APROXIMACIONES GENÉTICAS Y MOLECULARES PARA LA MEJORA DE LA AVENA POR RESISTENCIA A ESTRESES BIÓTICOS Y ABIÓTICOS

MOLECULAR AND GENETIC APPROACHES FOR OAT BREEDING FOR BIOTIC AND ABIOTIC STRESS RESISTANCE

TESIS DOCTORAL

GRACIA MONTILLA BASCÓN

ENERO 2013

TITULO: *Aproximaciones genéticas y moleculares para la mejora de la avena por resistencia a estreses bióticos y abióticos*

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DOCTORANDO

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Diciembre 2013

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PhD THESIS

MOLECULAR AND GENETIC APPROACHES FOR OAT BREEDING FOR BIOTIC AND ABIOTIC STRESS RESISTANCE

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Diciembre 2013

TÍTULO DE LA TESIS: Aproximaciones geneticas y moleculares para la mejora de la avena por resistencia a estreses bioticos y abióticos
(Molecular and genetic approaches for oat breeding for biotic and abiotic stress resistance)

DOCTORANDO/A: Gracia Montilla-Bascón

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

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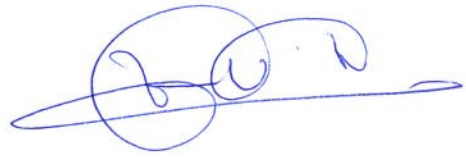

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Que el trabajo " APROXIMACIONES GENETICAS Y MOLECULARES PARA LA MEJORA DE LA AVENA POR RESISTENCIA A ESTRESES BIOTICOS Y ABIOTICOS" realizado Gracia Montilla Bascón bajo su codirección se considera ya finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

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A mis padres

RESUMEN

La avena es un cultivo de origen mediterráneo presente de forma tradicional como cultivo de grano y forraje, siendo el cuarto cereal en importancia en España, solo superado por trigo, cebada y maíz. Sin embargo, la sequía y enfermedades causadas por hongos biotrófos, tales como la roya de la corona (*Puccinia coronata* f.sp. *avenae*) y el oídio (*Blumeria graminis* f. sp. *avenae*), mitigan fuertemente su producción. El hecho que tanto la tolerancia a la sequía como la resistencia a ambas enfermedades sean extremadamente complejas, incluyendo un amplio rango de procesos entre los que están incluidos importantes interacciones entre moléculas señalizadoras, hacen que el conocimiento de las bases moleculares de la resistencia a dichos estreses sea fundamental para la mejora genética de este cultivo. Además, conocer un poco más sobre los efectos sinérgicos y antagónicos de las moléculas implicadas en la resistencia a ambos estreses es fundamental para promover una mejora genética de la avena más eficiente y con ello poder desarrollar plantas con una resistencia más amplia pudiendo hacer frente a diversos estreses.

Teniendo en cuenta esto, en el capítulo 1 de la presente tesis estudiamos el papel de las poliaminas en la resistencia a oidio. Así, se monitorizó el contenido de poliaminas en dos genotipos, resistente y susceptible, de avena a oidio durante la interacción huésped y no-huésped. Los resultados mostraron diferencias significativas en los niveles de algunas poliaminas solubles entre los genotipos resistente y susceptible en ambas interacciones, así como en los productos de degradación de las poliaminas relacionados con las especies reactivas de oxígeno (ROS). Esto sugiere un papel importante de las poliaminas en la resistencia a este hongo fitopatógeno. En el segundo capítulo se llevó a cabo el estudio del papel de las poliaminas durante la infección de roya en cultivares resistentes y susceptibles de avena y en particular en los diferentes mecanismos de resistencia. Se confirmaron aumentos de espermidina y espermina asociados a mecanismos de resistencia pre- y durante la penetración de la células del mesófilo, que se corroboró con el aumento de la resistencia en el cultivar susceptible tras la aplicación exógena de las mismas. En el cultivar resistente Saia observó además un aumento en la actividad DAO y PAO unida a pared y en la producción de DAP en etapas tempranas, lo que sugiere que la implicación de los mismos en la resistencia a la penetración o la contribución al endurecimiento de la pared celular o lignificación. En capitulo 3 se monitorizaron los niveles de óxido nítrico (NO) endógeno en cultivares de avena susceptible y resistente a la sequía confirmando una reducción de los niveles de NO asociados a la tolerancia. Para confirmar estos resultados se han utilizado líneas transgénicas de cebada

que sobreexpresan el gen de la hemoglobina HvHb1 reduciendo los niveles de NO y se ha estudiado el rol del NO durante la sequía en relación con el metabolismo de las poliaminas. Los resultados obtenidos muestran como una mayor resistencia de la línea de cebada transgénica frente a su "wild type", correlacionada con un aumento en el contenido de poliaminas específicas. Además se ha confirmado que el NO influye en la ruta de las poliaminas por varias vías, afectando al flujo de nitrógeno y contenido de aminoácidos clave en la biosíntesis de poliaminas, a nivel de expresión génica, influyendo genes claves de la ruta de la poliaminas y modificando postraduccionalmente algunas proteínas. Por otro lado, en el capítulo 4 se ha llevado a cabo un estudio de diversidad genética de 177 entradas, incluyendo variedades comerciales y de población de avena que componen nuestra colección con la finalidad de poder conocer la estructura y la similaridad genética de nuestra población para un uso más eficaz dentro del un programa de mejora. De acuerdo a este estudio, las entradas de la colección han sido clasificadas dentro de cuatro grupos principales que separaron claramente las variedades comerciales de las variedades población de avena roja y blanca. En el último capítulo se ha desarrollado un análisis de asociación para identificar secuencias génicas asociadas a la resistencia a roya y oídio en avena mediante "association mapping". Para ello 174 entradas de avena, incluyendo cultivares de avena roja y blanca y variedades población fueron evaluadas para resistencia a roya y oídio, y genotipadas usando marcadores microsatélites y DArTs para identificar secuencias génicas con caracteres de resistencia a dichas enfermedades. Cinco marcadores, dos de ellos altamente significativos en todos los modelos testados, resultaron asociados con resistencia a roya y un marcador resultó fuertemente asociado a resistencia a oídio. Así dichos marcadores fuertemente asociados, pueden ser utilizados como candidatos ideales en posteriores estudios y en futuras estrategias de selección asistida por marcadores.

ABSTRACT

Oat is a Mediterranean crop used for grain and fodder and ranking fourth in importance in Sapin, following wheat, barley and maize. However drought stress and fungal diseases such as the crown rust (*Puccinia coronata* f.sp. *avenae*) and the powdery mildew (*Blumeria graminis* f. sp. *avenae*) strongly constrain its yield. The high complexity of the resistance responses to both, drought and fungi, including the crosstalk with other signaling molecules, made that a deep knowledge of the molecular bases of the resistance to these stresses is crucial for breeding the crop. In addition, knowledge of the synergic and antagonic effects that different molecules involved in both kind of stresses may have is important to promote a more efficient breeding of the crop.

Taking this into account in the chapter 1 of the present thesis we studied the role of polyamines in the resistance to powdery mildew. Thus, polyamine content in two, powdery mildew resistant and susceptible oat genotypes was monitored during a host and non-host interactions. Results showed significant differences in the levels of particular soluble polyamines between resistant and susceptible genotypes in both interactions. In addition, resistant and susceptible genotypes differed in the content of important polyamine degradation products, suchas reactives oxygen species (ROS). This suggests an important role for polyamines in the oat resistance to these phytopathogenic fungi. In the second chapter, we carried out an study on the role of polyamines during the oat-rust interaction focusing in particular disease resistance mechanism. We confirmed an increase on spermidine and spermine associated with the pre- and penetration resistance. These results were supported with bioassays of exogenous polyamine application that increase the resistance in the susceptible cultivar. In addition in the resistant cultivar Saia, we observed an early increase in the cell-wll bound DAO and PAO activity and in the generation of DAP, suggesting its implication in the penetration resistance for instance in the cell-wall reinforcement or lignification. In chapter 3 we monitored the levels of nitric oxide (NO), in resistant and susceptible oat cultivars and confirmed an NO reduction associated with drought tolerance. In order to confirm the results and to determine the role of NO during drought in relation with polyamine metabolism, barley transgenic plants overexpressing the hemoglobin gen HvHb1 and hence with lower level of NO were used. Results showed a higher drought resistance in the barley lines overexpressing the hemoglobin gene compared with the wild type and modification in these plants in the level of specific polyamines. In addition we confirmed the influence of NO in the polyamine biosynthetic pathway at different levels: influencing nitrogen

fluxes and hence the content of key aminoacids of the polyamine biosynthetic pathway, at gene expression level, influencing key genes of the polyamine pathway and through post-translational modifications of proteins. On the other hand, in chapter 4 we carried out a genetic diversity study of 177 oat accessions including commercial varieties and landraces to determine the structure and genetic similarity of the collection, which is an important component for a more efficient breeding. According to our study, the different accessions of the collection were classified in four clusters that clearly separated the commercial varieties from the landraces and the red and white oats. In the last chapter and taking advance of this previous studywe carried out an association mapping study in order to associated specific genomic regions with resistance to rust and powdery mildew. To this aim, the 177 accessions of the oat collection including the white and red oat commercial varieties and landraces were screened for resistance to rust and powdery mildew and genotyped using SSR and DArTs markers. Five markers, two of them highly significant in all models tested, were associated with the resistance to rust, and one marker was strongly associated with resistance to powdery mildew. These markers can be used as candidates to future studies and strategies of marker assisted selection.

OBJECTIVES

The main objectives of this work are:

- Characterize the role of polyamines in the host and non-host interaction between oat and the powdery mildew fungus (*Blumeria graminis* f. sp. *avenae*).
- Determine the implications of polyamines on pre/penetration resistance and hypersensitive response of oat to crown rust (Puccinia coronata f.sp. avenae).
- Characterize the role of polyamines during tolerant responses to drought stress in cereals and the possible crosstalk with the signaling molecule nitric oxide.
- Study the genetic diversity of an oat collection of commercial cultivars and landraces.
- Perform a genome-wide association study for crown rust and powdery mildew resistance in an oat collection of commercial varieties and landraces.

INDEX

General introduction

GENERALITIES OF THE OAT CROP

The Oat Crop

Avena ssp. ranks sixth in world cereal production statistics, following wheat, maize, rice, barley and sorghum (FAO, 2013). Thus, world production in 2012 was 20.974.945 tons, 7.893.544 tons in Europe and 681.200 tons in Spain, the latter being the fifth country in European production (Fig. 1).

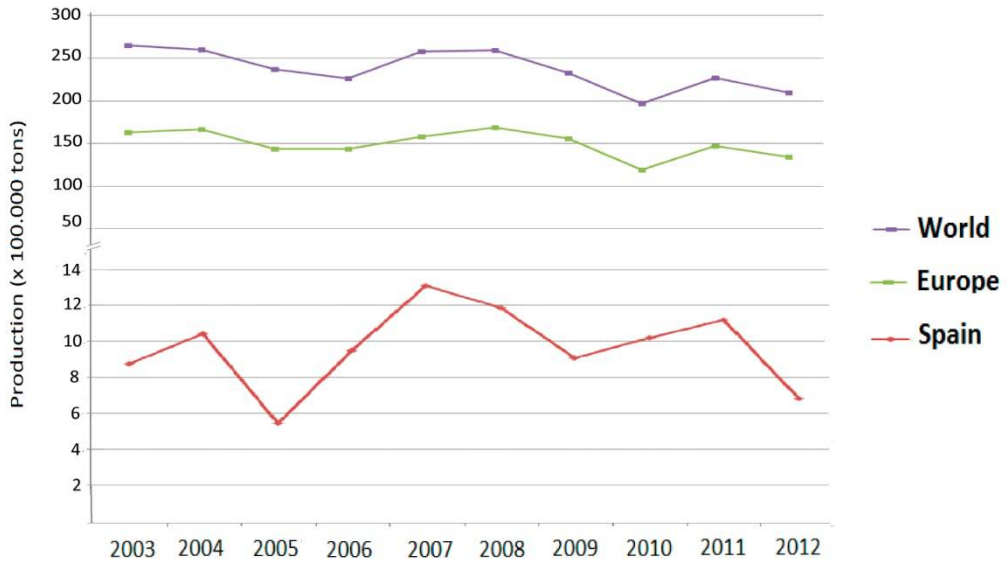

Figure 1. Evolution of the average production of oat (x 100.000 tons) in the World, Europe and Spain during the last ten years (FAO, 2013).

With respect to the cultivated area, oats are widely grown in temperate areas, with an increasing interest to expand the crop to subtropical areas, Mediterranean countries (Stevens et al., 2004) and northeast China (Islam et al., 2011). This is mainly due to its good adaptation to a wide range of soil types and because on marginal soils oats can perform better than other small grain cereals (Stevens et al., 2004). In 2012, the oat cultivated area in the world was 9.627.546 hectares; 2.665.677 in Europe and 441.600 has in Spain being the latter the fifth country in the world and the second in Europe (Fig. 2).

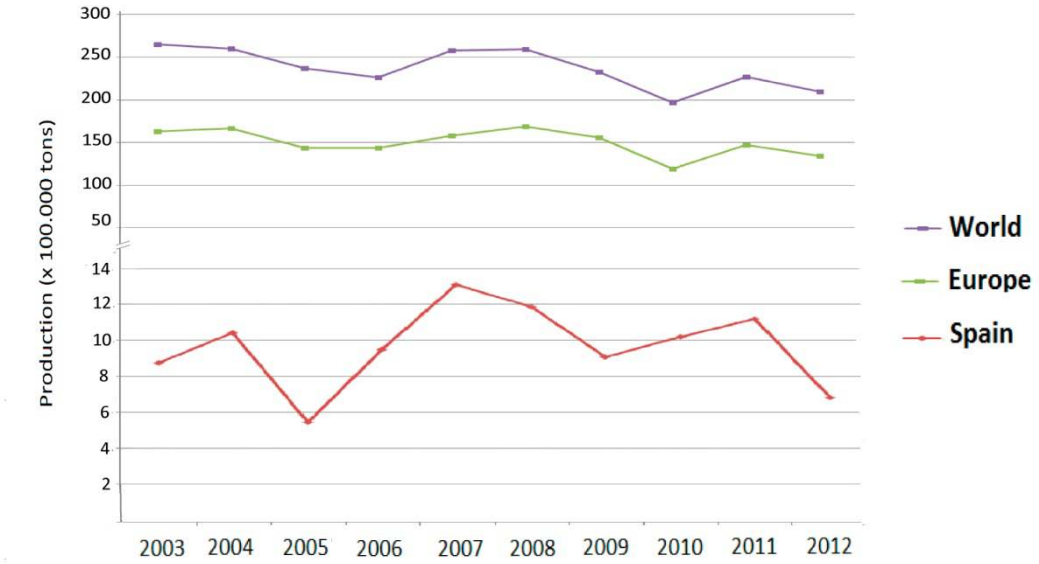

Figure 2. Evolution of oat area cultivated (x 100.000 hectares) in the World, Europe and Spain during the last ten years (FAO, 2013).

Taxonomy

Oats (*Avena sativa* L.) are members of the Gramineae (Poaceae) family along with other important grass species (Fig. 3; Draper et al., 2001). The base chromosome number of oats is seven and within the genus *Avena* we distinguish three ploidy levels according to their chromosome number (Chamla, 1984) and four karyotypes (A, B, C and D). Diploid oats (2n = 14) include *Avena strigosa* Schreb.; *Avena strigosa* var. *brevis* Roth.; *Avena clauda* Dur. ; *Avena longiglumis* Dur. Tetraploid oats (2n = 28) include *Avena barbaso subsp. wiestii* Steud.; *Avena barbata* Pott. Ex Link. Hexaploid oats (2n = 42) include *Avena* sterilis L. ; *Avena byzantina* C. Koch.; *Avena* fatua L.; *Avena sativa* L. (Table 1). All hexaploid oats have the same genome structure, AACCDD. The D genome has an unknown origin but it shows a close relationship to the A genome and may have arisen by duplication (Leggett and Thomas, 1975).

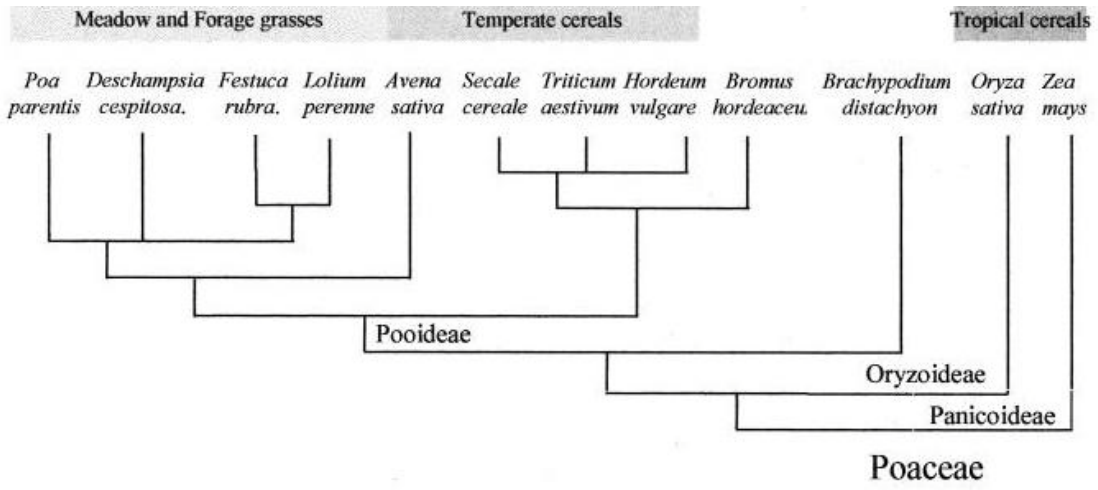

Fig. 3. Phylogenetic relationships within the Poaceae family (from Draper et al., 2001).

According to Harlan and De Wet (1971) and Leggett and Thomas (1975) *Avena* spp. are classified into three gene pools based on the ease of gene transfer (introgression) from the alien species into the cultivated hexaploid oats:

The Primary Gene Pool: Given the high inter-fertility in crosses, all the hexaploid oat taxa, including the most common wild oats *A. sterilis* and *A. fatua*, were grouped into a single biological species with cultivated oats (Ladizins and Zohary, 1971; Loskutov and Rines, 2011). Within this group we could achieve a good introgression of desired traits from the wild hexaploid oats by conventional crossing and backcrossing (Loskutov and Rines, 2011).

The Secondary Gene Pool: this group includes the AACC tetraploid species *A. magna* Murphy et Terrell. , *A. murphyi* Ladiz. and *A. insularis* Ladiz. Hybridizations with *A. sativa* produce highly self-sterile plants, but the F1 female fertility is enough to produce progeny. Subsequent crosses between tetraploid and hexaploid individuals are possible due to a correct pairing between chromosomes (Loskutov and Rines, 2011).

The Tertiary Gene Pool: Defined by Leggett and Thomas (1975) involves all the diploid *Avena* species and the tetraploids *A. barbata*, *A. vaviloviana* (Malz.) Mordv., *A. abysinnica* and *A. macrostachya*. Introgression of desired traits is complex since the F_1 progeny derived from the crosses with *A. sativa* are often sterile and the development of lines free of accompanying deleterious genes is difficult.

Table 1: Examples of ploidy levels within the Avena genus. (From Leggett, 1995)

Species	Chromosome Number	Genome
A. clauda	2x = 14	CC
A. eriantha	2x = 14	CC
A. ventricosa	2x = 14	CC
A. strigosa	2x = 14	AA
A. abyssinica	4x = 28	AABB
A. barbata	4x = 28	AABB
A. vaviloviana	4x = 28	AABB
A. maroccana	4x = 28	AACC
A. murpyi	4x = 28	AACC
A. atherantha	6x = 42	AACCDD
A. fatua	6x = 42	AACCDD
A. hybrida	6x = 42	AACCDD
A. occidentalis	6x = 42	AACCDD
A. sativa	6x = 42	AACCDD
A. sterilis	6x = 42	AACCDD
A. trichphylla	6x = 42	AACCDD

The hexaploid *A. sativa* is the most common cultivated oat. A. sativa varieties can be described as either winter or spring oats. Winter oats are sown in the autumn, over winter in the fields and are harvested in the summer. Spring oats are sown in early spring and harvested in late summer. Winter oats, like other winter cereals, have a significant yield advantage over spring oats with approximately 50% higher yield (SEERAD, 2005). However, a major limitation to growing autumn-sowing cereals is their susceptibility to freezing conditions during winter; this is particularly true for oat, that is considered the least winter-hardy of the winter cereals (Livingston et al., 2005). Winter oats also require a period of cold for vernalisation which results in a delay of flowering until spring-time. The vernalisation requirement ensures that flowering does not occur in the late autumn before the onset of winter. In contrast, spring oats flower and mature later in the summer, without vernalisation. The majority of oats grown in England and Wales are winter oats. However at northern latitudes, spring oats are used. There is a variation of *A. sativa* that was initially classified as the separate species *A. nuda* or the 'naked oat'. The difference between the two being that the groat (caryopsis) of the naked oat

threshes free from the hull (lemma and palea). However the 'naked' trait is controlled by a single gene with a few modifier genes so now naked oats are described as a variant of *A. sativa* (Rines et al., 2006).

The Origin of the Oat Crop

There is a great controversy about the center of origin and domestication of the oat crop. However, it seems clear that the domestication of the oat crop happened much later that for other cereals, such as barley and wheat. While the domestication of barley and wheat held at 12.000-8.000 years before present, oat domestication took place about 4000 years before present. This was so, because for many centuries, oats have spread as a weed in wheat and barley crops. Its constitution as crop with own identity did not occur until it was shifted to the north of the continent, where meteorological conditions are characterized by increased humidity and cold.

A good review of evolution and domestication of *Avena* species is summarized in Loskutov (2008). To date, it was accepted that the origin of *Avena* genus was the Fertile Crescent and its spread as weed within wheat and barley crop towards the rest of Europe finalized when the constitution of the oat crop by itself. We must differentiate between the origin of the genus *Avena* and the center of origin and diversification of cultivated oat. According to the analyses in the global diversity of local varieties available in the Vavilov Institute of Plant Industry (VIR) oat collection, the most likely center of origin of the genus *Avena* lies in the western part of the Mediterranean region, while the secondary center and origin of cultivated oat (*A*. *sativa* L.) is situated within the Asia Minor center (Loskutov, 2008).

The first morphogenetic center of oats, the Mediterranean center, was placed between Morocco, Algeria and Spain. From here, the center of diversity for the diploid species *A. strigosa* Schreb. was placed in Spain and Portugal towards Great Britain where naked forms designated as *A. nuda* appeared. The second morphogenetic center of oat is placed in the Fertile Crescent, the origin and domestication center of cultivated oat. From this South-West Asian Center (Turkey, Iran, Iraq and Syria) the hull-less forms (*A*. *sativa* subsp. *nudisativa* Husn.) were developed in Mongolia and China and *A. sativa* covar. *volgensis* (Vavilov, 1992) in Tatarstan region.

Morphology

The oat plant is described as having a main shoot or culm, which is usually the tallest; any side shoots that are produced are called tillers (Fig. 4). The stem is composed of a series of nodes (solid) and internodes (hollow in the maturity but solid during vegetative development). The leaves are solitary, alternate, two-ranked and sessile. Each leaf is composed of a sheath, which surrounds the culm, a ligule inserted at the apex of the sheath, and a leaf blade. Unlike the other grasses that have spikes, the inflorescence of oats is a panicle, equilateral (with spikelets arranged on both sides) or unilateral (all on one side) (Fig. 4). This is a highly branched inflorescence with a main axis called rachis, from which nodes arise other alternating lateral axes. Both the main axis and each lateral axes, culminate in a terminal spikelets, which is the individual floral unit. Each spikelet contains 2-3 flowers. The first two structures within the spikelet correspond to a pair of squamous glumes at the base which includes the rest of the floral unit. The flower itself is composed of the reproductive organs (ovary and three stamens) and two bracts, a lower, lemma, and an upper palea (Fig. 4).

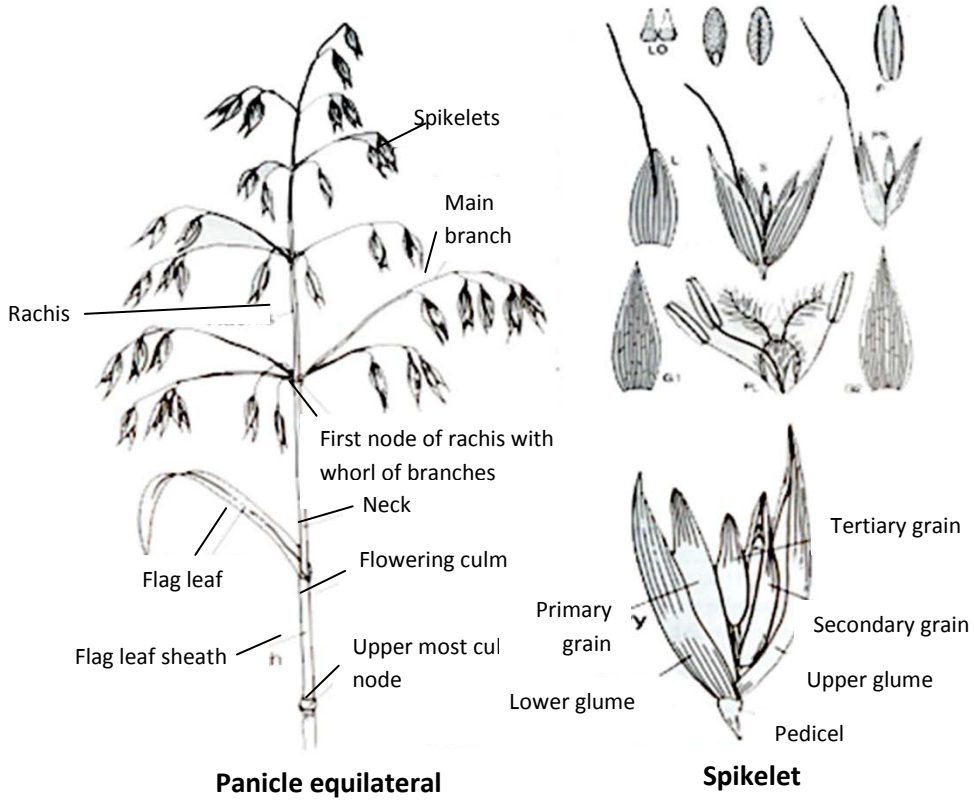

Fig. 4. A: Diagram of an oat panicle. B: Spikelet of A. sativa. Key: LO Lodicule; P. Palea; L. Lemma; S.Spikelet; FS, Florets; G1 Lower glume; G2. Upper glume; FL. Flower; CE caryopsis, dorsal viewwith embryo; CH, caropsis, ventral view showing groove. C: Detailed picture of a spikelet (from White et al., 2006).

Each developmental period of an oat plant is usually designated according to the growth stage of the Zadoks scale (Table 2; Zadoks et al., 1974). Initially the number of tillers produced is used to measure the growth stage of the crop. Stem extension is the next major class of growth stage classification. The subsequent growth stage classes are concerned with panicle emergence and development. Other key growth stages, complete flag leaf emergence, complete panicle emergence, beginning of anthesis, beginning of grain filling. These GS are the same as those used of barley and wheat (Zadoks et al., 1974). Unlike other cereal crops grown oats have a panicle rather than an ear.

Table 2: Stages of crop growth. (Zadoks et al., 1974)

GS Code	General Description	Explanation
00-09	Germination	Coleoptile emergence
10-19	Seedling growth	Leaf expansion
20-29	Tillering	Emergence of tillers
30-39	Stem elongation	Extension and formation of internodes
40-49	Booting	Swelling of flag leaf sheath
50-59	Inflorescence	Ear emerging
60-69	Anthesis	Anthers visible
70-79	Milk development	Content of milk in the grain
80-89	Dough development	Content of dough in the grain
90-99	Ripening	Grain hardening and loosening in panicle

Uses of the oat

Human food

Oats have numerous uses as human food. It is used as flakes, flour and meal. It is fairly low in gluten, so it is not suitable for making bread, although is suitable for use into oat-cakes. It is present at breakfast as cereal porridge and in special varieties of bread (FAO, 2011).

Oats has many proven health benefits for human health. The phytochemicals in oats mediate anti-oxidant activity (Sur et al., 2008) and when included in food they provide a means of preventing diseases such as cancer and coronary heart disease. ß-glucan is found in the cell walls of cereals. This compound has been found to lower blood

cholesterol levels, control blood sugar and enhance the immune system (Liu, 2007). Many studies have shown that the ß-glucan content of oats reduced the total cholesterol in the blood (Ripsin et al., 1992; Brown et al., 1999; Karmally et al., 2005; Queenan et al., 2006). Uncontaminated oats and oat products can be tolerated by the majority of people with celiac disease (Janatuinen et al., 2002; Hogberg et al., 2004; Garsed and Scott, 2007; Londono et al., 2013).

Use of whole-crop oats for animals

Oats are also used for animal feed but this is limited due to the fibrous husk which lowers the energy value of the grain. This has led to the development of cultivars of naked oats for in the animal feed industries. They were originally developed for specialized markets such as feed for premium horses but more recently their value for the avian market has been examined. Naked oats have a high oil content which provides high protein and energy grain when fed to poultry (HGCA, 2010). Naked oats had an overall better feed value when compared to wheat (HGCA, 2010). For ruminants, high oil husked lines are being developed. The low lignin husk has been found to be more digestible by rumen microbes and could have the potential to reduce methane emissions (HGCA, 2010).

BIOTIC STRESSES AFECTING OATS

Oats, as all plants, under both natural and agronomic conditions, are subjected to stresses; external conditions adversely affect growth, development or productivity of the plants. These stresses are classified in two groups depending on its origin: biotic stress when they are it is caused by the action of an organism or abiotic stress when it's caused by a physical or chemical agent (Azcón-Bieto and Talón, 2008). Biotic stresses can be produced by animals, other plants (allelopathy) and microorganisms such as bacteria, fungi and other plant pathogens causing disease. Oat diseases may cause direct damages and reduction of the fodder yield or produce indirect damages, compromising the quality of the product when they produce toxins in the grains and make them unsuitable for consumption either by animals or humans (FAO, 2011).In this work we will focus in the oat diseases caused by the rust and powdery mildew fungi.

Rust

Rust diseases are the most harmful diseases affecting cereals, and particularly oat. Cereal rusts are obligate biotrophic fungi with a complex life cycle, belonging to *Puccinia* genus. They are heteroecious and macrocyclic. To complete their life cycle, 5 types of spores and 2 different hosts are needed. On oats two spore stages of rust can occur, telial and uredial stages. The rest of spore stages to complete the life cycle must happen in the alternative host. Symptoms can appear in all aerial green parts of the plant (leaf sheaths, panicles, even floral structures) but especially in the leaves in the case of crown rust, and the stem in the stem rust. Although plants can be infected from seedling, is at the time of maturation and flowering when the disease usually impact in the crop.

The Crown Rust

Crown rust, caused by *Puccinia coronata* Cda. f. sp. *avenae* Eriks. is the most harmful disease affecting oats, causing high losses in yield and grain quality worldwide (Simons, 1985) particularly in the Mediterranean basin (Hemmami *et al.*, 2006) where populations are more virulent than in the center and north of Europe. Infection by the pathogen induces several structural, biochemical and physiological changes in its host. The more profound changes are brought about by intracellular invasion by the fungus and the formation of haustoria (Harder and Haber, 1992). Following inoculation of oat leaves with crown rust, the rate of whole-leaf gas exchange declines during the sporulation stage and photosynthesis is severely inhibited over the entire leaf (Scholes and Rolfe, 1996). This disease may reduce yield up to 40%. Its development is more rapid and harmful when weather conditions allows good oat crop growth and pathogen development, this is humidity and a range of temperature between 15-25 °C.

The life cycle explained here for *P. coronate* is valid for *P. graminis*, except for the sexual part. For *P. coronate* development, the alternative host is *Rhamnus* ssp. for *P. graminis* it is *Berberis* ssp. At the end of the planting season, coinciding with the lack of nutrients and the onset of oat senescence, uredia transform into telia that produce teliospores (Fig 5), a diploid-dark and thick-walled spore resistant to adverse environmental conditions that act as latent stage for the fungus. When environmental conditions are favorable, the germination of teliospores and subsequent meiosis results in the formation of four haploid spores, basidiospores, that is not able to infect the telial host and furthermore must travel to the aecial host, where its germination

produce a haploid colony called pycnia that can be of two or more mating types. Inside pycniospores are produced. This sexual recombination results in a higher frequency of new physiological races (Dhanda et al., 2004) particularly during summer in the Mediterranean region, where several species of *Rhamnus*, the alternate *P. coronata* host, are widespread (Vavilov, 1992).

The distribution of pycnidiospores carried out by insects or surface moisture distributes spores on the host surface facilitating the union of the opposite mating types and resulting in a dikariotic hyphae that profilate to form an aecial colony in the abaxial surface where dikaryotic aeciospores are produced . These aeciospores travel until the telial host and germinate.

After a uredospore lands on the leaf surface, its germination develops an appressorial germ tube. The germ tube growths towards stomata along the leaf surface guided by chemical and physical features of the host surface (Hoch and Staples, 1987). When finding a stomata, growth of the tube ceases and an appressorium is produced over stomata in response to some physical components of the stomatal structure (Hoch and Staples, 1987) (Fig. 6A).

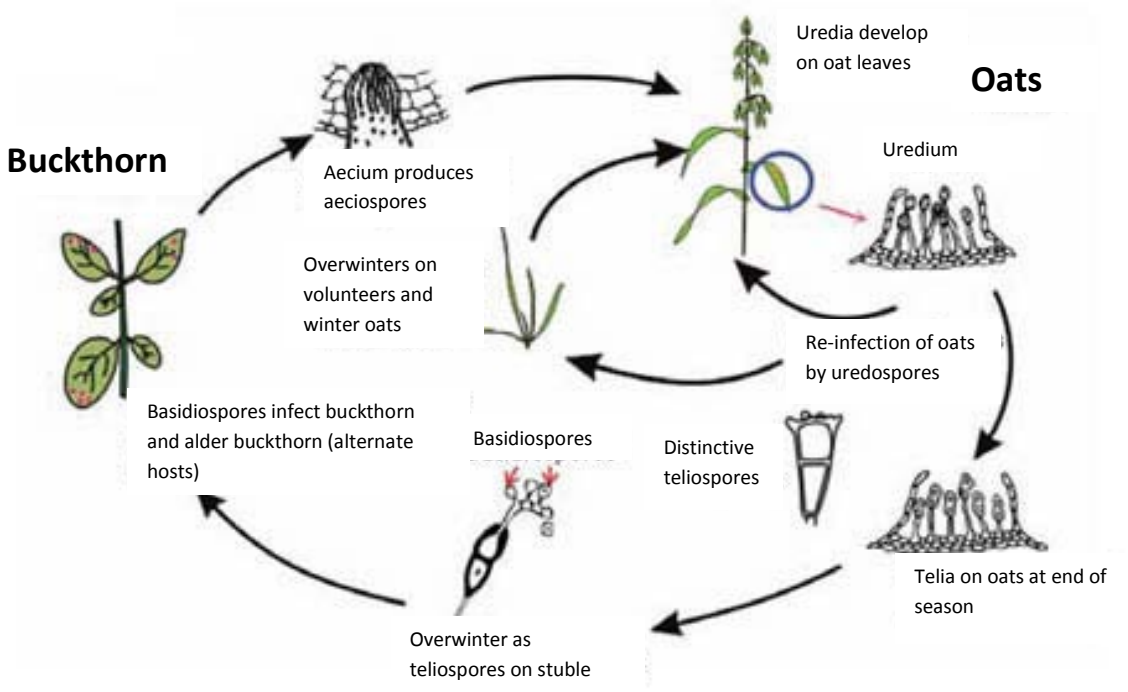

Figure5. Life cycle of *Puccinia coronata* f.sp. *avenae*

Beneath the appressorium, a penetration peg grows reaching the substomatal cavity, a cigar-shaped substomatal vesicle (SSV) is formed, from which a secondary hypha and a haustorium mother cell, at its tip, forms (Fig. 6B). Following contact between the haustorium mother cell and the mesophyll cell, an infection peg develops, penetrates the mesophyll cell and forms a feeding structure, the haustorium, which takes up nutrients for fungal growth (Parlevliet and Kievit, 1986) (Fig 6C). The haustorium is separated from the plant cytoplasm by an extension of the plant plasma membrane and is not truly intracellular but functions as the feeding organ of the fungus (Heath and Skalamera, 1997). As a result, a colony grows and develops pustules called uredinia, containing urediniospores (Fig. 6D). Beneath the appressorium, a penetration peg grows reaching the substomatal cavity, a cigar-shaped substomatal vesicle (SSV) is formed, from which a secondary hypha and a haustorium mother cell, at its tip, forms (Fig. 6b).

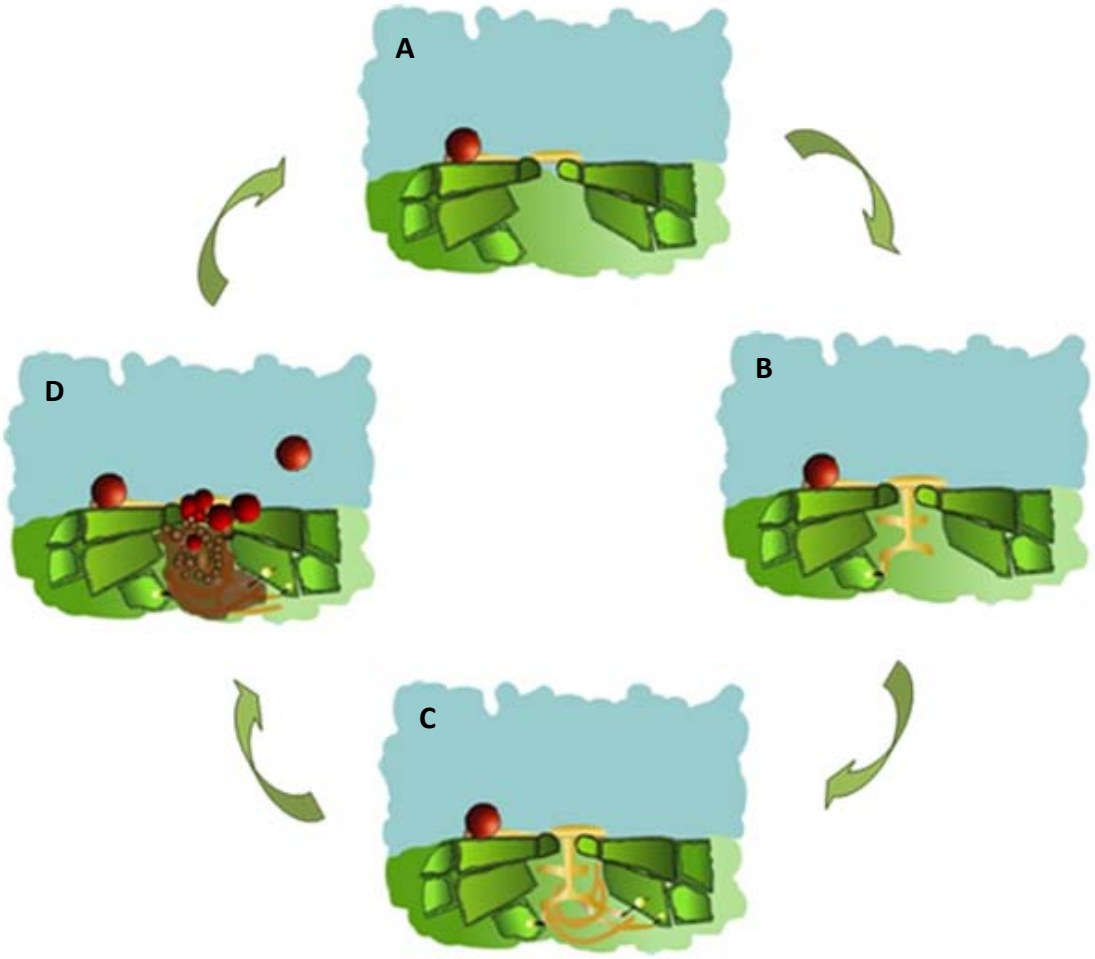

Figure 6. Infection process of oat crown rust. A) Appressorium formed over a stoma. B) Substomatal vesicle and secondary hyphae formation. C) Intracellular haustorium formed in mesophyll cells supporting the colony growth. D) Uredospores produced ready to be widespread.

Following contact between the haustorium mother cell and the mesophyll cell, an infection peg develops, penetrates the mesophyll cell and forms a feeding structure, the haustorium, which takes up nutrients for fungal growth (Parlevliet and Kievit, 1986) (Fig 6c). The haustorium is separated from the plant cytoplasm by an extension of the plant plasma membrane and is not truly intracellular but functions as the feeding organ of the fungus (Heath and Skalamera, 1997). As a result, a colony grows and develops pustules called uredinia, containing urediniospores (Fig. 6d).

Disease symptoms consist in yellow pustules containing masses of urediospores that are exposed after the rupture of the epidermis (Fig. 7). Lesions are circular or oblong and occur on both surfaces of the leaf and can reach other green parts of the plant, when the epidemic becomes more severe. After some weeks, the borders of the pustules can turn black, with teliospores formation. When the infected plants reach maturity, production of urediospores ceases and they are then replaced by teliospores.

