA + 1.0 mg C l^{-1} HS, 50µM NOA + 34 nM IAA, 50µM TIBA, 50µM TIBA + 1.0 mg C l^{-1} HS, 50µMTIBA + 34nM IAA, 50µM PCIB, 50µM PCIB + 1.0 mg C l^{-1} HS, 50µM PCIB + 34 nM IAA. Analyses were carried out in triplicate for each cDNA obtained from two independent RNA extractions. The specific transcript levels are expressed as arbitrary units (A.U.) of mean normalized expression. Bars represent standard error.

Discussion

The present paper shows that the HS contains a well-defined amount of IAA (34nM). Due to the complexity of the physiological responses to these substances, it seems that multiple signal cascades with different metabolic targets are involved. Only through a structural and molecular approach it is possible to clarify the signalling events governing the plant's response to HS. In previous papers the HS biological activity was related to the lower molecular fraction characterized by a high content in aromatic and carboxyl groups in acid (Muscolo et al., 1993, 1996, 2007; Nardi et al., 1994). In particular the large content of carboxylic groups, as suggested by Rubery (1981) and Napier (2004), are needed for auxin binding and control the modulation of auxin bioavailability in relation to plant necessity. The IAA presence in HS from earthworm faeces and from a wide range o forest soils has been shown in different studies (Muscolo et al., 1998; Nardi et al., 2000; Pizzeghello et al., 2001; Quaggiotti et al., 2004). How the IAA is bonded or is in association with HS is not well clarified (Shulten and Schnitzer 1998), even if a high concentration of organic acids from the Krebs cycle is required to release IAA from HS (Nardi et al., 1988, 2002, 2005; Piccolo et al., 1996). These findings, supported by recent results obtained by Dobbss et al., (2007), led us to hypothesize that auxin and other biological active compounds may be entrapped in the complex molecular structure of HS and that plants may modulate their bioavailability through the extrusion of specific molecules.

IAA and other plant-growth promoting substances are produced by many bacteria (Lambrecht et al., 2000) so it is not surprising that they may be entrapped in the molecular structure of HS. Nevertheless, the presence of other biological active compounds in HS cannot be excluded.

Our HS highlighted a great presence of carboxylic groups, sugar-like components and low content in aromatic substances similarly to those described by Canellas et al., (2002). Comparing the spectroscopic patterns of the high molecular size HS used in this work with the low molecular size (LMS) HS used in a previous study (Quaggiotti et al., 2004), some structural modifications can be seen. For instance, in our HS the strong enhancement of the band at around 1720 cm^{-1} could be due to a higher pK_a of the COOH groups, while in LMS (Quaggiotti et al., 2004) the contribution of carboxylate groups in acid dimers was dominant. Since the DRIFT spectra were recorded at the same pH it indicates that the

COOH groups in HS were not so extensively dissociated, as is the case of benzoic acids containing a large number of OH or, in general, electron-donor groups. A confirmation of the higher phenolic content in HS is given by the resonance at around 150 ppm, which may correspond to the phenolic carbons that are close to the quinonoid groups. The combination of carboxyl and aromatic groups in HS is a confirmation of common characteristics found out in HS from different origins (Muscolo et al., 2007). Moreover, the common structural characteristics arising from the results of Canellas et al., (2002) suggested that the HS deriving from earthworms are at an early stage of humification. On the basis of these results, chemical features are needed to explain the biological activity of HS. In fact, Schmidt et al., (2007) studying the effect of a HS on *Arabidopsis*, showed an unchanged expression for some auxin related genes, leading these authors to hypothesize an action of the HS utilized independent from auxin. The lack of details on the chemical composition of HS makes it very difficult to interpret these conflicting results. Moreover, the authors treated plants with a very high amount of humic matter (50 mg C L^{-1}) and they did not consider that the effect of HS on plant metabolism is selective and variable in relation to their concentration (Guminski et al., 1983; Vaughan and Malcom 1985; Maggioni et al., 1987, Varanini and Pinton 2001; Nardi et al., 2002).

To gain a deeper knowledge of the auxin-like activity of HS we have investigated their inductive action on a well-defined auxin-dependent process, lateral root development, in the model plant *Arabidopsis thaliana*. The dominant inducing role played by auxins on the process of lateral root initiation has been widely demonstrated (reviewed by Casimiro et al., 2003; De Smet et al., 2006). Therefore, in this study we have undertaken a comparative approach between auxin and HS, at a given known comparable auxin concentration, in inducing the formation of lateral roots.

For easier visualisation of the auxin response and of lateral root *primordia* from very early stages of development, the auxin responsive reporter DR5::GUS was used (Ulmasov et al., 1997). Our data show that HS induce lateral root formation and activate the DR5 reporter, already after 16 h of treatment. However, time-course experiments showed that HS appeared to induce lateral root formation faster and at higher rates than auxin after 24 h. This effect appeared to be reflected mainly by a higher number of *primordia* at early stages (stage I) suggesting that the faster and higher induction exerted by HS may depend on a

stronger initiation process. Interestingly, these differences were abolished by treatments with the inhibitor of auxin influx 1-NOA (Parry et al., 2001) or with the inhibitor of auxin signalling and action PCIB (Oono et al., 2003), when both the total number of lateral root *primordia* and of stage I *primordia* were considered, suggesting that humic substances act on lateral root initiation mainly relying on auxin transport and signalling. These findings were further supported by the finding that the treatment with HS rescued the lateral root phenotype of the *aux1* mutant. The mutation of the *AUX1* gene has been reported to result in a suboptimal auxin unloading, where lateral roots are initiated and has been shown to be rescued by exogenous feeding of auxin at nanomolar concentrations (Marchant et al., 2002). Here we show that 24 hours treatments with HS rescue the *aux1* lateral root initiation phenotype in a way that does not significantly differ from that of auxin. HS induce lateral root formation through auxin and, most probably, through the uptake into root cells of auxin bio-available in their structure and, in particular, in the functional groups. In addition the data suggest that an additive factor may be responsible for their action on lateral root induction. In fact, differently from the results obtained in the Col-0 background, treatment of the *aux1* mutant with humic acids did not result in a higher induction of LPR, and namely of stage I LRP, in comparison to auxin. This is in agreement with data obtained with 1-NOA, which exerts a similar inhibition of lateral formation in both IAA and HS treated plants, and it may be speculated that the additive effect of HS on lateral root formation requires *AUX1* to be functional and relies on AUX1-dependent auxin transport. In addition, HS-induced lateral root formation appeared less sensitive than the auxin-induced process to treatment with the inhibitor TIBA. This was evident mostly in terms of the induction of stage I lateral root *primordia* and may suggest that, besides the auxin-dependent action, an additive factor may be present in HS. In a recent paper, Zandonadi et al., (2007) also hypothesized an auxin-like activity of humic substances obtained from different soil sources and demonstrated their positive effect on lateral root development in maize. Our data taken together demonstrate that this effect mainly relies on the action of auxin contained in HS.

In a first attempt to further explore the molecular mode of action of HS and shed light on common or divergent regulatory aspects with IAA, we have studied HS effects on the time-course of expression of the auxin inducible genes *IAA5* and *IAA19* (Nakamura et al., 2003;

Oono et al., 2003). In fact, both DR5::GUS and the transcription of *IAA5* and *IAA19* has been shown to be induced also by brassinosteroids treatments (Bao et al., 2004; Oono et al., 2003; Nakamura et al., 2003; Yamazoe et al., 2005). However, AUX/IAA transcription is fastly and transiently up-regulated by auxin and is, on the contrary, enhanced in a more stable way by brassinosteroids (Oono et al., 2003; Nakamura *et al.,* 2003; Yamazoe et al., 2005). This evidence points out that the transcription of these genes can be used as a diagnostic tool to dissect the molecular mode of action of different hormones or molecules in relation to the IAA signal transduction pathway. Our results showed that *IAA19* transcript accumulation is induced after 30' of either IAA or humic acids treatment before returning to basal levels, already after two hours, in the presence of IAA but not of HS. This transient upregulation in the presence of IAA is in agreement with previous reports, in both terms of induction and temporal regulation (Nakamura et al., 2003), and further strengthens the hypothesis that humic substances can induce auxin inducible genes. However the lack of down-regulation of *IAA19* after two hours by HS may favour the hypothesis on the presence of additional factors other than IAA in HS. In our experimental conditions we could not show a significant induction of the *IAA5* gene in response to IAA or HS, however this is not in contrast with data found by Nakamura et al., (2003) showing that IAA significantly induce the expression of this gene only at concentrations higher than 100 nM, differently from IAA19 which appeared to be induced already by IAA concentrations as low as 10 nM (Nakamura et al., 2003).

When the expression of the two genes was studied in the presence of different auxin inhibitors, the existence of both divergent and overlapping mechanisms of regulation between HS and IAA appeared evident.

All together our data show that HS exert their action on lateral root development mostly through their auxin activity. This is based on independent experiments employing the induction, sensitive to different auxin inhibitors, of the DR5::GUS reporter and of lateral root initiation, on the rescue of the lateral root phenotype of the *aux1* mutant and on the early and transient transcriptional activation of an auxin responsive gene in *Arabidopsis*. At the same time, the presence of additional factors independent from auxin was evident based on *IAA5* and *IAA19* expression suggesting that more systematic approaches are needed to

unravel the molecular mode of action of HS. These aspects are currently under investigation in our laboratory.

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Chapter 3 – A cDNA-AFLP approach to isolate *Arabidopsis* genes involved in humic substances response

Abstract

HS, as component of soil organic matter, have been widely studied. Their positive effects on plant physiology have been further demonstrated, but there are no clear evidences about the direct as well as indirect mechanisms regulating these processes.

In this section a transcriptomic approach based on the detection of cDNA-AFLP markers was used to identify candidate genes potentially involved in the regulation of the response to HS in Arabidopsis thaliana. Using 160 primer combination, the cDNA-AFLP enabled to identify 133 genes putatively involved in plant-HS interaction. Sequence analysis and Gene Ontology classification indicated that a large number of genes involved in developmental and metabolic processes, as well as in transcription regulation or RNA metabolism were identified as HS-regulated.

Real-Time PCR analyses confirmed transcription levels of thirty two HS-regulated genes.

This study demonstrates that HS exert their effects on plant physiology in complex transcriptional networks.

Introduction

Soil is one of the most important natural resources for crop production. For satisfactory plant growth, it is essential that the soil provides a favourable physical environment allowing the root exploitation in order to recover water, nutrients and anchorage.

Humic substances (HS), as a component of soil organic matter, may influence the chemical and biological properties of the rhizosphere, being consequently an important factor of soil fertility (Nardi et al., 2005). Different theories about their molecular structures have been previously discussed (Nardi et al., 2002), and their functional properties on plant development and physiology have been matter of debate since nowadays (Canellas et al., 2008).

Several studies demonstrated that HS may positively influence plant growth in terms of dry and fresh weight of both shoot and root apex (Vaughan and Malcom, 1985; Varanini and Pinton 2001). Even if their physiological effects are detectable, no clear evidences about the direct as well as indirect mechanisms regulating these processes has been reported yet.

A widely studied physiological effect of HS concerns their positive action on plant metabolism, and, in particular, on the uptake of macro and micronutrients (Vaughan and MacDonald, 1976, 1971; Maggioni et al., 1987; Pinton et al., 1999; Quaggiotti et al., 2004).

As far as the metabolic processes are concerned, different humic fractions were observed to affect the enzyme activities related to glycolysis and tricarboxylic acid cycle (TCA) in different ways, depending on molecular size, molecular characteristics and concentrations (Nardi et al., 2007).

Fragmentary data have been published about the effect of HS on photosynthesis (Sladky, 1959). Moreover, such studies are principally focused on chlorophyll content, which is not necessarily linked to plant yield (Thomas et al., 1978). Ferretti et al., (1991) evidenced the relationship between HS and the photosynthetic sulphate reduction pathway, moreover a positive effect of HS has been detected on the basic photosynthetic metabolism in maize leaves (Merlo et al., 1991).

It has been further hypothesized that HS may exert their effects on plant physiology through auxinic activity. In fact, several studies demonstrated the effect of HS on specific

targets of auxin action, as for example the mha2 maize isoform of the plasmalemma H+-ATPase (Pinton et al., 1999; Canellas et al., 2002; Quaggiotti et al., 2004) and the pea phospholipase A2 (Russel et al., 2006). Moreover, the detection of indoleacetic acid (IAA) in a number of HS isolated from different sources supports this hypothesis (Muscolo et al., 1998; Pizzeghello et al., 2001; Canellas et al., 2002). In the previous chapter, we showed that HS induce lateral root formation mostly through their auxinic activity, but at the same time the expression profiles of two AUX/IAA genes suggested the existence of both divergent and overlapping mechanisms of regulation between HS and IAA. The same conclusion was also reported by Muscolo et al., (2007), showing that the amount of IAA detected in the humic substances could not be considered as the only factor contributing to the biological activity of HS, suggesting an important role for HS structure in plant-soil interaction (Muscolo et al., 2007). All together these findings remark the complexity of the relationship existing between humic substances and plant physiology and highlight the importance of a molecular approach to unravel the nature of this interaction.

Although there has been a noteworthy interest towards different auxin compounds or molecules that might affect plant physiology (Umehara et al., 2008; Nemhauser et al., 2006; Robertson et al., 2008), very little has been done to understand the molecular mechanisms involved in plant response to HS. Therefore, the identification of genes whose regulation is affected by HS treatments may give a relevant contribution to the research in this field, allowing to shed light on plant-HS interaction.

In this section, changes in steady state mRNA levels occurring in the plant during the treatment with humic substances were monitored and, consequently, HS-related differentially expressed transcripts identified in *Arabidopsis thaliana* by means of a cDNA AFLP transcriptome profiling.

This PCR-based technique does not require pre-existing information at the molecular level, thus allowing a wide study of mechanisms involved in the unclear HS-plant interaction. Moreover cDNA-AFLP is also able to detect low abundant transcripts, such as those encoding transcription factors and nucleotide binding proteins, putatively considered as regulators of the plant response to HS. The genes herein identified were annotated and sorted into gene ontology categories. Among all the isolated TDFs (transcript-derived fragments), 32 were chosen to validate the cDNA-AFLP expression profile by means of

Real-Time PCR.

Our data show that a short-term treatment with HS trigger the up and down-regulation of genes involved in important metabolic pathways, but also in transcriptional and post-transcriptional regulation.

Materials and methods

Plant material and growing conditions

Seeds of *Arabidopsis thaliana* Columbia ecotype (Col-0) were surface sterilised, sown on solid AM medium and germinated as described by Müller et al., (1998).

At the fourth day after germination plants were incubated for 30' or 120' minutes with, HS 1 mg C l^{-1} or IAA 34 nM, corresponding to the content of IAA measured in this humic fraction (chapter 2). Sterile mQ water was used as a control. Seventy seedlings were used per sample in each experiment that was repeated in triplicate.

RNA extraction

Total RNA was isolated using the "NucleoSpin RNA Plant" kit (Macherey-Nagel, Dure, Germany) following the protocol provided by the manufacturer. RNA concentration was calculated from the absorbance at 260 nm and the quality assayed by electrophoresis on Tris-acetate/agarose gel.

cDNA-AFLP

First-strand cDNA was synthesised from 1 μg of total RNA, using 200 u of MMLV Reverse Transcriptase (Promega, Milano, Italy) and oligodT as a primer, in 20 μl reactions, as described in Sambrook et al., (1989). Double-strand cDNA was used for cDNA-AFLP analyses as previously described (Botton et al., 2008). The final amplification was performed with 160 different combinations of EcoRI and MseI primers (Table 1). An equal volume of Gel Loading Buffer (98% formamide, EDTA 10 mM, 0.05%w/v of bromophenol blue and xylene cyanol) was added to the PCR reaction before a denaturing step of 5 minutes at 98°C. All the reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

The amplification products were separated in a 5% polyacrylamide gel with 8M urea at 90W constant power using the SequiGen GT system (Biorad, Hercules, CA, USA), dried-blotted onto a Whatmann 3MM paper at 80°C for 1 h and visualized by autoradiogram after overnight exposure on an Kodak MR-I X-ray film (Botton et al., 2005; Quaggiotti et al., 2007). All the experiments were carried out in triplicates.

Table 1 - Primers used in the cDNA-AFLP experiments. All the 160 possible combinations between EcoRI and MseI primers were adopted.

EcoRI primers ***(Eco=AGACTGCGTACCAATTC)***	***MseI primers*** ***(Mse=GACGATGAGTCCTGAGTAA)***
A Eco+AAT	01 Mse+AA
B Eco+ACC	02 Mse+AC
C Eco+ACT	03 Mse+AG
D Eco+AGG	04 Mse+AT
E Eco+TAA	05 Mse+CA
F Eco+CAC	06 Mse+CC
G Eco+CAG	07 Mse+CG
H Eco+CCA	08 Mse+CT
I Eco+CCG	09 Mse+GA
J Eco+GCA	10 Mse+GC
	11 Mse+GG
	12 Mse+GT
	13 Mse+TA
	14 Mse+TC
	15 Mse+TG
	16 Mse+TT

Purification and sequencing

The autoradiogram films were scannerized and acquired with KODAK 1D v 3.6 software (Scientific Imaging Systems, Eastman Kodak Company) to quantify the net intensity of the bands. Amplicons showing at least a 3-fold difference in terms of intensity between the two samples in all the replicates were excised from the blots and immersed overnight in 100 µL of PCR-grade water. Five microliters were used in a standard PCR reaction with the same

EcoRI and MseI primers adopted in the preamplification step described above. The re-amplified DNA was directly sequenced as PCR-derived product purified by the EXO-SAP enzymatic system (Amersham Biosciences, GE Healthcare, United Kingdom,). The sequencing reaction was performed at the BMR Genomics (University of Padova, www.bmr-genomics.com).

Bioinformatics and gene ontology (GO) analysis

Homologies for all TDF were searched in public databases with the BLASTN and BLASTX applications (Altschul et al., 1990) to compare nucleotide and translated sequences, respectively. For each of the selected candidate genes, the most significantly similar entries were selected.

The Mapping tool of Blast2GO software (Conesa et al., 2005) was used to obtain Gene Ontology (GO) information from retrieved database matches. Analysis was performed using either standard GO parameters or GO slims (plant-related subset of GO terms available at www.geneontology.org/GO.slims.shtml). Definitions and relationships for each GO term were obtained from the AmiGO browser of the gene ontology consortium website (www.geneontology.org). Statistics were developed for the three structured controlled vocabularies to describe TDFs in terms of their associated biological process, cellular component and molecular function in a species-independent manner. Results composed by a pool of GO terms and sequences found by using our TDFs as query were visualized by count-based histograms and acyclic graphs using a sequence cut-off = 0.

Real Time

Real-time PCR relative quantification was performed in a total volume of 10 µL using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with 3 pmol of every primer and 2 µL of a 1:10 dilution of cDNA. The gene-specific primers (Table 2) were designed with Primer3 software version 0.4.0 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the instructions reported in the SYBR® Green PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA). The specificity of amplification was assessed by subsequent subcloning and sequencing of the PCR products obtained under the same conditions adopted in the real-time experiments. The reaction

mixture was amplified in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the conditions described in Botton et al., (2008). After every PCR cycle, a data acquisition step was introduced to record the fluorescent signals at the optimum temperature, previously determined by melting point analysis of every specific amplification product (Table 2). Data were acquired, elaborated and exported with the software SDS Sequence Detection System v1.2 (Applied Biosystems, California), whereas all the final calculations were carried out with the automated spreadsheet Q-Gene designed by Simon (2003), using the modifications of the delta Ct method suggested by Pfaffl (2001). Two genes (*ACT2* and *18S*), giving similar expression values, were selected as housekeeping (Quettier et al., 2006; Goda et al., 2002). Gene expression values were normalized to the 18S gene (At2g01010) and reported as arbitrary units (A.U.) of Mean Normalized Expression (Pfaffl, 2001), using the equation 2 of Q-Gene. The correct size of the amplification products was checked by running each reaction in a 1.5% agarose gel stained with ethidium bromide and viewed under UV light.

Table 2 List of Gene Specific Primers used in RT experiments.

Gene		Forward	Reverse	Size (bp)	Tm (°C)
At1g08930	ERD6	5'-AATGTCGTGTTACCGCCTCT-3'	5'-CCTGATACGGCAACAAAGGT-3'	52	68,9
At1g09130	CLPR3	5'-TCTCCACGCTCGCTTCTATT-3'	5'-TAACCGGCCGATTGAGAA-3'	60	71,8
At1g09210	CRT2	5'-TAAGCCAATGTCCGCCAAA-3'	5'-GAGACTAACAACAAATTCTCAAAGG-	79	71,0
At1g12360	KEULE	5'-CGATCTCCAAATATGAGAATCCA-3'	5'-TCGGACAAACAATTTGGGTA-3'	146	70,1
At1g27770	ACA1	5'-GCTCTAGTTTGGTTGGAATGTAGAA-3'	5'-CAAAGTTACCACAACCATTTCACC-3'	78	71,1
At1g50030	TOR	5'-TGAGCGTGCCGTAGTTGTTA-3'	5'-GATTGCTCGGAATTGCAGAC-3'	83	71,9
At1g56140	LRR	5'-CTTGGAGCCCAGATGAATGA-3'	5'-GCCACCATATAGGGTCTTTGTC-3'	50	68,4
At1g56340	CRT1	5'-AACATTGTGCTCAGTGGTGG-3'	5'-TCATCATACTCGGCCTTGG-3'	67	65,0
At1g57720	eEF1B	5'-AAAGGCTCTTTGTATCTCCGTA-3'	5'-AAACCCGAGTACGATTCAAA-3'	96	70,7
At1g67090	RBCS1A	5'-TCGGATTCTCAACTGTCTGATG-3'	5'-ATTTGTAGCCGCATTGTCCT-3'	104	66,4
At2g01010	18S	5'-CGGCTACCACATCCAAGGAA-3'	5'-GCTGGAATTACCGCGGCT-3'	186	77,4
At2g21870		5'-AAGATCGTTCCCTTGCTGCT-3'	5'-GAGAGACCTTATTGAACGCCAAA-3'	50	66,8
At2g29630	THIC	5'-AAGCGCGGTTTGAGTTTAGA-3'	5'-GGTCCAACGACAGAGCAAAT-3'	51	68,2
At2g30950	VAR2	5'-AAGGCTTCTTTGGATGGGAAG-3'	5'-GCATTTGCTTTCCCACTTGC-3'	107	71,9
At2g38540	LTP1	5'-TGAGGTGATGAGCTAGCAACG-3'	5'-GATTCGAAACTTCCGGTAGTAGCAT-3'	54	68,5
At2g40940	ERS1	5'-GCTTGTCTTGTGGGAAATGG-3'	5'-CTTGTCTGTCTGCCGGAAA-3'	83	70,0
At2g42030	C3HC4	5'-GCTTTGGCAACCCAGAGAC-3'	5'-GACGACACCCTCCCATACAT-3'	50	71,7
At3g15540	IAA19	5'-GGCGTCGTTTCATCTGGTG-3'	5'-GCCGGCGAATCATTAACCT-3'	50	70,8
At3g16460	MBP1	5'-CGGACGAGCTGGTGAGTATG-3'	5'-TTGGGATTGTGTTGCAGGAG-3'	88	73,1
At3g18780	ACT2	5'- AACATTGTGCTCAGTGGTGG-3'	5'- TCATCATACTCGGCCTTGG-3'	206	76,8
At3g46000	ADF2	5'-ACACCTTCAACTTCATTCTACTTGG-3'	5'-CCATCACAAGGCACACATAACA-3'	68	66,2
At3g57390	AGL18	5'-GAAAGAAGCCGAAGATCGAA-3'	5'-GAAGCCACTTGACTCCCAGA-3'	55	69,9
At4g14950		5'-TCCTCATGCTCCAACATCAA-3'	5'-GTTTGTTGGCCCTTCAACTC-3'	60	67,7
At4g17800		5'-GCGAAGAATTTGCGAGAGTG-3'	5'-CAATGTCCGAAATCACGATG-3'	87	67,1