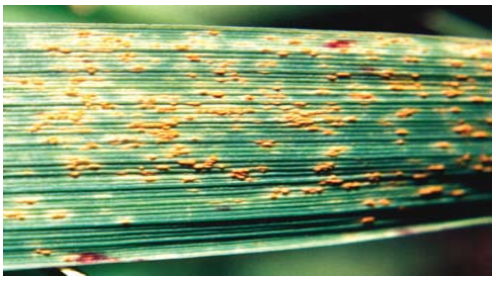

Figure 7. Pustules of crown rust on leaves caused by *Puccinia coronata* f. sp. *avenae*

- **Stem rust**

Stem rust is another harmful disease affecting oat that occurs wherever oats are grown. It is caused by *Puccinia graminis* f. sp. *avenae* Eriks. and Henn. and, although it could be a more destructive pathogen than crown rust fungi under favored weather conditions, the damages caused by stem rust are lower than with other rust diseases. However, these damages may seriously affect yield and quality of grain.

Disease symptoms appear on the stems and leaf sheaths like masses of urediospores called pustules (uredia), larger than those crown rust, oval or elongated, and dark-brown in color. They may appear in both surfaces on the leaf and the rupture of the epidermis expose masses of reddish-brown spores. The fungus can produce several

times this part of its life cycle until plant approach maturity, when teliospores transform into telia.

Powdery mildew

Although *Blumeria graminis* is considered to be a major disease of wheat and barley, in the case of oats, this consideration is restricted only in those areas with a cold and humid climate. In these regions, powdery mildew is the most important foliar pathogen of oats causing annual grain yield and total biomass losses (Harder and Haber, 1992). Jones (1977) reported that powdery mildew caused 11 to 40% oat grain yield losses depending on disease severity and cultivar. Taking into account the potential of the oat crop for fodder, it is interesting to note that a strong epidemic of the disease at the seedling stage results in 40 to 50% forage loss, albeit, the disease does not affect grain yield because later in the season stage, with the appearance of higher temperatures, the powdery mildew is arrested. Powdery mildew overwinters primarily as mycelium on volunteers and autumn-sown crops. The cleistothecia produced during late summer act as survival forms of the fungus, being resistant to low temperatures and drying out, allowing the fungus to survive for a time in the absence of a host.

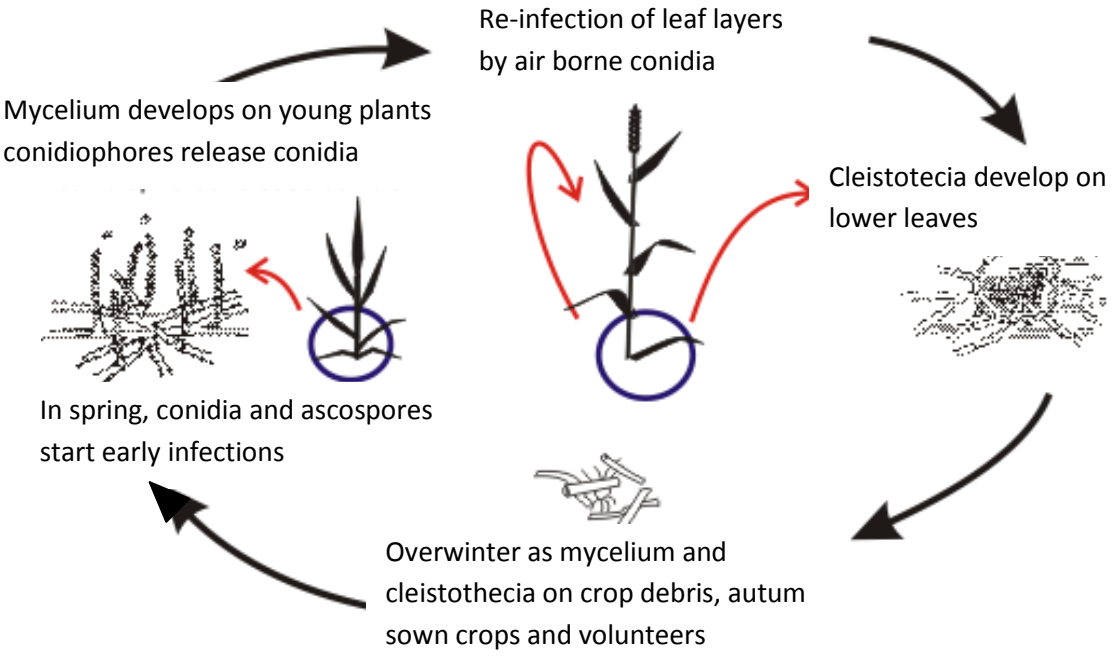

Fig 8. Life cycle of powdery mildew.

Under humid conditions, cleistothecia release the sexually produced ascospores which can initiate autumn infections. As temperatures rise in the spring, dormant mycelium starts to grow and spores are quickly produced. The asexual conidia of *Blumeria graminis* DC Speer f. sp. *avenae*, germinates and follows an ordered morphogenetic sequence (Green et al., 2002). Following deposition on the leaf surface, a short primary germ tube (PGT) emerges after contact with the epidermal cell surface and play three main roles as a prerequisite to appressorium formation (Carver et al., 2001): 1) attachment to the host surface, 2) gaining access to host water and 3) recognizing host surface features. After that, the appressorial germ tube (AGT) emerges, elongates and differentiates a hooked, apical appressorium (Kunoh, 2002) (Fig. 9a). When a functional appressorium is formed, a penetration peg emerging beneath the appressorium attempts to breach the plant epidermal cell wall, probably combining physical force and enzymatic degradation (Fig. 9b). The attack leads a cell response involving localized deposition of material into the inside surface of the cell wall, directly beneath the appressorium and penetration peg.

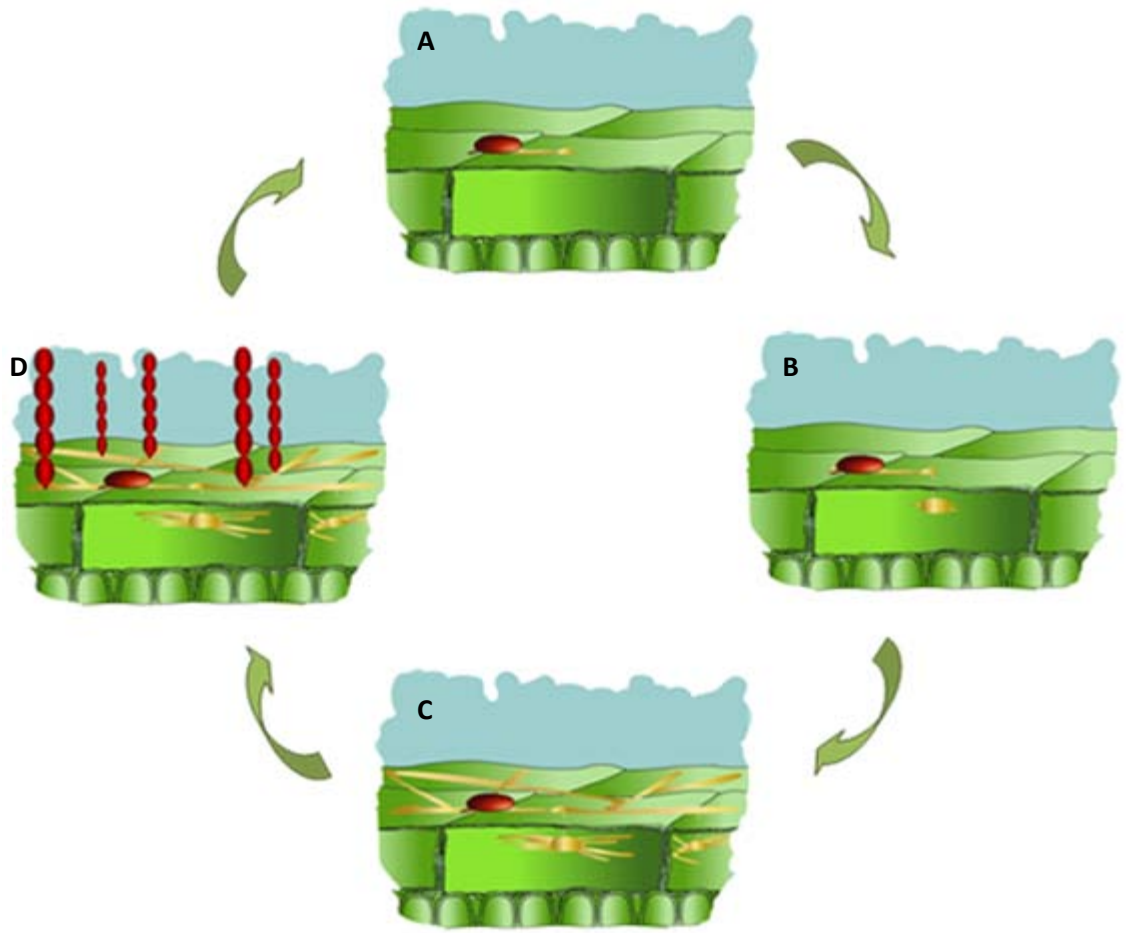

Figure 9. Infection process of *Blumeria graminis* f. sp. *avenae*, causal agent of oat powdery mildew. A) Apical hocked appressorium form on an epidermal cell. B) Penetration peg breach cell. C) Formation of an intracellular haustorium. D) Colony well-established. E) Beginning of formation of conidiophore and F) Chain of conidiophores

These cell wall appositions, called papillae, may arrest the infection process. If this first penetration attempt fails, the fungus, may forms another lobe, usually opposite the first lobe thought nearer to the conidia, and tries the penetration again. In this way, one AGT could develop 3-4 lobes in the attempt to penetrate the cell. On susceptible hosts, penetration tends to be successful and the peg penetrates into the host cell (12-15 h.a.i) and a primary haustorium is formed (15-24 h.a.i).The haustorium then develops digitate processes over the next 4-5 days (Fig. 9c). This haustorium absorbs enough nutrients to feed mycelia which develop subsequent generations of haustoria and conidiophores (Fig. 9d). At this point, the asexual lifecycle is completed.

The disease is most destructive when mild winters are followed by dry weather in spring and early summer, particularly when late-sown crops occur near infected winter oat (Jones and Griffiths, 1952). At the end of the season, volunteers and early autumn-sown crops may become infected, providing inoculum for the following crop. The symptoms appear as colonies with a grey-white superficial mycelium on the upper surfaces of the lower leaves of the plant, although colonies may develop also on the underside of the leaf (Fig. 10). The colonies could be surrounded by chlorosis and often join together to form large masses of fundal growth on the upper surface of leaves when conditions are favorable. At the disease progresses, the patches become grey or brown, and may eventually extended to upper areas in the plant if the weather conditions are favored. Severely diseased leaves collapse and die. Powdery mildew is most prevalent on lower leaves but can cause blighting of the upper leaves, heads and awns of susceptible cultivars.

Figure 10. Mycelium of powdery mildew on leaves caused by *Blumeria graminis* f. sp. *avenae*.

MAIN CONTROL MEASURES FOR OAT DISEASES

There are several methods of control against biotic stresses; however, no single practice is available to completely control all diseases. The biotic stress control should be done as part of an integrated pest management. An integrated control program is defined as the strategy that allows control of biological stress with no economic damage, using all appropriate techniques combined as possible, benefiting to the whole society. Thus the different control methods should consider not only the biological and agroecosystem factor but also related ecosystems and society that will use agricultural products (Pimentel, 1982; Kogan, 1998). The various control methods can be classified into four categories: agronomic, chemical, biological and genetic control.

Agronomic and cultural methods

The most important cultural methods include the crop rotation, agronomical changes to escape from the favorable disease conditions and practices that reduce the inoculums pool.

Crop rotation: In general, avoidance of monoculture is a good technique to reduce the impact of pests, diseases and weeds. In some areas where the same crop is sown repeatedly after years, the diseases related to it are established, incremental population and the damage is more severe.

Escape**:** Sometimes is possible avoid the coincidence between the period of maximum susceptibility of the plant and the highest abundance of the parasite through the use of early maturing cultivars or early date planting (Last, 1954). Thus, it has been reported a reduction of *P. coronata* damages in early ripening oats (Simons and Michel, 1968) and by an earlier planting date (Simons, 1966).

Inoculum reduction: Any cultural practice that minimizes the amount of over-wintering inoculum on volunteer plant should help to reduce disease levels of powdery mildew in oat (Harder and Haber, 1992). In those areas where *Rhamnus* host acts actively generating new sources of genetic variability for the pathogen, its eradication, through mechanical elimination or using foliar fungicides, could help reducing crown and stem rust epidemics. This method is applied in the dispersal part of the life cycle the pathogen.

Chemical control

Chemicals has been used for more than a century to control plant diseases, but acquired a special significance from the 40's with the development of broad-spectrum organic pesticides. Chemical control has been effective in controlling many pests and weeds and is still widely

used and necessary in intensive agriculture for high yields. However, in many cases it has been ineffective and presents a series of problems. Thus, it should be used wisely taking into account the proportion between advantages and disadvantages. Oat crop is usually considered a low input crop, with little economic margins as to justify widespread use of fungicides. Even so, Jones et al., (1985) assured that a systemic fungicidal seed treatment together with an adult plant resistant could be used controlling oat powdery mildew. Systemic fungicides have been successfully used against crown rust (Rowell, 1984). However, its use has been refused on economic grounds and difficulties to have a unique and effective formulation so its use is only recommended in very susceptible varieties under weather conditions that favored the epidemic (Soovaeli and Koppel, 2011).

Biological control

Biological control consists on the use of any living organism or substances derived there from, to control biotic factors that can damage a crop. Obviously the effectiveness of biological control depends on the combination between the agent employed and the pathogen. The use of biological must take into consideration the impact that parasites or predators may have in the ecosystem and their relationship to other crops in the area (Bélanger and Labbé, 2002).

Genetic resistance

The most desirable method for protecting plants from diseases is the use of resistant cultivars. Developing resistant cultivars requires the sought of sources of resistance, donors that restore the diversity lost in cultivated oats but that initially possessed their wild progenitors. This highlights the importance of maintain, screen and characterise genetic resources. About 220.000 oat accessions in ex situ collections have been estimated in the state of the world's plant genetic resources report (Loskutov and Rines, 2011). Large collections are held by the USDA, USA (20.000 accessions), the PGRC, Canada (30.000 accessions) and within the framework of the ECP/GR (34.146 accessions), namely by the Vavilov Institute of Plant Industry (VIR, Russia) (about 12.000 accessions), which has a collection of about 10.000 accessions of four cultivated and 2.000 accessions of 21 wild species. In FAO/VIEWS (htpp://apps3.fao.org/views/germplasm.htm), 29 collections listed maintain accessions of wild Avena species (Table 3) (Loskutov and Rines, 2011).

Some genebanks accumulate specific and geographic diversity of wild oat species not only by means of natural collection but also through seed exchange with and ordering samples from other gene banks (Table 4)

It should be noted that hexaploids species, *A. sterilis* (*A. ludoviciana*) and *A. fatua*, represent the main part of wild oat accessions in the ex situ collections because they are of great importance as breeding material and are easy to conserve and propagate in the field (Loskutov and Rines, 2011). Although acreage grown to oats is continuously declining in Europe, oat still plays an important part in the genetic resources work within the European Cooperative Programme for Plant Genetic Resources (ECPGR). This program has been established as a platform to strengthen cooperation of European ex situ collections already in 1980. The Avena Working Group has been established in 1984 as one of the original six Crop Working Groups (Germeier, 2008).

Table 3. Number of accessions of oat germplasm in the main genebanks

Institution	**Country**	**Number of accessions**
Agriculture and Agri-Food Canada, Plant Gene Resources of Canada, Saskatoon Research Center	Canada	14.935
USDA-ARS, National Small Grains Germplasm Research Facility	USA	10.908
N.I. Vavilov Research Institute of Plant Industry	Russia	2.001
Tel-Aviv University Institute Cereal Crop Development Lieberman Germplasm Bank	Israel	1.544
Agricultural Research Center, Australian Winter Cereals Collection	Australia	549
Aegean Agricultural Research Institute, Department of Plant Genetic Resources	Turkey	311
Institute for Plant Genetics and Crop Plant Research – Genebank	Germany	300
National Wheat Research Center	Brazil	254
National Plant Genetic Resources Center Plant Breeding and Acclimatization Institute	Poland	168
Agricultural Research Organization, Volcani Center, Israel Gene Bank for Agricultural Crops	Israel	117
Centro de Recursos Fitogenéticos	Spain	1405

Nowadays, the European Avena Database (EADB) has passport data of 32.910 accessions representing collections from 26 European contributors and nearly 170,000 characterisation and evaluation observation points for 3134 accessions. Besides, Avena is one of the four model crops represented in a European initiative for "An European Genebank Integration System" (AEGIS), which also represents the regional strategy for Europe.

In Spain, the Centro de Recursos Fitogenéticos (CRF-INIA) has 1405 accessions of *A. byzantina* K. Koch, *A. murphyi*, *A. prostata*, *A. sativa*, *A. sterilis* and *A. strigosa* Schreb. In the last 60 years, efforts have been done in order to incorporate resistance genes against different pathogens in cereals and also oat cultivars. The use of resistant varieties has some advantages over other control methods, especially over the use of chemicals (Niks *et al.*, 1993):

Table 4. Representation of wild Avena species in ex situ collections in the world (Germeier, 2008)

Species	Number of accessions
A. strigosa	697
A. abyssinica	615
A. barbata	2.526
A. fatua	2.341
A. sterilis	22.951

- Use of resistant cultivars represents an economic saving. If we have a complete resistance, it is not necessary the use of chemicals; if the resistance is partial, the dose employed is lower.
- The seed cost is the same compared with susceptible varieties.
- Resistant varieties are safer because its use avoid the risks of using chemicals for farmers and have no potentially harmful residues.
- Contrary to what happens with chemical control, use of resistant varieties is compatible with other control methods, such as biological control.

However, the use of resistant varieties is not a perfect solution.

- Developing resistant varieties, using conventional breeding methods, is a very long process.

- Resistant varieties may be low yielding due to the namely “resistance cost” discussed later.
- Resistant varieties are usually resistant to a specific pathogen or even isolate (race-specific resistance), while there are wide spectrum chemicals that act against several pathogens.
- The resistance usually is not durable in time, although this also occurs with the use of chemicals.

PLANT RESISTANCE RESPONSES TO PATHOGENS

It is now clear that there are, in essence, two branches of the plant immune system. One uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs), that therefore act before pathogen invade host cells. The second acts largely inside the cell, using the polymorphic NB-LRR protein products encoded by most R genes (Dangl and Jones, 2001). They are named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains. Pathogen effectors from diverse kingdoms are recognized by NB-LRR proteins, and activate defence responses leading to cell death. NBLRR-mediated disease resistance is effective against pathogens that can grow only on living host tissue (obligate biotrophs), or hemibiotrophic pathogens, but not against pathogens that kill host tissue during colonization (necrotrophs) (Jones and Dangl, 2006).

The plant immune system can be represented as a four phased ‘zig-zag’ model (Fig. 11). In a first phase, PAMPs (or MAMPs) are recognized by receptors, resulting in PAMP-triggered immunity that can halt further colonization. This phase is also named basal resistance and may act at different stage of the infection process. In phase 2, successful pathogens deploy effectors inside the cell contributing to pathogen virulence. However if a given effector is ‘specifically recognized’ by one of the NB-LRR proteins, an effector-triggered immunity leading to disease resistance and, usually, a hypersensitive cell death response (HR) at the infection site is developed (phase 3). Finally, in phase 4, natural selection drives pathogens to avoid host recognition either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress the previous reaction. In turn natural selection results in new R specificities so that the host effector-triggered immunity can be triggered again.

In this work we will focus on the resistance responses that plants may trigger as part of the basal resistance and the effector-triggered immunity. In addition a brief consideration will be also taken with those constitutive characteristic that may confer resistance without the induction of the immune system. Example will focus mainly on the rust and powdery mildew-plant interaction since they are the main target of our studies.

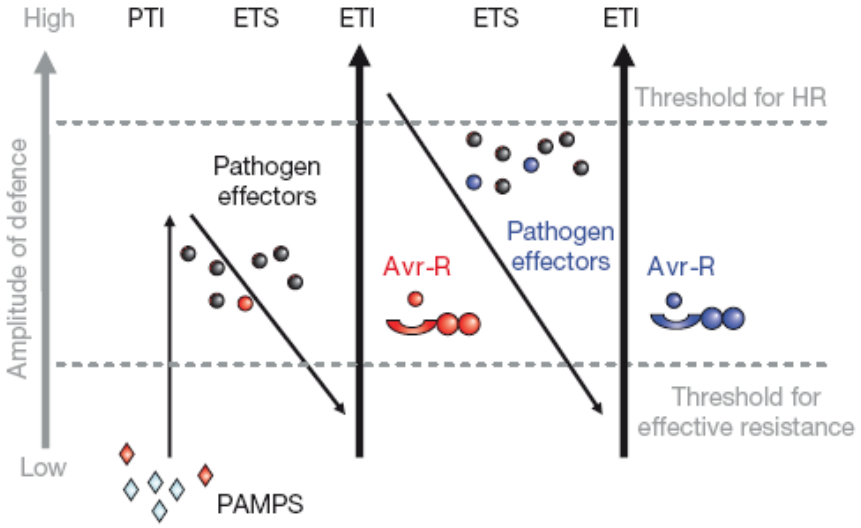

Figure 11. Zigzag model illustrating the quantitative output of the plant immune system. From Jones and Dangl, 2006

Constitutive defenses

Constitutive, physical leaf characteristics may influence pathogens germination, appressorial tube elongation, and appressorial formation. For both, powdery mildew and rusts germination adhesion of the germ tube to the leaf surface is necessary to start the infection process (Mendgen, 1978) so that features avoiding these early processes successfully contribute to plant resistance. In addition, failed stomata penetration, by germ tubes not forming appressoria or forming them away from the stomata is responsible for might contribute to resistance to rusts (Niks and Rubiales, 2002; Patto and Rubiales, 2009; Prats *et al.*, 2007; Rubiales and Moral, 2004; Rubiales and Niks, 1992; Sillero and Rubiales, 2002).

Waxes: waxes on leaf surfaces may form a water repellent surface avoiding the formation of a film of water necessary to germination. Thus, low appressorium formation by various leaf rusts of cereals (*P. triticina, P. hordei, P. recondita*) in some genotypes of *Hordeum chilense* and of other wild barleys (till 10 times fold reduction) have been reported, but unfortunately not in accessions of the cultivated barley, *H. vulgare* or any cultivated cereal (Rubiales and Niks, 1996). In addition a marked reduction in appressorium formation by *P. hordei* on some cer-mutants (Rubiales *et al.*, 2001) or in stomatal recognition by *P. striiformis* in some resistant wheat cvs. have been also found (Broers and Lopez-Atilano, 1996). Finally, Patto and Niks (2001) and Rubiales and Niks (1996) reported a decrease in appressorium formation due to wax accumulation (Fig 12).

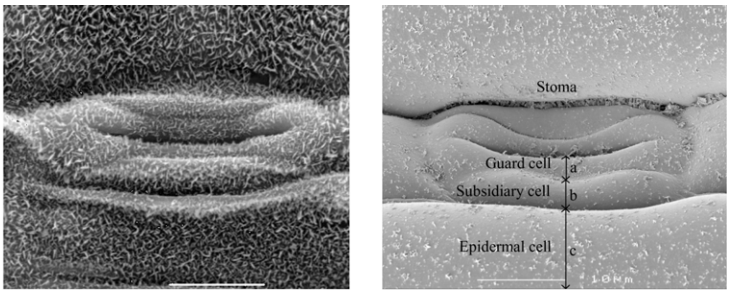

Figure 12. Ultrastructure of stomata of *H. chilense* line H1 (low avoidance) control (left) and wax removed (right) from (Patto and Niks, 2001).

Trichomes: Trichomes have been also reported to hamper the germ tubes in reaching a suitable penetration site, considering them as a physical barrier to infection (Martin and Glover, 2007) and in *Uromyces*, this can retard germination of the surface of bean leaves by trapping the spores (Mmbaga *et al.*, 1994). Finally, Chattopadhyay *et al* (2011) reported a powdery mildew resistance in field grown mulberry (*Morus* spp.) associated to high trichome density.

Basal resistance

In addition to these constitutive characteristics, PAMPs may induce a battery of mechanisms to avoid cell invasion by the pathogen.

Production of fungitoxic compounds: Fungitoxic compounds excreted to leaf surface may inhibit conidia germination, and appressorium formation. Thus, in rice, inhibition of germination and appressorium differentiation by *Magnaporthe grisea* has been reported to occur, probably by the accumulation of fungitoxic leaf diffusates accumulated in resistant and partial resistant cultivars (Pasechnik *et al.*, 1997). Studies by Prats *et al.*, (2002) demonstrated how coumarins accumulations (such as scopolin, ayapin and scopoletin) on sunflower leaf surface prevent rust germination tube growth and appressorium differentiation, describing the "lost" stage of the fungus.

Cell-wall modifications: The cell-wall constitutes the first line of defense against fungal pathogens. It is composed of a framework of cellulose microfibrils that are embedded in a matrix of hemicelluloses, pectins and structural proteins. During the infection process, microbes produce a number of cutinases and cell wall hydrolyzing enzymes, such as pectinases, cellulases, xylanases and polygalacturonases (PGs) that attack the cell wall polymers, to breach the cell wall and enter into the plant. Plants can exhibit inhibiting proteins of these enzymes, such as polygalacturonase-inhibiting proteins (PGIPs) that inhibit PGs conferring resistance against pathogens reviewed by De Lorenzo et al., (2001). Also, plants may exhibit some cell-wall modifications leading to plant cell wall strengthening. The materials involved in the thickening of the host cell wall range from minerals (silicon, calcium and sulphur) to more or less complex organic polymers, including callose or lignin.

- Papillae formation:

  Fungal penetration attempts may be hampered by encasement of the penetration peg in a localized deposition of material between the cell wall and the plasmalema, known as "papilla response" (Zeyen et al., 2002) (Fig 13.). Papillae are chemically complex appositions comprising inorganic and organic constituents including callose (a carbohydrate containing β-1-3, linked glucan as the most important constituent) and autofluorogenic phenolic (Prats et al., 2006). Papillae are deposited by the epidermal cell cytoplasmic aggregate onto its own inner wall surface, directly beneath the appressorium contact area (Zeyen *et al.*, 2002). Their deposition involves generation of NO (Prats et al., 2005) and H_2O_2 (Vanacker et al., 2000). H_2O_2 provides oxidative power necessary to drive protein cross-linking, polymerization or esterification of phenolic compounds (Zeyen et al., 2002) in the papilla area.

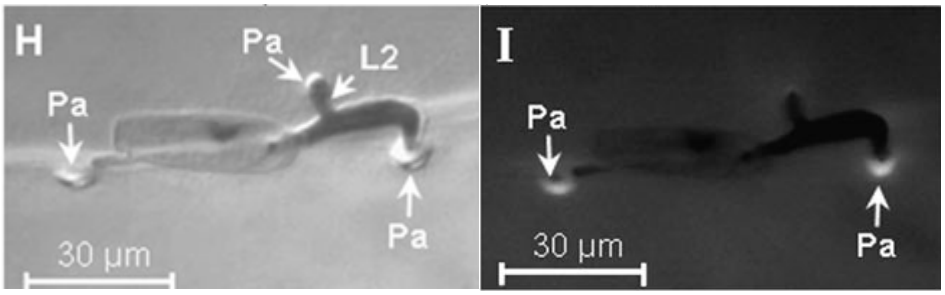

Figure 13. Transmitted light (left) and fluorescence images (right) of a germling that failed to penetrate a living plant cell from its first appressorial lobe and therefore formed a second lobe (L2). Refractive, autofluorescent papillae subtend both appressorial lobes (Pa) (Prats *et al.*, 2006).

- Cell wall lignification:

It makes the cell wall more resistant to mechanical pressure applied during penetration by fungal appressorial (Vance *et al.*, 1980). Additionally, lignin plays a role as an impermeable film and thus, less accessible to cell wall-degrading enzymes. Therefore, induced lignification represents an efficiency inducible structural barrier for plant pathogens.

- Crosslinking:

Protein cross-linking has been shown as a rapid and effective defensive response against intruding pathogens like bacteria, fungi or parasitic plants. Extensins and other Hydroxyproline rich glycoproteins (HRGPs), proline-rich proteins (PRPs), and glycine-rich proteins (GRPs) are structural proteins present in the cell walls. They can be rapidly insolubilized after wounding, pathogen penetration or elicitor treatment and it is a very fast response which enhances cell wall resistance within just a few minutes after pathogen attack. This process implies the formation of covalent cross-links and is mediated by H_2O_2 and peroxidases (Perez-de-Luque *et al.*, 2006).

Hypersensitive resistance (HR)

Successful pathogens that circumvent the basal resistance response usually triggered an HR like resistance response (Jones and Dangl, 2006). Then, this kind of resistance occurs after the pathogen reaches the cytoplasmic content (Heath, 1981). Harold Flor first described the dependence of the HR and resistance on *R* gene-interaction with pathogen encoded avirulence

(*avr*) gene production hence the term gene-for-gene interactions (Flor, 1956). Subsequently, a large number of *R* genes have been cloned and can be broadly classified into five classes (Martin *et al.*, 2003). A near ubiquitous feature of this resistance gene products (RGP) is the possession of variable numbers of leucine-rich repeats (LRR), and frequently nucleotide binding sites (NB).Those NB containing RGP that have either regions of homology to insect Toll or mammalian IL-1 receptors the TIR domain form the TIR-NB-LRR *R* gene classes. Another major class of *R* gene has a coil-coil motif instead of a TIR domain and is designated CC-NB-LRR.

Among the first signs of HR are H^+ and Ca^{2+} efflux from the apoplast and, within the attacked cell, the transient generation of nitric oxide and H_2O_2 (Prats *et al.*, 2006; Thordal-Christensen *et al.*, 1997). The whole cell subsequently shows autofluorescence, as phenolic compounds accumulate (Fig 14.). Apoptotic features in oat cells at and around the infection sites were observed to various oat pathogens: oat crown rust, the halo or stripe blights of oats and the blast fungus (Tada *et al.*, 2004). Some studies have reported the hypersensitive cell death in oat involved in resistance against crown rust (Tada *et al.*, 2001; Yao *et al.*, 2002) and victoria blight (Coffen *et al*., 2004).

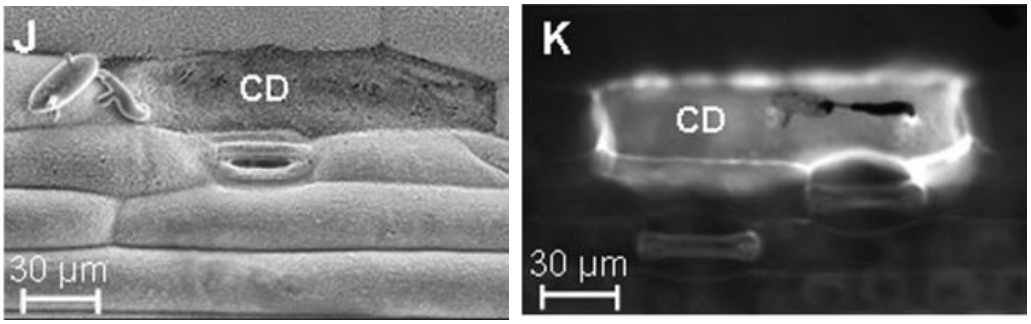

Figure 14. Epidermal cell death (CD) as a result of single gene-controlled hypersensitivity that prevents further pathogen growth. By SEM (left), dead epidermal cells are obviously collapsed while they show-cell autofluorescence viewed by fluorescence microscopy (right) (Prats *et al.*, 2006)

It is apparent that all nutrients necessary for elongating powdery mildew and rust secondary hyphae and colony growth must be absorbed via haustoria. Thus, in absence of hypersensitive response, restriction of haustorial development may result in limited fungal invasion. Restriction of haustorium growth may arise from several factors which include nutritional limitation or possibly physical restriction by the haustorial sheath which showed to be an

invagination of the host plasmamembrane separating it from the host cell (Bracker, 1968). As the sheath appears to be derived from host tissue, host genotype could exert an influence. Thus reduction in colony size could result from a lack of host nutrients suitable for colony growth or, as the sheath seems to behave like a semipermeable membrane through which nutrients must pass before being absorbed by the haustorium (Bushnell, 1972; Hirata, 1967), sheath structure may affect nutrient passage. Altogether, this resistance, only very poorly studied, lack for the selection pressure for pathogen change implicit in that kind of resistance causing a complete block to pathogen development such as hypersensitive response. Any restriction in size and/or efficiency of haustoria must necessarily affect such characters as latent period, sporulation capacity and colony size and so should reduce disease development. If the factors limiting colony growth can be combined with those limiting primary infection (i.e. penetration resistance), it should be possible to produce varieties with a high level of broadly based and hopefully durable resistance.

In conclusion several important factors are crucial for improving plant crops with durable resistance including availability of resistance sources, appropriate selection tools and characterisation of the resistance responses.

BREEDING OATS FOR RESISTANCE TO POWDERY MILDEW AND RUST

Over the last decades there has been a continuous debate on the classification of the forms of resistance and its relation with the breeding of resistance crops.

Race-specific resistance

Due to the monogenic nature of this resistance it has been the most widely used in breeding programs for years. However, under field conditions HR is almost invariably overcame by pathogens because mutation of *Avr* genes matching the R genes is favored by selection since this new pathogenic isolates avoid recognition and plant defence (Dangl and McDowell, 2006). In turn, new specificities of R genes may then be generated by variation of the leucine-rich repeats that they encoded in the plants.

Due to the relative easy management and despite the short durability of this form of resistance it has been widely use in oat resistance, particularly against rusts. Indeed, the use of race-specific (*Pc*) genes for rust resistance has been the primary mean of control. Currently,

more than 90 genes for crown rust resistance have been assigned with permanent designations (Chong *et al.*, 2000). For instance, from the primary gene pool, *A. sterilis*, the progenitor of cultivated oat, has been found to be quite a riche source of crown resistance genes allowing introgression of *Pc38, Pc39* and *Pc68* to develop hexaploids resistance oat cultivars (Brown *et al.*, 2001; McKenzie *et al.*, 1984; McKenzie *et al.*, 1981). However, the resistance was overcome and search for additional sources of resistance including new *A. sterilis* lines was necessary to do. Other *A. sterilis*-derived *Pc* genes for crown rust resistance include *Pc58* in TAM-0-301 (McDaniel, 1974a), *Pc59* in TAM-0-312 (McDaniel, 1974b) and finally *Pc60* and *Pc61* (Leonard and Martinelli, 2005). In addition, from the secondary gene pool, the resistance *Pc91* from *A. magna* has been used to develop the cultivar HiFi (McMullen *et al.*, 2005) and *A. strigosa* from the tertiary gene pool has been the source to introgress *the Pc15*, *Pc23* and *Pc94 genes*, to hexaploids *Avena* species (Dyck and Zillinsk.Fj, 1963; Rines *et al.*, 2007).

Although not as widely used as in rust resistance, sources of resistance to powdery mildew and specific resistance genes have been identified. The partially dominant gene *Eg-1* was found in a hexaploid oat derived from the cross between *A. sativa* and *A. ludoviciana* (Jones and Griffiths, 1952). Another powdery mildew resistant gene, *Eg-2*, was reported in the diploid *A. strigosa* ssp. *hirtula* by the same author. Later Hayes and Jones (1966) reported the powdery mildew resistance gene *Eg-3* traces to a wild oat, A. *sterilis* L. var. *ludoviciana*. This resistance has been transferred to many oat cultivars grown commercially in Belgium, France, Germany, Great Britain and other countries (Leonard *et al.*, 2004). Gene *Eg-4* was found in a tetraploid *A. barbata* and successfully transferred to a cultivated hexaploid oat by induced translocation (Aung *et al.*, 1977) or interfering with regular meiotic behaviour using the diploid specie *A. longiglumis* (Thomas *et al.*, 1980). Recently, a new powdery mildew resistance gene from *A. macrostachya*, *Eg-5* gene, has been successfully introgressed into hexaploid oat *A. sativa* (Frey, 1982*)*. A. sterilis has been reported as a source of resistance to oat powdery mildew (Roderick *et al.*, 2000) and although chromosomal heteromorphology between the diploid and tetraploid donors and *A. sativa* could make difficult the crosses, some successful introgression of resistance genes from *A. barbata, A. hitula, A. ventricosa, A. prostata* and *A. macrostachya* to hexaploids oat cultivars have been done (Thomas, 1968).

Unfortunately, as previously stated these genes have been usually defeated rapidly by new populations of the pathogens, because of selection pressure resulting from large-scale and long-term cultivation practices (Chong and Kolmer, 1993; Leonard *et al.*, 2004, 2005a; Leonard

et al., 2005b; Leonard and Martinelli, 2005). Thus, in the last year several strategies such as the use of multilines or pyramiding have been adopted to improve the durability of the resistance to crown rust. Gene pyramiding is based in the idea of combine in just one cultivar two or more single resistance genes. Thus, it would be necessary the occurrence of two or more simultaneous mutations to overcome the resistance. However, it's difficult to work with gene pyramiding due to the dominance and epistasis effect of multiple resistance genes. In addition, since two or more resistance genes may have similar reactions to numerous races it makes necessary the use of molecular markers to tag specific rust resistance genes (Chen *et al.*, 2007). Even so, it would take few years to gather in a single variety two or more genotypes because of the backcrosses needed. Multilines are mixtures of individual varieties that are agronomically similar (precocity, flowering time, mechanical harvesting, and grain quality) but differ in their resistance. Theoretically, the varieties would be obtained by parallel backcrosses and these varieties would be isogenic lines. However, in practice, the varieties used in the mixtures have often only a common phenotypic base. The mechanism involved in the protection of the multilines, is not completely understood. It seems clear that a fewer number of susceptible plants, would reduce the amount of inoculum available for development of the epidemic. In the same way, the presence of a resistant variety in the mixture could act as mechanical barrier, difficulting pathogen dispersion. Also, induced resistance during incompatible interactions may play a role in these mixtures. Thus, the use of multilines with *Pc51* and *Pc*52 genes has been successfully used in reducing rust severity in oat (Frey, 1982).

Broad-spectrum resistance

Unlike race-specific resistance, broad-spectrum resistance confers an incomplete, partial but more durable protection to the pathogen (Niks and Rubiales, 2002). Due to its complex genetic base it is difficult to improve cultivars with this form of resistance, mainly due to the lack of appropriate selection tools for selecting the appropriate individuals from the segregating populations.

One of the difficulties of managing the broad-spectrum resistance is the subjective assessment of the phenotypic resistance parameters. Recently, in order to avoid the subjectivity of such assessments, more objective methods based on digital image analysis (Diaz-Lago *et al.*, 2003) has been used in these evaluations. Although these phenotypic approaches can estimate the heritability and the weight of dominance and additive effects in the control of the traits, these

methods do not give information about the location and the number of genes involved (Fondevilla *et al.*, 2010).

The development of genetic maps and Quantitative Trait Loci (QTL) analyses has been a major breakthrough in the characterization of quantitative traits, enabling the identification of associated genomic regions and their contribution to the phenotypic variation. In addition, the mapping of QTLs is an useful tool to identify molecular markers linked to the resistance genes that could be used to assist breeding (Collard *et al.*, 2005).

Only a limited number of QTLs for partial resistance to crown rust have been identified and they have been determined by using the impact of the disease in agronomic traits (Diaz-Lago *et al.*, 2003) or macroscopic evaluation of disease symptoms such as disease severity and infection type (IT) (Acevedo *et al*., 2010; Jackson *et al*., 2007; Zhu *et al*., 2003) hence, the specific resistance responses linked to the QTLs were not elucidated. In chapter three "QTL association with resistance mechanism to crown rust" we aimed to identified QTLs for resistance to crown rust, and particularly with specific resistance responses related to basal resistance previously determined histologically in a mapping population Ogle x TAM 0-301 (Jackson *et al*., 2007).

Currently it is commonly accepted that together with a wide genetic resource base and appropriated selection tools, understanding the mechanisms underlying the plant resistance response is crucial in order to improve cultivars for durable resistance. Thus, there has been an increasing impetus for modern breeding strategies to employ the physiological, biochemical and molecular characteristics responsible for resistance phenotypes, which may better reflect lineage productivity, coupled with a requirement to integrate responses to environmental stress into any assessment (Araus, 1996; Richards, 1996; Slafer and Araus, 1998).

ABIOTIC STRESSES AFFECTING OATS

Abiotic stresses are the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Bray *et al.*, 2000). Abiotic stresses may have a physical nature: water stress (deficit or excess), temperature (high or low), salinity and UV radiation, or a chemical origin; heavy metals, toxins and alteration of the mineral components are the most representative.

However, among all of the resources necessary for plant growth and development, water can be considered the most important and limiting factor. In fact, currently drought is one of the main constrains preventing crops plants from expressing their full genetic potential (Farooq *et al.*, 2009) so that in the present work, we will focus on drought stress. The effects of drought in plants range from morphological to molecular levels and are evident at all phenological stages of plant growth at whatever stage the water deficit takes place. The knowledge of the impact of drought on plants is important since differences in the drought effect on the plants together with changes in the resistance responses may indicate drought tolerance and may be used as marker for selection.