At4g18880	HSFA4A	5'-GAGCCCTCCTCTTTCTTGCAT-3'	5'-AACCATCGGGCTCACAGTTC-3'	98	73,0
At4g19210	RLI2	5'-CGACACAGAAGGACCAGACA-3'	5'-TCTGAAACTTTGGGCTGATCT-3'	80	69,8
At4g24190	SHD	5'-TGCAATACCTGATGGATTACGAA-3'	5'-CCTTCCTTGGACACATTCTGG-3'	55	66,1
At4g31530		5'-GTGTTGGAACCCACTCGTTT-3'	5'-GGCATATACGCAACGACAAC-3'	71	67,6
At4g34220	LRR	5'-TGGTGCAAGTGTTGGAGAAA-3'	5'-TGCGAGGGTAAAGTAAGCATT-3'	136	69,1
At5g23720	PHS1	5'-TGCGCGGATCTTAATAAACC-3'	5'-CGGACACACTTTCATTGTCG-3'	91	72,2
At5g45680	FKBP13	5'-TTTGCCCAATTATCCTCATGT-3'	5'-TTTCGGCTAATGCAGAAACA-3'	94	68,8
At5g58900	MYB	5'-TTGTTGGAGATCAGCGTTCA-3'	5'-TGCCTGTGTTCCATTGTTGT-3'	71	71,7
At5g65310	HB5	5'-GAAGTCATATTGGGTCGGAAA-3'	5'-CCTCTCGCGTCATATTCCAT-3'	78	68,0

Gene expression data clustering

Global gene expression trends were examined by means of hierarchical clustering. Ratios were calculated on raw gene expression data at 30 and 120 minutes, and processed by means of Gene Cluster 3.0 software (de Hoon *et al.*, 2004). Data were log2-transformed, mean-centered, normalized, and hierarchically clustered using the uncentered correlation similarity matrix and the centroid linkage clustering method. Heat maps were generated with Java TreeView 1.1.3 (Saldanha, 2004).

Results

cDNA-AFLP analysis

The cDNA-AFLP-based transcriptome profiling allowed the identification of an average of 5 differentially expressed transcripts for each of the 160 combinations of primers (Figure 1). Changes in gene expression potentially related to HS response were searched among cDNA-AFLP patterns of treated and untreated samples at 30 and 120 minutes after the addition of HS. This procedure allowed the identification of 133 transcript-derived fragments (TDFs) whose expression was differentially modulated by HS and not dependent upon the time course developmental events. These amplification products were excised from gels, purified, re-amplified and sequenced. Verified and edited sequences of all polymorphic TDFs were used as a Blast query to identify the corresponding full-length sequence.

The whole sequences were FASTA formatted and used to automatically recover the related GO annotations by means of Blast2GO software (Conesa et al., 2005). According to the overall results, 119 genes out 133 had a putative function with the corresponding gene ontology terms, whereas fourteen of them encode still unknown proteins (Tables 3 and 4).

Regarding the transcription profiles detected by cDNA-AFLP, a high percentage of genes (75%) were up-regulated in the HS-treated samples with respect to the control (Table 4), whereas the remaining TDFs (25%) were down-regulated (Table 3). The positive or negative effects on transcription were already detectable after 30 minutes for some products

(65%), while other transcripts (35%) were affected later on. As a general remark, the majority of the former genes (70%) showed similar expression patterns also at the second time-point (120 minutes), showing a prolonged effect of HS on their transcription.

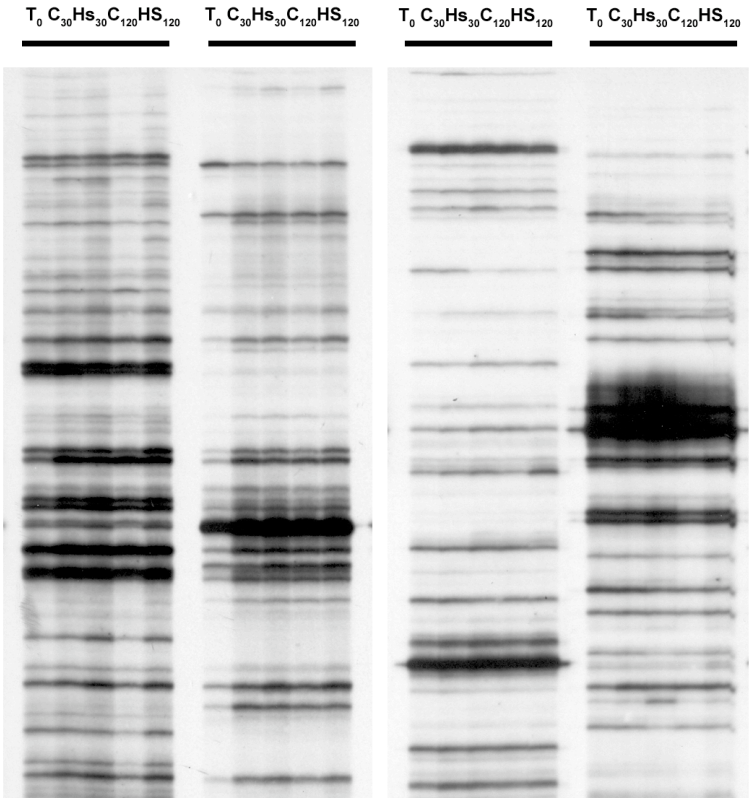


Figure 1 - Gene expression profiles generated by cDNA-AFLP with EcoRI and Mse primer combinations. C refer to control plants, while HS_{30} and HS_{120} refer to treatments for respectively 30 and 120 minutes with 1mg C L^{-1} HS.

Table 3 - List of Arabidopsis down-regulated TDFs isolated by cDNA-AFLP along with BLAST results

TDF	Length (bp)	AGI no.	Putative gene function	*E* value	Primers combination	Expression patterns
132	429	AT1G27770	Autoinhibited Ca2+-ATPase 1 (ACA1)	5E-13	I13	-
43	201	ATCG01090	NADH dehydrogenase subunit I (NDHI)	1E-04	G14	-
53	157	ATCG01100	NADH dehydrogenase subunit 1 (NDHA)	1E-05	G05	-
82	300	ATCG00700	Photosystem II reaction center N protein (PSBN)	5E-22	A12	-
131	474	AT5G12250	Putative tubuline beta6 chain (TUB6)	8E-41	I14	-
50	174	AT4G14960	Tubulin alpha-6 chain (TUA6)	7E-10	J03	-
33	430	AT3G50970	LOW TEMPERATURE-INDUCED 30 (LTI30 / XERO2)	1E-11	H14	-
100	330	AT2G16630	Proline-rich family protein	4E-30	I02	-
134	165	AT2G32900	Expressed protein; homologous to Drosophila ZW10, a centromere/kinetochore protein involved in chromosome segregation	3E-20	I14	-
52	118	AT1G65980	THIOREDOXIN-DEPENDENT PEROXIDASE 1 (TPX1)	9e-91*	J03	-
75	197	AT3G53900	Uracil phosphoribosyltransferase, putative/UMP pyrophosphorylase, putative/UPRTase putative	4E-20	A15	-
77	100	AT4G39800	Myo_inositol-1-Phosphate synthetase	2E-65	A15	-
20	111	AT5G53460	GLUCOSE TRANSPORTER 1, NADH-dependent glutamate synthase (GLT1)	2E-81	D03	-
39	361	AT4G25810	Xyloglucan endotransglycosylase-related protein (XTR6)	1E-87	G03	-
47	390	AT1G36170	Putative acetyl-CoA carboxylase (ACC1)	6E-33	J03	-
51	180	AT1G62750	Elongation factor G	6E-38	J03	-
34	233	AT3G17920	MEB5.14 protein binding	7E-21	H14	-
65	187	AT2G17360	30S ribosomal protein S4 (RPS4)	2E-37	A16	-
151	452	AT3G60770	40S ribosomal protein S13 (RPS13A)	2E-32	F08	-
30	239	AT4G13170	60S ribosomal protein L13A (RPL13aC)	5E-58	H03	-
1	274	ATCG00800	30S ribosomal protein S3 (RPS3)	2E-79	D01	-

152	257	ATCG01310	Chloroplast ribosomal protein L2 (RPL2)	1E-04	F07	-
109	142	AT2G25260	Unknown protein	2E-06	G02	
18	125	ATCG00860	Unknown protein. This gene is regulated by AtSIG6 transcriptionally (YCF2)	1E-09	A06	
102	271	AT5G55620	Unknown protein	9E-05	A08	

Table 4 - List of *Arabidopsis* up-regulated TDFs isolated by cDNA-AFLP along with BLAST results