Effects of drought on plants

Effects on crop growth and yield: Cell enlargement, differentiation and division are key phenomena involved in the plant growth. All of them are strongly influenced by water deficit since water is one of the physical forces involved in the cell enlargement. Impaired mitosis, cell elongation and expansion result in reduced plant height, leaf area and crop growth under drought conditions. Drought-induced yield reduction has been reported in many crop species by reduction the number of tillers, spikes or grains per plant. Moreover, the flower production and grain filling is hampered (Taiz and Zeiger, 2006). Decline in the rate of grain growth resulted from reduce sucrose synthase activity, while cessation of growth resulted from inactivation of adenosine diphosphate-glucose-pyrophosphorylase in water-stressed wheat (Ahmadi and Baker, 2001).

Assimilation partitioning: Drought stress frequently enhances allocation of dry matters to the roots, which can enhance water uptake due to a preferential accumulation of starch and dry matter in roots as an adaptation to drought (Singh and Gupta, 1993). Drought stress decreases the photosynthetic rate and disrupts the carbohydrate metabolism and level of sucrose in leaves that spills over to a decrease export rate. Limited photosynthesis and sucrose export to the sink organs and ultimately affect the reproductive development (Sinha, 1978).

Nutrients: Drought stress reduces the availability, uptake, translocation and metabolism of nutrients. A reduced transpiration rate due to water deficit reduces the nutrient absorption and efficiency of their utilization (Farooq *et al.*, 2009). Therefore, water stress affects plant mineral nutrition and disrupts ion homeostasis. It is difficult to identify the effects of drought on mineral uptake and accumulation in plant organs but in general, moisture stress induces an

increase in N, a decline in P and no effects on K. Briefly four main ions are affected by drought stress; calcium, potassium, phosphorous and nitrogen.

Water relations: Under drought stress, some important characteristics of the plant such as relative water content (RWC), leaf water potential, stomatal resistance, rate of transpiration, leaf temperature and canopy temperature are influenced. Exposure of plants to drought led to noticeable decreases in leaf water potential and RWC with an increase in leaf temperature in wheat plants (Siddique *et al.*, 2000). Change in leaf temperature may be an important factor in controlling water leaf status under drought stress. Canopy temperature, obtained by infrared thermometry, was proposed as a crop water indicator by (Jackson *et al.*, 1981) and widely used in drought studies since then. Also, numerous studies have associated increased stomatal resistance (or its opposite, diminution of stomatal conductance) with response to water deficit in cereals (Otoole and Cruz, 1980). As previously reported Medrano *et al.*, (2002), stomata close in response to drought before any change in leaf water content was detectable. This is attributed to the abscisic acid (ABA) root-to-leaf signaling promoted as the soil dries. The stomatal closure is followed by parallel decreases of net photosynthesis.

Photosynthesis: Photosynthesis is particularly sensitive to water deficit. In accordance with a decrease in relative water content (RWC) and leaf water potential, there is a decrease in photosynthetic rate (Lawlor and Cornic, 2002). However there are controversial reports about whether the origin of the photosynthetic decrease is the result of stomatal closure or a direct metabolic impairment (Sharkey, 1990; Tezara *et al.*, 1999). It´s evident that stomata close progressively with increased drought stress. Thus, water deficiency reduces the supply of carbon dioxide and photosynthetic carbon assimilation in favor of photorespiration. In addition, metabolic impairment may also contribute to photosynthetic rate decrease since: 1) under drought conditions, reduction in chloroplast volume may lead to desiccation within the chloroplast, which in turn leads to conformational changes in rubisco (Reddy *et al.*, 2004), furthermore, acidification of chloroplast stroma due to drought conditions inhibits rubisco activity (Vu *et al.*, 1987); 2) Water stress reduces activity of other photosynthetic enzymes such as phosphoenolpyruvate carboxylase, nicotinamide adenine dinucleotide phosphate-malic enzyme, fructose-1,6-bisphosphatase and pyruvate orthophosphate dikinase; 3) Inhibition of chlorophyll biosynthesis; 4) Impaired adenosine triphosphate synthesis (Tezara *et al.*, 1999).

Oxidative damage: As stomata close during progressive drought stress, an imbalance between excess incident light and the CO_2 available for reduction lead to reactive oxygen species generation (Foyer and Noctor, 2000). In fact, under conditions of photon excess, cyclic electron flow either through photosystem PSI or PSII to down regulate quantum yield. However, if there is more excitation than can be processed, photoinhibition occurs and highly reactive species are produced. In PSII highly reactive singlet oxygen can be produced via triplet chlorophyll P680 (Asada, 2006; Krieger-Liszkay, 2005). In PSI, under low $NADP^+$ concentration, Fd will reduce O_2 instead of FNR yielding O_2^-. O_2^- dismutates to H_2O_2 and dioxygen, in a reaction catalysed by superoxide dismutase (Cruz *et al.*, 2005). Increase of ROS, if not properly scavenge lead a denaturation of functional and structural macromolecules, including DNA, protein and lipids causing oxidative damage an impairing the normal functions of cells, overall enhancing peroxidation of membrane lipids, degradation of nucleic acids and both structural and functional proteins.

Mechanisms of adaptation to drought stress

Several morphological, physiological and molecular plant responses can contribute for coping with drought stress. These may be classified in three groups (Turner *et al.*, 2001): shortening of the crop duration to complete life cycle before stress, **escape**; increasing its ability to avoid damage, **avoidance mechanisms** and/or to maintenance of metabolic functions under water limiting conditions, **tolerance mechanisms**

Escape: Plants may complete the life cycle before the onset of severe drought by different ways:

- Early flowering time is probably the most representative trait associated with drought escape. Drought escape occurs when plant phenological development is successfully matched with periods of soil moisture availability avoiding stress period that could mitigate plant yield (Araus *et al.*, 2002). Developing varieties that mature before the onset of severe terminal drought has successfully increased the yield crop under drought-prone conditions (Kumar and Abbo, 2001). However, we must be careful reducing crop duration, because yield is correlated with it and an excessive decline in crop duration under favorable conditions could mitigate optimum yield (Turner *et al.*, 2001). Peuke and Rennenberg (2004) reported a selection, for earlier flowering *Avena barbata* germplasm under drought conditions.

- Early vigor. It is considered one of the most import drought escape mechanism. Early vigour should be combined with appropriate phenology for the target environment. Correlation between initial growth vigour and other characterises in recombinant imbred lines of chickpea showed that high growth vigour had significant negative correlation with days to first flower, flowering, first pod and maturity (Toker, 2006). In cultivated oat early planting, rapid germination and early emergence results in a competitive advantage over wild oat having a great effect of final yield (Willenborg *et al.*, 2005). In fact, the selection of oat genotypes with larger seeds suitable for sowing in areas displaying moisture stress will help to reduce the risk of poor stand establishment and will enable more homogenous growth under varying rainfall conditions (Medrano *et al.*, 2002).

Avoidance: It is the ability to avoid damage by keeping the water content high in the plant tissues. This may be achieved by:

- Closing stomata, reducing light absorbance through rolled leaves (Ehleringer and Cooper, 1992), dense trichome layer increasing reflectance (Larcher, 2000), steep leaf angles, decreasing canopy leaf area through reduced growth. Others traits are shedding of older leaves, production of smaller leaves, with more densely distributed stomata and leaves covered by epicuticular waxes or thicker cuticles.
- Maximising water uptake developing an adapted root system to drought conditions. Root thickness and root dry weight per tiller have been found to be implicated in drought avoidance (Champoux *et al.*, 1995). In fact, all root parameters could be considered as drought avoidance traits: biomass, length, density, depth, branching, diameter, and root to shoot ratio (Gowda *et al.*, 2011).
- Storing water on below ground organs.

Tolerance mechanisms: Understanding plant tolerance to drought is of fundamental importance and forms one of the major research topics in plants research. Osmotic adjustment, osmoprotection, antioxidation and a scavenging defense system have been the most important bases responsible for drought tolerance to counteract the previously state drought induced damages. However, the physiological basis of genetic variation in drought response is not clear; in part, because highly complex mechanisms are likely involved. Some of these mechanisms are described below (Farooq *et al.*, 2009).

At physiological level: Mechanisms such as improved water uptake under stress and the capacity of plant cells to hold acquired water, reduce water loss and water storage are important. Plants respond to water deficit using mechanisms as improved root traits (Price *et al.*, 2002) and by reducing water loss through reduced epidermal (stomatal and cuticular) conductance, reduced radiation absorption, and reduced evaporative surface (leaf area).

In addition, maintenance of turgor pressure through osmolite accumulation and cell wall elasticity. Popham *et al.*, (1993) reported that changes in cell wall rigidity are important in drought tolerance in cotton.

Biological membranes are the first target of many abiotic stresses (Levitt, 1980). Then, it's generally accepted that the maintenance of integrity and stability of membrane under water stress is a major component of drought tolerance in plants (Bajji *et al.*, 2002); indeed cell membrane stability is a physiological trait widely used for the evaluation of drought tolerance in wheat (Blum and Ebercon, 1981; Dhanda *et al.*, 2004; Singh *et al.*, 1992) and rice (Agarie *et al.*, 1995). The causes of membrane disruption are unknown; it supposed that a decrease in cellular volume causes crowding and increases the viscosity of cytoplasmic components. This increases the chances of molecular interactions that can cause protein denaturation and membrane fusion (Farooq *et al.*, 2009). Thus, the adaptation of oat seedlings to water stress has been associated with changes in the lipid composition of the plasma membrane of root cells increasing permeability for glucose and decreased permeability for protons as compared to control vesicles and highlighting the importance of the ratio phosphatidylcholine (PC) / phosphatidylethanolamine (PE), the levels of cerebrosides and free sterols and the possible interaction of these components for the plasma membrane in that acclimation (Mut and Akay, 2010).

At molecular level: Tolerance of environmental stress such drought arises from integration of events occurring at molecular and biochemical levels which are manifested at the physiological and morphological level previously seen. At the molecular level drought is perceives as signal that brings about changes in expression of genes and synthesis and modification of protein activity. At biochemical level drought tolerance responses are related with profound metabolism changes mostly leading to osmotic compound production.

Oxidative damage induced by drought may be alleviated by the join action of both enzymatic and non-enzymatic antioxidants systems. Among antioxidant enzymes are catalases,

superoxide dismutase, peroxidases, ascorbate peroxidases, glutathione reductase and monodehydroascorbate reductase. The non-enzymatic antioxidant systems include β-carotenes, ascorbic acid, α-tocopherol, reduced glutathione (Hasegawa *et al.*, 2000; Havaux, 1998; Prochazkova *et al.*, 2001).

Compatible solutes and osmotic adjustment through overproduction of compatible organic solutes (Serraj and Sinclair, 2002) help to maintain turgor in the cells; low-molecular-weight, highly soluble compounds contribute not only to osmotic adjustment, but also contributes to the detoxification of ROS, stabilization of membranes and native structures of enzymes and proteins. This involve accumulation of specific compounds such as sugars (i.e. from the raffinose family oligosaccharides, RFO) sugar alcohols (such as mannitol), amino acids (such as proline) and amines (such as glycine, betaine and polyamines) which allows the cell to decrease osmotic potential and hence increase the gradient for water influx and turgor. Thus, osmotic adjustment has been related to grain yield under water deficit environments (Moinuddin *et al.*, 2005) and it is considered as a selection criterion for drought tolerance in wheat (Dhanda *et al*., 2004).

In addition sugar and sugar alcohols such as galactinol and raffinose function as osmoprotectans during drought stress. Trehalose is a non-reducing disaccharide of glucose that functions as a compatible solute in the stabilization of biological structures under abiotic stress (Garg *et al.*, 2002). Trehalose enhance drought tolerance by stabilization of dehydrated enzymes, proteins and lipid membrane, as well as protection of biological structures under desiccation stress rather than regulation water potential (reviewed by Farooq *et al.*, 2009)

Accumulation of proline is one of the first plant responses to water stress. Proline has widely been studied because its accumulation provides drought tolerance in many species. It is thought to play a multifunctional role to cope water stress; participates in cellular homeostasis, including redox balance and energy status. Also, it acts as signaling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression which can be essential for plant recovery (Szabados and Savoure, 2010).

In addition to this and other aminoacids polyamines (PAs) such as putrescine, spermidine and spermine are accumulated under abiotic stress providing tolerance in many species such as rice or wheat (Erdei *et al.*, 1990; Yang *et al.*, 2007) playing as osmolites increasing leaf water

potential. Since we extensively focused in polyamine role during biotic and abiotic stress responses we will be devoted to these important molecules in the next section.

POLYAMINES IN BIOTIC AND ABIOTIC STRESS RESPONSES

Polyamines (PAs) are low molecular weight aliphatic nitrogen compounds positively charged at physiological pH (Groppa and Benavides, 2008) that are found in a wide range of organisms from bacteria to plants and animals (Alcazar et al., 2006).

They are protonated at cellular pH and because of this interact with macromolecules like DNA and RNA, as well as with other negatively charged molecules such as phospholipids and proteins (Walters, 2000). Thus, polyamines are known to stabilize macromolecular structures, but, in addition, recent studies indicate that polyamines may act as cellular signals in intricate crosstalk with hormonal pathways, such as abscisic acid, ethylene, hydrogen peroxide and nitric oxide (An et al., 2008; Toumi et al., 2010; Yamasaki and Cohen, 2006). Overall poliamines has been reported to act as regulatory molecules in many fundamental cellular processes including cell division, embryogenesis, senescence and in response to stress (Martin-Tanguy, 1997).

Several studies have demonstrated that the homeostasis of PAs in plants correlates with several important physiological functions, including the control of the N:C balance (Mattoo et al., 2006), stress responses (reviewed in Alcazar et al., 2011), xylem differentiation (Muniz et al., 2008; Tisi et al., 2011a; Tisi et al., 2011b), pollen tube growth (Wu et al., 2010), membrane fluidity, and protein regulation (Baron and Stasolla, 2008; Takahashi et al., 2010). Polyamines have also been implicated in plant responses to abiotic stress, with polyamine levels increasing several fold in plants responding to, for example, potassium deficiency, osmotic shock, drought and salt stress (Watson et al., 1998; Watson and Malmberg, 1996). PA catabolism, biosynthesis, conjugation, interconversion, and transport altogether contribute to PA homeostasis. The pathways of PA biosynthesis in higher plants have been well established: most genes for enzymes involved are cloned and transgenic and mutant plants with changed PA metabolism obtained (Bhatnagar et al., 2001; Kaur-Sawhney et al., 2003; Capell et al., 2004). Over-expression of different PA biosynthetic enzymes (cloned from bacterial, fungal, plant, and animal genomes) in different plant species have yielded biotic and abiotic stress tolerant plants (reviewed by Alcazar et al., 2010 and Hussain et al., 2011b). Thus PAs have been highlighted to be the substantial players in self-defense against various environmental

stresses which have been substantiated by gain-of-function and loss-of-function experiments on PA biosynthetic genes. For example, *Arabidopsis thaliana* plants overexpressing a *Cucurbita ficifoia* Spd synthase gene have been demonstrated to become tolerant to multistress factors such as low temperature, freezing temperature, salinity, drought, and herbicide Paraquat (Kasukabe et al., 2004).

In plants cells the polyamines could be found as free molecules and also exist as conjugates, associated with small molecules such as phenolics acids preferentially *p*-coumaric, ferulic and caffeic acids, (Martin-Tanguy, 1997). Conjugation is achieved by the formation of an amide linkage, using esters of CoA for provision of the activated carboxyl groups (Negrel, 1989). Putrescine and spermidine are conjugated by distinct transferases that differ in their specificities towards hydroxycinnamoyl CoA derivatives. Polyamine conjugates have long been associated with flowering (Martin-Tanguy 1997), and they have also been found to undergo marked changes in interactions between plants and viruses. Thus, Torrigiani et al., (1997) found that conjugated forms of putrescine and spermidine increased in tobacco leaves exhibiting a hypersensitive response (HR) to the tobacco mosaic virus (TMV).

Furthermore, PA catabolism/ interconversion generates reactive oxygen species (ROS), and particularly H2O2 which has been confirmed as a key player during stress responses. Stress-induced PA accumulation and their protective function against biotic and abiotic stresses are of special interest (Bouchereau et al., 1999).

Thus, a general phenomenon observed is that PAs can alter their titres in response to various types of environmental stresses such as water stress (Capell et al., 2004; Kasukabe et al., 2004; Ma et al., 2005), low and high temperatures (Hummel et al., 2004; Imai et al., 2004; Song et al., 2002) and salinity (Liu et al., 2006; Maiale et al., 2004; Roy et al., 2005). In another example, Waie and Rajam (2003), observed that transgenic tobacco plants over-expressing a human SAMDC gene had higher Spd and Put levels and exhibited tolerance to drought and salt stress. Franceschetti et al., (2004) also showed that over-expression of Arabidopsis SAMDC in tobacco plants resulted in increased SAMDC activity, accumulation of dcSAM, perturbation of PA levels and transgenic plants exhibited multiple stress tolerance. Recently, Peremarti et al., (2009) generated transgenic rice plants constitutively expressing heterologous SAMDC gene from Datura stramonium to dissect the roles of Put from higher polyamines Spd and Spm. Both transgenic and wild type plants showed identical symptoms when exposed to drought stress but transgenic plants recovered much more quickly on re-watering. Similarly, transgenic carrot

lines over-expressing mouse odc, which converts ornithine to diamine putrescine were able to withstand salt and osmotic stress over short period (Minocha and Sun, 1997). In another set of experiments, ADC expressing transgenic rice plants produced higher levels of Put, Spd and Spm and exhibited drought tolerance. Further, it has been proposed that Put may reflect the sub optimal growth conditions while Spd and Spm may help detoxifying free radicals (Larher et al., 2003). These results confirmed the involvement of polyamines in drought stress and further attributed individual roles to Put, Spd and Spm. Interestingly, introduction of a single polyamine biosynthesis gene has been shown to confer tolerance to multiple stresses. Examples of these are when Kasukabe et al., 2006; Kasukabe et al., 2004; and Wi et al., 2006 found broad spectrum tolerance to abiotic stresses—drought, chilling, freezing, salinity and oxidative stress—by over-expression of SPDS (Spermidine synthase) from Curcurbita ficifolia in Arabidopsis, sweet potato (Ipomoea batatas) or tobacco. A cDNA microarray analysis between chilled leaves of a transgenic line and wild type revealed that genes encoding transcription factors such as WRKY, B-box zinc finger proteins, NAM proteins, DREB2B and NAC domain proteins are up-regulated in transgenic plants. (Wen et al., 2008) demonstrated that overexpression of an apple MdSPDS1 gene in European pear substantially increased tolerance to multiple stresses by altering PA levels. Similarly, Prabhavathi and Rajam, (2007) demonstrated that transgenic eggplant harboring the oat ADC gene exhibited increased tolerance to drought, salinity, low and high temperature and heavy metals.

In addition, several studies have shown that polyamine accumulation occurs under biotic stresses in plants. For instance, early efforts found that in compatible interactions between barley and Puccinia hordei (brown rust fungus), polyamines particularly spermidine accumulate in infected leaves (Greenland and Lewis, 1984). Polyamine accumulation has also been reported in leaves of barley following infection by powdery mildew fungus (Blumeria graminis f. sp. hordei) (Walters et al., 1985; Walters and Wylie, 1986). The presence of PA in both plants and pathogenic fungi makes it difficult to identify their respective contribution to PA accumulation in infected organs. Another important observation regarding PA implication in the signaling pathway involved in biotic stress response has been postulated. Accumulation of H2O2 as a result of PA catabolism and nitric oxide due to induction by spermine/spermidine plays an important signaling role in plant–pathogen interactions (Romero-Puertas et al., 2004; Tun et al., 2006; Walters, 2003; Yamasaki and Cohen, 2006). These studies suggest that manipulation of key factors present upstream of polyamine biosynthesis or in the polyamine-induced signaling pathway could render the host plant resistant to biotic stresses.

During the last decade, knowledge on the importance of PAs to unfavorable growth conditions has increased considerably. Analyses of metabolic adjustments of plants with different levels of stress tolerance and transgenic approaches provide important complementing evidence for better understanding the role of PAs in adjusting to harsh environments. However, the molecular mechanism of how PAs act in these processes has remained unclear. Stress-induced signaling networks are well studied with new and exciting reports coming up every year. However, the signaling processes required for homeostasis of basic cellular and metabolic processes in adverse environments are just starting to emerge. Better understanding of how environmental changes are communicated via cellular signal transduction to induce a coordinated metabolic response, and how the function of PAs are adjusted by transcriptional and post-translational modifications, are of basic scientific interest and will contribute to meet the goal of increased plant stress tolerance and productivity in an ever-changing environment (Gupta et al., 2013).

CROSSTALK BETWEEN RESISTANCE RESPONSES TO BIOTIC AND ABIOTIC STRESSES

Plants encounter a wide range of environmental stresses during a typical life cycle and have evolved mechanisms to increase their tolerance through both physical adaptations and interactive molecular and cellular changes that begin after the onset of stress (Gupta et al., 2013). Thus, plant responses to biotic and abiotic stresses involve a network of molecular mechanisms that vary depending on the nature of the pathogen or stress signal (AbuQamar et al., 2009). It has been reported that exposure to abiotic stresses, in some cases, enhances plant resistance to pathogens indicative of crosstalk between biotic and abiotic stress signaling (Bowler and Fluhr, 2000). Induced resistance to both biotic and abiotic stresses has also been documented by Zimmerli et al., (2000), Jakab et al., (2005) and Ton et al., (2005). This may arise from the fact that the plant encounters stress combinations concurrently or separated temporally and must present an integrated response to them to survive. Thus, several biotic and abiotic stress pathways share common elements that are potential “nodes” for cross-talk. Cross-talk can also occur between pathways in different organs of the plant when a systemic signal moves from a stimulated cell into a another tissue to elicit a response (Knight and Knight, 2001).

The involvement of a wide battery of signaling molecules and hormones in plant responses to different stresses may allow plants to quickly adapt to their biotic and abiotic environment and

to utilize their resources in a cost-efficient manner (Pieterse et al., 2009). It is generally believed that hormone-regulated induced defense responses evolved to save energy under enemy-free conditions, as they only involve costs when defense are activated upon pathogen or insect attack (Walters and Heil, 2007). This cost arises from the allocation of resources to defense and away from plant growth and development. Trade-offs between plant growth rate and disease resistant has been well documented (Walters and Heil, 2007) and support the hypothesis that plant growth and defense are regulated by a network of interconnecting signaling pathways.

Phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) are endogenous, low-molecular-weight molecules that primarily regulate the protective responses of plants against both biotic and abiotic stresses via synergistic and antagonistic actions, and hence they are important components of the signaling crosstalk (Bostock, 2005; Lorenzo and Solano, 2005; Mauch-Mani and Mauch, 2005). Moreover, the generation of reactive oxygen species (ROS) has been proposed as a key process that is shared between biotic and abiotic stress responses (Apel and Hirt, 2004; Torres and Dangl, 2005). Rapidly accumulating data, resulting from large-scale transcriptome analyses with DNA microarray technology, strongly support the existence of such crosstalk between signaling networks (Schenk et al., 2000; Seki et al., 2002; Cheong et al., 2002; Narusaka et al., 2003; Davletova et al., 2005) with biotic and abiotic stresses regulating the expression of different but overlapping suites of genes.

So far, many examples of positive and negative crosstalk between SA, JA and ET signaling have been reported and are documented in a series of informative reviews (Kazan and Manners, 2008; Koornneef and Pieterse, 2008; Lopez et al., 2008; Lorenzo and Solano, 2005; Robert-Seilaniantz et al., 2007; Spoel and Dong, 2008).

In recent years, several proteins with an important regulatory role in SA-JA crosstalk have been identified in *Arabidopsis*. Mutation or ectopic expression of the corresponding genes were shown to have contrasting effects on SA and JA signaling and on resistance against biotrophs and necrotrophs (reviewed in Koornneef and Pieterse, 2008).

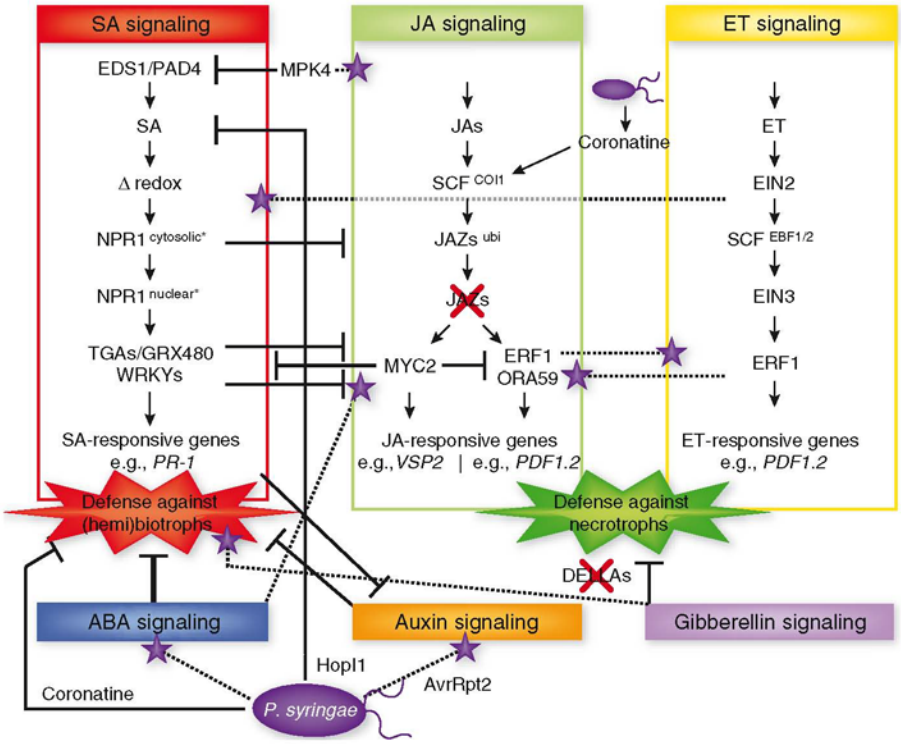

Fig 16. Networking by phytohormones in the plant immune response. Cross-communication between hormone signaling pathways provides the plant with a large regulatory capacity that may tailor its defense response to different types of attackers. On the other hand, pathogens such as *P. syringae* produce effector proteins (for example, coronatine, HopI1 and AvrRpt2) that manipulate the signaling network to suppress host immune responses and promote virulence. The SA, JA and ET signaling pathways represent the backbone of the defense signaling network, with other hormonal signaling pathways feeding into it. Only those signal transduction components that are relevant to this review are shown. ⊥, negative effect; purple stars, positive effect. (from (Pieterse et al., 2009).

ET has been demonstrated to be an important modulator of the plant's defense response to pathogen and insect attack (Howe, 2004; Loake and Grant, 2007; van Loon et al., 2006; von Dahl and Baldwin, 2007). For instance, from a study with ET-insensitive (Tetr) tobacco plants, it was concluded that ET is essential for the onset of SA-dependent SAR that is triggered upon infection by tobacco mosaic virus (Verberne et al., 2003). Moreover, ET was shown to enhance the response of *Arabidopsis* to SA, resulting in a potentiated expression of the SA-responsive marker gene *PR-1* (De Vos et al., 2006; Lawton et al., 1996). This synergistic effect of ET on SA-induced *PR-1* expression was blocked in the ET-insensitive mutant *ein2* (De Vos et al., 2006), which indicates that the modulation of the SA pathway by ET is EIN2 dependent and thus functions through the ET signaling pathway (Fig. 16).

Though the SA, JA and ET response pathways serve as the backbone of the induced defense signaling network, studies in *Arabidopsis* demonstrated that other hormone response pathways feed into it (Fig.16). ABA is commonly associated with plant development and abiotic stress, but its role in biotic stress is becoming increasingly evident (Asselbergh et al., 2008; Mauch-Mani and Mauch, 2005). ABA is connected to the SA-JA-ET network, as it was shown to attenuate JA/ET-dependent gene expression (Anderson et al., 2004) and to affect JA biosynthesis and resistance against JA-inducing necrotrophic pathogens (Adie et al., 2007; Flors et al., 2008). Moreover, ABA was demonstrated to antagonize the onset of SA-dependent defenses and SAR (Mohr and Cahill, 2007; Yasuda et al., 2008).

Auxins play a role in virtually every stage of plant development. The auxin response pathway is connected to the SA-JA-ET signaling network in different ways. For instance, auxin has been demonstrated to affect JA biosynthesis (Nagpal et al., 2005) and the expression of genes involved in JA production (Liu and Wang, 2006).

Recently, gibberellins were shown to hook up to the SA-JA-ET network as well. Gibberellins are hormones that control plant growth by regulating the degradation of growth-repressing DELLA proteins. Navarro *et al., (2008)* demonstrated that DELLA proteins promote susceptibility to biotrophic pathogens and resistance to necrotrophic pathogens by modulating the relative strength of the SA and JA signaling pathways.

Neverthless, it not well know the crosstalk between the polyamine and the other signals molecules implicated in plant resistance.

GENOMIC APPROACHES FOR BREEDING OATS FOR BIOTIC AND ABIOTIC STRESS RESISTANCE

Analysis of genetic diversity in crops by molecular markers is important for crop improvement and provides essential information to enable a more efficient use of available genetic resources in breeding programs (Mohannadi and Prasanna, 2003). Thus, diversity studies can be used in a number of applications including analysis of genetic variability between cultivars, identification of parents in a crossing program to maintain a wide genetic base (Souza and Sorrells, 1990), introgression of desirable traits from wild relatives (Feuillet *et al,* 2007) and cultivar fingerprinting (Manifesto *et al*, 2001).

Associations between traits and molecular markers (e.g., allozymes or RFLPs) have been widely use in oats in surveys of genetic diversity. For example, in a collection of wild oat, *Avena sterilis* L., allozymes of 6-phosphogluconate dehydrogenase were associated with differences in groat size and spikelet yield (S. Beer and T. Phillips, pers. comm.). When a large fraction of the associations between molecular and agronomic traits are caused by linkage, then such associations may be useful in identifying a subset of probes with enhanced potential as markers for quantitative (or simply-inherited) agronomic trait loci. Even after a linkage map has been developed for a crop species, selection of probes for subsequent studies will remain a challenge, especially in polyploids. Markers will be sought for traits not evaluated in the original mapping population, and new polymorphisms will likely map on different chromosomes. Given the expense of generating mapping data and the large numbers and kinds of molecular markers from which to select, efficient selection of markers will be an objective of any marker-assisted breeding program. While genome coverage is usually the first criterion used in selecting probes, putative linkage with quantitative trait loci might be used as an additional criterion.

The nature of the markers used in genetic diversity or linkage studies has been changing according to the development of new genomic approaches. The first markers used were biochemical markers such as enzymes or isozymes, which have been used in a number of species including oats (Souza and Sorrells, 1990) and morphological markers such as plant height, number of fertile tillers, maturity date and thousand grain weight also used in oats (Souza and Sorrells, 1991). However the most widely used markers are the molecular markers. These have an advantage over phenotypic markers in that they are potentially much more numerous and display variation in the DNA which is not seen in the phenotype e.g. differences in the length of a simple sequence repeat (SSR) or a single nucleotide polymorphism (SNP) (Jones et al., 1997). Another advantage of molecular markers is that they are not affected by the environment (Collard et al., 2005). There are many examples of the use of molecular markers in diversity studies of cereals including oats (Li et al., 2000, Fu et al., 2007), wheat (Manifesto et al., 2001, Soleimani et al., 2002), barley (Manninen and Nissila, 1997), and sorghum (Mace et al., 2008).

Molecular markers include random fragment length polymorphisms (RFLPs, Weber and May, 1989), amplified fragment length polymorphisms (AFLPs, Vos et al., 1995), microsatellite or simple sequence repeats (SSRs; Hearne, 1992) and diversity array technology (Jaccoud et al., 2001) markers. These markers have been used in oats to produce molecular maps (Groh et al.,

2001, De Koeyer et al., 2004), to map genes (Milach et al., 1997) and to monitor allele frequency (Fu et al., 2003). We will focuss in the SSR and DArT markers which have been used in this work.

Microsatellite markers (SSR)

Microsatellites (also known as simple sequence repeats or SSRs) are simple sequence tandem repeats of a relativity small size usually less than 100 base pairs (bp) in length (Hearne et al., 1992). They occur in large quantities throughout the genome in eukaryotic cells. Microsatellites can be amplified using PCR to show polymorphisms which occur when there is a difference in the number of repeat units (Hearne et al., 1992). The size of the product reflects the length of the forward and reverse primers, the length of the repeat and any additional DNA adjacent to the repeat depending on where the primers are designed. They can be detected on high resolution gels or on capillary sequencer, which can separate products which vary in size by as little as two base pairs. Microsatellites are co-dominant markers meaning they are able to distinguish between hetero- and homozygotes. These qualities make microsatellites ideal molecular markers for genetic mapping and diversity studies. The development of microsatellite markers has followed two main paths. The first involves the use of expressed sequence tags (ESTs) which are short, sequenced fragments of DNA developed from cDNA libraries. These are then screened for microsatellite repeats. Recently 216 oat EST-SSRs have been published in oats (Becher, 2007). The other method involves construction and screening of a small insert genomic library. In this, the whole genome is split into 300-900bp fragments using restriction enzymes and then screened with microsatellite probes. Microsatellite containing clones are then sequenced (Li et al., 2000).

In the last few years there have been a number of reports of the development of microsatellites in oats (Becher, 2007; Li et al., 2000). However, compared to other cereals such as barley, wheat and rice, relatively few oat microsatellites have been published. Microsatellites developed for other species have also been investigated for use in oats (Li et al., 2000) and used to help identify relationships between lines.

In most genetic diversity studies the map position of the SSR markers is known before the commencement of the study (Mantovani et al., 2008). In some cases a good genome-wide spread can be devised by choosing a set of markers from each linkage group (Hurtado et al., 2008).

DArTs markers

SSR markers are expensive to develop as they rely on library construction and sequence information which is time consuming to determine. Diversity Array Technology (DArT) markers do not require any sequence information and offer a low-cost, high throughout system of marker analysis providing a wide coverage of the genome (Jaccoud et al., 2001). These markers can detect insertion, deletion and rearrangement type DNA polymorphisms (Jaccoud et al., 2001) but more usually are a result of single-nucleotide polymorphisms (SNP) (Tinker et al., 2009). DArT marker discovery comprises a complexity reduction of a pooled DNA sample of the species of interest (Fig. 16). The resulting fragments are used to form a library, a selection of these are spotted onto glass slides to form a genotyping array. Individual DNA samples can then be prepared using the same complexity reduction method and hybridised to the genotyping array, the presence or absence of hybridisation is then recorded (Fig. 16). DArT markers can expand map coverage and enhance germplasm characterisation of oats (Tinker et al., 2009) and have been used successfully in wheat (Akbari et al., 2006), barley (Wenzl et al., 2004), sorghum (Mace et al., 2008) and pigeonpea (Yang et al., 2006). DArT markers are dominant so unlike SSRs they cannot distinguish between hetero- and homozygotes.

DArT markers are a relativity new technology being first described by (Jaccoud et al., 2001). To date there has only been one paper published which reports the use of DArT markers in oats (Tinker et al., 2009); this paper describes how three microarray slides were developed using a worldwide selection of oat germplasm originating from 60 oat varieties from 15 countries. These arrays were then used on 182 different accessions, including 19 used in this study, to identify the genetic similarity. Unlike microsatellites, DArT markers cannot be transferred across species. However now the oat microarrays have been formulated and they can be used on any oat population thus allowing integration of existing genetic maps into a single consensus map. Integrated mapping of DArT and SSR markers has been conducted in durum wheat (Mantovani et al., 2008) and barley (Hearnden et al., 2007) and very recently in oat (Oliver et al., 2013).

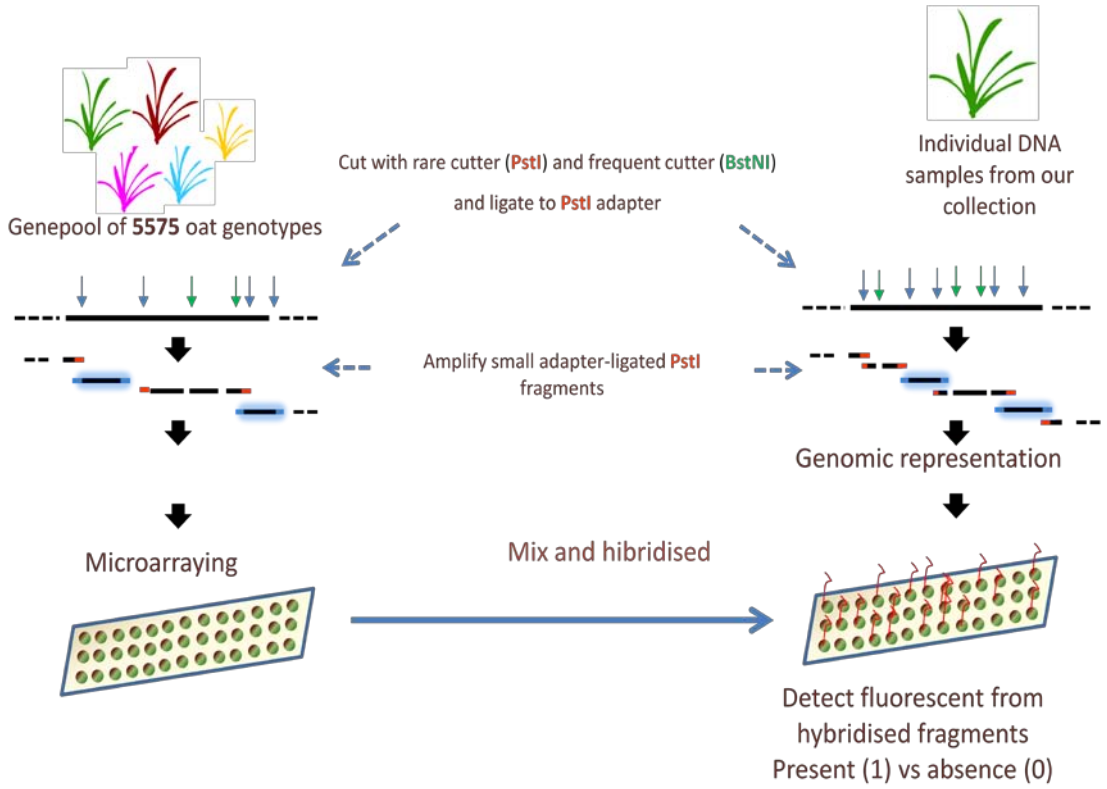

Fig 17. A schematic diagram of how DArT markers detect a DNA polymorphism. The first step involves the development of an array based on a mixture of DNA representing the genepool of the species being analysed. Fragments formed from a complexity reaction are spotted onto glass slides to create a genotyping array. The second step involves the hybridisation of the sample DNA to the array (DArT, 2009).

QTL identification by linkage mapping

Genetic markers have been proved useful for selection of desirable traits in several species including oats. Moreover, association of genetic markers with regions of the genome controlling different traits would enable efficient and precise transfer of useful alleles from landraces to modern cultivars.

Traditionally, genetic markers have been used with a number of experimental bi-parental oat populations to identify quantitative trait loci (QTL) associated with important agronomic traits including vernalization response, flowering time and heading date, quality traits including seed's tocopherol concentration and groat protein and oil content and resistance to stresses including winter field survival, crown freezing tolerance and crown rust resistance reviewed by (Holland, 2007; Rines et al., 2006). Although this has proven to be a powerful approach for QTL detection, it delivers low-resolution, population specific QTL, and samples only a small portion

of the allelic diversity present in the germplasm available (Zhu et al., 2008). Thus markers linked to these QTLs are not immediately available for use in breeding. QTL effects are required to be validated in other genetic backgrounds prior to widespread application of QTL-linked markers in marker-assisted selection (MAS). For example, Steele et al., (2006) found in rice that only one of four root-length QTLs were effective when transferred by backcrossing into a new rice variety. In some cases, this is due to the small effect of an allele transferred into elite varieties (Charcosset and Moreau, 2004). Often for QTL mapping experiments, parents that represent the extreme ends of a trait phenotype are selected. This increases the chance of detecting QTLs because QTL mapping is based on statistically different means of marker groups. The main disadvantage with this approach is that one (or even both) parent(s) may possess QTL alleles that are similar or even identical to the elite germplasm used in breeding programmes. In this case, the effect of a QTL may be insignificant when used for introgression into elite varieties. In other cases, the effect of a QTL may differ in different genetic backgrounds due to interactions with other loci or epistasis (Holland, 2001; Li et al., 2000).

Association mapping

Genome-wide association studies (GWAS) attempt to overcome the pitfalls associated with linkage mapping in bi-parental populations. Genome-wide association studies have the ability to identify useful allelic diversity and to map this diversity with high resolution within complex plant pedigrees that are typical of breeding programs (Jannink et al., 2001). From a practical perspective, GWAS have been applied in many grain crops, including rice, maize, barley, and wheat (Agrama et al., 2007; Kraakman et al., 2006; Zheng et al., 2009). Implementation of GWAS in oat for QTL detection could be valuable to the oat community.

The ability of GWAS to deliver high-power, high-resolution results is largely dependent on the extent of linkage disequilibrium (LD) within the working population. Also known as gametic phase disequilibrium, LD is defined as the non-random association of alleles at two loci (Falconer and Mackay, 1996) and is affected by mutation, admixture, selection, drift, population structure associated with breeding history, and reproductive biology (reviewed by Flint-Garcia et al., 2003). Additionally, since the mechanisms mentioned may differentially affect different genomic regions, this can introduce LD heterogeneity across the genome. This makes the power and resolution achieved in GWAS highly dependent on the species and the population being evaluated.

Alternatively, association mapping is a new QTL mapping approach that can use natural populations, the collection of cultivars released over years, and the material within a breeding program (Shi et al., 2011). These types of populations, or a subset of these may represent a smaller set of the available genetic diversity within a breeding program. Collections of these lines may provide great potential for applied association mapping experiments because they are routinely evaluated in the breeding programs and regional trials to assess their local adaptation or response to biotic and/or abiotic stresses (Shi et al., 2011).

Thus, association mapping is increasingly being utilized to detect marker-QTL linkage associations using plant materials routinely developed in breeding programs. Compared with conventional QTL mapping approaches, association mapping using breeding populations may be a more practical approach for cultivar development, considering that markers linked to major QTL can immediately be utilized in MAS, once new QTLs are identified.

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Chapter 1:

Changes in polyamine profile in host and non-host oat-powdery mildew interactions.

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ABSTRACT

Oat crop (*Avena sativa* L.) constitutes a rich source of biologically active secondary metabolites. Most of these compounds act as a chemical signals and defense metabolites and constitute a potential source for the development of pest control methods for specific diseases. Polyamines are low molecular organic cations involved in various physiological events particularly those related to abiotic stress resistance, i.e drought and cold, albeit recently their potential in disease resistance has being investigated. In this work we monitored the polyamine content on oat leaves in response to powdery mildew infection, both in a resistant and susceptible cultivar challenged with the appropriate fungal forma specialis (f.sp.), *Blumeria graminis* f.sp. *avenae* (*Bga*) and during the non-host interaction with *Blumeria graminis* f.sp. *hordei*. Our results show significant differences between the resistant and susceptible cultivars for specific free polyamine levels, and also with respect to the non-host interaction at crucial stages of the infection process. In addition, polyamine degradation products, such as 1,3-diamino propane increased following pathogen challenge, suggesting a role for reactive oxygen species derived from this pathway in resistance. Exogenous application of polyamines to leaf surface increased penetration resistance of oat against *Bga.* Overall, data support both, a direct and indirect role for polyamines in resistance responses of oat against appropriate and inappropriate powdery mildew f.sp.

Keywords: disease resistance, host and non-host interactions, oat, polyamines, powdery mildew,

INTRODUCTION

Plants produce a wide number of phytochemicals useful in its interaction with the environment including biotic and abiotic stress factors. Polyamines can be considered as one of the earliest known secondary metabolites in biochemistry (Galston and Sawhney, 1990) and are considered to be ubiquitous in all living cells. These low molecular weight compounds are positively charged at physiological pH and hence initially their biological function was associated with the capability of binding to negatively charged molecules (Cohen, 1998). However, in addition to stabilizing macromolecular structures, polyamines also act as regulatory molecules in many fundamental cellular processes including cell division, embryogenesis, as well as in senescence and in response to stress (Martin-Tanguy, 1997). Recent studies indicate that polyamines may act as cellular signals in intricate cross talk with hormonal pathways, such as abscisic acid and ethylene, integrated with processes of hydrogen peroxide and nitric oxide signaling (An et al., 2008; Toumi et al., 2010; Wi and Park, 2002; Yamasaki and Cohen, 2006).

Polyamine biosynthesis is initiated from the basic amino acids ornithine and arginine, which are decarboxylated by ornithine decarboxylase (ODC; EC 4·1.1·17) and arginine decarboxylase (ADC; EC 4·1.1·19), respectively, to yield the diamine putrescine. Putrescine then serves as the substrate for the formation of the tri- and tetra-amines spermidine, spermine and other derived polyamines. The earliest reported changes in polyamines were associated with the response to abiotic stresses as reviewed by Alcazar et al., (2010). However, recent studies also show polyamine accumulation in response to pathogens (Walters, 2003). However the physiological significance of these responses, the dynamics of polyamines at the very early stages of the infection, or whether same polyamines are commonly used during resistance to related fungi in other plant species remains unclear. Furthermore, very little is known about the role of polyamines in a non-host interaction with the sole evidence of their involvement during the attempt of infection of bacteria to non-host tobacco plants (Yoda et al., 2009).

Oat powdery mildew (*Blumeria graminis* f. sp. *avenae, Bga*) is a biotrophic fungus that develops reasonably synchronously through a highly ordered morphogenetic sequence slightly delayed with respect barley powdery mildew (*Blumeria graminis* f. sp. *hordei, Bgh*) reviewed by Green, (2002). Emergence of a short primary germ tube is followed by that of the second, appressorial germ tube that elongates and differentiates a hooked, apical appressorium. A penetration peg emerging beneath the appressorium (14-16 hours after inoculation; h.a.i.)

attempts to breach the plant epidermal cell wall. If successful, it enters the cell lumen where its tip swells and differentiates (20-24 h.a.i.) a mature haustorium. This absorbs nutrient from the epidermal cell to support further fungal growth. Alternatively plants may hamper the infection process to limit fungal multiplication. The best known mechanisms by which cereal defend against powdery mildew are by forming papillae - cell wall appositions deposited on the inner surface of epidermal cell walls directly beneath appressoria that impede fungal penetration within the epidermal cell, and by the death of penetrated cells.

The objective of this work is to reveal changes on specific free polyamines during the resistance response of oat against *Blumeria graminis* f.sp. *avenae* focusing in the very early changes occurred following inoculation, and the role of each specific polyamine in the different resistant mechanisms (i.e. penetration resistance, cell death). Furthermore, we investigated the role of polyamines during the non-host interaction between oat and the barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*).

MATERIALS AND METHODS

Plant material and inoculation

Seedlings of the oat (*A. sativa*) cultivars (cvs) Charming (resistant to powdery mildew) and Selma (susceptible to powdery mildew) were used. Seedlings were grown in plastic pots with peat:sand (3:1) in a growth chamber with 20 ºC, 65% relative humidity and under 12 h dark/12 h light with 200 µmol m^{-2} sec^{-1} photon flux density supplied by high-output white fluorescent tubes.

For host interaction studies both oat cvs were inoculated with *Blumeria graminis* f.sp. *avenae* (*Bga*) race 5. For non-host interactions cv Selma was inoculated with *Blumeria graminis* f.sp. *hordei* (*Bgh*) isolate CC1 according to Prats et al., (2005).

Microscopic observations

For histological studies leaves were fixed at 48 h.a.i. and cleared as described by (Carver et al., 1994). Fungal structures were stained with aniline blue in lactoglycerol (0.1%) according to Lyngkjaer and Carver, (1999). Observations were made with a Leica DM LS phase contrast microscope (Leica Microsystems) fitted with differential interference contrast and incident

fluorescence attachments (blue exciter filter, max transmittance 480 nm; dichroic mirror and barrier filter transmittance >530 nm).

Polyamine quantification

The standard polyamines, putrescine (Put), spermidine (Spd), spermine (Spm) and 1-3,diaminopropane (Dap) were obtained as their hydrochlorides (Sigma) whereas agmatine (Agm) was obtained as its sulfate (Sigma) and norspermidine (Norspd) was used as free base (Aldrich). At 12, 24 and 48 h.a.i. oat leaves were fixed in liquid nitrogen and stored frozen until use. Plant extract were obtained by homogenizing the plant tissue in perchloric acid (0.1 w/v) according to Flores and Galston, (1982). Standards and plant extracts were benzoylated according to Redmond and Tseng, (1979). High performance liquid chromatography analysis of benzoyl-PAs was performed according to Slocum et al., (1989), using an Agilent 2100 Series HPLC.

Analysis of ADC activity

ADC activity in the leaf extract was determined as previously described by (Tiburcio et al., 1986), using L-[U-^{14}C]arginine (Amersham, UK) as substrate, and measuring the $^{14}CO_2$ resulting from the specific decarboxylation of arginine catalysed by ADC. Enzyme was expressed as nmol $^{14}CO_2$ released/h mg protein. Protein was determined according to the method of Bradford (Bradford, 1976). Bovine γ-globulin (Sigma) was used as a standard.

Polyamine bioassay

To assess the effects of polyamines on the different resistance mechanisms, a 1mM solution of each polyamine with 0.1% Tween 20 was sprayed over the entire plant until surface runoff was observed. Control plants were similarly treated with 0.1% Tween 20 in water. Treatment was applied twice a day during two days and the third day plants were inoculated and fixed for microscopic inspection.

In addition a second polyamine application was performed by removing the abaxial leaf epidermis and floating the leaf segments on solution, to bathe the mesophyll and facilitate a wide access of polyamine solution to the adaxial (inoculated) epidermis (Lyngkjær et al., 1997; Zeyen et al., 2002b). Therefore, the abaxial epidermis was removed and the central 30 mm leaf segment was excised and floated adaxial (intact) surface up, in a randomised design, on individual 10 ml aliquots of the appropriate solution 1mM of each polyamine contained in

wells of multi-compartment boxes. Segments were held for 1 h for uptake before inoculation using a settling tower placed directly over the floating segments. Transparent lids were fitted to the boxes which were placed in the growth cabinet for 36 h incubation before segments were fixed as stated above.

Statistic

Five replications were used for experimentation. For statistical analysis, percentages from microscopy studies were transformed to arcsine square roots to normalize data and stabilize variances throughout the data range. Data were subjected to analysis of variance using GenStat 7th Edition, after which residual plots were inspected to confirm data conformed to normality. Significances of mean differences were assessed following contrast analysis (Scheffe's).

RESULTS

Microscopic characterization of resistance responses to Bga attack

Histological characterization shown in Table 1 (controls) confirmed the susceptibility of cv Selma with approximately 70% of established colonies. No cell death was evident, and failed attempted penetration due to papilla formation was observed in the 30% of the cases. In contrast, cv Charming was resistant with only 9% established colonies, with a combination of penetration resistance (44%) and hypersensitive response (HR). Approximately 47% of cells triggered a HR leading to the death of the cell. From this, 26.7% of the cells showed death symptoms before any haustorium could be observed and the response was considered an early and rapid HR whereas in 20% of the cells a small haustorium was observed in the dead cell and the response was considered a late HR.

Polyamine content and ADC activity in oat leaves following Bga attack (host interaction)

Detailed quantification of polyamines showed significant differences between the resistant and the susceptible cv, regarding specific polyamines and time-frame during the infection process (Fig. 1). Putrescine levels were slightly but significantly increased in both cvs at 24 h.a.i. In addition, an early increase at 12 h.a.i. in DAP was observed in Charming following *Bgα* inoculation (Fig.1). Selma overall showed higher levels than Charming (P<0.001) albeit no changes with inoculation were observed. By contrary Charming showed significantly higher

levels of the polyamine spermidine than Selma (P<0.01) and in addition showed a significant increase at 24 h.a.i. of more than 40% respect to constitutive levels (P<0.05).

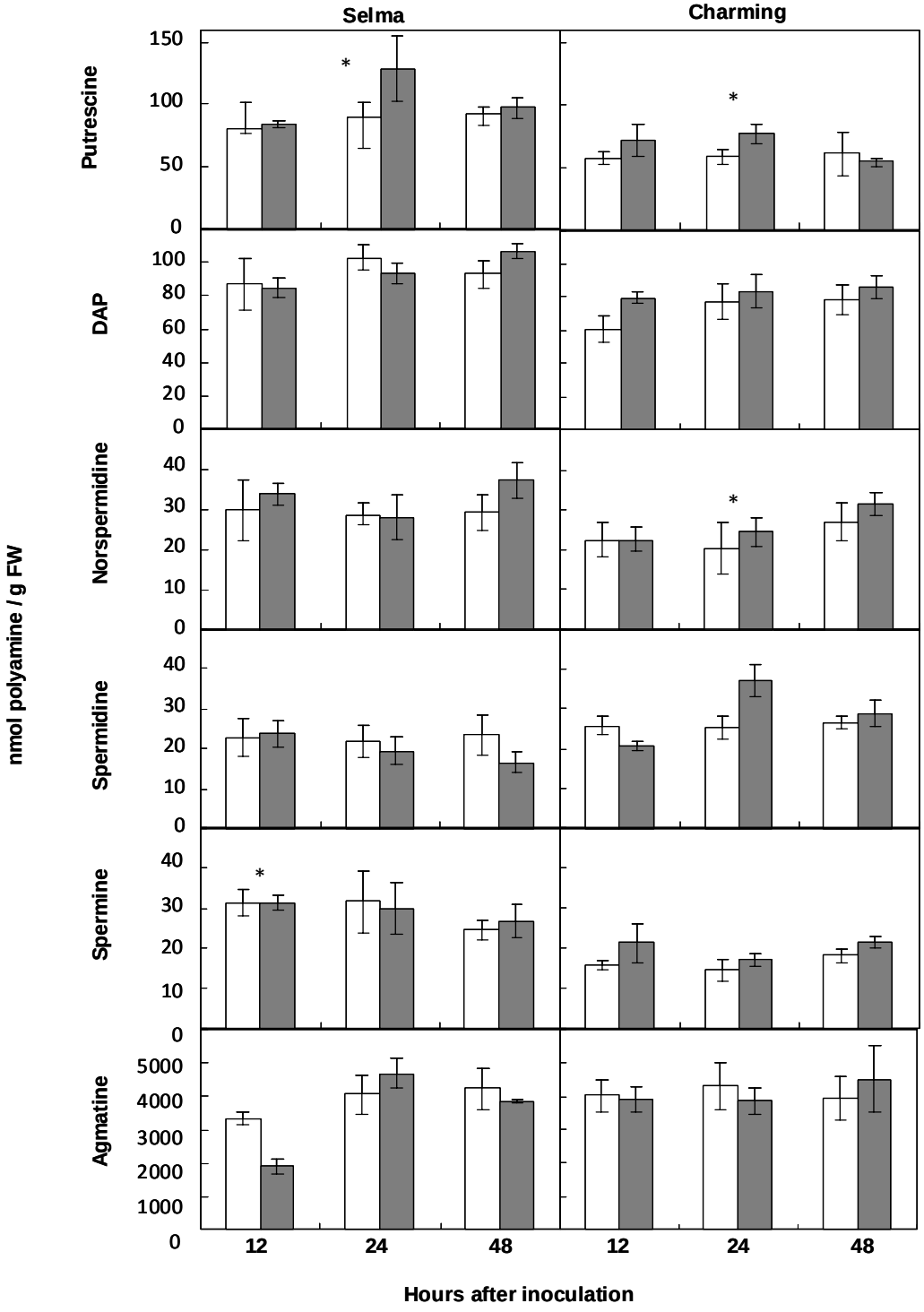

Figure 1. Polyamine content in Selma and Charming cultivars during the host interaction oat-*Bga*. Putrescine, DAP, Norspermidine, Spermidine, Spermine, and agmatine were quantified in susceptible Selma and resistant Charming plants during a time course following inoculation with the host fungus *Bga*. Data are mean of 5 replicates ± standard error. White bar = control, healthy plants; Grey bars = plants inoculated with *Bga*. *, **, *** indicate significant differences at p<0.05, 0.01 and 0.001 respectively between control and inoculated plants; absence of stars indicates no significant differences.

Arginine decarboxylase activity (ADC) was significantly higher in Charming compared with Selma with a mean of 55.6 and 46.3 nmol $^{14}CO_2$ h^{-1} mg $prot^{-1,}$ respectively. Following inoculation ADC activity was significantly higher in inoculated Charming leaves compared to Selma at 12 and 24 h.a.i. although a significant decrease was observed at 48 h.a.i. (Fig. 2A).

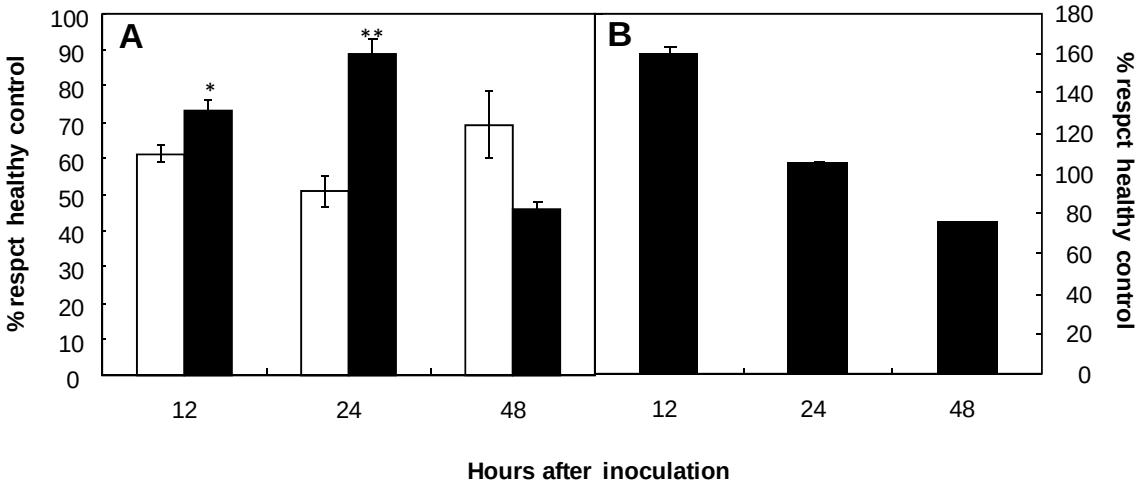

Figure 2. ADC activity in oat leaves during the host and non-host interaction. **A.** ADC activity in Selma and Charming cultivars during the host interaction oat-*Bga.* ADC activity of inoculated Selma (white bars) and Charming (black bars) cvs, respect to healthy plants during a time course following inoculation with the host fungus *Bga*. **B.** ADC activity in Selma plants during the non-host interaction oat-*Bgh.* Bars indicate the ADC activity of inoculated Selma respect to healthy plants during a time course following inoculation with the non-host fungus *Bgh*. Data are mean of 5 replicates ± standard error. *, ** indicate significant differences at $p<0.05$ and 0.01 respectively between genotypes; absence of stars indicates no significant differences.

Polyamine content and ADC activity in oat leaves following Bgh attack (non-host interaction)

Following non-host inoculation of oat leaves with *Bgh* an increase in putrescine was observed during the early stages of the interaction (12 and 24 h.a.i). These changes were correlated with an increase in agmatine also at very early stages of the oat-*Bgh* interaction (Fig. 3). Interestingly also an increase in norspermidine was observed at late stages of the interaction (Fig. 3). No changes in DAP, spermine or spermide were observed following inoculation of the oat leaves with the inappropriate powdery mildew f.sp. ADC activity of healthy plants was by mean 51 nmol $^{14}CO_2$ h^{-1} mg $prot^{-1}$. Increases in agmatine were correlated with increases of ADC activity of the inoculated plants respect to their controls at 12 h.a.i. Levels of ADC activity of

inoculated leaves were similar to those found in healthy plants at 24 h.a.i and slighty lower at 48 h.a.i. (Fig 2B).

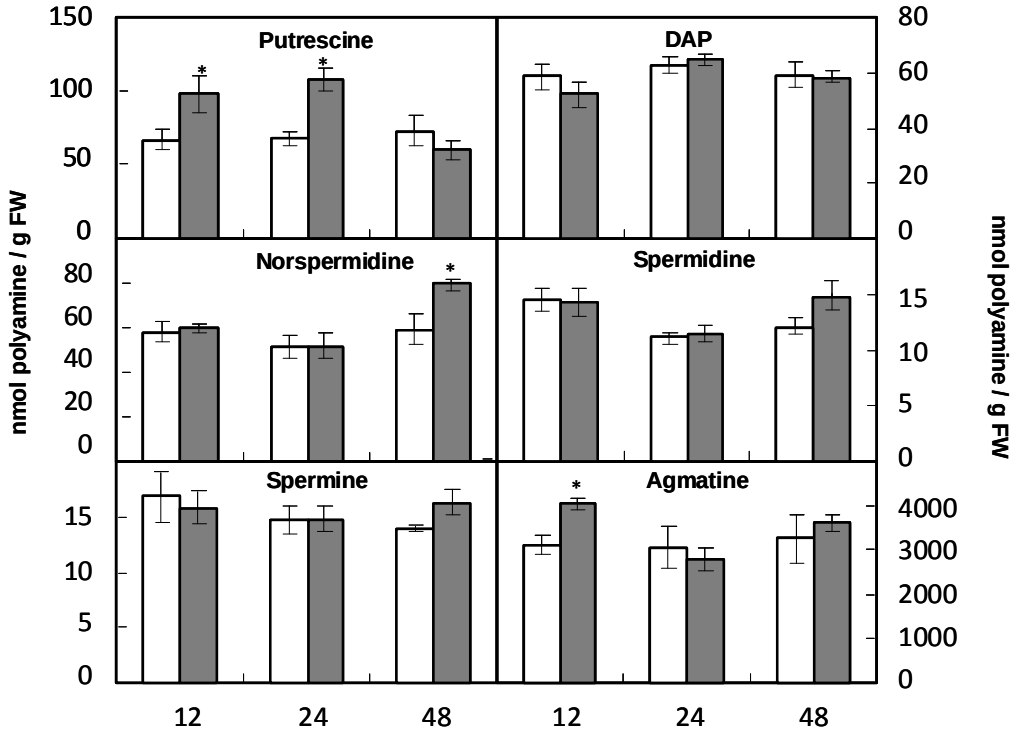

Figure 3. Polyamine content in Selma and Charming cultivars during the non-host interaction oat-*Bgh*. Putrescine, DAP, Norspermidine, Spermidine, Spermine, and agmatine were quantified in Selma plants during a time course following inoculation with the non-host fungus *Bgh*. Data are mean of 5 replicates ± standard error. White bar = control, healthy plants; Grey bars = plants inoculated with *Bgh*. *, **, *** indicate significant differences at p<0.05, 0.01 and 0.001 respectively between control and inoculated plants; absence of stars indicates no significant differences.

Effect of exogenous polyamine application on the different resistance responses of oat to powdery mildew

Exogenous spraying of a 1mM solution of each independent polyamine on leaves previous fungal inoculation increased the resistance response of the susceptible cv Selma but not of the resistant Charming (Table 1). Interestingly, the effect of all polyamines was observed regarding the penetration resistance. Thus, all assayed polyamines increased the percentage of penetration resistance in Selma up to the levels observed in Charming (Table 1). This lead to a significant decrease in the percentage of established colonies following polyamine spraying. No toxic symptoms in the leaves or in the fungal development were observed following this application. No effect of polyamine application was observed with respect to the percentage of HR in any of the cvs.

Table 1. Effect of polyamine application on resistance responses of oat cvs Selma, susceptible, and Charming, resistant, to powdery mildew (*Bga*) infection.

Treatment	Pen Res[a]	Early HR	Late HR	Total HR	Established
			Selma		
Control	29,7	0,0	0,0	0,0	70,2
Agmatine	44,0***	0,0	0,0	0,0	56,0***
Putrescine	45,2***	0,0	0,0	0,0	54,7***
Spermine	42,5***	0,0	0,0	0,0	57,5***
Spermidine	41,0***	0,0	0,0	0,0	59,0***
			Charming		
Control	44,0	26,7	20,2	9,0	47,0
Agmatine	$40,7^{ns}$	$30,5^{ns}$	$20,2^{ns}$	$8,5^{ns}$	$50,7^{ns}$
Putrescine	$41,0^{ns}$	$29,5^{ns}$	$18,7^{ns}$	$10,7^{ns}$	$48,2^{ns}$
Spermine	$46,0^{ns}$	$28,2^{ns}$	$17,5^{ns}$	$8,2^{ns}$	$45,7^{ns}$
Spermidine	$44,5^{ns}$	$23,5^{ns}$	$20,2^{ns}$	$11,7^{ns}$	$43,7^{ns}$

[a]Data obtained at 48 h.a.i. The percentage of germlings that reached different developmental stages (passing from one stage to the next), a) formed an appressorium but not penetrated the cell (Pen Res), b) penetrated the cell but a rapid hypersensitive response avoid haustorium development (Early HR) c) penetrated the cell and a late hypersensitive response develop but allowing haustorium development (Late HR) d) penetrated the cell and establish a colony (Est) not associated with cell necrosis, were scored from 100 infection units. Analysis of variance was applied to transformed replicate data. *** indicate a significant difference between genotypes at $P<0.001$. Data are mean of 5 replications.

Interestingly, when polyamines were applied to stripped-epidermal leaf segments allowing solutions to bath mesophyll cells a toxic effect was observed. In Charming the rate of abnormally germinated conidia (i.e. formation of multi germ tubes, long germ tubes or very thin appresorial tubes) of control plants was 25.3%. However, application of agmatine, putrescine, spermine and spermidine increased significantly ($p<0.001$) the number of abnormally germinated conidia up to 69.4, 49.5, 39.4, and 43.75, respectively. Similarly, whereas in Selma the rate of abnormally germinated or conidia was 15.83, application of agmatine, putrescine, spermine and spermidine increased significantly ($p<0.001$) these percentages to 64.6, 35.2, 45.2, and 46 respectively.

DISCUSSION.

Traditionally it has been assigned a role for polyamines during resistance to abiotic stresses. However, a few reports have recently highlighted the modulation of polyamines profiles during compatible and incompatible interaction of several plants species and their pathogens (Carver et al., 1992; Cowley and Walters, 2002b; Christopher-Kozjan and Heath, 2003; Krippner-Heidenreich et al., 2001). Increased levels of free putrescine and spermine have been associated with the resistance response of barley to powdery mildew (Cowley and Walters, 2002a, b) but also in the green islands that form in compatible responses around mildew pustules during the later stages of infection (Coghlan and Walters, 1990). Particularly it has been reported an increase of free putrescine and spermine in barley plants of cv Delibes carrying the genes *Ml1al* and *Ml(Ab)* conferring HR to *Bgh* (Cowley and Walters, 2002b). However, the lack of histological characterisation, made difficult to assign this polyamine increase to the HR or the penetration resistance present in different rates in all genotypes. In this sense, our data showed an early (24 h.a.i.) increase in putrescine in both resistant and susceptible oat cvs infected with *Bga*. Since even the susceptible cv Selma showed a moderate level of penetration resistance, the increase in putrescine could be associated with this resistance mechanism. Supporting this, exogenous application of putrescine to Selma leaves increased the level of penetration resistance up to the level observed in the resistant Charming. No additional effect of putrescine on penetration resistance could be observed in Charming which could indicate that polyamine content in Charming could be near to the maximum threshold at which it exerts its influence. Furthermore an increase in spermidine was also observed in Charming and its exogenous application also lead to an increase in penetration resistance. Thus, the combined levels of putrescine and spermidine or a synergistic effect might explain at least in part the higher penetration resistance observed in Charming. Since *Bga* development is slightly delayed with respect to *Bgh*, the time at which the increase in polyamines were observed fit with the time at which penetration mechanism are engaged. Interestingly, an increase in DAP was observed earlier, at 12 h.a.i. Since DAP is formed by spermine oxidation mediated by polyamine oxidase, data suggest that the H_2O_2 generated might contribute to the localized oxidative burst which occurs directly beneath the region of attempted penetration (Huckelhoven, 2007; Vanacker et al., 2000). This leads to the rapid accumulation of hydrogen peroxide at the site of papilla formation that is involved in the oxidative cross linking of the papilla component in the callose matrix. Alternatively, the earliness at which DAP was increased in Charming point out to a role for H_2O_2 as messenger for the papilla assembling. Indeed, H_2O_2 together with nitric oxide and have been proposed as the

earliest signals triggering both penetration resistance and HR (Huckelhoven and Kogel, 2003; Prats et al., 2005). Overall our data showed a role for polyamines, particularly for putrescine, spermidine and DAP in the penetration resistance response of oat to its appropriate powdery mildew f.sp, *Bga*. No significant increases in any of the assessed polyamines were observed at later stages of the infection processes correlating with the HR. In addition, exogenous application of polyamines did not increase the percentage of cell death, early or late, in any of the cultivars.

A direct toxic role could also be attributed to polyamines since high abundance of these compounds in epidermal cells following bath of mesophyll cells in the polyamines solutions lead to abnormal fungal development at the very early stages. Since one of the functions of the PGT is to gain access to water and other host components directly through epidermis (Carver and Bushnell, 1983), polyamines could entry early into the fungus and exert a toxic effect. Polyamines are important regulators of growth and differentiation in higher eukaryotic organisms including fungi (Pegg, 1988; Walters, 1995). It has been described that whereas polyamine depletion in fungal cells results in growth cessation, excessive intracellular accumulation of polyamines may be cytotoxic (Valdes-Santiago et al., 2012). Thus, an excess of polyamine uptake during this treatment might be responsible for a deregulation of the polyamine metabolism leading to the fungal growth abnormalities observed. However this direct fungicide effect does not appear to be the cause of the reduction of disease following polyamine spraying where normal fungal development, including appressorium and haustorium formation was observed.

Attempted infection of a cereal by an inappropriate fungal pathogen f. sp. is commonly arrested by papilla deposition at attempted penetration site and if cell is penetrated, the challenged epidermal cell dies before haustoria are formed or mature (Bushnell and Bergquist, 1975; Niks and Rubiales, 2002). The frequency of these responses appears influenced by plant genus and fungal f. sp., and also by genotypic variation in host. However, inappropriate relationships rarely allow fungal development to proceed to sporulation. During the non-host interaction between Selma and the inappropriate powdery mildew *Bgh* approximately 50% of the penetration attempts were successfully hampered by penetration resistance mechanisms and the penetrated cells developed a programmed cell death so no sporulation of the fungus was observed (data not shown, Carver et al., 1992). Our data shows a very fast increase in the polyamine putrescine from 12 h.a.i. that might contribute to the penetration resistance as observed during the host interaction between oat and *Bga*. The earliness of the response

might account for the higher penetration resistance observed during the non-host interaction since the speed at which the resistance machinery is triggered as well as the speed of deposition and compaction of papilla is crucial for successful defense (Huckelhoven, 2007; Prats et al., 2005; von Ropenack et al., 1998).In addition, a significant increase in norspermidine and slight increase in spermidine at later stages, i.e. 48 h.a.i. might contribute for cell death during the non-host interaction. Norspermidine levels were not changed during the host interaction oat-*Bga*. However, evidence suggests that processes leading to cell death differ between *R*-gene controlled cell death and non-host cell death (Christopher-Kozjan and Heath, 2003). Increased levels of putrescine and spermidine have been reported during the non-host interaction of tobacco plants with the bacteria *Pseudomonas cichorii* which lead to an extensive cell death reaction. These polyamines were shown to serve as the source of hydrogen peroxide during the non-host cell death (Yoda et al., 2009). The increase in agmatine observed at 12 h.a.i. together with the increase in ADC activity respect to non-inoculated plants at this time point, suggest the involvement of this pathway in the increase of polyamine observed while we cannot rule out the involvement of the ODC enzime in the increase of polyamines observed.

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Chapter 2:

Implications of polyamines on pre/penetration resistance and hypersensitive response of oat to crown rust (*Puccinia coronata* f.sp. *avenae*).

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ABSTRACT

Polyamines are low-molecular-weight polycations implicated in a wide range of regulatory processes including growth, cell division, DNA replication, cell differentiation and stress responses. Although in recent years a wealth of studies have focussed on the role of polyamines during plant-environmental stress interaction, relatively little work has been carried out in incompatible interactions between plants and pathogens, and much of that was related to the hypersensitive response. In this work we examined the involvement of polyamines during the oat-crown rust interaction focusing in the resistance mechanisms acting before, during and post mesophyll cell penetration. Increased in spermidine and spermine were associated with increased pre-penetration resistance. This role was supported by the inhibition of appressorium formation observed following exogenous polyamine application to the susceptible cultivar Araceli, which increased its resistance. Elevated levels of spermidine and spermine together with increases of wall-bound DAO and PAO activities in resistant cultivar Saia at early stages of the infection process and concomitant increases of oxidation product DAP suggest and involvement of polyamine derived H_2O_2 during the penetration resistance by mean or contributing to the cell wall strengthening and lignification.

INTRODUCTION

Polyamines (PAs) are low-molecular-weight organic polycations positively charged at physiological pH. This property allows PAs to interact with negatively charged macromolecules such as DNA and RNA, proteins and phospholipids and in this way they are involved in the regulation of physical and chemical properties of membranes, nucleic acids structure and function and modulation of enzyme (Galston and Sawhney, 1990). As result, polyamines are implicated in a wide range of regulatory processes such as promotion of growth, cell division, DNA replication, cell differentiation and stress responses (Evans and Malmberg, 1989).

Polyamine biosynthesis pathway is under complex metabolic and developmental control and such control is necessary for efficient regulation of cell metabolism (Hussain et al., 2011). The diamine putrescine (Put), the triamine spermidine (Spd) and the tetramine spermine (Spm) are the main PAs found in all living cells. In plants, putrescine is synthethized from ornithine by ornithine decarboxylase (ODC, EC 4.1.1.17) or from arginine by arginine decarboxylase (ADC, EC 4.1.1.19) via agmatine. Spd and Spm are synthesized by addition of aminopropyl groups, arisen from the decarboxilation of S-adenosylmethionine through decarboxylated S-adenosylmethionine (dcSAM), to putrescine and spermidine respectively, by spd and spm synthases. Free PAs level in plant cells depends not only on their synthesis but also on their transport, degradation and conjugation. Polyamines are catabolized through the activity of one or more diamine oxidases (DAO) and polyamine oxidases (PAO) (Bagni and Tassoni, 2001). Thus, putrescine degradation is catalyzed by diamine oxidase (DAO; EC 1.4.3.6), whereas Spd and Spm are oxidized at their secondary amino groups by a flavin-containing polyamine oxidase (PAO; EC 1.5.3.3) (Flores, 1985). There is evidence that DAO and PAO are predominantly located in the cell wall (Angelini et al., 1993; Sebela et al., 2001) and that DAO can be released into the apoplast (Moller and McPherson, 1998).

In the last years, there has been a growing interest in the study of PAs involvement in the defense reaction of plants against several environmental stresses (Bouchereau et al., 1999; Kasukabe et al., 2004; Kumar et al., 1997). Stress-induced accumulation of PAs often correlates with the improvement of plant tolerance in a wide range of environmental stresses (Bouchereau et al., 1999; Kasukabe et al., 2004; Kumar et al., 1997; Liu, 2007). Increase of these molecules have been observed under several abiotic stresses such as drought, high salinity, mechanical injury, potassium deficiency and cold (Alcazar et al., 2006; Armengaud et al., 2004; Hummel et al., 2004; Perez-Amador et al., 2002; Urano et al., 2003). However,

relatively little work has been carried out on free polyamines and their metabolism in incompatible interactions between plants and pathogens (Walters, 2000), and much of what has been conducted relates to the hypersensitive response (Walters, 2003).

Puccinia coronata f.sp. *avenae* is an obligated oat fungal pathogen that cause crow rust. It is the most important disease of the oat crops with high yield and grain quality losses worldwide (Simons, 1985), particularly in the Mediterranean basin (Hemmami et al., 2006) where populations are more virulent than in the center and north of Europe. The rust infection process starts with the germination of the urediospores on the leaf surface. When the germ tube contacts a stoma, an appressorium develops over the guard cells and then a penetration hypha penetrates through the stomata (Hoch and Staples, 1987; Prats et al., 2002). The penetration hypha develops a substomatal vesicle from which a secondary hypha and a haustorium mother cell, at its tip, forms. Following contact between the haustorium mother cell and the mesophyll cell, an infection peg develops, penetrates the mesophyll cell and forms a feeding structure, the haustorium, which takes up nutrients for fungal growth (Parlevliet and Kievit, 1986). Attacked plants may trigger a battery of resistance mechanisms that can act before, during, or after cell penetration to arrest rust development. Prestomatal penetration mechanisms include poor germling adhesion to the leaf surface (Mendgen, 1978; Wynn and Staples, 1981), deviating micromorphology of the epidermal surface that serves as cues in guiding the thigmosensing germ tube toward stoma (Wynn and Staples, 1981) stomatal guard cell morphology (Wynn, 1976), leaf pubescence (Mmbaga and Steadman, 1992) and leaf chemical compounds excreted to the leaf surface that interfere with appressorium development (Choi et al., 1998; Prats et al., 2007). If despite these barriers the fungus reach the stomata, papillae, apoplastic cell wall appositions deposited by host cells at site of attempted penetration, may be formed and act as physical or chemical barriers to hamper the rust infection process (Skalamera and Heath, 1996; Zeyen et al., 2002). If penetration resistance fails, a second defense mechanism involving race-specific, programmed cell death (PCD-hypersensitivity) (Heath, 2000) may be triggered to arrest pathogen growth. Such PCD has been described in many pathosystems including the cereal rusts (Niks and Dekens, 1991; Niks and Rubiales, 1994; Tiburzy and Reisener, 1990). Whenever resistance mechanisms are lacking or they are insufficient to hamper fungal development, disease symptoms appear. These consist on yellow pustules containing masses of urediospores which are exposed after the rupture of the epidermis. Lesions are circular or oblong and occur in both surfaces of the foliar sheet and can reach other green parts of the plant, when the epidemic becomes more severe. After some weeks, the borders of the pustules can turn black, with teliospore

formation. When the infected plants reach maturity, production of urediospores ceases and they are then replaced by teliospores (Simons, 1985).

The present work aim to unravel the role of polyamines, during the different resistance responses engaged by oats against *Puccinia coronata* f.sp. *avenae.* To this, we determined polyamine content at key point of the infection process in different oat genotypes resistant and susceptible to the crown rust, characterized different enzymatic activities related to polyamine biosynthesis and catabolism and performed targeted bioassays of exogenous polyamine application complemented with histological studies.

MATERIAL AND METHODS

Plant material

For this study 3 commercial varieties supplied by the Andalusian Network of Agriculture Experimentation (RAEA) were used. Oat varieties studied were Araceli, Kankan y Saia that had previously shown to be susceptible and resistant to the rust isolate used and their resistance mechanisms characterized (Sanchez-Martin et al., 2012). Seedlings were grown in 5 L trays filled with peat:sand (3:1) in a growth chamber with 20ºC, 65% relative humidity and under 12 h dark/12 h light with 250 µmol m^{-2} sec^{-1} photon flux density supplied by high-output white fluorescent tubes. All experiments used fully expanded first-formed leaves of 11 day-old plants (their second-formed leaf was unrolled).

Inoculation

P. coronata f.sp. *avenae* (*Pca*) isolate Co-04, was used for inoculation (Sanchez-Martin et al., 2012). Urediospores were multiplied on plants of oat cvs. Araceli which were highly susceptible to this isolate. One day before experimental inoculation, spores were collected and kept overnight in a desiccator.

When plants had the first leaf completely expanded they were inoculated with urediospores mixed with pure talcum (1:1, w/w) by dusting them over the plants to give approximately 30 spores mm^{-2} (checked by counts made from glass slides laid adjacent to leaves). Homogeneous inoculation was ensured by placing the leaves attached to the plant horizontally with the help of metallic clips. After inoculation, plants were incubated for 9.5 hours in darkness at 100 % RH and 18^{o}C, and thereafter at 20 ^{o}C under a 14 h photoperiod with 150 µmol m^{-2} sec^{-1} photon flux density.

Microscopic observations

For microscopic assessment of fungal development, middle segments of 1-3 cm^2 from each inoculated leaf still attached to the plant (four leaves per treatment) were excised at 24, 48 and 72 hours after inoculation (h.a.i.) and stained with Uvitex (Ciba, Barcelona, Spain), according to (Niks and Rubiales, 1994)and non inoculated plants fixed at the moment of the inoculation were used as control (T0). These were observed by ultraviolet light incident fluorescent microscopy (330 nm excitation/380 nm emission) using a Leica DM LS phase contrast microscope (Leica Microsystems, Wetzlar, Germany; 100x objective).

Percentages of germinated urediospores were determined from 100 random urediospores per leaf segment. Percentages of germtubes forming an appressorium over a stoma were determined from 100 germinated urediospores per leaf segment. In addition, on each leaf segment, 100 infection units (growth arising from individual urediospore) that successfully formed an appressorium on a stoma were scored and classified according to their developmental stage i.e whether they formed the substomatal vesicle, penetrate the mesophyll cell, and establish a colony. Accordingly, early aborted infection attempts due to penetration resistance, were those that formed a substomatal vesicle and one or more primary infection hyphae but forming less than six haustorial mother cells and where colony growth had ceased. Infection units with six or more haustorial mother cells were considered established. The presence of host cell death associated with early aborted or established colonies was recorded. Death of plant cells was recognized by yellow whole-cell fluorescence under violet incident light (420 nm excitation/490 nm emission).

Polyamine quantification

The standard polyamines, putrescine (Put), spermine (Spm), spermidine (Spd), and 1-3,diaminopropane (DAP) were obtained as their hydrochlorides (Sigma) whereas agmatine (Agm) was obtained as its sulfate (Sigma). HTD (1,7-diaminoheptane) was used as internal control (Sigma).

For total polyamine quantification, plant tissue was pulverized in liquid N_2 and homogenizing in cold 5% $HClO_4$ in (0.1 w/v) according to Flores and Galston, (Flores and Galston, 1982). Extracts were incubated at 4ºC during 1 h and centrifugated at 13000 g at 4ºC during 20 min. Resulting supernatant were collected in a plastic tube was stored at -20ºC until use. In addition and in

order to quantify the excreted polyamine to the leaf surface, leaves were washed twice with the help of a pipette with 4 ml of 5% perchloric acid 5% (Prats et al., 2002).