TDF	Length (bp)	AGI no.	Putative gene function	E value	Primers combination	Expression patterns
40	253	ATCG00150	ATPase complex CF0 subunit (ATPI)	0.021	G03	+
54	276	AT2G21870	Putative ATP synthase. Unknown protein	0.096	G08	+
142	119	AT4G19210	Member of RLI subfamily. ABC transporter (ATRLI2)	0.001	I09	+
46	356	AT1G08930	EARLY RESPONSE TO DEHYDRATION 6 (ERD6)	2E-11	J02	+
81	60	AT3G23660	Transport protein, putative (GAD2)	1E-21	A09	+
61	314	ATCG00730	Cytochrome b6/f complex subunit IV (PETD)	0.87	B05	+
119	75	AT5G23190	Cytochrome P450, family 86, subfamily B, polypeptide 1 (CYP86B1)	1E-13	I02	+
11	68	ATCG00680	Photosystem II 47 kDa protein (PSBB)	2E-08	D02	+
42	209	AT1G79040	Photosystem II subunit R (PSBR)	8E-17	G03	+
110	147	ATCG00020	Chlorophyll binding protein D1 (PSBA)	4E-27	I02	+
112	140	AT5G54270	LIGHT-HARVESTING CHLOROPHYLL BINDING PROTEIN 3 (LHCB3)	1E-14	I02	+
60	143	AT1G67090	RuBisCO small subunit 1 (RBCS-1A)	2E-07	B05	+
66	140	AT5G45680	FKBP-type peptidyl-prolyl cis-trans isomerase3 (AtFKBP13)	1E-26	A16	+
139	239	AT1G09130	ClpP protease complex subunit (ClpR3)	4E-11	I09	+
4	206	AT4G24190	SHEPHERD (SHD)	2E-05	D02	+
9	131	AT3G46000	ACTIN DEPOLYMERIZING FACTOR 2 (ADF2)	1E-14	D02	+
137	268	AT3G17850	IRE homolog; protein kinase-like protein	2E-04	I09	+
111	147	AT1G50030	TARGET OF RAPAMYCIN (TOR)	3E-15	I02	+
113	135	AT3G57390	AGAMOUS-LIKE 18(AGL18)	1E-23	I02	+
114	167	AT5G23720	PROPYZAMIDE-HYPERSENSITIVE 1 (PHS1)	2E-57	I02	+
128	71	AT1G12360	KEULE	2E-04	I08	+

93	154	AT1G53380	Unknown protein (Similar to UNE1 (unfertilized embryo sac 1 At1g29300))	2E-05	C04	+
29	304	AT2G40940	ETHYLENE RESPONSE SENSOR 1 (ERS1)	1E-04	H03	+
10	84	AT3G16460	Jacalin lectin family protein	4E-08	D02	+
31	236	AT3G60370	Immunophilin/FKBP-type peptidyl-prolyl cis-trans isomerase family protein	1E-04	H03	+
21	491	AT1G63940	Monodehydroascorbate reductase, putative	1E-26	D03	+
86	113	AT1G50320	THIOREDOXIN X (ATHX)	2E-65	A10	+
138	248	AT1G45145	THIOREDOXIN H (ATH5)	1e-20*	I09	+
107	169	AT3G52960	Peroxiredoxin type 2, putative	5E-50	I02	+
62	101	AT2G29630	Thiamine biosynthesis family protein/thiC family protein	2E-29	B05	+
136	270	AT4G13930	Serine hydroxymethyltransferase, maximally expressed in root (SHM4)	0.011	I09	+
78	360	AT1G79550	Phosphoglycerate kinase (PGK)	6E-44	A09	+
87	61	AT5G09660	Peroxisomal NAD-malate dehydrogenase (PMDH2)	1E-38	A10	+
5	196	AT2G44620	MITOCHONDRIAL ACYL CARRIER PROTEIN1 (mtACP-1)	2E-24	D02	+
35	95	AT4G31530	Arabidopsis thaliana binding/catalytic/coenzyme binding	3E-07	H14	+
118	73	AT5G39320	UDP-glucose 6-dehydrogenase, putative	9E-10	I02	+
121	431	AT1G69830	ALPHA-AMYLASE-LIKE 3 (AMY3 / ATAMY3)	4E-12	I08	+
135	154	AT1G43710	EMBRYO DEFECTIVE 1075 (EMB1075)	1E-27	I14	+
90	61	AT1G65960	Glutamate decarboxilase 2	3E-28	A10	+
140	201	AT4G15560	1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE (CLA1)	0.001	I09	+
146	423	AT4G35830	Aconitate hydratase, cytoplasmic/citrate hydro-lyase/aconitase (ACO)	4E-48	F01	+
25	471	AT2G26080	Arabidopsis thaliana glycine decarboxylase p-protein 2 (ATGLDP2)	7E-10	B02	+
143	255	AT4G18880	Arabidopsis thaliana heat shock transcription factor A4A (AT-HSFA4A)	1E-11	F04	+
120	71	AT2G42030	Zinc finger (C3HC4-type RING finger) family protein	3E-04	I02	+
147	391	AT5G65310	ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 5 (ATHB5)	2E-47	F01	+

150	99	AT5G58900	Myb family transcription factor	4E-18	F01	+
113	135	AT3G57390	AGAMOUS-LIKE 18(AGL18)	1E-23	I02	+
129	61	AT4G17800	DNA-binding protein-related	5E-82	I08	+
24	634	AT1G57720	Putative elongation factor 1B gamma	0.025	D15	+
122	357	AT3G59940	Kelch repeat-containing F-box family protein	6E-38	I08	+
115	94	AT4G34220	Leucine-rich repeat transmembrane protein kinase, putative	8E-09	I02	+
76	182	AT1G56140	Leucine-rich repeat family protein/protein kinase family	6E-33	A15	+
71	300	AT1G73660	Protein kinase family protein	2E-08	A14	+
139	239	AT1G09130	ClpP protease complex subunit (ClpR3)	4E-11	I09	+
137	268	AT3G17850	IRE homolog; protein kinase-like protein	2E-04	I09	+
111	147	AT1G50030	TARGET OF RAPAMYCIN (TOR)	3E-15	I02	+
114	167	AT5G23720	PROPYZAMIDE-HYPERSENSITIVE 1 (PHS1)	2E-57	I02	+
16	194	AT2G30950	Zinc dependent protease	3E-04	D04	+
12	66	AT5G55220	Trigger factor type chaperone family protein	2E-19	D02	+
125	125	AT2G20460	Transposable element gene	9e-75*	I08	+
141	125	AT2G38540	Nonspecific lipid transfer protein 1 (LP1)	4e-04*	I09	+
14	117	AT1G09210	Calreticulin 2 (CRT2)	8E-29	D04	+
69	400	AT1G56340	Calreticulin 1 (CRT1)	7E-04	A14	+
98	418	AT4G23460	Beta-adaptin putative involved in intracellular protein transport, protein complex assembly, vesicle-mediated transport	6e-19*	I02	+
145	120	AT1G04520	33 kDa secretory protein-related	5E-14	F04	+
128	71	AT1G12360	KEULE	2E-04	I08	+
36	126	AT2G39390	60S ribosomal protein L35 (RPL35B)	6E-35	G04	+
79	75	AT2G39460	Ribosomal protein L23A (ATRPLR23A)	3E-05	A09	+
32	102	AT3G27850	50S ribosomal protein L12-C (RPL12-C)	9E-04	H03	+

116	92	ATCG00160	30S ribosomal protein S2 (RPS2)	2E-98	I02	+
94	74	ATCG00820	Ribosomal protein S19 (RPS19)	6E-18	C04	+
91	157	ATCG00905	Chloroplast gene encoding ribosomal protein S12 (RPS12)	0.66	C04	+
26	383	ATMG00020	Mitochondrial 26S ribosomal RNA protein	2E-04	B12	+
3	244	AT4G13500	Unknown protein	2E-06	D02	+
126	119	AT4G14950	Unknown protein	2E-67	I08	+
123	287	AT4G19160	Unknown protein	2E-11	I08	+
80	78	AT5G23610	Unknown protein	7E-05	A09	+
13	335	AT3G48200	Unknown protein	7E-31	D04	+
70	300	AT1G32920	Unknown protein	0.30	A14	+
8	162	AT3G09860	Unknown protein	2E-08	D02	+

Gene Ontology analysis

In order to identify the major biological processes involved in the response to HS, the AFLP-derived ESTs were classified by means of the Gene Ontology (GO), which consists of three structured vocabularies (i.e. ontologies) describing genes, transcripts and proteins of any organism in terms of their associated cellular component, biological process and molecular function in a species-independent manner (Pal, 2006; Gene Ontology Consortium, 2006). Blast2GO software (Conesa et al., 2005) was used for GO terms recovering, allowing the functional annotation of 94 sequences out of 133. Different levels of ontology were chosen according to the number of sequences described. Therefore, level 2 for both biological process and molecular function, and level 3 for cellular component were reported in the annotation charts, and subdivided according to the pattern of expression of the associated genes (i.e. up- or down-regulation). Annotations were 239 for cellular components, 75 for molecular functions and 154 for biological processes.

As pointed out in figure 2, among the cellular component annotations no marked differences between the up- and down-regulated transcripts were observed. The majority of TDFs were in both cases related to the 'intracellular component', followed by 'intracellular part' and 'intracellular organelle'.

Regarding the up regulated transcripts, 'binding' was the GO term most frequently encountered (24%) in the molecular function annotations, followed by 'catalytic activity' (18%), 'protein binding' (8%), 'nucleotide binding' (6%), 'transferase activity' (6%), and 'transporter activity' (6%). As far as the biological process ontology is concerned, the majority of annotations analyzed for inducted transcripts, were related to 'metabolic process' (34%), and 'cellular process' (34%) followed by the 'response to stress' and 'response to stimuli' (9%) and 'localization' and 'establishment of localization' (8%).

Concerning the down-regulated sequences, the 'catalytic activity' was the most numerous category of the molecular function annotations, reaching the 41% of the total. It was followed by the 'binding' (34%), 'structural molecule activity' (19%), 'transporter activity' (6%). Whereas, in the biological process classification, 33% was related to 'cellular process', 33% to 'metabolic process', 8% to 'localization', 'response to stimuli' and 'establishment of localization', followed by 'developmental process and 'multicellular

organismal process' (16%).