Standards and plant extracts were benzoylated according to Redmond and Tseng, (Redmond and Tseng, 1979); one ml 2 N NaOH was mixed with 500 uL of $HClO_4$ extract. After addition of 10 uL benzoyl chloride, vortexing for 10 s, and incubation for 20 min at room temperature, 2 mL saturated NaCl were added. Benzoyl polyamines were extracted in 2 mL diethyl ether, vortexing for 15 s and incubating 5 min at 4ºC. Thereafter 1 mL of the ether phase was collected, evaporated to dryness under a stream of warm air, and redissolved in 100 uL methanol (MERCK; HPLC grade). Standards were treated in a similar way, with up to 50 nmol of each polyamine in the reaction mixture. The benzoylated samples were stored at -20°C. High performance liquid chromatography analysis of benzoyl-PAs was performed according to Slocum et al., (Slocum et al., 1989), using an Agilent 2100 Series HPLC. The benzoylated extracts were eluted at room temperature through a 4.6 x 250 mm, 5-,um particle size reverse-phase (C18) column (Altex-octadecylsilane) and detected at 254 mm. 20 uL of benzoyl-PAs were manually injected at a flow rate of 1 mL/min. The solvent system consisted of methanol:water, run isocratically at 50 to 100% methanol following the Programme I described by Slocum et at., (Slocum et al., 1989).

Polyamine bioassay

To assess the effect of polyamines on the different resistance responses, 1mM solution of each polyamine with 0.1% Tween 20 was sprayed over the entire plant until surface runoff was observed. Control plants were similarly treated with 0.1% Tween 20 in water. Treatment was applied twice a day during two days and the third day plants were inoculated and fixed for microscopic inspection at 84 h.a.i.

ARN extraction and cDNA amplification

Total RNA from was extracted from 100 mg of ground leaf tissue using previously reported protocols (Chomczynski and Sacchi, 1987; Raeder and Broda, 1985). RNA was cleaned by means of RNeasy® Minelute Cleanup Kit (QIAGEN). Contamination of residual genomic DNA in all RNA samples was verified by conventional PCR amplification on total RNA using the designed primers listed in Table 1. RNA samples containing DNA were further DNase treated until no PCR amplification of RNA samples was obtained. Prior to retrotranscription experiments, the concentration and integrity of RNA were verified by an optical density at 260

nm (OD260)/OD280 absorption ratio in a NanoDrop ND- 1000 spectrophotometer (Thermo scientific).

First and second-strand of complementary DNA (cDNA) were synthesized using SuperScript® III First-Strand (Invitrogen) and DNA Polymerase I (BioLabs), respectively. cDNA was cleaning by QUIquick PCR Purification Kit (QIAGEN and DNase treated by the RNase-Free DNase Set (Qiagen), according to the manufacturer's recommendations. Conventional RT-PCR and PCR assays followed by gel electrophoresis were performed to verify the amplification of cDNA using the designed primers. Quality and quantity of cDNA was determined by running aliquots in agarose gels and by spectrophotometric analysis in a NanoDrop ND- 1000 spectrophotometer (Thermo scientific).

Gene expression analysis by real-time QRT-PCR.

Relative expression of ADC activity was quantified by using the following primers: 5'-TACGGCGATGTGTACCATGT-3' and 5'-GTCCTTGTTCACGGCAAAGT-3'); designed with the Universal Probe Library Assay Design Center (Roche applied Science) from the sequence published in NCBI database (X56802.1; (Bell and Malmberg, 1990). Previous to test the expression of ADC, four additional genes were tested for using as reference genes; glyceraldehyde-3-phosphate dehydrogenase (GADPH), beta-tubulin (TUBB), alpha-tubulin (TUBA) and 18S ribosomal RNA (10S rRNA) according to Jarasova and Kundu (Jarosova and Kundu, 2010). Following preliminary assay, GADPH was selected as the endogenous normalization measure for the relative quantification of the ADC gene due to the high stable expression showed in our oat samples. Real-time qRT-PCR was performed for the ADC gene and for GADPH on at least 3 independent biological plus 3 technical replicated cDNA templates in StepOne Real-Time PCR System (Applied Biosystems) using FartStart Universal SYBR Green Master (Rox) (Roche) according to the manufacturer's recommendations. The reaction mixture contained: 10 µl of SYBR Green master mix, 6 µl of each primer set (Table 1), and 4 µl of cDNA or standard solution as template. The amplification conditions were 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s, 60°C for 1 min,. Following amplification, a melting curve program 95ºC for 15 seg, 60ºC for 1 min and 60 to 95°C with a heating rate of 0.3°C/min. The melting point analysis was performed at the end of the real-time RT-PCR to confirm the amplification of a unique product for each gene. The fold changes of ADC gene transcripts in different treatments versus control (i.e., non-inoculated plants) were normalized using the CT and efficiency obtained for the GADPH amplification run on the same cDNA templates according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Analysis of ADC activity

ADC activity was determined in leaf extracts in which fungal structures has been removed with latex (Prats et al., 2005) according to (Tiburcio et al., 1986), using L-[U-^{14}C]arginine (Amersham, UK) as substrate, and measuring the $^{14}CO_2$ resulting from the specific decarboxylation of arginine catalysed by ADC. Enzyme was expressed as nmol $^{14}CO_2$ released/h mg protein. Protein was determined according to the method of Bradford (Bradford, 1976). Bovine γ-globulin (Sigma) was used as a standard.

Analysis of DAO/PAO activity

Diamine oxidase (DAO) (EC1.4.3.6.) and polyamine oxidase (PAO) (EC1.4.3.4.) activities were estimated using the first leaf as source of enzyme, following fungal removal with latex (Prats et al., 2005). Activity was measured spectrophotometrically based on the colorimetric assay ofΔ -pyrroline using putrescine (Put) and spermidine (Spd) as substrates for DAO and PAO activities, respectively (Asthir et al., 2004; Holmstedt et al., 1961a). Briefly, as a cleaning step each leaf was homogenized 1:2 (w:v) in 100 mM K-phosphate buffer (pH 7) containing 5mM dithiothreitol at 4ºC, and centrifuged at 16,000g for 20 min at 4ºC. In order to measure soluble DAO/PAO activities the pellet was extracted 100 mM K-phosphate buffer (pH 7) containing 20 mM EDTA (1:1, p:v), incubated 10 min at 4ºC and centrifuged 20 min at 16,000g at 4ºC supernatant collected. This step was repeated twice. The remaining pellet was used directly to quantify wall-bound DAO/PAO activities. For the assay, extract (0.75 uL of supernatant for soluble or 0.30 mg of the pellet for wall-bound activity) was combined with 50 units of catalase, 75 uL of 2-aminobenzaldehyde (0.1%) and 75 uL of substrate: 10 mM Put in 50mM K-phosphate buffer (pH 7.5) for DAO or 10 mM Spd in 50mM K-phosphate buffer (pH 6) for PAO. The reaction was carried out at 30ºC for 3h, then 10% perchloric acid (v:v) was added and the mix was incubated 15 min at RT in order to stop the reaction. The mix was then centrifuged at 5,000 rpm for 15 min at RT and the supernatant was used to determinate the formation ofΔ -pyrroline product by reading the absorbance at 430 nm. Inactivated enzymes by heating at 95ºC for 5 min were used as control reactions. Enzyme activity was expressed in ngΔ -pyrroline g^{-1}FW min^{-1}.

Statistical analysis

Four leaves (from four different plants) per genotype were studied in a complete randomized block design. For statistical analysis, data recorded as percentages were

transformed to arcsine square roots (transformed value = 180/π x arcsine√[%/100)]) to normalize data and stabilize variances throughout the data range. Data were then subjected to analysis of variance using SPSS software after which residual plots were inspected to confirm data conformed to normality. In addition Shapiro-Wilk test and Bartlett's test were performed to test normality and homogeneity of variances respectively. Multiple comparison among all genotypes were carried out according to Tukey test ($p<0.05$).

RESULTS

Microscopic characterization of resistance responses of oat cultivars to *Puccinia coronata* f.sp. *avenae* attack

Histological characterization confirmed the susceptibility of cv Araceli with 68.5% of established colonies and a lower number of early aborted colonies (Table 1). Pre-penetration resistant mechanisms, those engaged previous to mesophyll attempt penetration, and penetration resistance mechanisms (early aborted colonies) of cv Kankan were not significantly different to Araceli, however post-penetration resistant mechanisms leading to cell death were strongly enhanced in this genotype so that the final number of established colonies decreased up to 15%. A detailed histological caharacterisation showed that out of the 50% of cells that died following rust attack, 20% died rapidly, before haustorium formation, and c.a. 30% showed a late cell death allowing the growth of a limited number of hyphae. Cultivar Saia showed also a high resistance response as Kankan but this was not based on cell death response but in mechanisms engaged before mesophyll cell penetration. Thus, 34% of rust attacks were hampered during the early stages of the infection process, before mesophyll cell penetration , and 37% were hampered during the penetration attempt leading to early aborted colonies.

Table 1: Microscopic characterisation of resistance response to *Puccinia coronata* attack.

Genotype	Pre-penetration	EA	EA-N	Est-N	Est
Araceli	24.02 ± 1.26	7.44 ± 1.45	0.00 ± 0.00	0.00 ± 0.00	68.54 ± 2.08
Kankan	21.78 ± 2.08	10.38 ± 1.36	19.42 ± 1.94	32.58 ± 3.11	15.84 ± 1.19
Saia	33.87 ± 4.06	37.07 ± 5.04	3.03 ± 1.84	7.74 ± 5.02	18.29 ± 1.87
	l.s.d 4.4	4.7	3.8	5.8	3.8

Polyamine content

At most crucial times of the infection process significant differences were observed in levels of particular polyamines between the oat cultivars.

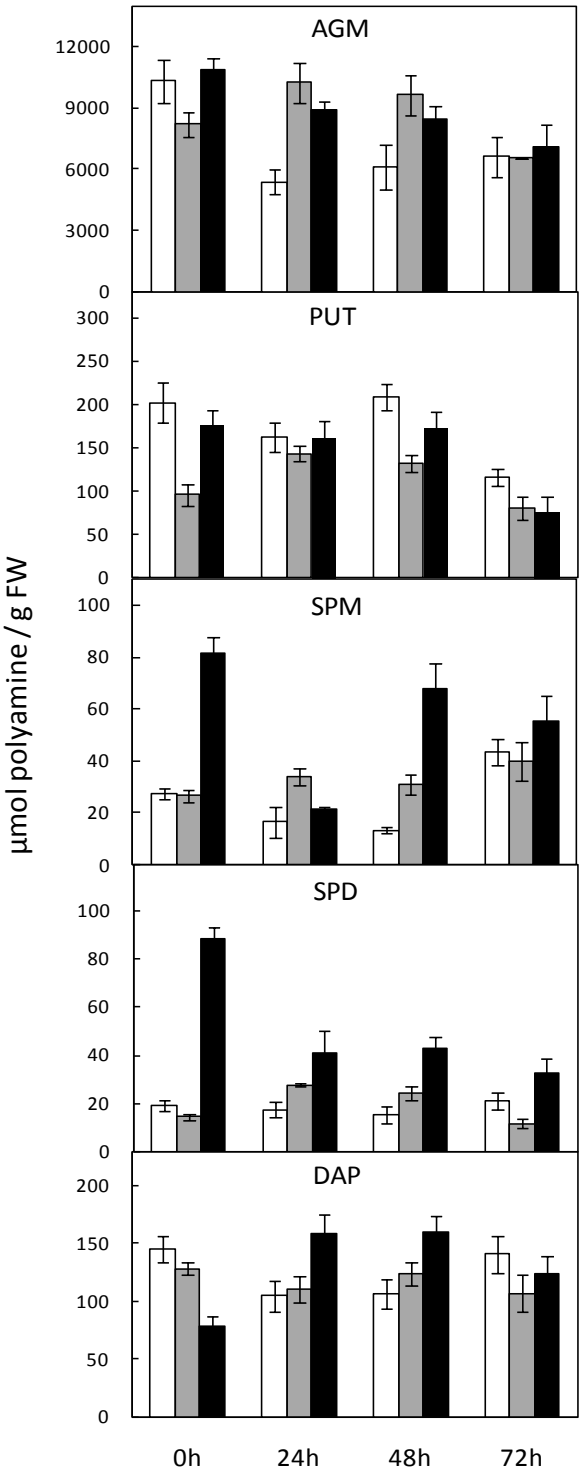

Figure 1. Total polyamine content in rust susceptible and resistant oat cultivars. Agmatine (AGM), putrescine (PUT), spermidine (SPD), spermine (SPM) and 1,3-diaminopropane (DAP) were quantified in susceptible Araceli (white bars) and resistant Kankan (grey bars) and Saia (black bars) plants during a time course following inoculation with the host fungus *Puccinia coronata* f. sp. *avenae*. Data are mean of 5 replicates ± standard error.

Overall, the highest significant differences among genotypes were found for Spm, Spd, Agm and Put ($p<0.001$; Fig 1). Thus, Saia displayed significantly, 4 fold higher, constitutive levels of

Spd and Spm, than the susceptible genotype Araceli. Although Kankan did not showed this high Spd and Spm constitutive levels, it showed significant higher levels of both polyamines respect Araceli at 24 and 48 hai. Agmatine content was also overall higher in Saia and Kankan respect to Araceli ($p=0.013$). By contrast Put content was overall higher in cultivar Araceli than in the two resistant Kankan and Saia ($p<0.001$). In addition statistical analysis showed interaction between genotype and time post inoculation for most polyamines indicating a different trend between the susceptible and resistant cultivars. Decrease in the levels of Spm and Spd 24 and 48 hai after inoculation in cv Saia correlated with a increase of DAP at these time points (Fig 1).

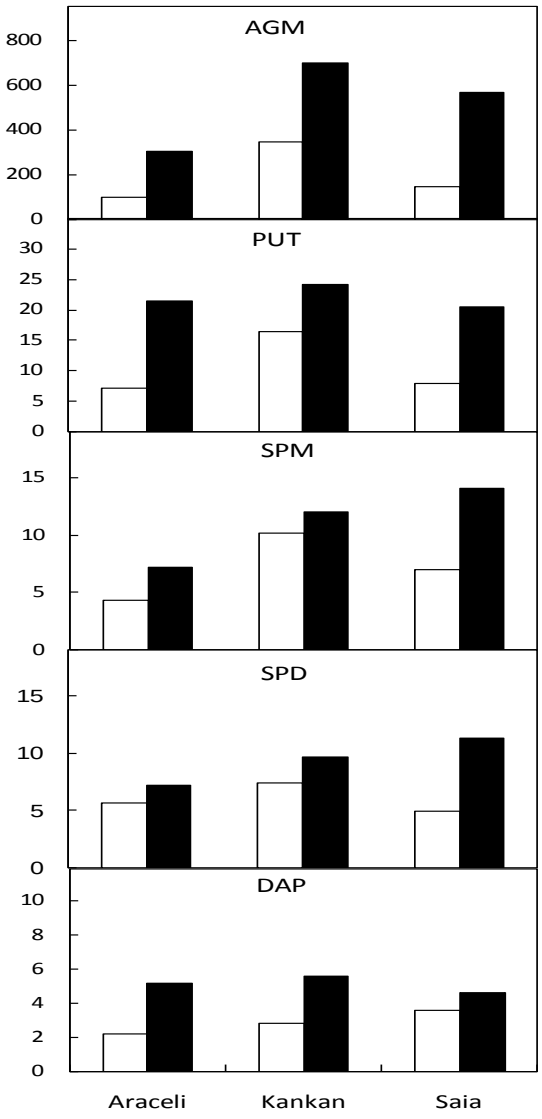

Figure 2. Excreted polyamine to leaf surface in rust susceptible and resistant oat cultivars. Agmatine (AGM), putrescine (PUT), spermidine (SPD), spermine (SPM) and 1,3-diaminopropane (DAP) were quantified in susceptible Araceli and resistant Kankan and Saia plants 12 hours following inoculation with the host fungus *Puccinia coronate* f. sp. *avenae*. Data are mean of 5 replicates ± standard error. White bar = control, healthy plants; Black bars = plants inoculated with *Puccinia coronata* f. sp. *avenae.*

In general levels of polyamines excreted to the surface was less than 10% of the total polyamine content for all polyamine assessed. Interestingly excreted polyamines to the leaf surface increased 24 hai inoculation in both susceptible and resistant genotypes compared with non inoculated controls ($p<0.001$). However, there was a significant interaction for most polyamines between genotypes and treatment indicating that not all cultivars respond in a similar proportion to the rust attack. Cultivar Saia showed the highest increase in excreted Spm and Spd after inoculation, showing significant differences respect to the susceptible Araceli. Particularly whereas no significant increase of excreted Spd was observed in Araceli, more than two fold increase was observed in Saia. In addition both Saia and Kankan showed 2 and 2.3 fold increased respectively in excreted agmatine respect to Araceli. No significant differences were found between genotypes respect to excreted DAP.

Exogenous polyamine bioassay

Since levels of particular excreted polyamines showed higher in cultivar Saia with increased pre-penetration resistance that in Araceli, polyamines were applied exogenously to susceptible cultivar Araceli in order to test the effect in arresting early fungal developmental stages.

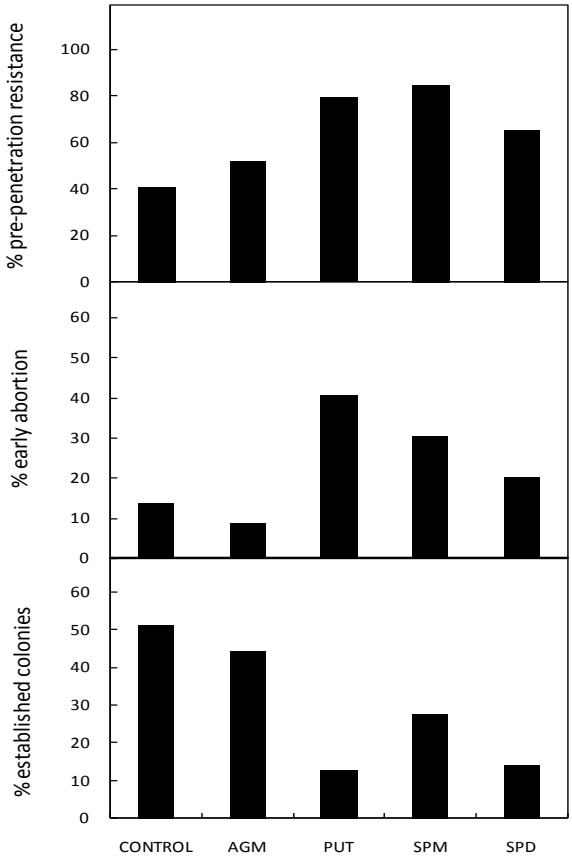

Figure 3. Effect of exogenous application of polyamines on *P. coronata* f.sp. *avenae* development and plant resistance responses. All parameters were microscopically evaluated on the second leaf 84 hours after inoculation. Values are the means of five independent replicates ± standard error.

Exogenous application of polyamines showed an increase of pre-penetration resistance in the susceptible genotype Araceli for all polyamines applied particularly for Spm, Spd and Put ($p<0.001$). In addition, the percentage of early aborted colonies increased following leaf treatment with Put, 4 fold, and Spm, 3 fold, respect to the control. Thus, a significant decrease of established colonies were observed following polyamine application in susceptible cultivar Araceli, showing Put, Spm, and Spd the highest reduction in colony establishment. Cell death response was not modified following polyamine application.

Relative expression of ADC gene

Quantitative RT-PCR experiments were carried out to assess ADC regulation at transcript level during the responses to rust attack in the different cultivars. Surprisingly, ADC expression was overall down-regulated in all genotypes following rust inoculation (Fig 4A). However, significant differences were observed between cultivars. Thus, genotype Araceli showed the lowest expression of ADC gene and this was maintained during the first 72 hai. Cultivar Kankan showed relatively higher ADC expression than Araceli at 24 hai but then the gene expression was down-regulated to reach similar levels to that observed in Araceli by 72 hai. Interstingly although ADC expression was dow-regulated at 24 hai in Saia, it significantly increased at 48 hai ($p=0.002$) whereas at 72 hai it was similar to control, non-inoculated levels (Fig 4A).

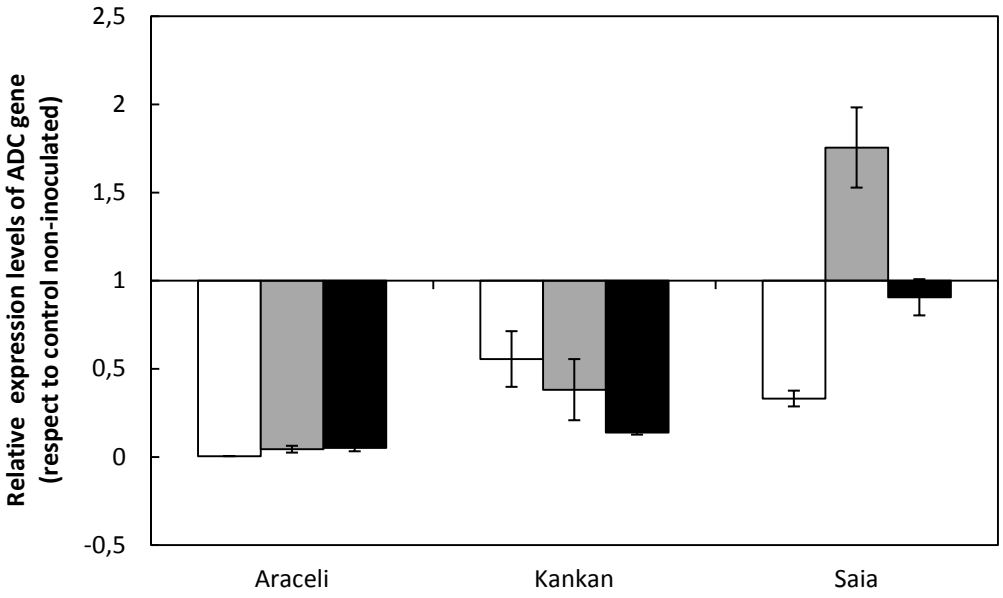

Figure 4. Relative expression of ADC gene. Real time RT-PCR was carried out in oat leaves of susceptible (Araceli) and resistant (Kankan and Saia) during a time course following inoculation with the host fungus *Puccinia coronata* f. sp. *avenae* at 24 (white), 48 (grey) and 72 (black) hours after inoculation. Data are mean of 3 biological replicates and 3 technical replicates ± standard error.

ADC activity

Assessment of ADC activity showed steady state levels in susceptible Araceli during 24, 48 and 72 hai. Overall, Kankan and Saia showed significantly higher ADC activity than Araceli ($p<0.001$) following inoculation. Particularly, Kankan showed the maximum levels at 48 hai showing at slight decrease at 72 hai. Saia showed significantly increased ADC activity at earlier timepoints after inoculation with approximately 3 fold higher ADC activity at 24 hai than Kankan or Araceli ($p<0.001$). This activity also decrease in Saia at 72hai reaching similar levels than those observed in Araceli (Fig 4B).

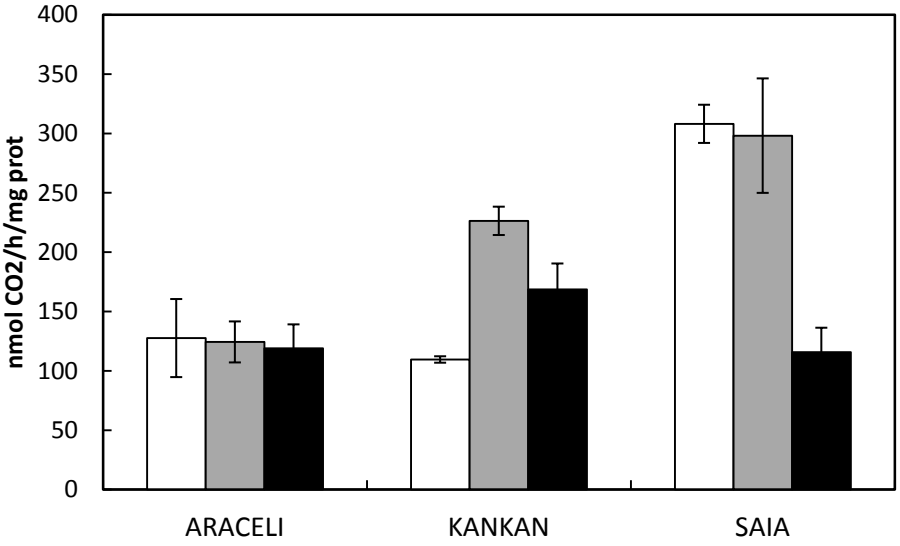

Figure 5. ADC activity in oat leaves. ADC activity was measured in oat leaves of susceptible (Araceli) and resistant (Kankan and Saia) during a time course following inoculation with the host fungus *Puccinia coronata* f. sp. *avenae* at 24 (white), 48 (grey) and 72 (black) hours after inoculation. Data are mean of 5 biological replicates ± standard error.

DAO and PAO activities

No significant differences were observed between susceptible and resistant genotypes after inoculation with respect to soluble DAO activity. However significant differences were observed in bound-wall DAO activity between cultivars. Thus, although the different cultivars showed similar non-inoculated activity levels, a 4 fold increase in bound-wall DAO activity was observed in resistant Saia at 48 hai respect to control non-inoculated plants ($p=0.01$). In addition, Kankan showed a slight increase in bound-wall DAO at 48 hai and more than 4 fold increase at 72 hai ($p=0.004$). Cultivar Araceli also significantly increase the bound-wall DAO activity at 72 hai ($p=0.014$) although in a lower proportion than Kankan (Fig. 5).

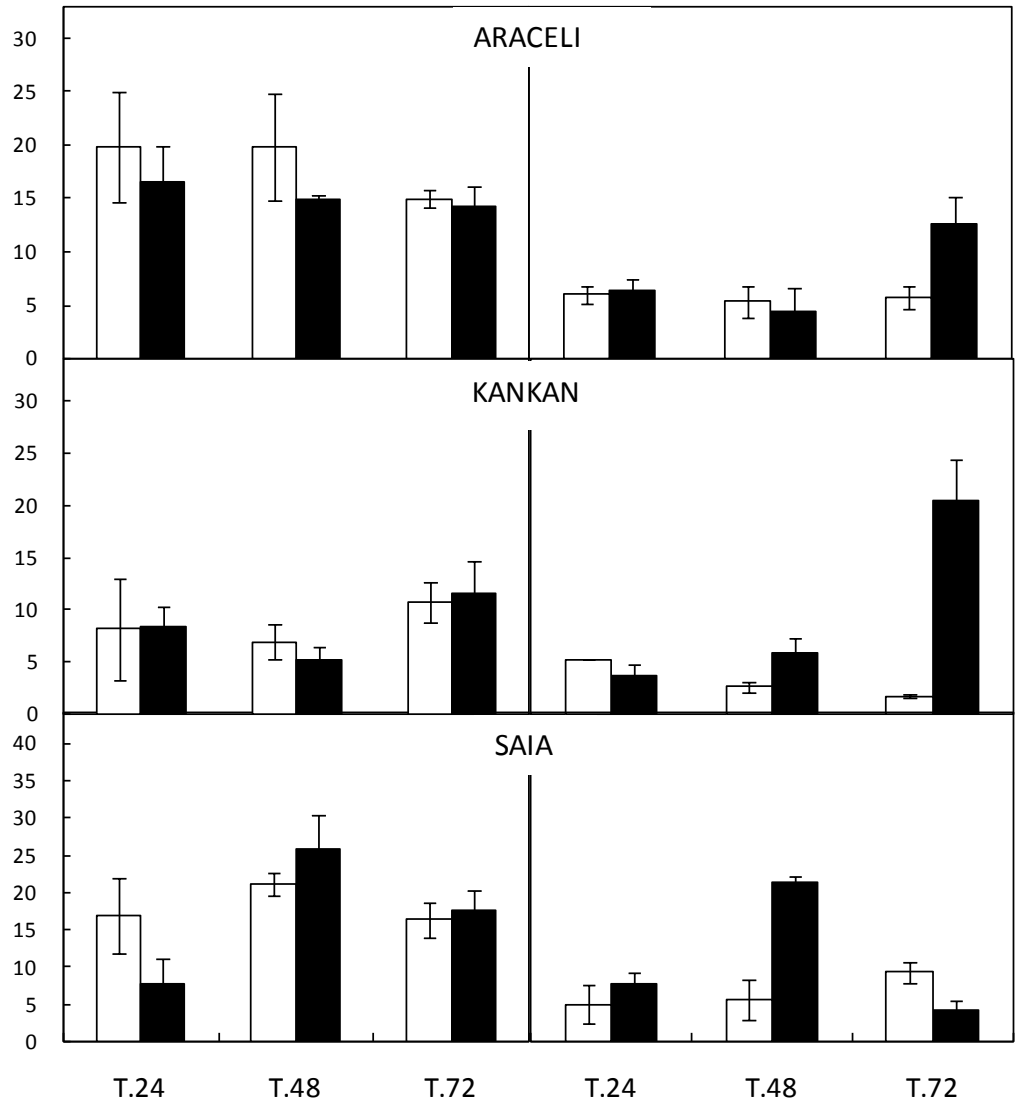

Figure 6. DAO activity in oat leaves. Soluble and cell-wall bound DAO was measured in oat leaves of susceptible (Araceli) and resistant (Kankan and Saia) during a time course following inoculation with the host fungus *Puccinia coronata* f. sp. *avenae.* Data are mean of 5 biological replicates ± standard error. White bar = control, healthy plants; Black bars = plants inoculated with *Puccinia coronata* f. sp. *avenae*

Overall no significant differences were also observed between genotypes respect to soluble PAO activity (Fig. 6). However, slight increases of bound-wall PAO activity were observed in Saia at 24 and 72 hai.

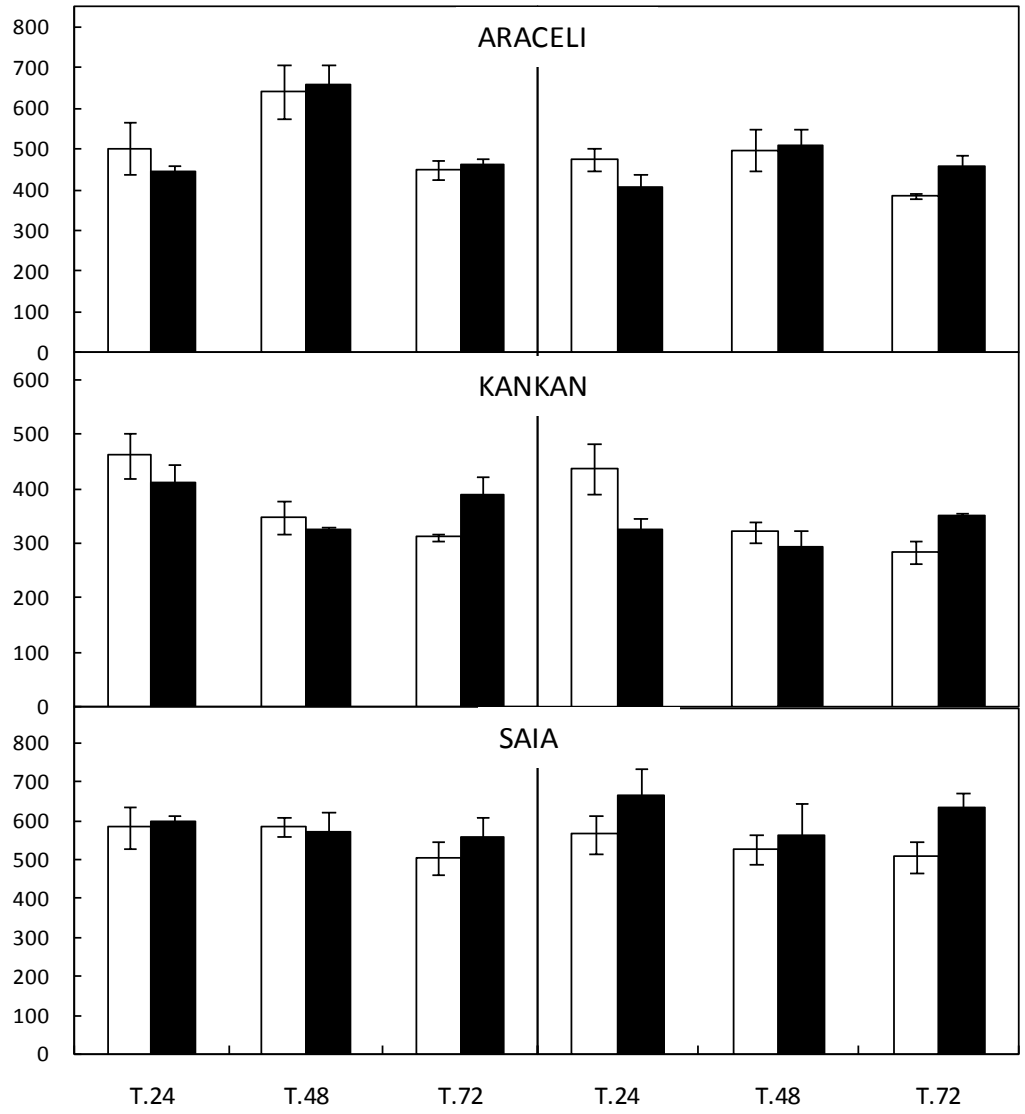

Figure 7. PAO activity in oat leaves. Soluble and cell-wall bound PAO was measured in oat leaves of susceptible (Araceli) and resistant (Kankan and Saia) during a time course following inoculation with the host fungus *Puccinia coronata* f. sp. *avenae.* Data are mean of 5 biological replicates ± standard error. White bar = control, healthy plants; Black bars = plants inoculated with *Puccinia coronata* f. sp. *avenae*

DISCUSSION

Despite the interest in polyamine metabolism in plants exposed to abiotic stresses (Gupta et al., 2013) little information exists on polyamine metabolism during plant-pathogen interaction (Walters, 2003). Our data in oat show increases in specific free polyamines, particularly Agm, Spd and Spm at crucial times of the crown rust infection process in cultivars Kankan and Saia characterized by hypersensitive response and pre/penetration resistance responses, respectively but not in the susceptible cultivar Araceli. Previously, Cowley and Walters (Cowley

and Walters, 2002b) reported increased levels of free and conjugated forms of putrescine, spermidine and spermine 1-4 days after inoculation with the powdery mildew fungus. Increased levels of free and conjugated spermidine were also observed in an incompatible interaction between barley and powdery mildew that involved a penetration based resistance (Cowley and Walters, 2002a). Further, work in barley inoculated with a compatible powdery mildew fungus also showed increased concentrations of putrescine, spermidine and spermine in the infected leaves (Walters, 2000). In wheat infected with the leaf rust fungus *Puccinia recondita* increases in putrescine and spermine levels were observed in both susceptible and moderately resistant near-isogenic lines (Bharti et al., 1996) whereas in wheat infected with *Puccinia graminis* f.sp. *tritici*, spermine concentrations remained unchanged in moderately resistant and susceptible plants. Thus, although changes in levels of polyamines have been observed during plant-pathogen interaction, the topic deserves further studies.

Our data showed particularly elevated levels of polyamines in cultivar Saia, characterized with a strong resistance response not based on hypersensitivity. These resistance mechanisms acting before or during mesopyhll cell penetration may prove more durable and difficult to be overcome than resistance based on a single mechanism or mechanisms governed by a single gene as occur in the HR and hence are desirable from a breeding point of view. Pre-penetration resistance mechanisms might be particularly relevant during the plant-rust interaction due to the relatively extense growth of the rust germ tube until reaching a stomata for appressorium formation (Prats et al., 2007). It is known that topographical signals mediate the processes leading to appressorium formation (Hoch and Staples, 1987). However, physical features are not the only important determinant of rust germ tube growth and appressorium formation. Specific plant chemical signals may also influence appressorium differentiation. Thus, it has been found that phenolic compounds excreted from dead cells of outer scales to the surface in resistant onion varieties prevent urediospore germination of *Colletotrichum circinans* (Walker and Stahmann, 1955) and coumarins excreted to sunflower leaf surface prevent germination and appressorium formation of *Puccinia helianthii*. In order to determine whether the higher pre-penetration resistance observed in Saia could be due at least in part to differential polyamine content on the leaf surface, we quantified the levels of excreted polyamines. Interestingly all genotypes increased the levels of one or more polyamines following rust inoculation and this might explained the basal levels of pre-penetration resistance observed in all genotypes including the susceptible Araceli (Table 1). However, Saia showed significantly higher levels of spermidine and spermine compared with Araceli. Further, these two polyamines showed highly efficient at the concentration tested to increase pre-

penetration responses in leaf bioassays. Increase of the pre-penetration resistance following exogenous polyamine application was mainly due to a poor growth of the germ tube that often did not reach the stoma, because disoriented germ tube growth or continued germ tube growth across stomata and the inability of the germ tube to form an appressorium when finding a stoma. No correlation was found between a particular polyamine and the prevalence of one of these responses. These observations agree with previous work on *Magnaporthe grisea in vitro* showing that polyamines did not affect conidial germination but specifically impaired appressorium formation (Choi et al., 1998). These authors observed high levels of polyamines, particularly of spermidine, in freshly collected spores, decreasing during conidial germination and growth. Thus, altered levels of polyamines within the fungus, provoked by the excreted polyamines from the host might lead to the inhibition of the appressorium formation observed in cultivar Saia.

Polyamine content in Saia remained higher during the earliest stages of the rust infection process compared to that of the susceptible check. This together with the increase of early aborted colonies following exogenous polyamine application, suggest a further role for polyamines during mesophyll penetration resistance. This is supported by the induction in the DAP in Saia at 24 and 48 hai together with increases in ADC expression and in the ADC and DAO and PAO wall-bound activities. There is evidence that DAO and PAO are predominantly located in the cell wall (Sebela et al., 2001). Particularly in oat leaf 80% of the total PAO could be accounted for in the cell wall debris (Kaursawhney et al., 1981). As a result of DAO activity, putrescine can be oxidised whereas PAO catalised the oxidation of spermidine and spermine in both cases yielding H_2O_2 (Bagni and Tassoni, 2001). H_2O_2 is required in oxidative cross-linking of components such as phenolics and proteins into the papilla and associated cell wall region (Pellegrini et al., 1994; Vanacker et al., 2000) to enhanced penetration resistance to pathogen infection (Angelini et al., 1993). The H_2O_2 generated by the bound-wall DAO and PAO could be utilised by cell wall peroxidises for cell wall strengthening and lignification (Angelini et al., 1993; Yoda et al., 2009). Thus, our data suggest an involvement of polyamine derived H_2O_2 during the penetration resistance as it has been demonstrated recently during the hypersensitive response in host and non-host interaction (Yoda et al., 2009).

In agreement with previous results that showed and increase of spermidine and spermine during hypersensitive response in barley 1-4 days following inoculation with the powdery mildew fungus, our results also showed such an increase during the oat-rust interaction. This

was accompanied by increases in the ADC activity and DAO wall-bound activity at 48 and 72 hai supporting the role of polyamines during HR.

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Chapter 3:

Reduced nitric oxide levels during drought stress promote drought tolerance in oat and barley by regulation of the polyamine pathway.

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ABSTRACT

Understanding plant tolerance to drought is of fundamental importance for plant breeding of improved drought tolerant plants. However drought tolerance responses are extremely complex and involved a wide range of processes including intricate signaling crosstalk. Polyamines are low-molecular-weight polycations that may act as hormones or regulatory molecules in many fundamental cellular processes. Nitric oxide (NO) is a key biological messenger implicated in fundamental plant processes such as flowering, germination, growth and development or responses to biotic and environmental stresses. Although increasing numbers of studies have addressed the role of NO during plant-drought stress interaction, many questions remain yet unanswered. We here monitored the endogenous NO levels in drought susceptible and resistant oat cultivars during a drought time course and further used transgenic barley lines overexpressing the barley hemoglobin gene HvHb1 to dissect the role of NO during drought tolerance responses in relation with polyamine metabolism. Our data showed an in vivo reduction of NO levels in the oat drought resistant cultivar. Further work showed that transgenic HHb barley lines were also more tolerant to drought than the wild type. This tolerant phenotype correlated with increases in the content of specific polyamines in the HHb lines. Further work showed that NO influenced polyamine pathway in several ways. Thus, HHb lines showed an increase of the direct aminoacids precursor of polyamines, and regulate the expression of several polyamine biosynthesis genes. In addition our data showed a differential S-nytosylation and nitration pattern between HHb and WT although the identity of the S-nytrosilated and nitrated proteins remains to be elucidated.

INTRODUCTION

Drought is considered the most important stress contributing to yield and economical losses in many regions worldwide (Farooq et al., 2009). Understanding plant tolerance to drought is therefore of fundamental importance and forms one of the major research topics. Drought, as other environmental stresses lead to profound changes in the plants for adaptation to the adverse conditions at gene, protein and metabolite level. Polyamines are low molecular weight metabolites considered to be ubiquitous in all living cells. These molecules are positively charged at physiological pH and hence initially their biological function was associated with their capability of binding to negatively charged molecules, such as nucleic acids, phospholipids, and proteins. These ionic interactions, which are reversible, lead to the stabilization of DNA, RNA, membranes and some proteins (Bachrach, 2005; Cohen et al., 1998). In addition to stabilizing macromolecular structures, polyamines also act as hormones or regulatory molecules in many fundamental cellular processes including replication, transcription, translation and enzyme activity modulation in cell division, embryogenesis, as well as in senescence and in response to stresses (Kuznetsov and Shevyakova, 2007a; Martin-Tanguy, 1997). The di-amine Putrescine (Put), the tri-amine spermidine (Spd) and tetra-amine spermine (Spm) are the most common polyamines in plants although agmatine, a putrescine precursor, cadaverine and thermospermine are also found. A protective role for spermine against drought stress has been reported in Arabidopsis, where spermine, by modulating the activity of certain ion channels, increases Ca^{2+} regulating stomatal closure (Yamaguchi et al., 2007). In cereals, changes in polyamines contents have also been reported in barley and rice during drought stress (Turner and Stewart, 1986; Yang et al., 2007).

Over the last decade the free radical nitric oxide (NO) has gained special interest due to its involvement in an increasing number of signaling pathways controlling processes that range from growth and development to biotic and abiotic stress responses (reviewed in (Mur et al., 2012; Wendehenne et al., 2001). At the crossroad between developmental and abiotic stress responses lies the regulation of the stomatal aperture by NO (Hancock et al., 2011; Mur et al., 2012). Thus, during the induction of stomatal closure it has been demonstrated an ABA induced NO generation together with an increase in cytoplasmic pH and H_2O_2 (Bright et al., 2006; Neill et al., 2002). According to this, stomatal closure provoked by exogenously applied NO donors (i.e. sodium nitropruside, SNP) correlated with tolerance to rapid dehydration in wheat seedling (Garcia-Mata and Lamattina, 2001). However, NO can also appear to be a redundant element in stomatal regulation in conditions of rapid dehydration (Ribeiro et al.,

2009). Thus, despite some evidences of NO involvement in stomatal-based drought tolerance by exogenous applications of NO donors, the *in vivo* NO generation in plants undergoing water stress has not been determined as neither other possible roles of endogenous generated NO during drought.

Evidences indicated that NO might interact with polyamines during developmental and stress responses. Thus, NO production has been observed in plants exposed to exogenously applied polyamines (Tun et al., 2006). However, whether polyamines act as substrates, cofactors, or signals for promoting NO synthesis needs to be determined (Freschi, 2013). In addition, a possible effect of NO on polyamine byosinthesis have been also shown in some studies (Fan et al., 2013) but not in others (Arasimowicz-Jelonek et al., 2009) consequently this topic still deserve further research efforts.

It is known that NO interact with phytohormones such as auxins, ABA, ethylene, giberelins, cytoninins, or salicylic acid (Freschi, 2013). Furthermore, the polyamine biosynthetic pathway share common elements with that of NO and hence polyamines could be particularly influenced by the NO levels (Supplemental Fig 1). For instance L-arginine is a common precursor in the biosynthesis of both putrescine and NO (Gao et al., 2009) and nitrate/nitritre are common precursors of NO and several aminoacids directly related with the polyamine biosynthesis.

In addition to this direct influence of NO on polyamine metabolism, as a signaling molecule, NO may be able to modulate elements controlling the plant hormone level and distribution (Freschi, 2013). This modulation may occur at transcriptional and/or post-translational level. Thus, NO likely interacts with a wide range of target proteins via direct modification of protein structure triggering changes in their activities and functions (Paris et al., 2013). Among the NO-depended post-translational modification, S-nytrosilation of cysteine residues is emerging as key mechanism for transduction of bioactivity of NO in plants (Mengel et al., 2013). This reversible post-translational modification has been involved in the control of plant processes such as cellular architecture, photosynthesis, genetic information processing, protection against oxidative stress, defense responses to biotic and abiotic stresses and hormonal signal (Astier and Lindermayr, 2012; Astier et al., 2011; Lindermayr et al., 2005; Romero-Puertas et al., 2008). In addition, NO may react with reactive oxygen species (ROS), such as superoxide (O_2^-), resulting in the production of NO derived species such as peroxynitrite ($ONOO^-$)which can covalently modify tyrosine residues by a process known as tyrosine nitration (Ferrer-Sueta and

Radi, 2009). This NO post-translational modifications have been involved in processes such as photosynthesis, respiration, and nitrogen metabolism (Cecconi et al., 2009; Chaki et al., 2009; Lozano-Juste et al., 2011; Tanou et al., 2012). Furthermore, it has been shown that redox-sensitive transcription factors are also nitrosylated and that NO influences the redox-dependent nuclear transports of some proteins, implying a role for NO in the regulation of the transcription and/or general nuclear metabolism (Mengel et al., 2013). Thus, several studies, based on plants with altered NO-levels, have provided genetic evidences for the relevance of NO in gene induction (reviewed in (Besson-Bard et al., 2009; Grun et al., 2006).

An important and frequently neglected aspect that influences NO metabolism and signaling are the NO scavenging mechanisms. NO may be removed by reacting with ROS and others chemicals such as urate, which have been shown to prevent NO toxicity (Alamillo and Garcia-Olmedo, 2001). In addition, enzymatic NO removal by GSNO reductase and non-symbiotic hemoglobins (nsHb) have been reported for more selective regulation of NO levels. Plants Hbs are able to regulate several of the NO effects, as recently reviewed by Hill (Hill, 2012). Out of the three different classes to which Hbs evolutionary belong, class 1 Hbs possess an extremely high affinity to oxygen and their main function seems to be related to scavenge NO (Gupta and Igamberdiev, 2011). Thus, this Hb class have been shown to regulate the NO-induced expression of genes involved in phosphatidic acid synthesis and sphingolipid phosphorylation during plant acclimation and tolerance to cold (Cantrel et al., 2011). Class 1 Hb have been also found to modulate salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens (Mur et al., 2012) and modulates the NO emission and hyponasty under hypoxia-related stress in Arabidopsis thaliana (Hebelstrup et al., 2012). Class 2 Hbs are mainly devoted to facilitate oxygen supply to tissues and include the Hbs found in association with nitrogen-fixing bacteria in root nodules (“symbiotic hemoglobins”). Class 3 Hbs are truncated Hbs with a very low affinity for O_2 and albeit their function is obscure, it might be related to regulation of oxygen delivery at high O_2 concentrations (Watts et al., 2001).

Early work used over-expressed bacterial Hb *hmpX* in transgenic lines as a useful method to reduce NO production and show the roles of NO in the hypersensitive response, responses to UV-B, symbiotic interactions and senescence (Boccara et al., 2005; del Giudice et al., 2011; Mishina et al., 2007; Tossi et al., 2009; Zeier et al., 2004). We here monitored the endogenous NO levels in drought susceptible and resistant oat cultivars during a drought time course and further used transgenic barley lines overexpressing the barley hemoglobin gene HvHb1 to

dissect the role of NO during drought tolerance responses in relation with polyamine metabolism.

MATERIALS AND METHODS

Plant Material

We used a transgenic barley line (*Hordeum vulgare* var. Golden Promise) expressing cDNA of the barley hemoglobin gene HvHb1 (accession number: U94968), donated as a plasmid (CA2α) from Dr. Robert D. Hill, University of Manitoba (Duff et al., 1997). The cDNA was cloned into the vector pUCEHordD::USER::NOS or pUCEUbi::USER::NOS by USER cloning (Hebelstrup et al., 2010). The generation of independent transgenic lines (HHb) with overexpression of HvHb1 by the barley hordein-D promoter is described in Hebelstrup et al. (Hebelstrup et al., 2010).

Experiments were carried out at seedling stage (3 week old plants) (Gong et al., 2010; Hao et al., 2010; Sanchez-Martin et al., 2012). Seedlings were grown in 0.5 L pots filled with peat:sand (3:1) in a growth chamber at 20 ºC, 65 % relative humidity and under 12 h dark/12 h light with 150 µmol m^{-2} sec^{-1} photon flux density supplied by white fluorescent tubes (OSRAM). During growth, trays carrying the pots were watered regularly. At day 21, water was withheld from those plants selected for drought treatment, (Gong et al., 2010; Hao et al., 2010). Control plants were watered as described above throughout the whole experiment. During the drought treatment the relative water content of the soil was monitored daily reaching a level of 20% by day 18 (Gong et al., 2010)

In vivo NO measurements

NO production was measured using a QCL (quantum cascade laser)-based spectrometer, equipped with an astigmatic multipass absorption cell for wavelength modulation spectroscopy on NO (Cristescu et al., 2008). For online concentration measurements and data analysis, LabVIEW program (National Instruments) was used (Cristescu et al., 2008). The detector was calibrated using a certified calibration mixture with 100 ppbv NO (National Measurement Institute, Delft, The Netherlands). Four glass cuvettes (150 ml volume) containing one barley plant each were used per replication. NO was allowed to accumulate in the headspace for 75 min, and, thereafter, the cuvette was flushed with purified air at a flow rate of 1.66 l/h, and accumulated NO was quantified. The four cuvettes were measured sequentially. One cuvette contained the control WT plant, the second cuvette the control HHb plant, a third and four, with the water stress samples. For the following three replications on

independent plants the order of the cuvettes was changed randomly. Soil and pots were autoclaved to prevent the growth of microorganisms which could modify the NO balance.

Visual assessment of drought symptoms

From the time at which water was withheld for drought treatment (from now on T0) all plants were visually evaluated daily according to the following scale: 0 = vigorous plant, no leaves shows drought symptoms; 1 = one or two leaves show slight drought symptoms (less turgor) but most leaves remain erect; 2 = most leaves show slight levels of drought stress, however one or two leaves still show no drought symptoms; 3 = all leaves show drought symptoms but these are no severe; 4 = all leaves show severe drought symptoms including incipient wilting; 5 = the whole plant is wilted with all leaves starting to dry, rolled and or shrunken (Online Resource 1). Five plants per accession were assessed. Drought severity values daily assessed according to this scale were used to calculate the area under the drought progress curve (AUDPC) for each oat accession similarly to the area under the disease progress curve widely used to disease screenings (Jeger and Viljanen-Rollinson, 2001) using the formula:

$$AUDPC = \sum_{i=1}^{k} \tfrac{1}{2}\left[(S_i + S_{i+1})(t_{i+1} - t_i)\right]$$

where Si is the drought severity at assessment date i, ti is the number of days after the first observation on assessment date i and k is the number of successive observations. Measurements were performed on ten independent plants per genotype and treatment.

Relative water content

RWC was measured in ten plants per genotype and treatment according to (Barrs and Weatherley, 1962). Measurements were carried out in the second leaves at time 0, 6, 9, 12, 15 and 18 days after withholding water (daww). Six hours after the onset of the light period, leaf blade segments were weighed (fresh weight; FW), floated on distilled water at 4 ºC overnight and weighed again (turgid weight; TW). They were then dried at 80 ºC for 48 h. After this, the dry weight (DW) was determined. RWC was then calculated as RWC = (FW - DW) (TW -DW)-1 x 100.

Leaf water potential

Leaf water potential (Ψ) was measured at midday with a pressure chamber (Soil Moisture Corp., Santa Barbara, CA, USA). Measurements were performed on ten independent plants per genotype and treatment.

Transpiration assessment

Transpiration expressed in per leaf unit area was measured gravimetrically in 10 plants per genotype and treatment. The pots were covered from both ends with 2 polythene bags that were fixed to the pot with elastic bands. A small slit was made in the top bag to allow the plant to go through. Control pots without plants showed minimum water loss. The initial and final (after each time point) pot weight was taken and transpiration was calculated by subtracting the final pot weight from the initial weight. Leaf area was calculated with software ImageJ after scanning the leaves fixed on a sheet of paper.

Polyamine quantification

The standard polyamines, putrescine (Put), spermidine (Spd), spermine (Spm) and 1-3,diaminopropane (Dap) were obtained as their hydrochlorides (Sigma) whereas agmatine (Agm) was obtained as its sulfate (Sigma). When soil reach 20% RWC, leaves were fixed in liquid nitrogen and stored frozen until use. Plant extract were obtained by homogenizing the plant tissue in perchloric acid (0.1 w/v) according to Flores and Galston, (Flores and Galston, 1982). Standards and plant extracts were benzoylated according to Redmond and Tseng, (Redmond and Tseng, 1979). High performance liquid chromatography analysis of benzoyl-PAs was performed according to Slocum et al., (Slocum et al., 1989), using an Agilent 2100 Series HPLC. HTD (1,7-diaminoheptane) was used as internal control (Sigma).

Aminoacids quantification

Amino acids standards and the internal standard, norvaline were obtained from Sigma. Standard solutions were prepared from a stock solution by dilution with 0.1 M HCl. When soil reach 20% RWC, leaves were fixed in liquid nitrogen and stored frozen until use. Plant extract were obtained by homogenizing the plant tissue in 0.1 M HCl according to Herbert et al., (Herbert et al., 2000). Analysis were carried out using an Agilent 2100 Series HPLC and a column Merck Lichro-CART®250-4 Superspher®100 RP-18 endcapped (25 cm x 4.6 mm; 5 mm particles) at 42 °C. Briefly, 40 µL of leaf extract was mixed with 200 µL borate buffer (0.4M, ph 10.6), 200 µL OPA reagent and 40 µL FMOC reagent prepared according to (Herbert et al.,

2000). The reaction mixture was allowed to stand for 2 min at 42 ºC and then 20 µL was injected. The chromatographic separation was made using a binary gradient elution (Herbert et al., 2000). Mobile phase A was a 20 mM sodium acetate solution, with 0.018% (v/v) triethylamine, 0.3% (v/v) tetrahydrofurane, and 0.010% (v/v) of a 4% (m/v) solution of EDTA. The pH was adjusted to 7.20 with a 0.1% (v/v) solution of acetic acid. Mobile phase B was a solution with 20% (v/v) of a sodium acetate solution (100 mM, pH 6.0), 40% (v/v) of acetonitrile, 40% (v/v) of methanol, and 0.018% (v/v) triethylamine. Excitation/emission wavelengths were respectively 340/450 nm for primary amino acids and 237/340 nm for secondary amino acids. The latter was used to enhance the sensitivity of proline detection. The change in wavelengths was made at 115 min.

Primer design

All primers used in this study (Table 1) were designed using the Universal Probe Library Assay Design Center (Roche applied Science) based on mRNA sequences deposited in GenBank. The specificity of the primers was checked by alignments with the original GenBank sequences using the standard nucleotide-nucleotide BLAST (blastn; provided online by NCBI).

ARN extraction and cDNA amplification

When soil reach 20% RWC, leaves were fixed in liquid nitrogen and stored frozen until use. Total RNA from was extracted from 100 mg of ground leaf tissue using previously reported protocols (Chomczynski and Sacchi, 1987; Raeder and Broda, 1985). RNA was cleaned by means of RNeasy® Minelute Cleanup Kit (QIAGEN). Contamination of residual genomic DNA in all RNA samples was verified by conventional PCR amplification on total RNA using the designed primers listed in Table 1. RNA samples containing DNA were further DNase treated until no PCR amplification of RNA samples was obtained. Prior to retrotranscription experiments, the concentration and integrity of RNA were verified by an optical density at 260 nm (OD260)/OD280 absorption ratio in a NanoDrop ND- 1000 spectrophotometer (Thermo scientific).

First and second-strand of complementary DNA (cDNA) were synthesized using SuperScript® III First-Strand (Invitrogen) and DNA Polymerase I (BioLabs), respectively. cDNA was cleaning by QUIquick PCR Purification Kit (QIAGEN and DNase treated by the RNase-Free DNase Set (Qiagen), according to the manufacturer's recommendations. Conventional RT-PCR and PCR assays followed by gel electrophoresis were performed to verify the amplification of cDNA

using the designed primers. Quality and quantity of cDNA was determined by running aliquots in agarose gels and by spectrophotometric analysis in a NanoDrop ND- 1000 spectrophotometer (Thermo scientific).

Gene expression analysis by real-time QRT-PCR

Previous to test the expression of the polyamine-associated genes, four additional genes were tested for using as reference genes; glyceraldehyde-3-phosphate dehydrogenase (GADPH), beta-tubulin (TUBB), alpha-tubulin (TUBA) and 18S ribosomal RNA (10S rRNA) according to Jarasova and Kundu, (Jarosova and Kundu, 2010). Following preliminary assay, GADPH was selected as internal control as it showed a highly stable expression in our barley samples. Real-time qRT-PCR was performed for each of the polyamine-associated genes and for GADPH on at least 3 independent biological plus 3 technical replicated cDNA templates in StepOne Real-Time PCR System (Applied Biosystems) using FartStart Universal SYBR Green Master (Rox) (Roche) according to the manufacturer's recommendations. The reaction mixture contained 10 µl of SYBR Green master mix, 6 µl of each primer set (Table 1), and 4 µl of cDNA or standard solution as template. The amplification conditions were 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s, 60°C for 1 min,. Following amplification, a melting curve program 95ºC for 15 seg, 60ºC for 1 min and 60 to 95°C with a heating rate of 0.3°C/min. The melting point analysis was performed at the end of the real-time RT-PCR to confirm the amplification of a unique product for each gene. The fold changes of polyamine-associated gene transcripts in different treatments versus control (i.e., well watered plants) were normalized using the CT and efficiency obtained for the GADPH amplification run on the same cDNA templates according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Protein analysis

For protein extraction, frozen leaves were ground to a fine powder (about 500 mg) in a mortar placed in a liquid nitrogen bath. Then, leaves were homogenized in lysis buffer (50 mM Tris-HCl, 300mM NaCl, 5 mMEDTA, 0.1 mM Neocuproine; 1% Triton X-100, pH 7.4) containing complete protease inhibitor cocktail (Sigma, St. Louis) (Martinez-Ruiz and Lamas, 2005). The extract was centrifuged at 4 ºC for 20 min at 10000 g, and the protein concentration in the supernatant was measured using a Bradford assay (BioRad, Hercules, CA).

S-nitrosylated proteins were detected by using the biotin-switch method (Jaffrey and Snyder, 2001), with slight modifications. Briefly, samples containing 200 µg of protein were diluted in

TABLE 1. Primers designed and used in real-time reverse transcription-polymerase chain reaction (RT-PCR) for amplifying polyamine-associated genes of barley plants.

Target	Primer name	Fw Primer	Rv Primer	Accession	Reference
ADC	ADCHor	CATCATCGTGTTGGAGATGG	AGCTTGTTGCTCTGGTCGAT	AK249293.1	Sato, et al., 2009
ODC	ODCHor	CGGCTCCAACTTCAATGG	GTCAGCTGGAGTAGGCCAAG	AK367967.1	Matsumoto, et al., 2011.
MAT	MAT123Hor	CTTCACCAAGCGTCCAGAA	GCATCAGCTCAGGGGTCTC	D63835.1 AK354757.1 AK358278.1	Mori,and Takizawa, unpublished; Matsumoto et al., 2011.
AdoMetDC	AdoMetDCHor	GGCTCTCTCATCTACCAGAGCTT	GATCTTGGCGACCCACTG	AK368996.1	Matsumoto et al., 2011
ACS1	ACS1Hor	GTCTCCTCCCAGACGCAGTA	TGCGGGTGAAGTCCTTGT	JX046052.1	Dahleen et al., 2012
ACS2	ACS2Hor	GAGTTCAGACAGGCGATGG	GTCAAACCTGGCCTTCCAC	JX046053.1	Dahleen et al., 2012
ACS5	ACS5Hor	GAGCTGCTCACGTTCATCCT	CAAAACCCGGGTAGTACGG	JX046054.1	Dahleen et al., 2012
ACS6	ACS6Hor	TCCTCCAGCTCTACATCAAGC	GAGGAGGAGGCCGAAGTG	JX046055.1	Dahleen et al., 2012
GADPH	GADPH	TGTCCATGCCATGACTGCAA	CCAGTGCTGCTTGGAATGATG	AK251456	Sato et al., 2009

HENS buffer (with 2.5% SDS) to obtain a 1:1.5 (µg protein/µl) proportion. Samples were then incubated with the S-nitrosylating agent GSNO (500 µM), or DTT (10mM) in the dark at room temperature for 30 min with regular vortexing. Reagents were then removed by two precipitations with two volumes of cold acetone. Protein extracts were then incubated with 20 mM methyl-methanethiosulfate (MMTS) at 50 ºC for 1h with frequent vortexing to block free cysteines. Excess MMTS was removed by precipitation with two volumes of cold acetone, and proteins were resuspended in 0.1 ml HENS buffer (25 mM HEPES, 1 mM EDTA, and 1% SDS, pH 7.7) per milligram of protein. After the addition of 1 mM HPDP-biotin (Pierce, Rockford, IL) and 1 mM ascorbic acid, the mixture was incubated 1 h at room temperature in the dark with intermittent vortexing. Reagents were removed again by precipitation with two volumes of cold acetone. Proteins were then subjected SDS-PAGE and Western blot analysis using an antibiotin antibody.

For analysis of nitrated proteins leaf extracts were precipitated in an acetone:methanol solution (8:1 v/v) to remove interfering compounds/molecules (including chlorophyll).

SDS-PAGE was performed according to (Laemmli, 1970) using 12% acrylamide gels. Briefly, protein extracts were diluted 1:1 with Laemmli's sample buffer (62.5mM Tris-HCl, pH 6.8, 25% v/v glycerol, 2% w/v SDS, 0.01% w/v bromophenol blue and 5% β-mercaptoethanol), and boiled for 3min. The applied amount of protein sample per lane was 50 mg; the bioBlu prestained protein ladder (gTPbio) was used as a standard for molecular weight determination. The electrophoresis was conducted in a PROTEAN 2 Cell (Bio-Rad) with Tris/glycine/SDS running buffer (192mM glycine, 0.1% w/v SDS and 25 mM Tris to pH 8.3) by setting 25 mA until the tracking dye bromophenol blue, penetrated in the running gel and then 200V, until it reached the anodic end of the gels. The experiment was repeated twice.

For western blot analysis, proteins were electrophoretically transferred to nitrocellulose membranes (BioTrace^{TM} NT, Pall Corporation) using a Mini Trans Blot Cell (Bio-Rad) at 30 V overnight. Membranes were stained with Ponceau S (Sigma–Aldrich) to confirm equal protein loading and then blocked by incubating with 1% w/v bovine serum albumin in TTBS (0.2% v/v Tween- 20 Tris-buffered saline; 20mM Tris, 150mM NaCl, pH 7.6) for 1 h at RT. After 2x5 min washes in TTBS, the membranes were incubated with either an antibiotin antibody (1:10 000 dilution; Sigma) or a monoclonal antibody against nitrotyrosine (1:2500 dilution; Alpha diagnostic Int) for detection of *S*-nitrosylated or nitrated proteins, respectively, for 1 h at RT in

blocking solution. The membranes were then washed six times in TTBS and probed with ECL anti-mouse IgG HRP-linked (Santa Cruz Biotechnology) at 1:10 000 dilution for 1 h at RT in blocking solution. The immunocomplexes were detected by chemiluminescence (ECL Advance, Amersham Biosciences) on ETNA Firefly system (ETNA Sciences). The experiment was repeated twice.

RESULTS

In vivo NO generation in susceptible and resistant oat plants under water stress

Assessment of *in vivo* NO production in oat plants revealed a contrasting trend between the susceptible, Flega, and the resistant, Patones, oat cvs. Thus, the resistant cv Patones reduced by 50% the levels of NO at mild and high water stress (between 60% and 30% of soil relative water content, sRWC), whereas susceptible Flega increased its NO level respect to control, well watered plants, showing significant differences respect to Patones (Fig. 1). No differences between the levels of control and treated plants were observed at 90-95% sRWC, when plants still had sufficient water availability. Levels of NO decreased in Flega plants at the highest water stress tested (15-20% sRWC), although at this point it did not show significant differences respect to Patones plants (Fig 1).

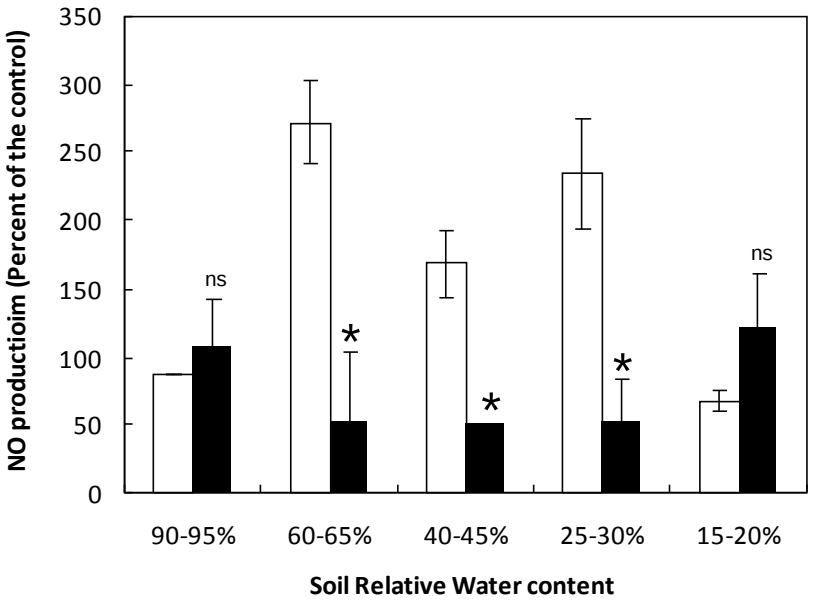

Figure 1. In vivo NO generation in Flega and Patones oat plants under drought. NO generation was measured in total plant in susceptible cultivar Flega (white) and tolerant cultivar Patones (black) during a time course drought course. Data are mean of 3 biological replicates ± standard error.

HHb barley plants showed reduced levels of NO compared with WT

In order to dissect the role of NO during water stress, hemoglobin overexpressing barley lines were assessed, as oat genotypes affected in NO pathway were not available to us. According to Hebelstrup et al., (2010) the HHb barley line overexpressing the HvHb1 gene used in this study, showed c.a 2000 fold increased relative expression of the HvHb1 gene with respect to the wild type. To confirm reduced NO levels in the HHb lines respect to WT, *in vivo* NO measurements were carried out in both, the wild type and HHb line under well watered and water stress conditions. Under well watered conditions, the HHb barley genotype showed significantly reduced levels of NO generation with respect to the wild type (Fig 2). Interestingly, under water stress wild type plants slightly but significantly reduced the production of NO whereas HHb plants dramatically reduced its NO levels (Fig. 2). This confirmed the efficiency of the HHb line in scavenging important amounts of the NO generated by the plant.

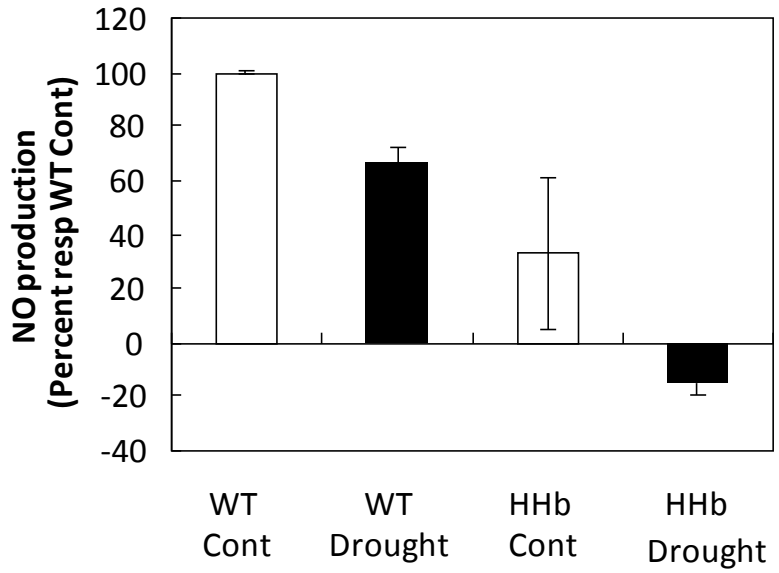

Figure 2. NO content in barley HHb line compared with Golden Promise (20% sRWC) NO generation was measured in total plant in barley wild type Golden Promise and in HHb line in control (white) and drought (black) conditions. . Data are mean of 3 biological replicates ± standard error.

HHb barley plants showed increased drought tolerance compared with WT

To confirm the effect of NO reduction in ameliorating drought stress symptoms, WT and HHb plants were evaluated during a water stress time course. HHb plants started to show visual

drought symptoms several days later than the wild type and showed a better aspect during the complete time course (Fig. 3). Indeed the area under the drought progress curve was greatly, 3-fold, and significantly reduced (*P*=0.003*)* in the HHb plants compared with the WT.

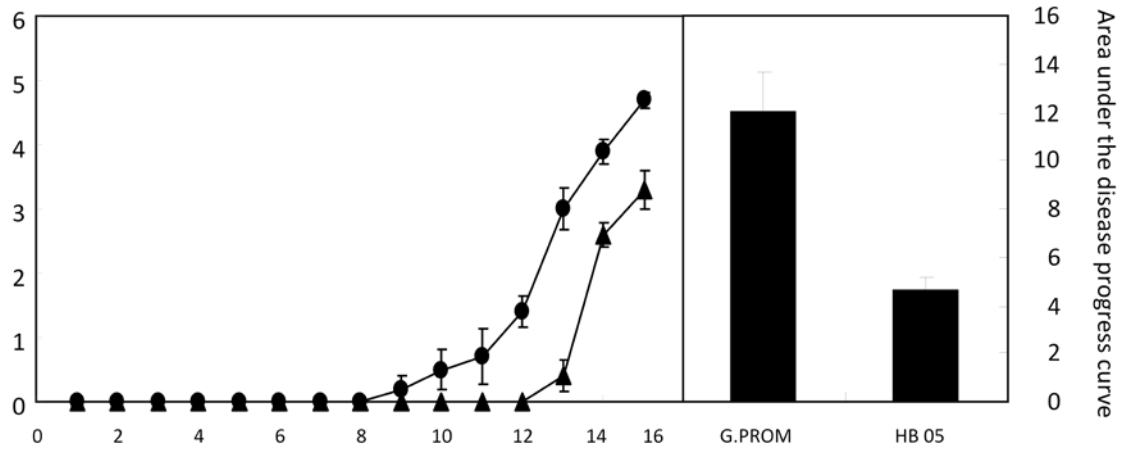

Figure 3. Drought tolerance response of barley HHb lines. Measure of drought tolerance response of barley HHb lines (–▲–) and in wild type Golden Promise (–●–) using a visual escale during the time course of drought Data are mean of 3 biological replicates ± standard error.

To confirm the drought tolerance phenotype of the HHb plants, assessment of several physiological parameters related to water balance was carried out at 20-25% sRWC (Fig 4). Both, wild type and HHb plants showed reduced levels of leaf relative water content under water stress respect to its respective well watered controls (*P*<0.001 and 0.05 respectively for WT and HHb plants). However, the HHb plants showed a significant higher lRWC than the wild type under drought (Fig 4A). As expected, no differences in the leaf relative water content were observed between well watered plants of both genotypes.

Assessment of the midday leaf water potential showed a similar trend than with lRWC albeit differences between WT and HHb plants were clearer. Both genotypes reduced significantly (*P*<0.001) the water potential under drought stress respect to its watered controls but whereas the WT doubled the negative water potential respect to well watered plants, HHb only slightly reduced its levels showing significant differences respect to WT plants (*P*<0.001; Fig 4B). Assessment of transpiration during the central part of photoperiod showed no differences between well watered. As expected both genotypes reduced transpiration under water stress (*P*<0.001). However, a slightly higher but significant (*P*<0.001) transpiration level in HHb plants subjected to drought stress respect to WT plants was observed (Fig 4C).

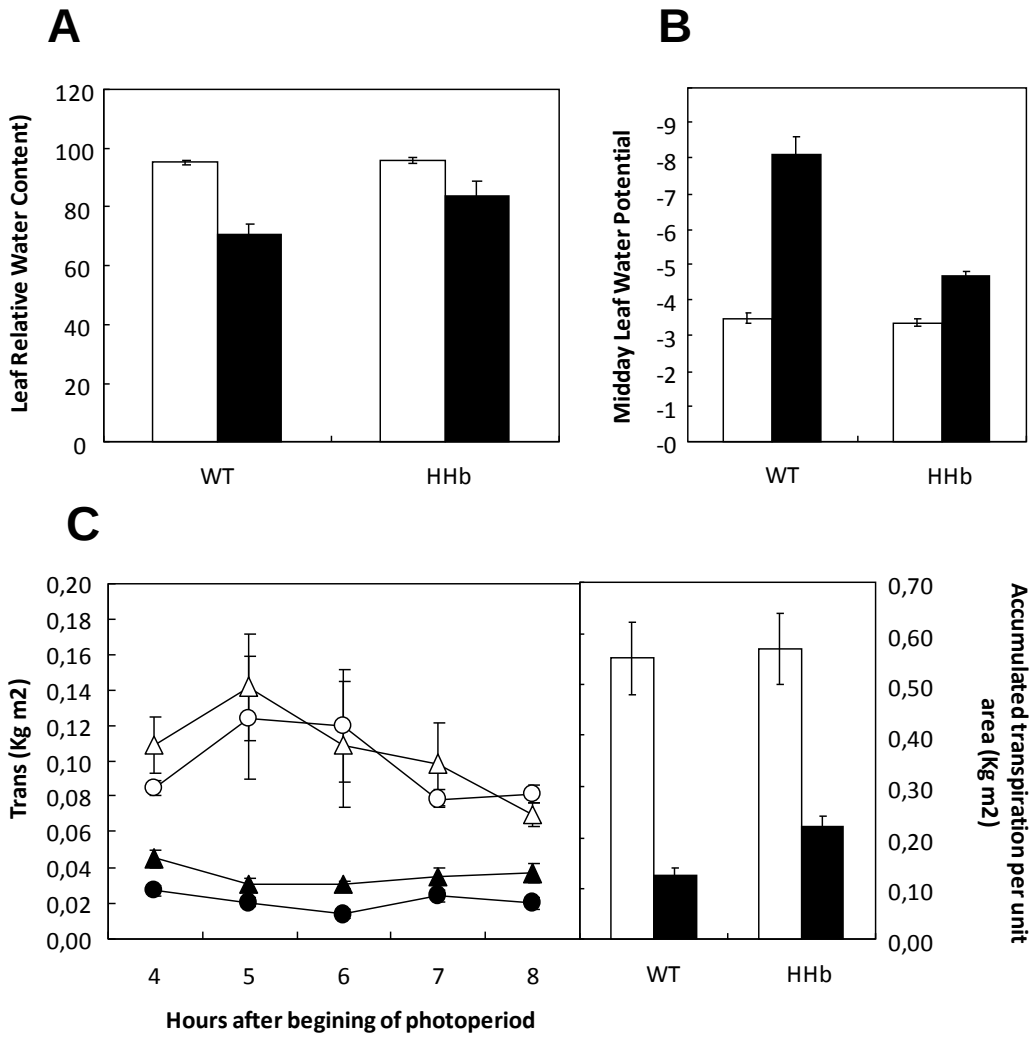

Figure 4. Water related parameters in barley HHb lines under drought. Different water related parameters; leaf relative water content, midday leaf water potential, transpiration and accumulated transpirations per unit area were measure in barley HHb lines and in wild type Golden Promise in control (white) and drought condition (black). Data are mean of 5 biological replicates ± standard error.

Levels of specific polyamines were increased in HHb lines respect to WT under drought

Polyamines are well known metabolites largely associated with drought and abiotic stress tolerance (REF). Recently a possible linkage between polyamines and NO have been reported (Tun et al., 2006). Thus, we explored the levels of polyamines in HHb and WT plants in order to determine if the drought tolerance response observed in the HHb plants might be at least in part due to changes in polyamine levels. Overall, HHb plants showed higher constitutive levels of putrescine, spermidine and spermine than WT plants (Fig 5). Following water deficit treatment, the content of these polyamines increased in both genotypes. The levels of

putrescine dramatically increase in the WT plants respect to control level and it also increased albeit moderately in HHb plants (Fig 5). Spermine also increased in both genotypes reaching similar levels. By contrast, spermidine did not significantly increase in WT plants following drought stress whereas it increased in HHb plants ($p<0.05$). Thus, the content of spermidine in HHb plants under drought stress was near 2-fold the content of WT plants. Increases of Agmatine and DAP were also observed in both WT and HHb plants subjected to drought with slighty higher levels observed in HHb plants (Fig 5). These results, confirmed a role for NO influencing polyamine content in HHb plants in control conditions and also under drought stress.

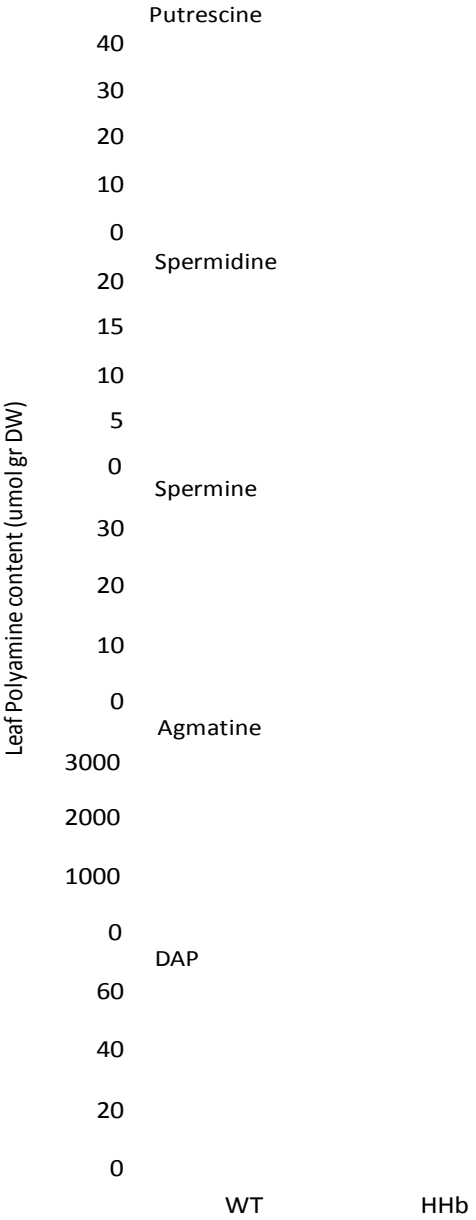

Figure 5. Poliamine content in HHb lines under drought Putrescine, spermidine, spermine,agmatine, and 1,3-diaminopropane (DAP) were quantified in in barley wild type Golden Promise and in HHb line in control (white) and drought (black) conditions. Data are mean of 5 biological replicates ± standard error.

Levels of aminoacids linked to the polyamine pathway differed between HHb and WT plants

Several aminoacids are directly involved in the polyamine biosynthesis pathways (Supplemental Fig 1 (modified from Alcazar et al., 2010). Alteration of NO levels, could affect their concentration either by modifying the nitrogen fluxes or through the regulation of their particular biosynthetic pathways affecting ultimately polyamine content.

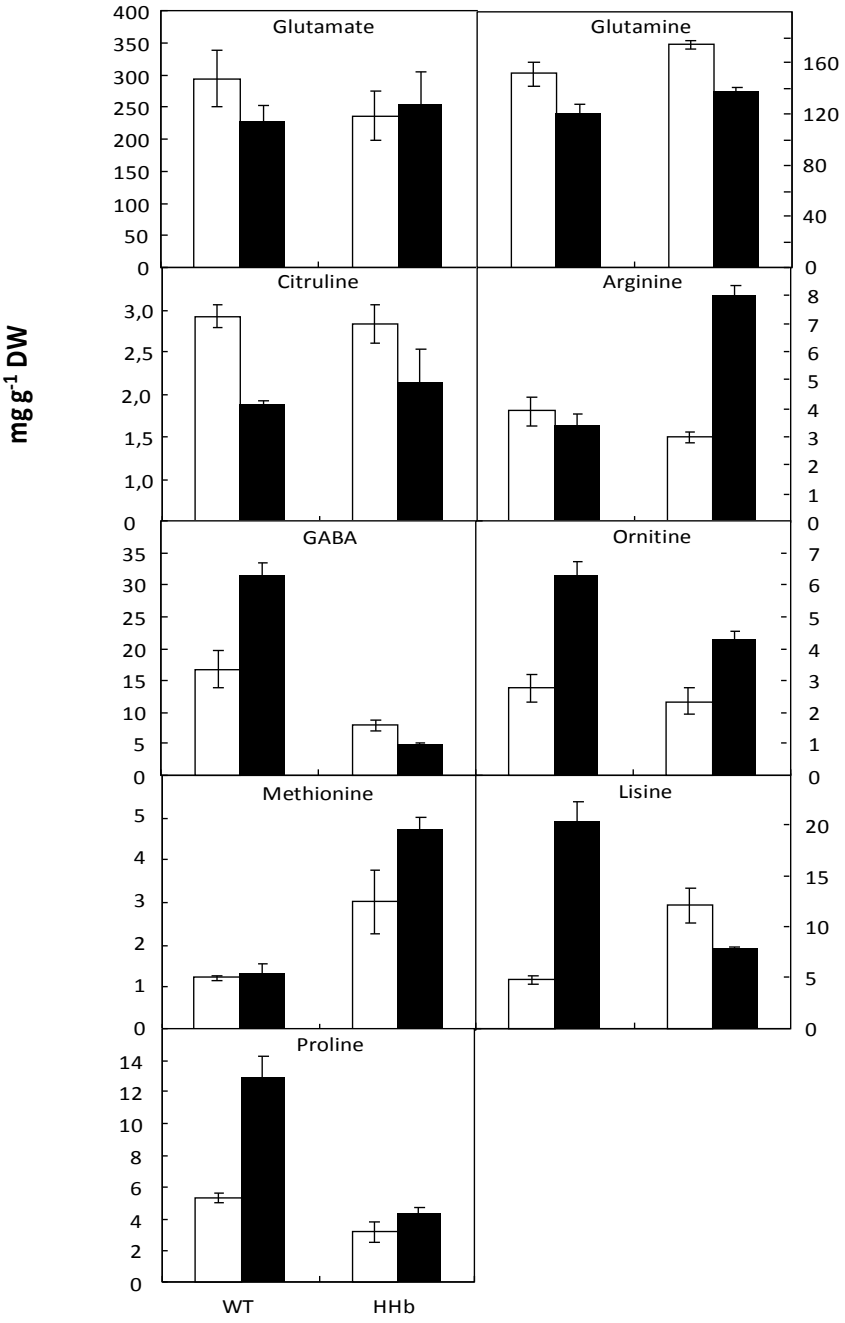

Figure 6. Content of aminoacids involved in poliamine pathway in barley Hb05 lines under drought. Glutamate, glutamine, citruline, arginine, GABA, ornitine, methionine, lysine and proline were quantified in in barley wild type GoldenPromise and in HHb line in control (white) and drought (black) conditions. Data are mean of 5 biological replicates ± standard error.