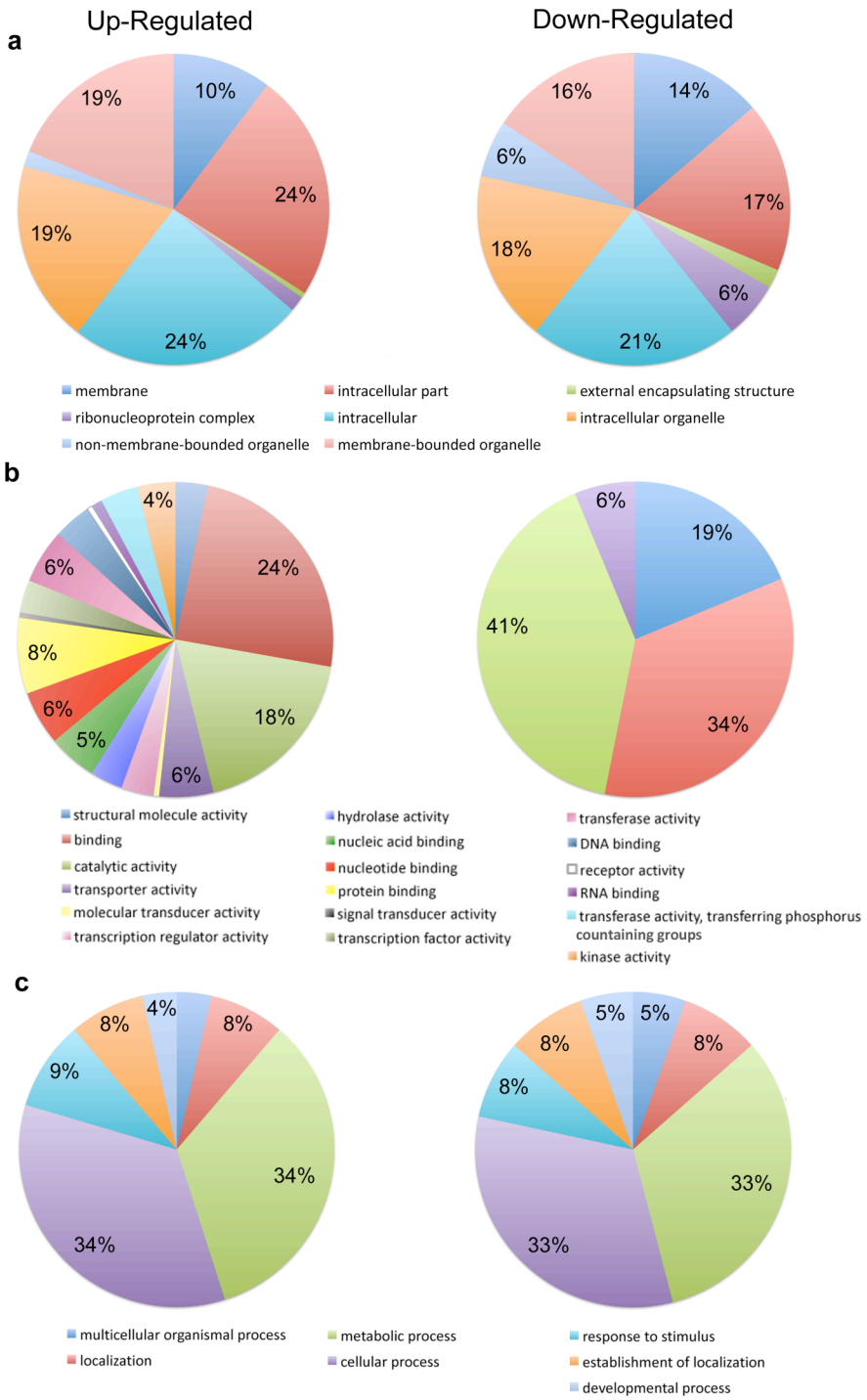


Figure 2 - Gene Ontology classification of genes that were up- or down- regulated in *Arabidopsis thaliana* treated with 1 mg C l^{-1} HS according to the **(a)** cellular component, **(b)** molecular function, and **(c)** biological process. GO codes are reported between brackets. For the classification according to cellular component, level 3 terms were chosen, whereas for the biological process and molecular function categories, level 2 GO terms were used to construct the pie charts.

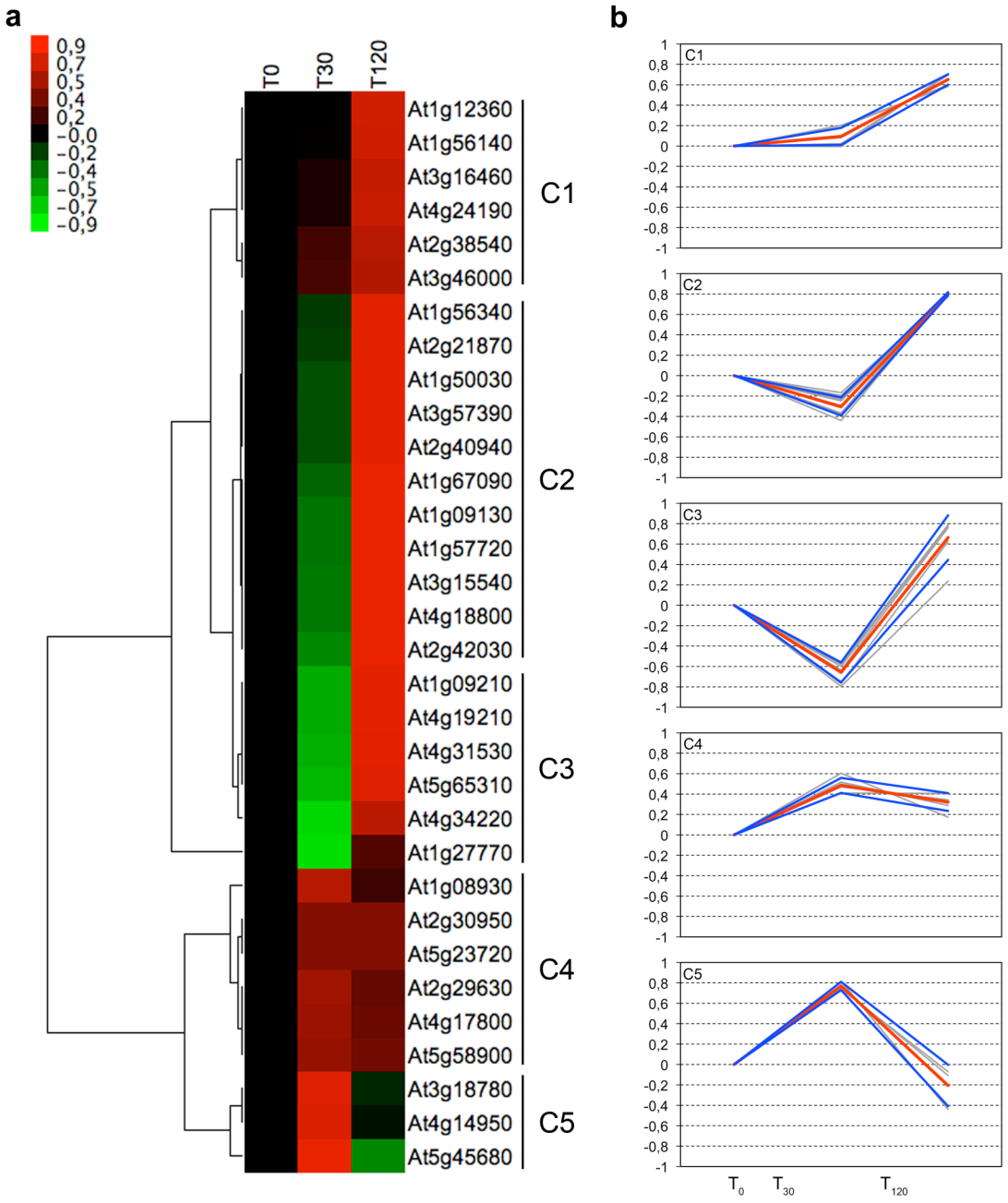


Figura 3 - Global gene expression trends on raw gene expression data at 30 and 120 minutes examined by means of hierarchical clustering are rapresented gene by gene in the heats maps (**a**) and cluster by cluster in the linear graphic(**b**).

Gene Expression analysis

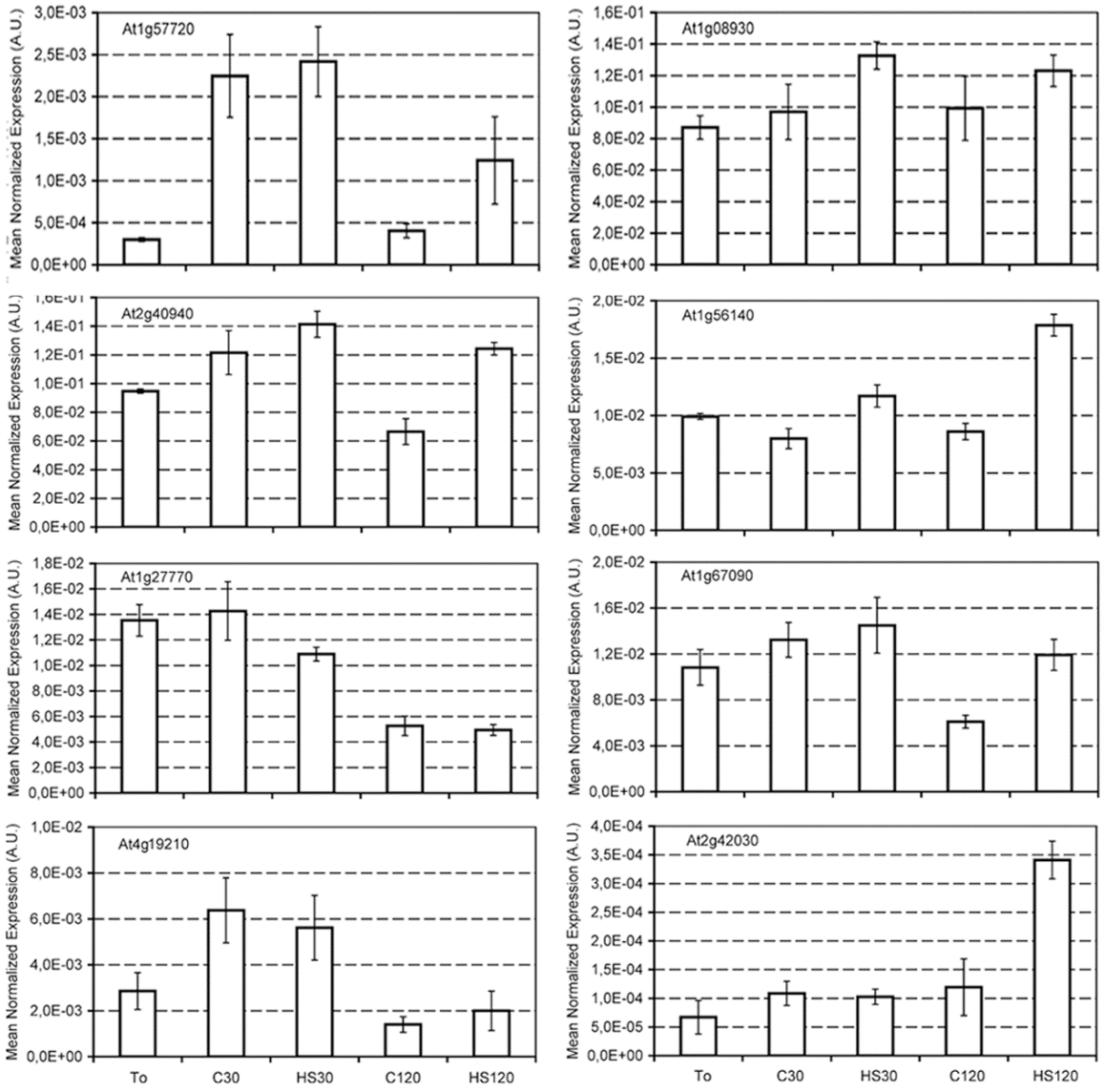
The expression levels of 32 HS-modulated transcripts were analyzed further by Real Time

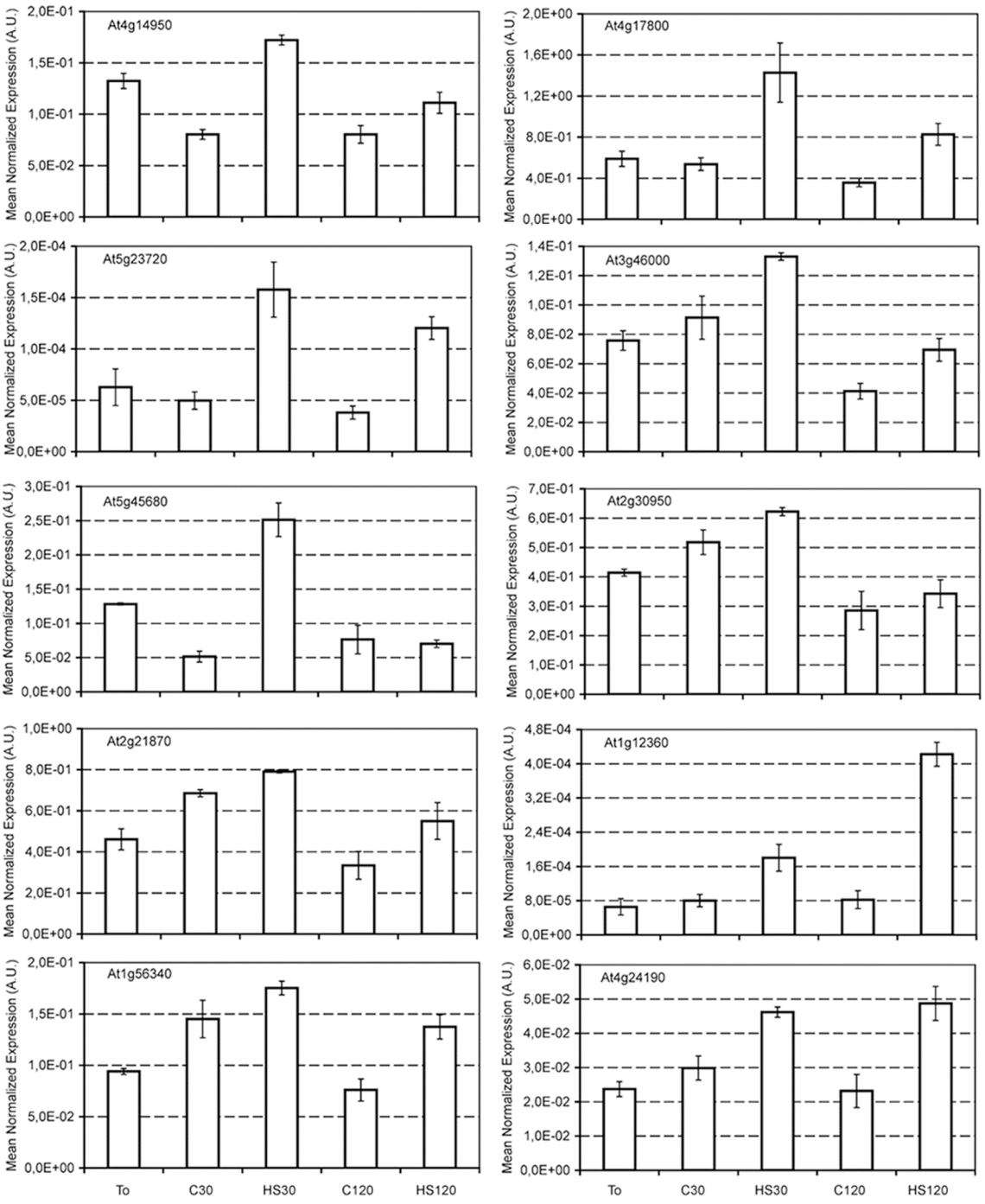
PCR to validate cDNA expression profiles. Genes were selected because of their AFLP profiles as well as of their putative physiological role. Mean normalized expression data confirmed almost completely the profiles detected by cDNA-AFLP.

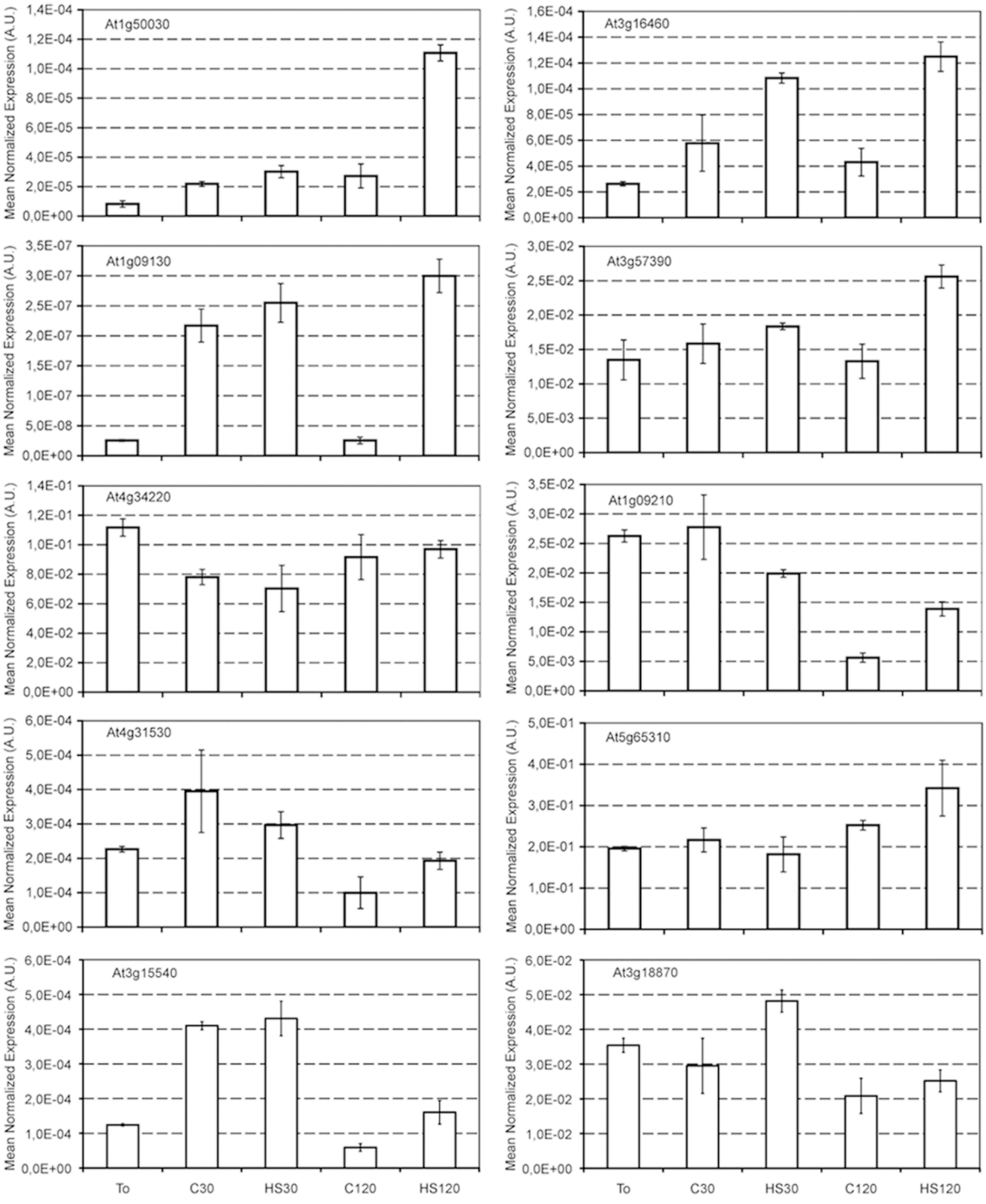
In order to group genes with similar expression profiles, a hierarchical clustering was performed, which allowed to outline five major groups. Two different graphical representations were adopted to describe gene-by-gene (heat maps in Figure 3a) and cluster-by-cluster (Figure 3b) expression trends. For the six genes belonging to the first cluster, 30 minutes of treatments did not induce any strong variation with respect to the control, whereas a weak induction could be detected after 120 minutes. Cluster C2 is characterized by eleven genes slightly down-regulated after 30 minutes and subsequently induced after 120 minutes. Transcripts showing the strongest induction after 120 are six and are grouped in the third cluster, while C4 hold together six transcripts slightly up-regulated after both 30 and 120 minutes of treatment. Finally the 3 transcripts grouped in C5 showed a strong induction of their expression already after 30 minutes, being then rapidly repressed after 120 minutes.

Figure 4 shows in details the expression profile of each gene analyzed by means of RT-PCR, and some examples for each major expression profile detected are commented below in details. For instance, after 120 minutes of HS treatments, At1g09130, encoding a ClpR3 subunit of the ClpP proteolitic complex, increased its transcript accumulation 12 fold with respect to the control, and KEULE (At1g12360), a gene coding for a protein involved in endosome trafficking and in citokinesis, showed a 5 fold induction of its transcript. At5g23720, encoding PHS1 (Propyzamide-Hypersensitive 1) similar to a MAPK-phosphatase, evidenced a 3 fold induction after both 30 and 120 minutes. A similar increase was also observed for At2g42030, encoding a zinc-finger protein, after 120 minutes of treatments, whereas SHD (SHEPHERD, At4g24190), AGL18 (AGAMOUS-LIKE 18, At3g57390) and a Leucine-rich repeat protein (At1g56140) showed a twofold transcript accumulation with respect to the control in response to the same treatment. For At4g17800, encoding a protein of unknown function, a 200% and a 100% increment were detected, respectively, after 30 and 120 minutes, while At5g45680 (a FKBP-type isomerase) transcript showed a twofold induction at 30 minutes of treatment, returning to the control level after 2 hours. The transcription of both At1g50030 and At1g57720, encoding the

TOR-kinase and a putative elongation factor 1B gamma, respectively, was strongly induced by HS only after 120 minutes. The same pattern of expression in response to HS treatment was also evidenced for other three genes: At2g29630, involved in the thiamine biosynthesis, At3g16460, a gene of the jacalin lectin family protein, and At1g56140, encoding a leucine rich repeat family protein.




At4g14950
Mean Normalized Expression (A.U.)
2,0E-01
1,5E-01
1,0E-01
5,0E-02
0,0E+00
At4g17800
2,0E+00
1,6E+00
1,2E+00
8,0E-01
4,0E-01
0,0E+00
At5g23720
2,0E-04
1,5E-04
1,0E-04
5,0E-05
0,0E+00
At3g46000
1,4E-01
1,2E-01
1,0E-01
8,0E-02
6,0E-02
4,0E-02
2,0E-02
0,0E+00
At5g45680
3,0E-01
2,5E-01
2,0E-01
1,5E-01
1,0E-01
5,0E-02
0,0E+00
At2g30950
7,0E-01
6,0E-01
5,0E-01
4,0E-01
3,0E-01
2,0E-01
1,0E-01
0,0E+00
At2g21870
1,0E+00
8,0E-01
6,0E-01
4,0E-01
2,0E-01
0,0E+00
At1g12360
4,8E-04
4,0E-04
3,2E-04
2,4E-04
1,6E-04
8,0E-05
0,0E+00
At1g56340
2,0E-01
1,5E-01
1,0E-01
5,0E-02
0,0E+00
At4g24190
6,0E-02
5,0E-02
4,0E-02
3,0E-02
2,0E-02
1,0E-02
0,0E+00
To
C30
HS30
C120
HS120