To support this hypothesis, aminoacids concentration in HHb and WT plants were determined. Figure 6 showed leaf content of those aminoacids directly linked to the polyamine pathway according to Alcazar et al., (2010) in HHb and WT plants under well watered and drought conditions.

For several of the aminoacids both, WT and HHb plants followed a similar trend under water stress. Thus, both genotypes decreased the levels of glutamine and citruline and increased the levels of ornithine, the latter slightly higher in the WT, under water stress. However, a quite different trend between genotypes was observed for key aminoacids such as Arginine, γ-Amibutiric acid (GABA), Methionine, Lisine and Proline (Fig. 6). HHb plants showed a significant increase in arginine and methionine particularly under drought stress compared with WT plants. By contrast, WT showed a significant increase in GABA, lisine and proline under drought stress with respect to HHb plants (Fig 6). These results support a role for NO influencing the concentration of the aminoacid precursors of polyamines.

NO regulate the expression of several polyamine biosynthesis genes

Several studies have recently provided genetic evidence for the importance of NO in gene regulation (Grün et al., 2006). Thus, we investigated whether NO might influence polyamine levels through regulation of several key genes of their biosynthesis pathway. Thus, quantitative RT-PCR experiments were carried out to determine the expression of key enzymes of the polyamine pathway, ADC, ODC, MAT, AdoMetDC and ACS - from which 4 different isoforms has been described in barley (Fig. 7). Expression of ADC increased significantly in both, WT and HHb plants under drought stress. However the increased was by far significantly higher (P<0.001) in the WT compared with the HHb plants (Fig. 7). No differences were observed between genotypes in well watered conditions. The level of the expression of ADC was significant (P=0.003) and positively (r^2=0.87) correlated with the level of drought symptoms (AUDPC) observed in the plants.

ODC expression followed a different trend in WT and HHb plants. Thus, whereas ODC expression up-regulated in WT plants it down-regulated in HHb lines. No significant differences were observed with respect to MAT expression in WT and HHb plants. Interestingly, a significant (P<0.01) downregulation of AdoMetDC was observed in WT plants subjected to drought stress compared with HHb plants in the same conditions. Finally a strong up-regulation of ACS gene, ACS1, ACS2 and ACS5, of up to more than 100-fold was observed in WT

plants subjected to water stress, whereas no significant differences or slightly higher increases in the expression levels (3.5-fold) were observed in HHb plants. No differences in ACS6 expression were observed between genotypes (Fig. 7).

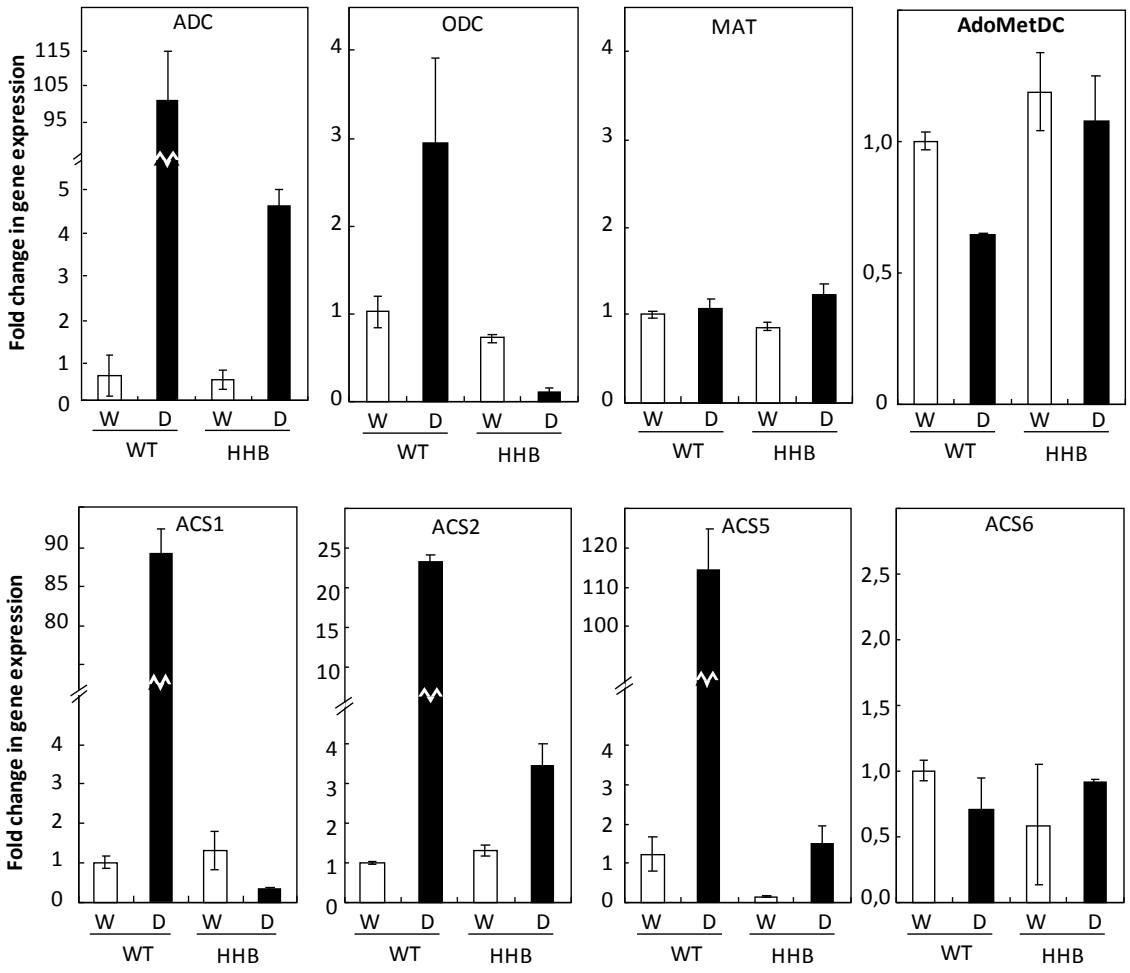

Figure7. Expression of several poliamines pathway genes in HHb under drought. Real time RT-PCR was carried out in barley wild type GoldenPromise and in HHb line in control (white) and drought (black) conditions. Data are mean of at least 3 biological replicates and 3 technical replicates ± standard error.

Different nitration and S-nitrosylation pattern of proteins between WT and HHb plants

Nitration and S-nitrosylation processes have emerged as key post-translational modifications of proteins in animal and plants. Thus, we explored the possibility of NO affecting the activity of proteins involved in the polyamine byosinthesis pathway, through *S*-nitrosylation and nitrosilation of cystein and tyrosin residues, respectively. Western blots assayed with anti-

nitrotyrosine antibodies showed one protein of approximately 22 KDa nitrated in WT plants, both well watered and under drought stress, that were not observed in HHb plants (Fig. 8A). Following the biotin-switch assay anti-biotin antibodies were used to detect *S*-nytrosilated proteins (Fig. 8B). One protein of approximately 18KDa that was not observed or with a very weak signal in WT and HHb under control conditions was S-nitrosylated under drought stress in WT but not in HHb plants. A similar trend was observed for another protein of approximately 8 KDa. Spots corresponding to both proteins increased the signal following extract incubation with the NO donor GSNO, and signal disappears in the controls without biotin.

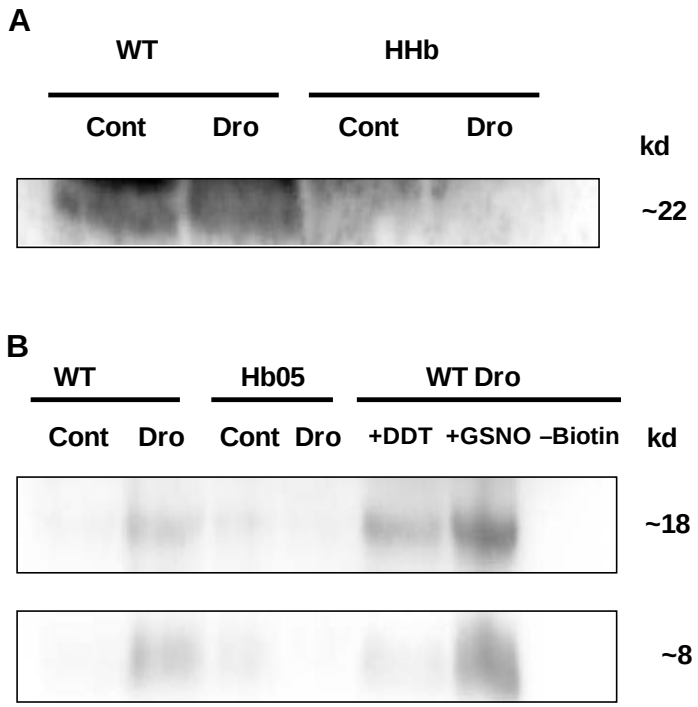

Figure 8. Nitration and nitrosilation of proteins. *S*-nitrosylated and nitrated proteins were detected in barley wild type Golden Promise and in HHb line under control and drought conditions by using the biotin-switch method.

DISCUSSION

Reduced NO levels lead to drought tolerance in oat and barley

Our work shows that during drought stress oat resistant cultivar Patones reduced NO levels respect to the susceptible cultivar Flega. This would suggest and involvement of NO in drought tolerance by which low NO levels correlate with the tolerance phenotype. To support this

hypothesis, further work in barley showed that the transgenic barley line HHb with reduced NO levels had increased drought tolerance respect to the WT plants with normal NO levels. Previous work by (Garcia-Mata and Lamattina, 2001)had reported an effect of NO conferring water deficit tolerance when it was exogenously applied as SNP. Thus, 150 µM SNP increased the RWC of detached wheat leaves subjected to different period of drought for no longer than 3 h. This higher RWC was associated to a decrease in the water loss achieved by stomata closure. It is known that ABA induced NO generation induced stomatal closure (Bright et al., 2006; Neill et al., 2003) and stomatal closure would be a crucial factor to maintain RWC in detached leaves in absence of water so it could be expected that NO induced the higher RWC in this system. However, although reduction of stomatal openings is a common feature of plants suffering stress periods of decreasing soil water availability, fine modulation of stomatal aperture, not only tight closure, is necessary to maintain a delicate equilibrium between saving water, maintenance of photosynthesis and avoiding oxidative damage. This fine stomatal modulation have been previously reported in Patones, whereas in Flega, rapid and tight stomatal closure resulted in a lack of a circadian rhythm, an increase of leaf temperature, increased electron flow-associated oxidative stress and altered photosynthethic activity leading to drought susceptibility (Sanchez-Martin et al., 2012). Similarly, HHb plants, which showed higher drought tolerance, although reduced the transpiration rate per leaf unit area under drought compared with well watered controls, they maintained slightly but significant higher transpiration than the WT plants. This likely aided to maintain lower oxidative stress and higher root hydraulic conductance than the WT plants contributing to the better water status observed in HHb plants (i.e. higher RWC and lower leaf water potential). This higher transpiration rate might be promoted by the lower NO levels observed but clearly, this is not only mediated by NO, as cross-talk between NO, SA, ABA and other hormones such auxin, ethylene, and jasmonate are also crucial in determine the size of stomatal apertures (Acharya and Assmann, 2009).

Moreover, contrasting results of SNP application in wheat seedlings have been also reported. According to Tian and Lei (Tian and Lei, 2006) 0.2 mM SNP enhanced wheat seedling growth under drought whereas 2 mM aggravated the stress. Thus, care should be taken when analyzed the results of NO donors (Floryszak-Wieczorek et al., 2006). Indeed, since we showed that the reduced NO levels had an effect on polyamine metabolism, we also tested whether exogenously applied SNP (Prats et al., 2005) to Flega and Patones oat plants could also affect polyamine production. Surprisingly exogenous SNP at the concentration tested increased the levels of putrescine in both cultivars and spermidine in Flega (Supplemental Fig 2) which could

be promoted for instance by alteration of the nitrogen fluxes and alteration of the direct polyamines precursor aminoacids such as arginine involved in both NO and polyamine biosynthesis (Supplemental Fig 1). This again highlights precaution when supporting hypothesis based in the use of NO donors.

Endogenous NO influence polyamine content by altering polyamine precursor content and gene expression

Polyamines have been largely defined as protective compounds involved in abiotic and also biotic stresses resistance (Kuznetsov and Shevyakova, 2007b; Walters, 2000). Our results showed an involvement of NO in polyamine content. Particularly HHb plants showed increased levels of putrescine and spermidine constitutively and spermidine under drought respect to WT plants. WT plants showed a dramatic increase of putrescine under drought which was accompanied by a near 100 fold increase of *adc* mRNA far higher than the 5-fold increase observed in the HHb plants. The lower spermidine levels observed in the WT plants under stress were associated with the down-regulation of the AdoMetDC gene and reduced methionine levels observed in WT plants. The production of dcSAM by the action of AdoMetDC is an essential step in polyamine production, so AdoMetDC is a key node controlling polyamine content. In addition the aminopropyl groups for spermidine or spermine synthesis are ultimately derived from methionine aminoacid (Pegg et al., 1998) so the reduced methionine content observed in WT plants would also contribute to the lower spermidine level of the WT plants. Furthermore, dramatic increase in the ACS1, ACS2 and ACS5 transcripts would contribute to a diversion of the methionine to the ethylene pathway instead to that of polyamines (Supplemental Fig 1). Thus, putrescine levels would be increased in Wt plants under drought favoured by the up-regulation of the *adc* gene. However, reduced levels of methionine, down-regulation of AddoMetDC and up-regulation of ACS would reduce its conversion to higher polyamines, spermidine and spermine. Using oat leaf system (Galston et al., 1997) showed that upon onset of osmotic stress, activation of *adc* gene transcription induced the accumulation of a putrescine inactive precursor. This precursor was post-translationally cleaved in the N-terminal fragment to produce the 24-kDa C-terminal fragment containing the ADC active site promoting the putrescine formation (Malmberg and Cellino, 1994; Malmberg et al., 1992). The increased levels of putrescine on the leaves lead to a chlorophyll loss and accelerated senescence (Capell et al., 1998). Exougenously applied spermine (Capell et al., 2004; Capell et al., 1998) and spermidine (Capell et al., 1998) are able to inhibit the post-translational processing decreasing putrescine accumulation and the

associated senescence. Our data agreed with these and also showed a senescence related phenotype under drought associated with the high putrescine accumulation in (Yang et al., 2007) suggested that high levels of putrescine at an early stage of drought is necessary for plants to adapt to stress by triggering the conversion of Put to the higher polyamines. Further, mass accumulation of putrescine extending beyond its involvement as higher polyamine precursor would be toxic for the plants. Our data reflect this point since the higher constitutive and also induced levels of putrescine might account for the increase in spermidine observed in HHb plants whereas the massive increase observed in WT might contribute to the drought induced senescence observed in WT.

In addition, a massive increase of the transcript levels of various member of the ACS family was observed in WT plants under drought. ACS catalyzes the first committed step in ethylene biosynthesis in higher plants. Thus, the induction of ACS transcription by NO in WT under drought, not observed in HHb plants, would greatly contribute to an increase in ethylene accelerating the drought induced senescence in this genotype as has been previously reported in maize (Young et al., 2004). Controversy exist about the synergistic or antagonistic relationship between NO and ethylene. However, this probably comes from the exogenous application of the NO as different NO donors and concentrations. Despite this, SNP infiltration has been shown to stimulate ACS expression (Ederli et al., 2006; Mur et al., 2008) and GSNO stimulate the transcript not only of ACS gene but also other key enzymes involved in ethylene biosynthesis (Garcia et al., 2011). Our data agree with these and demonstrate the influence of endogenous produced NO during drought stress stimulating ACS transcripts and suggesting an induction of the drought induced senescence mediated by ethylene.

Post-translational NO modifications under drought

A rapidly increasing number of substrates for *S*-nitrosylation in plants have been reported in the last years including protein kinases, phosphatases, ion channels, metabolic and regulatory enzymes, cytoskeletal and structural proteins, transcription factors, oxidoreductases, and respiratory proteins (review in (Hess et al., 2005; Wang et al., 2006). Much less information is available on protein nitration albeit recent studies also shown its relevance during plant abiotic and biotic interaction (Corpas et al., 2009; Radi, 2004). Our data showed differences in the nitration and *S*-nitrosylation pattern of WT and HHb plants under drought. In particular WT plants showed two nitrosylated proteins that appeared under drought stress in WT plants but did not appear or in a very low concentration in the HHb plants. Interestingly one of them might correspond to the AdoMetDC, which is synthesized as a proenzyme subsequently

cleaved in aproximately 30 (a) and 8 kDa (b) subunits (Park and Cho, 1999). The small subunit is part of the active enzyme complex, and in fact it contains residues which play critical roles in both processing and catalytic activity (Yerlikaya and Stanley, 2004). AdoMet DC has been described as a target for inactivation by NO since its activity was lost upon incubation in vitro with NO donors such as GSNO and SNAP (Hillary and Pegg, 2003). This post-translational inhibition of AdoMetDC by NO would add to the down-regulation of the enzyme above demonstrated, reducing spermidine and/or spermine biosynthesis in WT plants. Not only AdoMetDc, but also ODC (Bauer et al., 2001), MAT and additional polyamine handling enzymes are regulated by S-nitrosylation (Perez-Mato et al., 1999). Unfortunately it did not prove possible the identification of the nitrated/nitrosylated proteins so the detected proteins could belong to the polyamine biosynthesis or other metabolic pathway. A detailed study for the identification of the nitrosilated and nitrated proteins under drought in WT and HHb will be carried out to further dissect the nature of these proteins.

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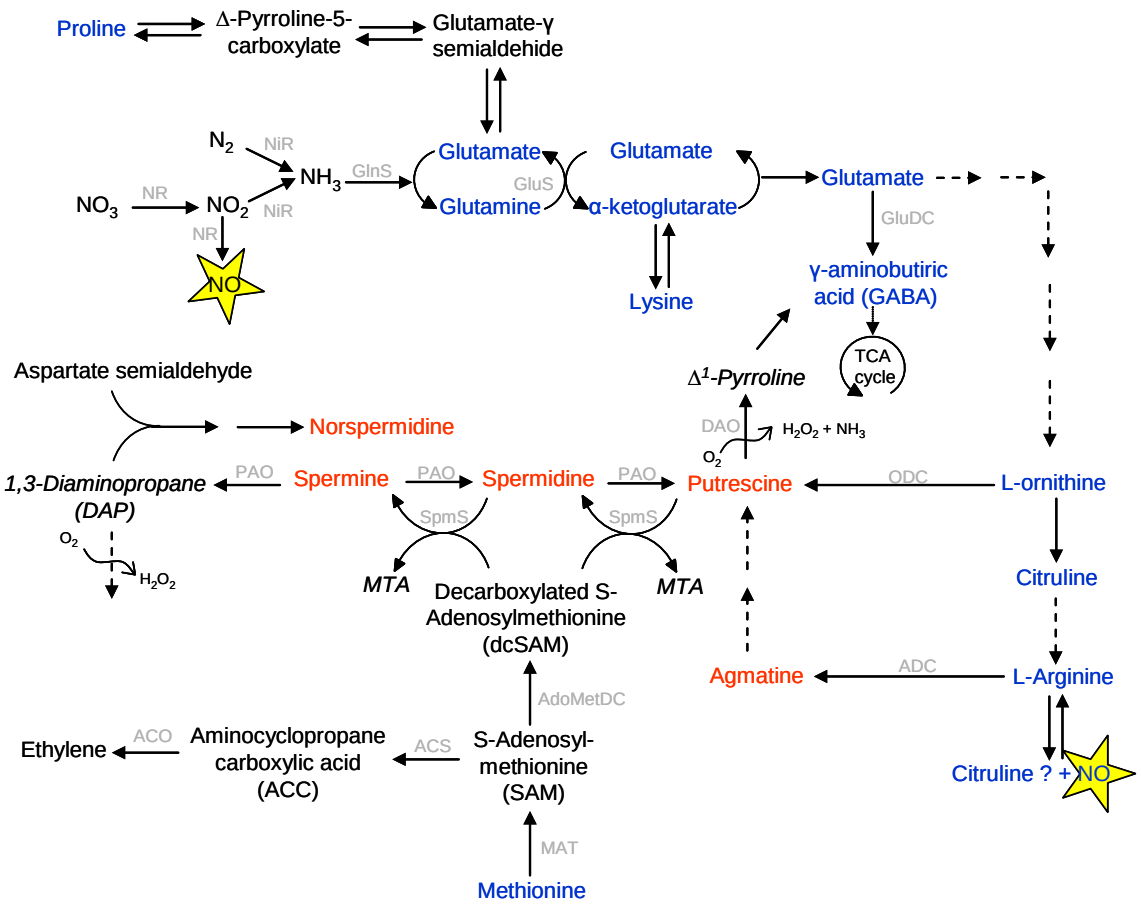
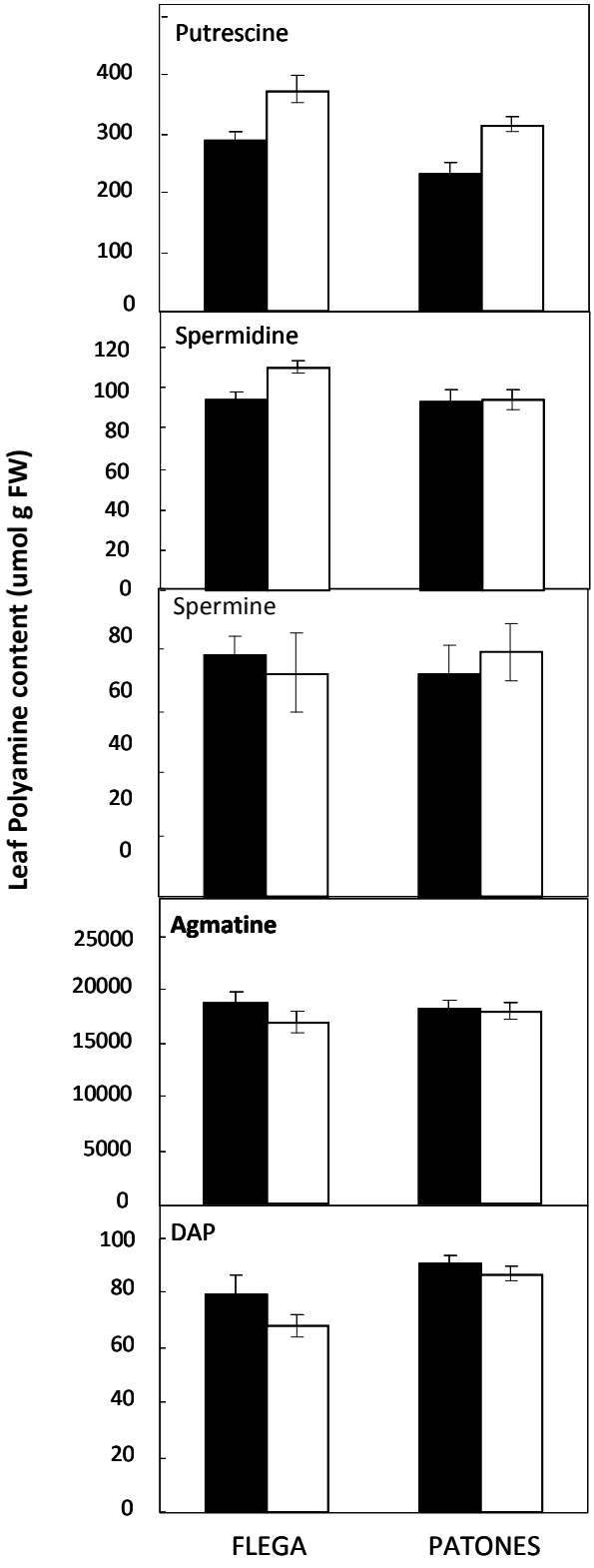
Supplemental Figures



Supplemental Figure 1. Biosynthetic polyamine pathway (Modified from Alcazar et al., 2010)



Supplemental Figure 2. Effect of the NO donor SNP in poliamine content in Hb05 lines under drought. Polyamines were quantified in in in Flega (susceptible) and Patones (resistant) plants, well watered (white) or subjected to drought treatment (black) conditions. Data are mean of 5 biological replicates ± standard error.

Chapter 4:

Genetic Diversity and Population Structure Among Oat Cultivars and Landraces.

Montilla-Bascón G, Sánchez-Martín J, Rispail N, Rubiales D, Mur LAJ, Langdon T, Griffiths I, Howarth CJ, Prats E.

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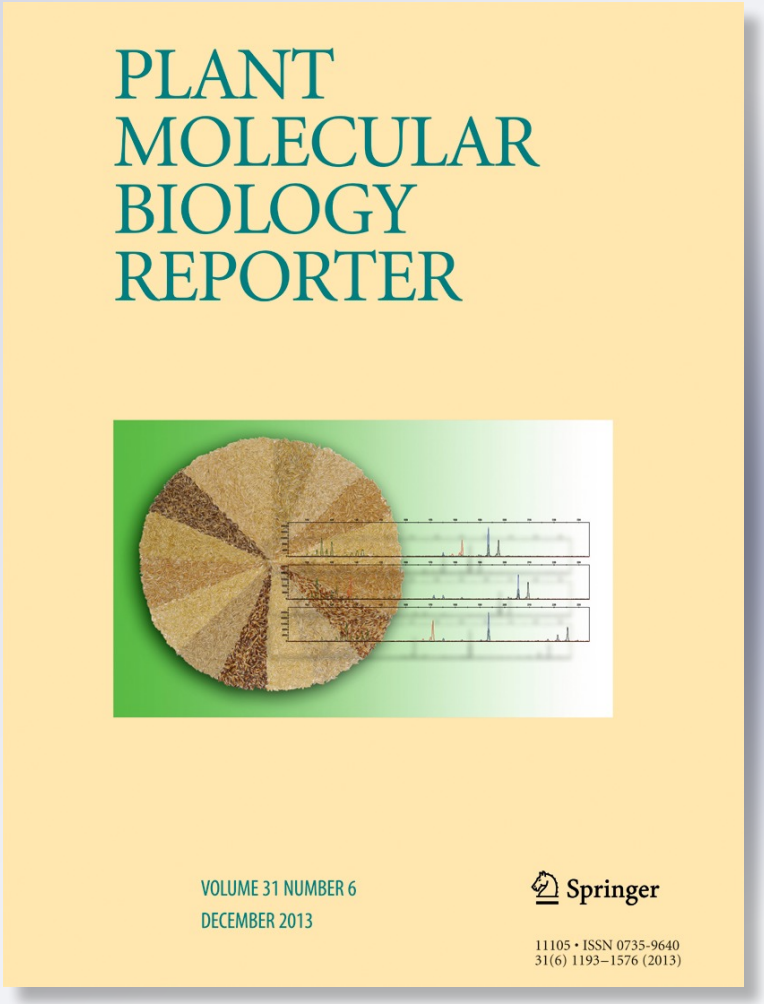

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ORIGINAL PAPER

Genetic Diversity and Population Structure Among Oat Cultivars and Landraces

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Abstract In this study, genetic diversity among 177 oat (*Avena sativa* L.) accessions including both white and red oat landraces and 36 commercial cultivars was studied for simple sequence repeat (SSR) loci. Thirty-one genomic and expressed sequence tags (EST)-derived primer pairs were selected according to high polymorphism from an initial 66 SSR batch. Markers revealed a high level of polymorphism, detecting a total of 454 alleles. The average gene diversity for the whole sample was 0.29. Genetic similarity, calculated using the Dice coefficient, was used for cluster analysis, and principal component analysis was also applied. In addition, population structure using a Bayesian clustering approach identified discrete subpopulation based on allele frequency and showed similar clustering of oat genotypes in four groups. Accessions could be classified into four main clusters that clearly separated the commercial cultivars, the red oat landraces and two clusters of white oat landraces. Cultivars showed less diversity than the landraces indicating a reduction of genetic diversity during breeding, whereas white oat landraces showed higher diversity than red ones. The average polymorphic information content of 0.80 for the SSR loci indicated the usefulness of many of the SSR for genotype identification. In particular, two markers, MAMA5 and AM04, with a total of 50 alleles and a high discrimination power (>0.90), were sufficient to discriminate among all commercial cultivars studied highlighting their potential use for variety identification.

Keywords A. *sativa* · Genetic diversity · Oat · Simple sequence repeat (SSR)

Introduction

Oat is a cereal crop of Mediterranean origin (Stevens et al. 2004). *Avena sativa* L. is the main cultivated oat including the white and red oats. White oats are preferred for milling and are used for human food and for fodder, especially for poultry and horses. Red oats (formerly known as *A. byzantina* K. Koch) are preferred for hay (Stevens et al. 2004).

During the twentieth century, landraces have increasingly been replaced by modern cultivars, resulting in significant reduction in genetic diversity (Warburton et al. 2008; Reif et al. 2005; Roussel et al. 2004) and contributing to the stability in genetic diversity of wheat, barley, and maize cultivars in recent years (Christiansen et al. 2002; Donini et al. 2000; Koebner et al. 2003). Thus, the loss of genetic diversity has become an important problem both in natural plant populations and in important crop species. This loss led to calls for the genetic conservation of crop germplasm (Frankel and Bennett 1970). Current molecular characterization of ex situ plant germplasm has placed more emphasis on cultivated gene pools and less on exotic gene pools representing wild relative species (Karp 2002). Although these modern cultivars may be higher yielding under high-input systems, landraces have considerable potential for use in improving disease and abiotic stress tolerance. Transfer of beneficial traits from landraces is relatively straightforward in that there is no barrier to crossing, as there can be with the use of crop wild relatives. Thus, several studies suggest that landraces may be a good source of new allelic diversity for breeding programs. However, better characterization of

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exotic germplasm is needed to facilitate its use in plant breeding and in research (Hawkes 1990; Jellen and Leggett 2006), so as to the introgression of exotic germplasm into a plant breeding program.

Genetic diversity studies, assessed by various tools including DNA markers, provide important information both for genetic conservation and for use in efficiently breeding new commercial varieties. To date, genetic studies in hexaploid oat has been more difficult than in other species, mainly due to large genome size (Bennett and Smith 1976) and polyploidy causing inherent complexities for mapping including large numbers of linkage groups, detection of multiple loci by a single probe, and comigration of fragments from different loci that can impede interpretation of allelic relationships and genetic analyses (Iannucci et al. 2011). Amplified fragment length polymorphisms (AFLPs) (Achleitner et al. 2008; Fu et al. 2005; Fu and Williams 2008), random amplified polymorphic DNA (RAPDs) (Baohong et al. 2003; Paczos-Grzeda 2004), and microsatellites have been previously used in oat for assessment of genetic diversity. In particular, microsatellites have been used to dissect genetic diversity in several *Avena* spp. (Li et al. 2000, 2007) and to examine allelic diversity changes over 100 years of oat breeding in both Nordic countries (Nersting et al. 2006), Canada (Fu et al. 2007), and North Europe (He and Bjornstad 2012). Association of genetic markers with regions of the genome controlling different traits would enable efficient and precise transfer of useful alleles from landraces to modern cultivars while minimizing linkage drag of nonbeneficial alleles.

To date, identification of oat cultivars has relied on morphological and phenological characteristics that may be influenced by environmental factors and require trained staff and large-scale growth experiments of mature plants under uniform conditions for evaluation. In addition, some cultivars are morphologically similar, making difficult to distinguish between them visually. SSR profiles can be used as a DNA fingerprint for registered cultivars to avoid redundancy of identical cultivars as well as to protect breeders' rights.

Here, we studied the genetic diversity of 141 white and red oat landraces together with 36 currently grown oat cultivars for potential use in breeding programs. Furthermore, we tested the potential of SSRs for molecular identification of the oat cultivars studied.

Materials and methods

Plant material

A germplasm collection of landraces consisting of 141 *A. sativa* accessions (110 white and 31 red oats) originally collected from 1944 to 1997 in southern Spain, when they were used locally in agriculture (Online resource 1), was provided by the "Plant Genetic Resources Center" (CRF-INIA, Madrid, Spain). In addition, 36 commercial cultivars were supplied by the Andalusian Network of Agriculture Experimentation (RAEA) selected for their adaptation to southern Spain agroecological conditions. For simplicity, germplasm bank codes were substituted for the codes included in Supplementary Table 1 (Sanchez-Martin et al. 2011a). White oat cultivars studied were Ac1, Acebeda, Adamo, Aintree, Alcudia, Anchuela, Araceli, Brawi, Caleche, Canelle, Chambord, Chapline, Charming, Cobeña, Condor, Cory, Edelprinz, Flega, Fringante, Fuwi, Hammel, Kankan, Kantora, Karmela, Cassandra, Kazmina, Mirabel, Mojacar, Norlys, Orblanche, Pallini, Patones, Prevision, Primula, and Rapidena. In addition, the *A. strigosa* cultivar Saia was included for comparison.

Seedlings were grown in 0.5-L pots filled with peat:sand (3:1) in a growth chamber at 20 °C, 65 % relative humidity, and under 12 h dark/12 h light with 150 μmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes. Sites and year of landraces sampling together with other characteristics of the site are recorded in Online resource 1, and year of registration, origin, and genealogy, if known of cultivars, are recorded in Online resource 2.

DNA extraction, SSR markers, and PCR procedure

Ten leaves from 12-day-old seedlings were harvested and DNA was extracted according to the CTAB protocol (Murray and Thompson 1980). Sixty-six SSR primer pairs derived from genomic and EST libraries of oats and barley were selected from previous reports to test for polymorphism (Becher 2007; Jannink and Gardner 2005; Li et al. 2000; Liu et al. 1996; Pal et al. 2002; Wight et al. 2010). In addition, three SSRs were developed from EST sequence information from *Avena barbata* and *Festuca* by using the following primers: Barb2-40 (5′-CCATCTCAACCTTTGCTTCTCTCCT-3′ and 5′-GTTCTTGAGCTCCTTGACCTTGAGC-3′), Barb4-10 (5′-GCTGAGCAATCTCA TCAGCTCAACT-3′ and 5′-GAGGTGATCCGAGCTTACTTCATCA-3′), and Fesc12 (5′-GTCGCCGGAGAAGAGAAGAG-3′ and 5′-AACGCTAGCCGTGATGACTT-3′). Following preliminary assays in a subset of 46 samples, a final set of 31 primer pairs (Table 1) were chosen because of their consistency in amplification and polymorphism in our oat genotypes and/or because they had been mapped in a mapping population developed from two winter oat cultivars Buffalo and Tardis (data not shown) and displayed reasonable genome coverage.

Amplification reactions were set up for 40 cycles with an initial denaturing step of 10 min at 95 °C. Each cycle consisted of denaturation at 94 °C for 1 min, followed by primer-specific annealing for 1 min (temperature specified in Table 1), and extension at 72 °C for 1 min. After 35 cycles, there was a final extension step of 10 min at 72 °C. The 10 μL reaction mix comprised of 6.05 μL sterile-distilled

water, 0.05 μL Taq polymerase (Roche Applied Sciences, Mannheim, Germany), 1 μL of 10× PCR buffer with $MgCl_2$, 1.3 μL of dNTP (5 mM equimolar solution of each dATP, dCTP, dGTP, and dTTP), 0.3 μL each of forward and reverse primers (10 μM solution), and 1 μL of template DNA (30 ng μL^{-1}). Reactions were stopped with 95 % formamide loading dye. Amplification products from markers AME097, AME105, AME168, AME176, AME192, BarbSSR_2-40, and BarbSSR_4-10 were separated on 4.5 % polyacrylamide denaturing gel (Bio-Rad, CA, USA, Sequi-Gen GT, 38× 50 cm) using 73-well comb and visualized by silver staining (Promega Silver Sequencing system, WI, USA). Relative movement of different amplicons and standard molecular-weight marker were used to estimate the sizes of amplified fragments using regression. The remaining markers were run on the ABI 3137 capillary sequencer. PCR, using AmpliTag gold, was conducted as for polyacrylamide gel analysis, except that one primer was labeled with a fluorescent dye and the concentration of DNA was 20 ng μL^{-1}. The size standard GeneScan 500 LIZ™ (orange) was included with each sample and used to determine the sizes of the PCR products detected. All primers and the size standards were supplied by Applied Biosystems (ABI). Data were analyzed using GeneMapper (ABI). Presence or absence of each amplified band was scored as 1 and 0, respectively, for all markers to generate a binary data matrix. The genetic diversity of each microsatellite locus was assessed by calculating the frequency of the microsatellite alleles based on polymorphic information content (PIC) following (Botstein et al. 1980) using the equation:

$$\mathrm{PIC} = 1 - \mathrm{J} = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of the jth allele for the ith marker. Estimates of genetic similarity (GS) were calculated for all possible pairs of genotypes according to Dice similarity coefficient (Nei and Li 1979). In addition, frequencies of incidence of all polymorphic alleles for each SSR marker were calculated and used for determination of statistical parameters. Confusion probability (C_j) and discriminating power (D_j) of each marker were estimated according to Tessier et al. (1999). Cluster analysis based on unweighted pair-group method with arithmetic average (UPGMA) was performed on a matrix of GS estimates using GenStat 7th Edition and a dendrogram was constructed. The correlation coefficient between the similarity matrix and the cophenetic values matrix was computed to test the goodness of fit of the cluster analysis. NTSYS-pc 2.02j software (Biostatistics Inc., USA, Rohlf 1998) was used for these statistical analyses.

Population structure and percentages of admixture

Population structure was inferred by the software STRUCTURE 2.3.3. We set most parameters to their default values as advised in the user's manual (Pritchard and Wen 2003). Specifically, we chose the admixture model and the option of correlated allele frequencies between populations, as this configuration is considered best in cases of subtle population structure (Falush et al. 2003). Similarly, we let the degree of admixture alpha be inferred from the data. Each simulation included 10,000 burn-in and 100,000 iterations. Longer burn-in or MCMC did not change significantly the results. Ten independent simulations per k value were run and the mean estimate across runs of the log posterior probability of the data for a given k, $\Pr(X \mid k)$, called $L(k)$, was plotted for each k category on a graph to determine the k value of the population as the value of k for which the distribution of $L(k)$ plateaus or continues to increase, but much more slowly. Because this point is known to be difficult to determine, we also used Δk, an ad hoc quantity proposed by Evanno et al. (2005) related to the second-order rates of change of the likelihood function with respect to k that is supposed to show a clear Δk at a true value of k.

Results

The 31 primer pairs used to characterize and evaluate the genetic diversity in the landraces and commercial varieties of the oat collection showed a high level of polymorphism, displaying a total of 454 alleles. The total number of alleles per marker ranged from three for AME168 to 42 for MAMA05 with a mean of 14.65 (Table 1). PIC varied from 0.46 (AME168 and AM112) to 0.96 (AM04) with a mean of 0.80. Based on PIC values obtained, most SSRs, with the exception of AM112, AME168, AME176, BarbSSR_2-40, and BarbSSR_4-10, were considered informative markers (PIC>0.7), indicating the potential use of this set of SSR markers for cultivar identification (Table 1). Allelic frequencies observed ranged from 0.001 to 0.69 with a mean of 0.062. One hundred thirty-eight alleles out of 454 detected were classified as "rare" due to their low frequency (<0.03), 194 were classified as "common," with frequencies between 0.03 and 0.2, and 122 were classified as "more frequent" with frequencies >0.2 (Table 1). Rare and common alleles were detected at 26 and 27 SSR loci studied, respectively. Rare alleles per locus ranged from 0 to 16 (MAMA05), whereas the number of common alleles per locus ranged from 0 to 28 (AM04) and the more frequent from 0 to 9 (AM30) (Table 1). High values of discriminating power ($D_j \geq 0.81$) and PIC≥0.81, and low values of confusion probability ($C_j \leq 0.19$) were obtained for 20 of the markers evaluated (64.5 %) (Table 1).