At1g50030
Mean Normalized Expression (A.U.)
1,4E-04
1,2E-04
1,0E-04
8,0E-05
6,0E-05
4,0E-05
2,0E-05
0,0E+00
At3g16460
1,6E-04
1,4E-04
1,2E-04
1,0E-04
8,0E-05
6,0E-05
4,0E-05
2,0E-05
0,0E+00
At1g09130
3,5E-07
3,0E-07
2,5E-07
2,0E-07
1,5E-07
1,0E-07
5,0E-08
0,0E+00
At3g57390
3,0E-02
2,5E-02
2,0E-02
1,5E-02
1,0E-02
5,0E-03
0,0E+00
At4g34220
1,4E-01
1,2E-01
1,0E-01
8,0E-02
6,0E-02
4,0E-02
2,0E-02
0,0E+00
At1g09210
3,5E-02
3,0E-02
2,5E-02
2,0E-02
1,5E-02
1,0E-02
5,0E-03
0,0E+00
At4g31530
6,0E-04
5,0E-04
4,0E-04
3,0E-04
2,0E-04
1,0E-04
0,0E+00
At5g65310
5,0E-01
4,0E-01
3,0E-01
2,0E-01
1,0E-01
0,0E+00
At3g15540
6,0E-04
5,0E-04
4,0E-04
3,0E-04
2,0E-04
1,0E-04
0,0E+00
At3g18870
6,0E-02
5,0E-02
4,0E-02
3,0E-02
2,0E-02
1,0E-02
0,0E+00
To
C30
HS30
C120
HS120

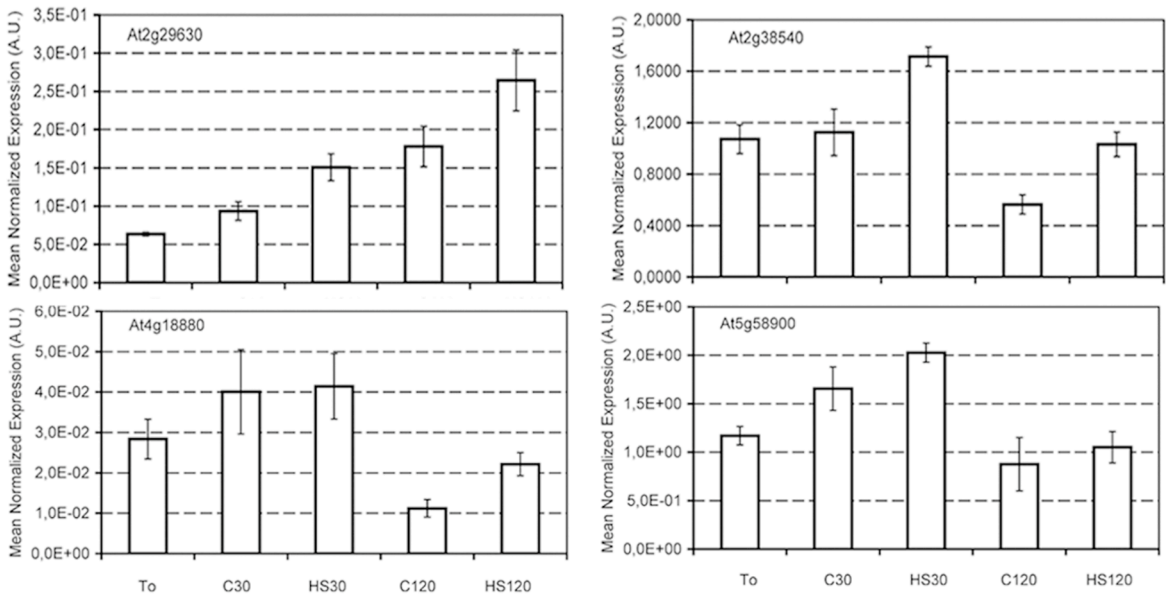


Figura 4 - Real-Time PCR expression profiles of 32 selected transcripts showing up- or down- regulated patterns in response to 30 and 120 minutes treatments with HS. C refers to the control, while HS30 and 120 refer to samples treated for 30 and 120 minutes respectively with 1mg C l^{-1} HS.

Discussion

Previous reports demonstrated the stimulatory effects of HS on physiological processes related to growth and metabolism, such as lateral root development and nutrient uptake (Zandonadi et al., 2007; Canellas et al., 2008, 2002; Varanini and Pinton, 2001; Quaggiotti et al., 2004, chapter 2). Despite the considerable amount of physiological and biochemical data, no information is available on the global effects that HS exert on genes transcription. In the present study, cDNA-AFLP was employed to identify HS-responsive genes in *Arabidopsis thaliana* with the aim of shedding more light on the potential molecular mechanisms through which HS exert their physiological effects.

The spectroscopic profile of the humic fraction used in this study was previously characterized in section 2 of this thesis. This analysis showed typical features of substances at an early stage of humification, such as a great presence of carboxylic groups, sugar-like components and low content in aromatic substances. Moreover, in the same study, the presence of a well-defined amount of indol-3-acetic acid in this HS fraction was proved by means of an immunological approach.

Transcriptomics may represent a powerful tool for the global analysis of plant-soil interaction, and thus we employed this strategy to provide the first large-scale investigation on the overall effects of humic substances on *Arabidopsis* physiology. In the present study, cDNA-AFLP technique was aimed at identifying HS-regulated genes. *Arabidopsis* seedlings were exposed to HS treatments for 30 and 120 minutes to detect genes early responding to HS. Treatments time-course and concentrations were chosen on the basis of previuos studies concerning the auxin-like activity of HS (chapter 2), in which the authors evidenced that the transcription of IAA19, an early auxin inducible gene, is rapidly and transiently induced already after 30 minutes of HS treatments. A T_0 point has been included as an internal control to the time course analysis, to discriminate among HS specific actions and effects due to stress, either mechanical or abiotic.

The AFLP analysis evidenced a widespread but slight modulation of the transcriptional activity, since no strongly affected profiles of gene expression were observed after HS treatments. This finding suggests that the physiological effect of HS may rely only partially on gene transcription regulation and that some different mechanism of action may also contribute to their overall biological activity.

All the 133 TDFs were classified according to Gene Ontology terms. The bioinformatic analysis was carried out independently on positively and negatively regulated transcripts, in order to better identify the mechanisms involved in the plant's response to HS. Regarding the biological process, the majority of annotations for both up- and down-regulated transcripts were attributed to 'metabolic processes' and 'cellular processes', indicating that HS treatments may affect the primary metabolism of plants by acting on gene transcription. This finding was also supported by the classification observed for the cellular component annotations, in which the intra-cellular components annotations were collected as the more representative. These results confirm previous reports concerning HS biological activity, reporting various physiological effects on plant metabolic pathways, such as glycolysis and Krebs cycle (Flaig and Saalbach 1955; Nardi et al., 2007), photosynthesis (Ferretti et al., 1991; Merlo et al., 1991), and nitrate assimilation (Muscolo et al., 1999; Sessi et al., 2000).

In terms of molecular functions, among transcripts being up-regulated by HS those encoding transcription factors or involved in protein binding activities were more significantly represented than among the down-regulated ones. These findings support the

idea that HS may exert their biological effects through transcriptional mechanisms of regulation, already hypothesized in different studies (Quaggiotti et al., 2003; Vaccaro et al., 2009; chapter 2), but highlight also the importance of post-transcriptional regulation in the response to these substances. A recent research, aimed at studying the effect of similar HS on protein patterns in maize root, identified 42 differently expressed proteins, giving further strength to our results (Carletti et al., 2008).

Information obtained from the GO analysis, along with the expression profiles and the putative function of the genes herein identified have been further used as criteria to select trascripts for the Real-Time expression investigations, which validated almost completely the cDNA-AFLP expression patterns. The results confirmed that the use of a low cut-off in terms of differential expression together with the RT-PCR validation is a good strategy to discard false positives and to isolate 'true' differentially expressed transcripts.

According to protein functions, a number of transcripts selected by cDNA-AFLP were found to be related to photosynthesis. The HS influence on photosynthetic processes supports previous results (Ferretti et al., 1991), even if specific studies on the effect of HS on photosynthesis are still lacking and further information is required.

Considering the role of HS on growth promotion (Canellas et al., 2002; Schimdt et al., 2007) and the lack of information concerning their physiological mechanisms of action, in this paper we mainly focused on genes related to developmental processes, to investigate the role of HS on regulation of meristem formation and organization, cell cycle, microtubule organization and cytokinesis.

It is well known that plant growth and development are sustained by meristems. Stem cells in the root meristem generate transit-amplifying cells, which undergo additional division in the proximal meristem, and differentiate at the meristem transition zone that encompasses the boundary between dividing and expanding cells in the different cell files (Dello Ioio et al., 2007). For meristem maintenance, the rate of cell differentiation must equal the rate of generation of new cells. According to this evidence, cell division and differentiation are key events in meristem development, and the hormone auxin has been further demonstrated to strongly affect these processes (Skoog and Tsui, 1948; Skoog and Miller, 1957; Ross and O'Neill, 2001). In our study the expression of several genes directly or indirectly related to meristem development and organization was shown to be HS-regulated, with 75% of them

showing enhanced expression in response to HS treatments. Among them *SHD*, which encodes the *Arabidopsis* ortholog of an HSP90-like protein (designated as the glucose-regulated protein of 94 kDa), showed a significant increase of transcription after two hours of HS treatment. The case of *SHD* appeared to be very intriguing, since *Arabidopsis grp94* mutants show an aberrant phenotype in both shoot and root meristem, and during pollen tube growth (Ishiguro et al., 2002). SHD functions as a chaperon protein in the post-transcriptional regulation of developmental processes, taking part in the CLV1/CLV2 complex activation for CLV3 binding (Ishiguro et al., 2002). Moreover, its expression is stimulated by auxin (https://www.genevestigator.ethz.ch/gv/index.jsp) and, in our experiments, by HS. Further studies will exploit this key factor to unravel the action of HS during meristem formation.

Another gene involved in the meristem formation (*IRKI*) showed a strong increase in its transcript accumulation after HS treatment, giving us a further evidence of the HS action on this process. Meristem maintenance and differentiation is regulated by intercellular communication by receptor-like kinases (RLK) (Clark et a., 1997; van der Berg et al., 1998; Hattan et al., 2004). *IRKI* encodes an interactor of inflorescence and root apices receptor-like kinase (IRK), which is expressed in several tissues in which cells are actively dividing, it is induced by exogenous auxin, and repressed by inhibitors of polar auxin transport. In particular, it does not function as a component of common cell proliferation, but is linked to multi–cellular organization of plants, acting downstream of *SLR* (SOLITARY ROOT) (Fukaky et al., 2002) in the auxin-regulated lateral root formation (Hattan et al., 2004).

A transcript herein identified by cDNA-AFLP encodes a TOR-kinase (target of rapamycin), belonging to the family of the phosphatidylinositol 3-kinase related kinases. Several components of the TOR signalling pathway are present in *Arabidopsis*, and are ideal candidates for operating the link between nutritional sensing and the regulation of growth (Menand et al, 2004). The TOR pathway can play an important role in the generation of the shape of multicellular organisms during embryonic and adult development by relaying the perception of nutrients furnished by source tissues into growing (sink) zones (Crespo et al., 2002). Disruption of the *TOR* gene leads to embryonic arrest, indicating a crucial role of TOR during early development in both *Drosophila* and *Arabidopsis* (Zang et al., 2000; Oldham et al., 2000). Moreover, *AtTOR* expression patterns are limited to the zones where

cell proliferation is coupled to cytosolic growth, such as primary apical and root meristems. Silencing of *AtTOR* (Deprost et al., 2007) showed that it is required for post-embryonic growth, and might act as a relay for ABA signalling between environmental information and the post-germinative growth processes. Others components of the plant TOR pathway are auxins and cytokinins, which are known to affect growth and cell proliferation (Stals et al., 2001). HS, as both nutrients and auxinic compounds (chapter 2) could also affect plant growth (Deprost et al., 2007) by signalling through the TOR pathway.

Normal embryo development governs correct seedling formation and preliminary results obtained in this research suggest that HS may interfere with plant growth also by targeting embryo morphogenesis. In *Arabidopsis*, the origin of seedling structures can be traced back to cell groups in the young embryo, due to the invariant pattern of cell division during early embryogenesis (Jürgens, 2001). In the present research, a gene encoding an Acetyl-CoA carboxylase (ACC1) showed an increased expression in response to treatment with humic substances. Acetyl-CoA carboxylase (ACCase) catalyses the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate, and *acc1* mutants are impaired in embryo morphogenesis (Baud et al, 2003). Moreover, the *Arabidopsis gurke* and *pas3* mutants were identified as new *acc1* alleles. A significant metabolic complementation of the *acc1* phenotype could be obtained with exogenous malonate, suggesting that the lack of malonyl-CoA is responsible for the developmental defect observed in *acc1, gk* and *pas3* mutants (Baud et al., 2004).

Moreover, in this study ClpR3, a member of ClpPR gene family, which plays a crucial role in post-translational regulation events, showed an increased transcript accumulation after HS supply. Its expression seems to be critical for embryo and seedling development, plastid size, number and function of plastids (Kim et al., 2008).

Besides the genes related to meristem and embryo development, several transcripts involved in cytoskeleton organization were also identified as HS-regulated. The actin-depolymerizing factors (ADF) are parts of the ADF/cofilin group, a family of ubiquitous, low molecular mass (15–20 kD) actin-binding proteins that include cofilin, destrin, depactin, and actophorin in eukaryotic cells (Quirk et al., 1993; Staiger et al., 1997). They are essential for many cellular processes, and play critical roles in regulating actin polymerization and depolymerization (Allwood et al., 2002; Vantard and Blanchoin, 2002).

Mutations in ADFs/cofilins from different species have been associated with lethality (Moon et al., 1993; Gunsalus et al., 1995; Chen et al., 2002), arrest in cell proliferation, and disorganized actin cytoskeletons (Chen et al., 2002). Moreover, given that actin is a structural component of most chromatin remodelling complexes and interacts with all three classes of RNA polymerase (Jockusch et al., 2006), ADFs could be responsible for shuttling actin to the nucleus and indirectly facilitating chromatin remodelling or gene expression. Thus the stimulation of its expression in response to HS is a further evidence of HS exploitation of molecular regulative mechanisms to influence plant development and growth.

A gene encoding a novel protein with a domain remarkably similar to a group of serine/threonine kinase domains (IRE) evidenced an increase of expression in response to HS. IRE protein may associate microtubules and function as a tip growth regulator through the cytoskeleton. It is noteworthy that microtubules are likely to regulate the directionality and stability of root hair growth, but are unlikely to affect the growth rate (Bibikova et al., 1999). Although the function of IRE is unclear, it has been suggested that phosphorylation/dephosphorylation of proteins in roots is important for the growth of root hairs (Smith et al., 1994). IRE-related activity might be necessary to general cell growth as well as tip growth. In our cDNA-AFLP experiments *IRE* was up-regulated by HS treatments, highlighting the interaction of microtubule activities and HS growth regulation.

Moreover in this study a gene coding for a phosphatase involved in phosphorylation cascades controlling the dynamics of cortical microtubules in plant cells (Naoi and Hashimoto, 2004) was also isolated. PHS1 is one of the five potential MAPK phosphatases represented in the *Arabidopsis* genome, and either localizes in the nucleus or shuttles between the two cellular compartments (Theodosiou and Ashworth, 2002). It may be involved in the activation or suppression of MAPs leading to modulation of the microtubule organization and stability.

Nevertheless, our results may indicate that humic substances influence plant development by interacting also with cytokinesis, in particular through cell cycle regulation. HS could act upstream the expression of genes involved in cell cycle regulation, forcing cell division and thus tissue growth. In fact, *KEULE*, which appears to have a role in cytokinesis in all somatic cells (Bergmann, 2001) showed a 5 fold increase of its transcript after 2 hours of

HS treatment. *keule* plants arrest as stunted seedlings with radial swelling, and the individual cells of the plants are bloated and disorganized. *KEULE* encodes a key regulator of vesicle trafficking, a Sec1 protein, that interacts genetically and biochemically with the syntaxin KNOLLE (Waizenegger et al., 2000; Assaad et al., 2001).

In both cytokinesis and root hair morphogenesis vesicle trafficking is tightly regulated, with respect to cell cycle cues in the case of cytokinesis and of developmental, hormonal, and environmental signals in the case of root hair morphogenesis (Miller et al., 1997; Ryan et al., 2001). Concerning root hair formation, *keule* mutants have a striking phenotype or absent root hairs (Assaad et al, 2001), suggesting a potential role for KEULE in root hair morphogenesis (Söllner et al., 2002).

Results obtained so far showed a marked biological activity exerted by HS on auxin targets (Russel et al., 2006; Canellas et al., 2002) From the overall transcriptomic results we may hypothesize that HS exert their function on plant physiology and development predominantly by means of their well demonstrated auxin activity (Dobbss et al., 2007; chapter 2), putatively interfering with vesicle trafficking, transport mechanism, gene transcription, protein regulation and possibly also with Ca^{2+} signalling. Therefore auxin activity of HS could be a possible way by which HS act on molecular regulation. This feature could be an important regulative instrument in plants crop species, which could easier adapt and react to nutritional stress conditions without demand for additional synthetic fertilizers. Further experiments are needed to assess the role played by these and other differentially expressed transcripts isolated through mRNA profiling in the orchestration of global response to HS in plants.

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Chapter 4 - Conclusion

General conclusion

Humic Substance, as the major component of soil organic matter, have been widely studied in varous areas of agriculture, such as soil chemistry, fertility and plant physiology. Recently they have been recognized as a possible tool in facing environmental problems.

Many of their positive effects on soil and plant growth have been demonstrated to rely on their chemical composition, but progress in research on HS has been considerably hampered by the lack of characterization of the humic fractions being used. The present study represents the first report concerning the global molecular mechanisms that plants act in response to Humic Substances and has gave a great amount of information about the molecular mechanisms involved in the HS-plant interaction.

A very detailed chemical characterization of the humic fractions used in this research had been undertaken by DRIFT and CP-MAS ^{13}C-NMR spectroscopy, to make the results obtained usable and comparable to other data present in litterature. HS highlighted a great presence of carboxylic groups, sugar-like components and low content in aromatic substances. DRIFT spectra reveals an high phenolic content, which may correspond to the phenolic carbons that are close to the quinonoid groups.

The combination of carboxyl and aromatic groups is a confirmation of common characteristics found out in HS from different origins and suggests that the HS deriving from earthworms are characterized by an early stage of humification.

Moreover the content of IAA in the HS was quantitatively determinated by means of an ELISA assay. In fact soils, and especially rizhosphere, are commonly known to contain variable amount of auxin, according to several factors, such as their geographical localization and the flora and microfauna hosting. In our study the IAA content in 1 mg C L^{-1} HS was found to correspond to a 34nM concentration. A precise data about the IAA concentration of the humic substances extracted from the soil analyzed is an essential prerequisite to investigate its hormonal activity, and make possible any further comparison with different humic fractions.

The auxinic activity of HS, demonstrated in the first chapter of this PhD thesis, is probably the main biological factor responsible for the positive effects exerted by HS on plant physiology. The activity of HS during lateral root development has been investigated, being

this developmental process a well known target of auxin action.

The stimulatory effect on *Arabidopsis* lateral root development observed in response to HS, has been found mainly in the first stages, when cells start to divide, suggesting that HS response may involve mechanisms as the stimulation of cell division and differentiation, which it is know to be under the control of auxin. Moreover, physiological and molecular data suggest Brassinosteroids as an additional factor throught which HS could exert their effects on plant development.

This finding has been further supported by the transcriptomic results. A great amount of the genes selected by means of cDNA-AFLP have been demonstrated to be auxin regulated and related to developmental process, as differentiation and organization of meristems, embriogenesys, citokynesis and microtubules organization.

All togheter these results provide evidence that HS need auxin transduction pathway to establish their action on plant physiology, but evidenced also the existence of different signalling cascades involved in the global physiological response of plants to these substances (Figure 1). This could be considered a starting point in elucidating mechanisms that occur in plant at molecular level in response to humic substances. Further studies are needed to assess the molecular targets and signalling pathways involved in the cross-talk between HS and plant cells.

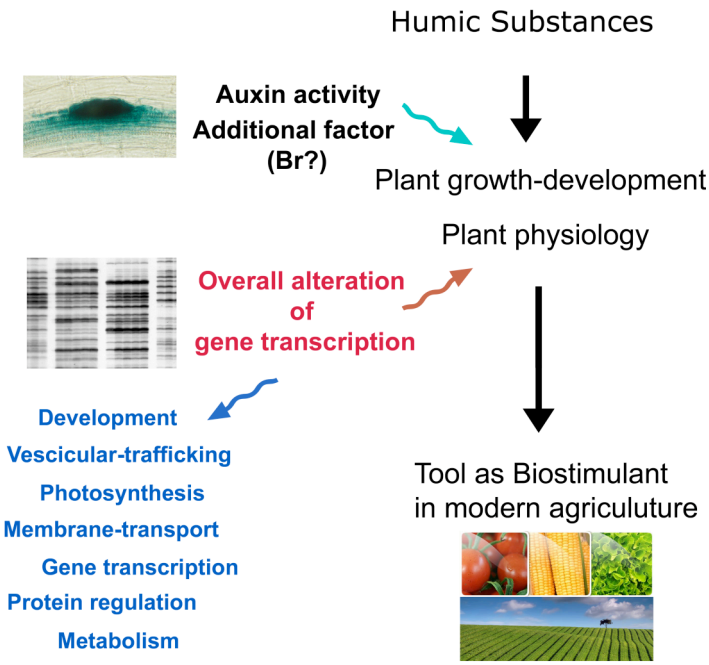

Figura 1 – Schematic representation of Humic substances impacrt on plant biology

Future perspectives

To further investigate the relationship between humic substances and plant physiology, the research program will involve firstly a detailed study aimed at better characterizing the brassinosteroids-dependents signalling pathways in the plant response to humic substances. To this aim, *Arabidopsis* mutants for brassinosteroids (*bri1, brz1, bin2*) will be treated with humic substances and their phenotype analyzed in comparison to those of control plants. This will allow unravelling divergent and overlapping mechanisms of regulation among HS, IAA and BR.

Moreover, a more systematic approach to detect global transcriptional changes occurring in the *Arabidopsis* transcriptome in response to different treatments with humic substances (in term of typology, concentration and time of exposure) will be performed.

Once gene targets, whose expression change in response to the different treatments, will be identified, a detailed study of the kinetics of this alteration will be undertaken by quantitative RT-PCR (Real Time), to select the most interesting. Specific studies should be, then, aimed at the functional characterization of the genes selected, by both silencing and knock-down approaches.

Furthermore, transcriptional profiling together with the identification of specific target genes for humic substances will help the identification of *Arabidopsis* mutants that will be used to shed more light on the mechanisms of action of these substances.

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