From the dendrogram generated, the 177 accessions could be classified into four main clusters that clearly separated the commercial cultivars, the red oat landraces and two clusters of white oat landraces (Fig. 1). The *A.*

Table 1 Characteristics of 31 microsatellite markers selected for use in the study. Size range, number of alleles (rare, common, and most frequent), confusion probability (C_j), discriminating power (D_j), and polymorphic information content (PIC)

Marker name	SSR type	T_m	Detection	Size (pb)	No. of alleles	Rare alleles (<0.03)	Common alleles (0.03–0.2)	Frequent alleles (>0.2)	C_j	D_j	PIC
AM01	Genomic	55	ABI	154–240	27	10	14	3	0.11	0.89	0.89
AM03	Genomic	58	ABI	249–298	22	7	15	0	0.06	0.94	0.93
AM04	Genomic		ABI	78–180	41	7	28	6	0.04	0.96	0.96
AM07	Genomic	55	ABI	146–195	25	8	13	4	0.08	0.92	0.92
AM102	Genomic	55	ABI	160–217	10	2	3	5	0.18	0.82	0.82
AM112	Genomic		ABI	227–255	7	3	3	1	0.54	0.46	0.46
AM14	Genomic	55	ABI	98–134	17	4	5	8	0.10	0.90	0.90
AM30	Genomic	55	ABI	178–230	17	3	5	9	0.11	0.89	0.89
AM42	Genomic	58	ABI	165–208	13	4	1	8	0.14	0.86	0.86
AM87	Genomic	55	ABI	92–171	15	6	3	6	0.17	0.83	0.83
AM89	Genomic	53	ABI	173–201	10	3	2	5	0.22	0.78	0.77
AME097	EST	52	Silver	145–155	4	0	0	4	0.26	0.74	0.74
AME105	EST	52	Silver	140–190	10	0	5	5	0.13	0.87	0.87
AME168	EST	52	Silver	200–220	3	0	1	2	0.54	0.46	0.46
AME176	EST	52	Silver	90–110	4	0	1	3	0.32	0.68	0.68
AME192	EST	52	Silver	300–345	6	1	0	5	0.22	0.78	0.78
BarbSSR_2-40	EST	60	Silver	195–220	4	0	0	4	0.37	0.63	0.63
BarbSSR_4-10	EST	60	Silver	270–310	5	1	1	3	0.36	0.64	0.63
CDO187	EST	55	ABI	104–152	9	3	0	6	0.18	0.82	0.82
Fesc12	EST	61	ABI	124–194	21	7	12	2	0.10	0.90	0.90
HVM20	Genomic	53	ABI	103–154	21	9	11	1	0.10	0.90	0.90
HvXan	EST	50	ABI	93–206	26	15	6	5	0.15	0.85	0.85
MAMA01	Genomic	55	ABI	183–215	12	3	7	2	0.18	0.82	0.81
MAMA03	Genomic	55	ABI	351–403	12	2	8	2	0.14	0.86	0.85
MAMA05	Genomic	55	ABI	62–274	42	16	20	6	0.07	0.93	0.92
MAMA07	Genomic	55	ABI	322–371	12	5	5	2	0.29	0.71	0.70
MAMA08	Genomic	55	ABI	548–623	18	5	12	1	0.10	0.90	0.90
MAMA09	Genomic	55	ABI	401–491	14	6	4	4	0.16	0.84	0.84
MAMA11	Genomic	55	ABI	124–183	9	4	3	2	0.24	0.76	0.75
MAMA12	Genomic	55	ABI	297–321	8	2	3	3	0.22	0.78	0.78
OL0410	EST	55	ABI	256–281	10	2	3	5	0.19	0.81	0.81
Mean					14.65	4.45	6.26	3.94	0.20	0.80	0.80
Total					454	138	194	122			

ABI Applied Biosystem ABI3137

strigosa genotype, Saia, did not cluster with the *A. sativa* entries. Genetic similarity estimates calculated among the oat collection varied from 0.16 to a maximum of 0.99 (between Gen130 and Gen131) with a mean similarity of 0.29 (Fig. 1). Cluster 1 included most of the commercial cultivars together with the landraces Gen141 and Gen17. Cultivars with the highest genetic similarity (0.88) were Chapline and Cobeña. Cluster 2 comprised the red oat landraces along with the commercial red oat cultivars Cassandra and Prevision. In addition, four landraces, Gen139, Gen64, Gen27, and Gen106, which are all described as white oats, were included in this cluster. The red oat landrace Gen84, however, grouped in cluster 3. The third and fourth clusters included most of the white oat landraces. The third cluster contained 51 genotypes, with Gen5 and Gen 13 being the most related landraces with a GS of 0.84. The fourth cluster was the largest with 53 genotypes. Genetic similarity within each cluster was similar with values of 0.50, 0.55, 0.50, and 0.54 for clusters 1, 2, 3, and 4, respectively, but when comparing the white oats, commercial cultivars had a slightly

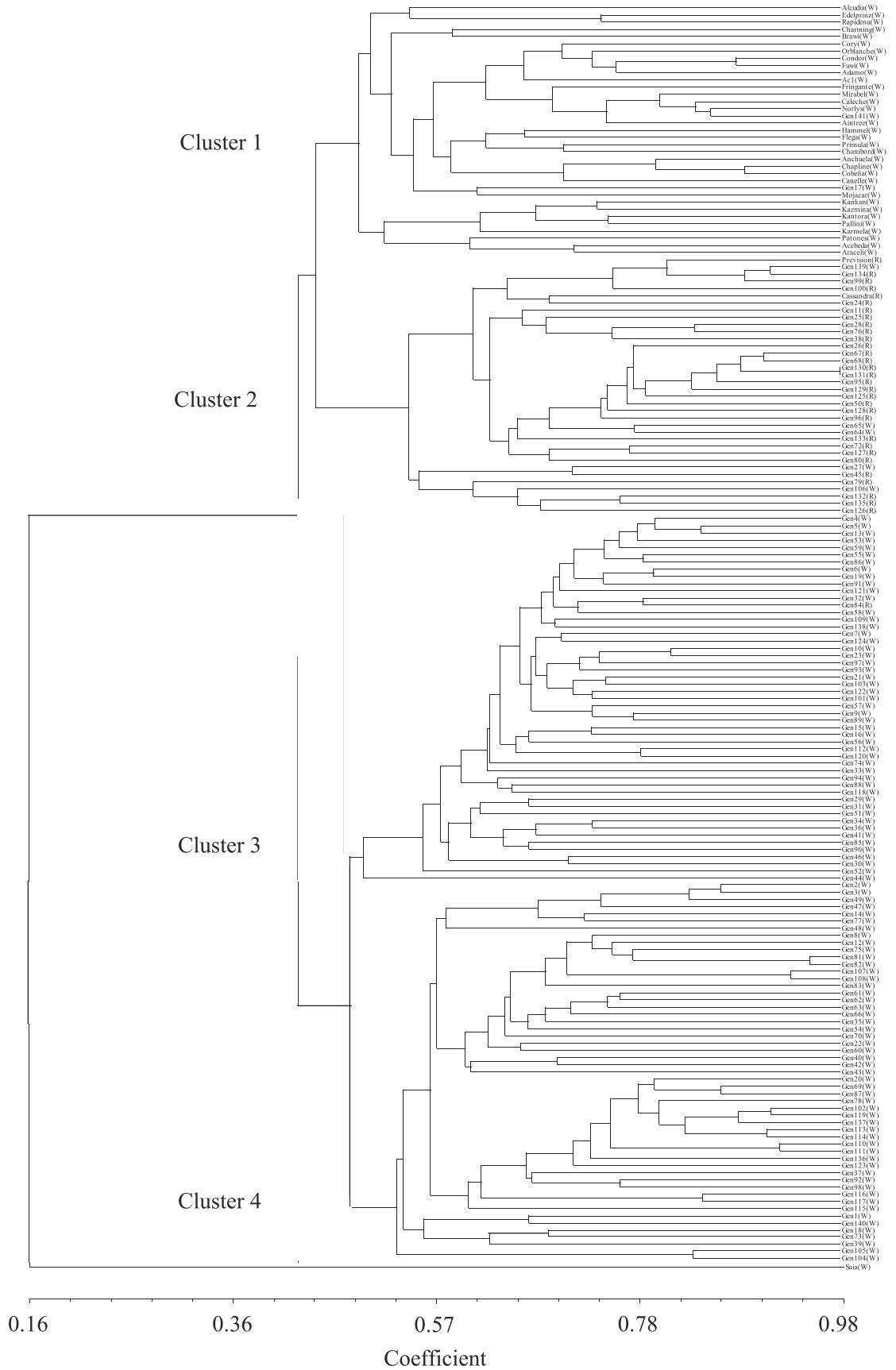

Fig. 1 UPGMA dendogram of 176 oat accessions based on Dice distance for 31 SSR markers

higher GS compared with landraces. Most clusters showed particular alleles characteristic for that group. For example, most genotypes of cluster 1 had the 209-bp allele from AM01 marker, all genotypes of cluster 2 had the 137- and 368-bp alleles from AME097 and MAMA3 markers, all genotypes from clusters 3 and 4, except one, had the 232-pb allele amplified with AM112 marker, but only genotypes from cluster 3 had the 161-pb allele amplified with AM87. Overall, comparison of the white oats revealed 22 unique alleles belonging to commercial cultivars and 129 to landraces. In addition, 11 alleles were assigned exclusively to the red oat landraces. Analysis of the allelic frequency obtained for each marker showed that often clusters 2, 3, or 4 associated with landraces had a significantly higher allelic frequency than cluster 1 (Table 2). In particular, markers AME105 and MAMA 9 showed significantly higher allelic frequencies in clusters 2, 3, and 4 compared with cluster 1. Only for marker MAMA11 was the allelic frequency of cluster 1 higher than that of clusters 2, 3, and 4 (Table 2). Comparison between the cophenetic matrix and the similarity matrix was significant with r^2=0.876 (p<0.01), indicating a high goodness of fit between both matrices.

Interestingly, when assessing independently the oat cultivars, a subset of 11 markers (HvXan, AM01, AM30, AM14, AM87, AM07, MAMA08, AM42, MAMA5, AM04, and FESC12) showed a high polymorphism with 167 alleles and high PIC (>0.80). This subset of SSRs was able to group the white oat cultivars from cluster 1 showing a similar genetic relationship among them as obtained when assessed with the complete SSR set. Furthermore, among these SSRs, two of them MAMA5, with 23 alleles and a discriminating power of 0.90, and AM04, with 27 alleles and a discrimination power of 0.94, were sufficient to discriminate between all cultivars.

Table 2 Comparison of allelic frequency between subpopulations

	Alellic frequency				Significance
Primer	Cluster 1	Cluster 2	Cluster 3	Cluster 4	
AM01	0.12^{a}	0.11^{a}	0.11^{a}	0.12^{a}	ns
AM03	0.05^{ac}	0.06^{ad}	0.07^{bd}	0.05^{c}	***
AM04	0.12^{a}	0.13^{a}	0.13^{a}	0.10^{b}	***
AM07	0.09^{ab}	0.10^{a}	0.08^{b}	0.10^{a}	ns
AM102	0.21^{a}	0.31^{b}	0.23^{a}	0.16^{c}	***
AM112	0.18^{a}	0.18^{a}	0.15^{bc}	0.15^{c}	***
AM14	0.21^{a}	0.20^{a}	0.25^{b}	0.22^{ab}	*
AM30	0.33^{a}	0.43^{b}	0.38^{c}	0.33^{a}	***
AM42	0.32^{a}	0.31^{a}	0.36^{b}	0.31^{a}	**
AM87	0.14^{a}	0.16^{a}	0.18^{b}	0.21^{c}	***
AM89	0.33^{a}	0.34^{a}	0.33^{a}	0.34^{a}	ns
AME097	0.36^{ab}	0.43^{a}	0.34^{b}	0.33^{b}	***
AME105	0.14^{a}	0.26^{b}	0.24^{b}	0.24^{b}	***
AME168	0.39^{a}	0.44^{a}	0.41^{a}	0.53^{b}	***
AME176	0.48^{a}	0.48^{a}	0.63^{b}	0.59^{b}	***
AME192	0.36^{a}	0.43^{b}	0.43^{b}	0.38^{a}	**
BarbSSR_2-40	0.32^{a}	0.49^{b}	0.45^{ab}	0.52^{b}	*
BarbSSR_4-10	0.40^{a}	0.45^{b}	0.43^{ab}	0.41^{a}	***
CDO187	0.38^{a}	0.43^{bc}	0.46^{b}	0.40^{ac}	**
FESC12	0.09^{a}	0.09^{a}	0.08^{a}	0.09^{a}	ns
HVM20	0.06^{a}	0.07^{a}	0.08^{a}	0.08^{a}	ns
HvXan	0.16^{a}	0.15^{a}	0.17^{ac}	0.19^{bc}	*
MAMA01	0.10^{a}	0.09^{ac}	0.08^{bc}	0.10^{a}	*
MAMA03	0.10^{a}	0.11^{a}	0.10^{a}	0.16^{b}	***
MAMA05	0.09^{a}	0.10^{ab}	0.11^{b}	0.08^{c}	***
MAMA07	0.11^{ab}	0.12^{a}	0.11^{ab}	0.10^{b}	ns
MAMA08	0.07^{ac}	0.08^{ab}	0.06^{c}	0.06^{c}	ns
MAMA09	0.09^{a}	0.13^{b}	0.14^{b}	0.12^{b}	***
MAMA11	0.14^{a}	0.11^{b}	0.11^{b}	0.12^{b}	***
MAMA12	0.14^{a}	0.17^{a}	0.16^{a}	0.22^{b}	***
OL0410	0.32^{a}	0.35^{a}	0.40^{b}	0.34^{a}	***

Different letters in a row indicate significant differences at $p \leq 0.05$, $p<0.01$, and $p \leq 0.001$ according to *, **, and *** respectively for that marker; *ns* not significant differences

Structure of the population

The criteria used to define the number of subpopulations in the oat collection, which are the position of a break point in the $L(k)$ curve and a peak in the Δk distribution, supported values of k=2 and k=4 (data not shown). For both k values, most accessions were assigned by STRUCTURE to a subpopulation. With k=2, it was possible to distinguish between the white oat landraces and the rest of the collection. With k=4, a finer subgrouping corresponding to the commercial varieties, the red oat landraces, and two groups of white oat landraces was obtained. Figure 2 shows the four subpopulations detected by STRUCTURE with the percentage of admixture of each genotype in the corresponding subpopulation. The results of the assignments showed a very good congruence between the two methods. Indeed, only three out of the 177 genotypes assessed, Saia, Gen51, and Gen61, were assigned to different clusters by UPGMA and STRUCTURE approaches, and in the three cases, STRUCTURE coefficients indicated a membership lower than 80 % in the corresponding population. According to STRUCTURE, subpopulation 1 showed the lowest degree of admixture with only 16.2 % of the genotypes with less than 80 % of membership to this subpopulation followed by subpopulation 2 with 28.6 % of genotypes with less than 80 % of membership in this group. Subpopulations 3 and 4, with 31.4 and 34.6 % of genotypes with less than 80 % membership to

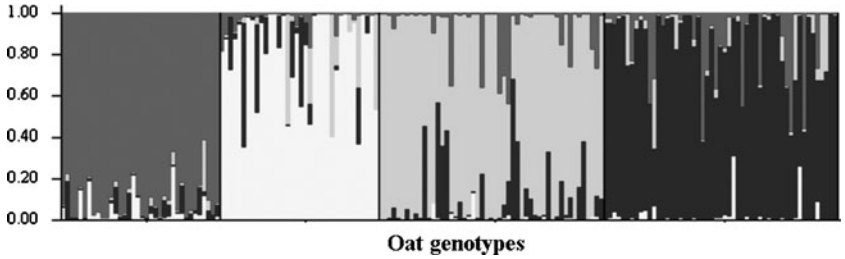

Fig. 2 Estimated population structure of oat genotypes according to STRUCTURE software. Each individual is represented by a *thin vertical segment*, which can be partitioned into 4 *gray-scale colored segments* that represent the individual estimated membership to the 4 clusters

the corresponding subpopulation, were the subpopulation with higher admixture. In addition, the germplasm collection was also subjected to multivariate analysis based on principal component analysis (PCA) and discriminant function analysis (DFA) that revealed clear separation of four subpopulations that indicate high consistency of the data (Fig. 3).

Discussion

Genetic diversity analysis of the oat collection including landraces and cultivars revealed high variability among accessions. However, this variability was more evident within the white oat landrace collection with 129 exclusive alleles, followed by the red oat landraces, and the cultivars, with 11 and 22 exclusive alleles, respectively. This indicates the considerable genetic variation that exists in landraces that is not present in the cultivars and offers opportunities for breeding new cultivars by exploiting the genetic diversity existing in the landraces. Our findings on clear distinctions between white and red oat landraces are in agreement with previous reports (Fu et al. 2005; Newell et al. 2011; Odonoughue et al. 1994) and support the hypothesis that white and red oats were domesticated independently of each other (Zohary and Hopf 2000). White oat landraces were more distinct from the white oat cultivars; however, red oat landraces grouped together with the red oat cultivars analyzed (Fig. 1), suggesting either lower improvement of the red oat cultivars compared with the white or the involvement of the nearest genetic similar red landraces in their genealogy.

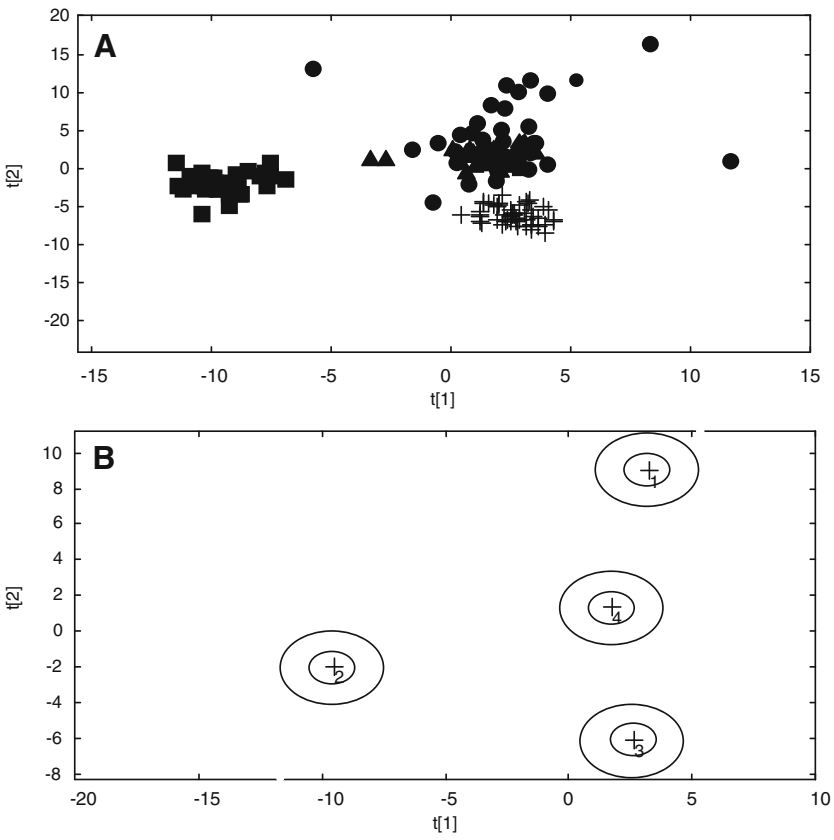

Fig. 3 Scatterplot of discriminant function analysis scores of components 1 and 2 based on 31 SSR markers used in this study. **a** Represented are the genotypes belonging to cluster 1 (*circles*), cluster 2 (*squares*), cluster 3 (*crosses*), and cluster 4 (*triangles*). **b** Magnification of the central part of graph a with the groups assigned according to the DFA scores

In general, most of the accessions closely related by pedigree and/or derived from germplasm having specific traits clustered together. Thus, those cultivars with the highest genetic similarity from cluster 1 shared common ancestors. For instance, Mirabel, Aintree, Caleche, and Norlys shared Fringante as one of their parent. In addition, Norlys and Caleche shared other common ancestors. Other closely related cultivars such as AC1 and Orblanche had Mostyn as a common ancestor. Furthermore, Orblanche together with Condor, Fuwi, and Adamo comprise a subcluster with a genetic similarity of 0.7. This result may be explained by their common genealogy, since Orblanche and Adamo had Condor as an ancestor, and Adamo and Fuwi shared the cultivar Manod in their genealogy. There is less information about ancestry for the landrace collection, but geographic location of the collection sites is known (Online resource 1). Cluster 2 grouped most red oat accessions but also four landraces described as white oats, Gen139, Gen64, Gen27, and Gen106, which also shared the 235-pb allele amplified by AM112 exclusive to this group. A detailed analysis of the morphological characters that differentiate white and red oats (Magness et al. 1971) showed that indeed Gen139, Gen64, and Gen27, in addition to the color of the seeds, had the typical white oats' morphological characters such as no basal scar in the spikelets from the separation from pedicel, twisted awns, and small glumes. However, Gen106 was morphologically nearer to red oat showing weak and nontwisted awns. The geographic proximity of these white oat accessions with their most related red oat landraces suggests a possible cross between the landraces so that the white landraces would have acquired some of the exclusive alleles of cluster 2. Indeed, Gen64 (white oat) and Gen65 (red oat) were sampled from the same locality, while Gen106 (white oat) and Gen132 (red oat) were sampled at sites with only 8 km between them. Interestingly, the white oat landraces grouped in two clusters, 3 and 4. A detailed analysis of alleles showed high differences between these two clusters. For instance, more than 95 % of genotypes of cluster 3 had the alleles BarbSSR4_10-4, MAMA3-2, AM87-15, and AM04-11, whereas less than 15 % of the genotypes of cluster 4 had them. On the contrary, more than 95 % of genotypes from cluster 4 had the alleles BarbSSR_4_10-2, AME192-3, and AME192-5, whereas less than 30 % of genotypes of cluster 3 had them. In order to determine the possible causes of these differences, we plotted the geographic distribution of the landraces. No differences in latitude or longitude could be inferred between the two clusters but landraces of cluster 3 were distributed in locations with significantly higher altitude than those of cluster 4 (average of 537 m altitude for cluster 3 and 377 m for cluster 4; $P=0.01$). This suggests a different evolution for the two clusters, with cluster 3 better adapted to higher altitudes and their associated cooler temperatures and probably poorer soils than cluster 4.

The narrow separation observed in this study among the *A. sativa* cultivars bred in European countries suggests that a rather small proportion of the available genetic variation from this species is currently used for oat improvement, as seen by Achleitner et al. (2008) in an oat collection of worldwide origin. A similar lack of diversity was also detected within sets of Canadian and Chinese oat varieties (Baohong et al. 2003; Fu et al. 2004) leading Fu et al. (2004) to identify an urgent need to broaden the genetic variation for sustainable oat improvement in Canada. The reduction of genetic diversity may have consequences both for the vulnerability of crops to new pests and pathogens and for their ability to respond to changes in climate and agricultural practices (Fu et al. 2003). Most of the landraces studied in this work have been characterized for disease and abiotic stress resistance (Sanchez-Martin et al. 2011a, b) revealing accessions with interesting resistance that would be valuable to include in European cultivars. Study of mechanisms underlying resistance in selected landraces and varieties has been also performed (Sanchez-Martin et al. 2011a, b), and the oat collection was also tested under a variety of Mediterranean environments for agronomic adaptation (unpublished results). The increased use of these accessions in European and/or Mediterranean breeding programs could simultaneously increase diversity and improve levels of valuable traits. From crosses of genetically divergent parents (i.e., a high yielding cultivar crossed with a landrace showing disease and drought resistance), novel varieties with improved traits might be selected.

Methods such as UPGMA presented here, which do not assume predefined structure, are only loosely connected to statistical procedures allowing the identification of homogeneous clusters of individuals. For this reason, the oat germplasm collection was also analyzed using an alternative model-based method implemented in the software STRUCTURE (Pritchard et al. 2000), which uses a Bayesian approach to simultaneously determine k (the number of subpopulations in a collection), and estimate for each accession the proportion of its genome that originates from each subpopulation, also called percentage of admixture. The model accounts for the presence of Hardy–Weinberg assumption or linkage disequilibrium by introducing population structure and attempts to find population groupings that (as far as possible) are not in disequilibrium (Pritchard et al. 2000).

Structure was detected in this germplasm collection using both classical multivariate and Bayesian analyses. The patterns obtained with the two methods were very similar. Population structure is the primary obstacle to successful association studies in any organism (Buckler and Thornsberry 2002). Model-based clustering suggests that a large amount of the allelic diversity can be described by subdividing the accessions into four discrete populations, where each subpopulation has a unique set of allele frequencies. This method is clearly a simplification of the observed data; however, it can be used to compare with other methods of clustering and to test models of association analysis that would account for genetic associations arising from structure presence. The congruence of patterns obtained with Bayesian and multivariate analyses suggests that

the estimates of these admixture proportions are reasonably reliable.

In other cereal crops such as corn (Gunjaca et al. 2008) and rice (Bonow et al. 2009), molecular profiles associated with the description of a cultivar have been used to enforce the rights granted to breeders. The two selected markers, MAMA5 and AM04, show great potential for identifying cultivars, since they were able to discriminate between the 36 cultivars tested, some of them with relatively high genetic similarity and sharing common genealogy. Thus, our findings suggest that microsatellite markers can play an important role as a source of additional information in oat to supplement the morphological descriptors recommended by the International Union for the Protection of New Varieties of Plants (UPOV).

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Chapter 5:

Genome-wide association study for crown rust and powdery mildew resistance in an oat collection of commercial varieties and landraces .

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ABSTRACT

Biotic stresses such as rust and powdery mildew constitute major constraints for oat crops. Efficient utilization of genetic resources in breeding programs might be greatly facilitated by proper information generated by genetic studies. However, linkage mapping studies are restricted to specific genetic backgrounds limiting their usefulness. To overcome this limitation, association analysis which exploits the variation in a collection of genetically diverse materials has emerged as a promising tool to identify QTL in plants. The purpose of this work was to identify elite alleles for rust and powdery mildew resistance in oat by association mapping. To this aim, 174 oat accessions including white and red oat cultivars and landraces were evaluated for disease resistance and further genotyped with 31 simple sequence repeat (SSR) and 15,000 Diversity Arrays Technology (DArT) markers to reveal association with disease resistance traits. After removing markers with more than 20% missing data, a minimum allele frequency (MAF) lower than 1%, and combining markers in near perfect linkage disequilibrium, 1712 polymorphic markers were considered for association analysis. Principal component analysis and a Bayesian clustering approach were applied to infer population structure. Five different general and mixed linear models accounting for population structure and/or kinship corrections and two different statistical tests were carried out to reduce false positive. Five markers, two of them highly significant in all models tested were associated with rust resistance. Interestingly, these DArT sequences shared homology with an autophagy-related protein 2 and an anthocyanin 5-aromatic acyltransferase. We did not identify strong association between any marker and powdery mildew resistance at seedling stage. However, one DArT sequence, oPt-5014, was strongly associated with powdery mildew rust resistance in adult plants. Overall, the markers showing the strongest association in this study provide ideal candidates for further studies and future inclusion in strategies of marker assisted selection.

Keywords: association analysis, drought, oat, powdery mildew, resistance, rust

INTRODUCTION

Oat is a crop of Mediterranean origin used as feed grain, green or conserved fodder and, more recently, as a winter cover crop in no-till rotations (Stevens et al., 2004). *Avena sativa* L. including the white and red (former *A. byzantina*) oat is the main cultivated oats. Several pathogenic fungi can infect oats and drastically reduce its yield. Among them, the biotrophic pathogens such as the powdery mildew *Blumeria graminis* f.sp. *avenae* and the crown rust *Puccinia coronata* are important oat production constraint. These fungi have very efficient spreading mechanisms, hampering its control by crop management measures such as rotation and the use of resistant varieties is one of the best control alternatives (Stevens et al., 2004).

Genetic markers have been proved useful for selection of desirable traits in several species including oats. Thus, they have been used with a number of experimental bi-parental oat populations to identify quantitative trait loci (QTL) associated with important agronomic traits including vernalization response, flowering time and heading date, quality traits including seed's tocopherol concentration and groat protein and oil content and resistance to stresses including winter field survival, crown freezing tolerance and crown rust resistance (Jackson et al., 2010). However, the parental genotypes used in these studies are often not representatives of the germplasm pool that is actively used in breeding programs and the markers linked to QTL are not always transferable to other genetic backgrounds, limiting their usefulness in marker-assisted selection (MAS) (Snowdon and Friedt, 2004).

Association analysis is a promising approach to overcome the limitations of conventional QTL mapping that has received increased attention of plant geneticists during the last few years (Breseghello and Sorrells, 2006; Gupta et al., 2005; Kraakman et al., 2004; Stracke et al., 2009) following the success in dissecting human diseases (Cardon and Bell, 2001). Unlike linkage analysis, where bi-parental mapping populations are used, association analysis relies on unrelated individuals to create population-wide marker-phenotype associations (Jannink et al., 2001). Association analysis is based on linkage disequilibrium, defined as the non-random association of alleles at two loci (Falconer, 1996). Linkage disequilibrium is affected by mutation, admixture, selection, drift and population structure and is related with breeding history and reproductive biology (Flint-Garcia et al., 2003; Newell et al., 2011). Thus, association analysis utilizes historic patterns of recombination that have occurred within a sample of individuals to detect correlations between genotypes and phenotypes within these individuals (Zondervan and Cardon, 2004). Although association analysis shows great promise

as an efficient and valuable tool for gene discovery it must account for the presence of population structure. Failure to do so can cause the detection of spurious associations between traits and unlinked markers.

In recent years, association studies have been performed in several plant species. Marker-trait associations were identified for flowering time in maize (Thornsberry et al., 2001), yield traits in rice (Agrama et al., 2007), agronomic traits in sugar beet (Stich et al., 2008) and beta-glucan concentration in oat (Newell et al., 2012). Few studies focussed on the association of markers with stress resistance traits including marker association with disease resistance in Arabidopsis and potato (Aranzana et al., 2005; Malosetti et al., 2007), or with iron deficiency in soybean (Wang et al., 2008). In oats, only a few association analysis studies have been reported (Achleitner et al., 2008) to identify molecular marker associated to yield and quality traits and none attempted to identify marker-trait association with respect to some of the most important biotic constraint of this crop, the powdery mildew and rust pathogenic fungi. In this work, we performed an association analysis in an oat collection of commercial cultivars and landraces based on SSR and Diversity Arrays Technology (DArT) markers following a detailed study of population structure and linkage disequilibrium. As a result, we found several markers associated with rust and powdery mildew resistance some of them identified as resistance related genes in data base.

MATERIALS AND METHODS

Plant material

For this study, a germplasm collection of landraces consisting of 141 *A. sativa* accessions (110 white and 31 red oats) kindly provided by the "Centro de Recursos Fitogenéticos", INIA, Madrid, Spain, and 36 commercial varieties supplied by the Andalusian Network of Agriculture Experimentation (RAEA) was used. For simplicity, germplasm bank codes were substituted for the codes included in Sánchez-Martín et al., (2011a). Oat cultivars studied were: Ac1, Acebeda, Adamo, Aintree, Alcudia, Anchuela, Araceli, Brawi, Caleche, Cannele, Chambord, Chappline, Charming, Cobeña, Condor, Cory, Edelprinz, Flega, Fringante, Fuwi, Hammel, Kankan, Kantora, Karmela, Kassandra, Kazmina, Mirabel, Mojacar, Norly, Orblanche, Pallini, Patones, Prevision, Primula, Rappidena and Saia. Seedlings were grown in 0.5 L pots filled with peat:sand (3:1) in a growth chamber with 20 ºC, 65% relative humidity and under 12 h dark/12 h light with 250 µmol m^{-2} sec^{-1} photon flux density supplied by high-output white fluorescent tubes. Sites of

landraces sampling and commercial cultivar's owners and origin, are recorded in Sánchez-Martín et al., (2011a).

Genotyping

Forty leaves from 12-day-old seedlings were harvested and DNA extracted according to the method prescribed by Diversity Arrays P/L, Camberra, Australia and described by Tinker et al., (2009). SSR primer pairs derived from genomic and EST libraries of oats and barley were assessed as previously (Montilla-Bascón et al., 2013). DArT marker analysis was performed by Diversity Arrays P/L, as described in (Tinker et al., 2009).The high density oat array with 15000 hybridized oat DArT markers was used.

Data curation

To remove possible errors and redundancies in markers that may cause false associations, data cleaning was performed. According to (Miyagawa et al., 2008) this process include four steps previously described as necessary for GWAS. Initially, the data set consisted of 177 accessions and 2086 polymorphic DArT and SSR markers. First, markers with call rates lower than 0.8 and hence, likely containing errors, were removed. Second, markers with minor allele frequency (MAF) of less than 0.01 were removed, as they do not contribute substantially to variation in the data. Third, markers that diverged less than 1% across the genotypes lines were merged, thus combining markers that were in near perfect LD. Finally, inspection were performed to determine accessions that differed by less than 1% on the markers to remove any redundant accession.The highest similitude was found between Gen130 and 131. However they differed in more than 1% of the markers so no accessions were removed following this step.

Genetic distance, population structure and kinship

Estimates of genetic distance were calculated according to Nei's parameter (Nei and Li, 1979) with Arlequin software. Population structure was inferred by the software STRUCTURE 2.3.4 (Pritchard et al., 2000). using the admixture model and the option of correlated allele frequencies between populations, as this configuration is considered best by (Falush et al., 2003) in cases of subtle population structure. Similarly we let the degree of admixture alpha be inferred from the data. Each simulation included 20 000 burn-in and 100,000 iterations. Longer burn-in or MCMC did not change significantly the results. 10 independent simulations per k value were run and the mean estimate across runs of the log posterior probability of the data for a given k, Pr(X|k), called L(k) were plotted for each k category on a graph to determine the

k value of the population as the value of k for which the distribution of L(k) plateaus or continues to increase but much more slowly. Because this point is known to be difficult to determine, we also used Δk, an *ad hoc* quantity proposed by Evanno et al., (2005) related to the second order rates of change of the likelihood function with respect to k that is supposed to show a clear Δk at true value of k. The percentages of admixture of each accession (Q matrix) given by the software were used as cofactors in the association analyses. For trait analyses per subpopulation, an accession was assigned to a subpopulation when it showed more than 80% membership in this subpopulation de Alencar Figueiredo et al., (2010). Principal component analysis (PCAs) was also performed as an alternative method to infer the structure of the collection with the software package PAST (Hammer et al., 2001)

The kinship coefficient approach proposed by (Yu et al., 2006) allows taking possible family relatedness into account and can help removing additional false positives. We computed these coefficients (K matrix) with the software TASSEL 4.1.27 (Bradbury et al., 2007) and used the two matrices (Q + K) in the variance analyses for tentative model comparisons.

Linkage disequilibrium

There are three common methods to infer LD: through the metric *D*, which is a quantitative measure of allelic association, *D´* which normalize allelic association, to some extend, with respect to allele frequencies, and r^2, obtained dividing D^2 by the product of the allele frequencies at the two loci (Gaut and Long, 2003). In this work we used r^2 since it is not as highly influenced by small sample sizes and low allele frequencies (Flint-Garcia et al., 2003) and is relevant for QTL mapping as it relates the amount of variance explained by the marker to the amount of variance generated by the associated QTL (Zhu et al., 2008). r^2 was calculated by software Tassel 4.1.27 to each marker pair together with the significance of the parameter. The disequilibrium matrix summarising pair wise measures of LD was also performed by Tassel software.

Phenotyping

Crown rust resistance assessment: The *P. coronata* f.sp. *avenae* (*Pca*) isolate Co-04, previously multiplied on the susceptible check Araceli was used (virulence described in Sánchez-Martín et al., 2012). Four plants per accessions were grown in a growth chamber at 65% RH and 20 ^{o}C under a 14 h photoperiod with 150 µmol m^{-2} sec^{-1} photon flux density. When plants had the first leaf completely expanded they were inoculated with urediospores mixed with pure talcum

(1:1, w/w) by dusting them over the plants to give approximately 30 spores mm^{-2} (checked by counts made from glass slides laid adjacent to leaves). After inoculation, plants were incubated for 9.5 hours in darkness at 100% RH and 18 ^{o}C, and thereafter at 20 ^{o}C under a 14 h photoperiod with 150 µmol m^{-2} sec^{-1} photon flux density. Infection frequency (IF) was determined as previously described (Prats et al., 2002). Infection frequency scores were converted into relative values, expressed as percentage of the reading of the susceptible check and referred to as the Relative Infection Frequency (RIF).

Powdery mildew resistance assessment: Four plants per accessions were grown in a growth chamber at 65% RH and 20 ^{o}C under a 12 h photoperiod with 150 µmol m^{-2} sec^{-1} photon flux density. When the second leaf was fully expanded (12 days), the first leaf was inoculated using a settling tower (Lyngkjær et al., 1997) to give about 30 conidia mm^{-2} with one isolate of *B. graminis* f.sp. *avenae* race 5 maintained on seedlings of oat cv. Selma, in a spore proof glasshouse. After inoculation, plants were maintained in the above mentioned growth chamber 8 days before assessment of the percentage area covered by powdery mildew on the inoculated leaf. Disease scores were converted into relative values, expressed as percentage of the reading of Selma check and referred to as the Relative Disease Severity (RDS) (Martinez et al., 2007; Rubiales et al., 1993). For assessment of adult plant resistance 5th leaves were inoculated and macroscopically assessed as above without excising the leaves from the plant.

Statistical analyses

For phenotype assessments the experimental design was arranged according to randomized complete blocks. For ease of understanding, means of raw percentage data are presented in tables and figures. However, for statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = 180/π x arcsine √(%/100)]) to normalize data and stabilize variances throughout the data range, and subjected to analysis of variance using GenStat 11th Edition, after which residual plots were inspected to confirm that data conformed to normality. Significance of differences between means was determined by contrast analysis (Scheffe's). The percentage of variation of each trait explained by the structure was computed through multiple linear regression of the phenotypes on the percentages of admixture using R (Ihaka and Gentleman, 1996) with GenStat software.

Association analysis

The tests of associations between molecular markers and phenotypes were computed using the software package TASSEL 4.1.27 (Trait Analysis by aSSociation, Evolution and Linkage) (Bradbury et al., 2007). Five models were used: a simple General Linear Model (GLM) which does not account for population structure as a potential cause of the genotype-phenotype relationship, a GLM model using the percentages of admixture of each accession (Q matrix) as cofactors to take population structure into account (GLM-Q), a GLM model using the PCAs covariates as cofactors (GLM-PCA), a GLM model using both Q matrix and PCAs covariates (GLM-Q-PCA) and a Mixed Linear Model (MLM) using both the percentages of admixture and the kinship coefficients as cofactors (Q and K matrices). All GLM procedures tested fixed-effect models in which mean phenotypes of a given trait were predicted by the independent variables. The tests were run with 1,000 permutations allowing the determination for each marker the site-wise *p* value, which is the probability of a greater F value under the null hypothesis that the polymorphic site is independent of phenotype. The Benjamini and Hochberg, (1995) false discovery rate (FDR) criteria at $q = 0.25$ was used to control for multiple testing (Newell et al., 2012) after estimation of the *q* values of each *p* values with the software QVALUE (Storey, 2002).

Sequence homology

Several of the DArT markers used here, have been previously sequenced (Tinker et al., 2009). To further characterise the identified markers, the nr protein database was searched for potential homologous sequence of significant markers using the function BlastX of the BLAST algorithm (Altschul et al., 1990) implemented in the ncbi webserver (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Data curation

From the initial 15,000 DArT markers assessed, 1,587 showed polymorphism in the oat collection. In addition 499 SRR alleles were also polymorphic. From the total 2086 polymorphic markers, 11 markers that showed a call rate lower than 80% and 56 markers that showed a minimum allele frequency (MAF) <0.01 were removed. A total of 476 redundant markers were also merged in 169 groups representing these markers. Following data curation a total of 1,712 markers were used for association purposes in the oat collection of 174 white and red commercial varieties and landraces.

Structure of the population

A previous genetic diversity study of the oat collection with only SSR markers revealed a structure of 4 subpopulations (Montilla-Bascón et al., 2013). In the present study the number of markers was increased to more than 1,500 and STRUCTURE software indicated the same number of subpopulations (Fig. 1). Indeed, the correlation between SSR and DArT+SSR results was high with a correlation coefficient of 0.84 (P<0.001). However, slight modifications of the genotype-cluster assignation and the corresponding percentage of admixture were observed. According to both analyses approximately 30% of the accessions showed less than 80% of membership for a particular cluster. The differences of genotype-cluster assignation were always related to these accessions and when they were discarded from the analysis the correlation coefficient increase up to 1. According to STRUCTURE, subpopulation 1 showed the highest degree of admixture with 75.6% of the genotypes with less than 80% of membership to this subpopulation followed by subpopulation 3 and 4 with 18% of genotypes with less than 80% of membership in these groups. Subpopulation 2 with 15% of genotypes with less than 80% membership to the corresponding subpopulation, was the subpopulation with lowest admixture.

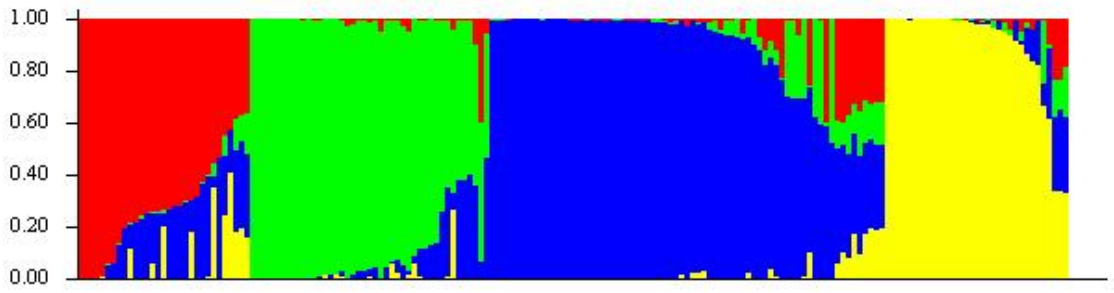

Figure 1 Estimated population structure of oat genotypes according to STRUCTURE software. Each individual is represented by a thin vertical segment, which can be partitioned into 4 grey-scale colored segments that represent the individual estimated membership to the 4 clusters

Multivariate analysis based on PCAs also revealed a separation of 4 subpopulations which indicate a high consistency of the data (Fig. 2). Cluster analysis was implemented on the first four principal components explaining approximately 50% of the variation with 23.3, 13.8, 8.11 and 4.5 % for each of the components, respectively. Although separation between clusters was clear, a continuity derived from accessions connecting all clusters was also observed (Fig.

2). The number of lines per cluster ranged from 33 to 64. The first cluster included mainly the white commercial varieties, cluster 2 the red oats, cluster 3 the white oat landraces characteristic to high altitude locations, and cluster 4 white oat landraces more adapted to low altitude locations.

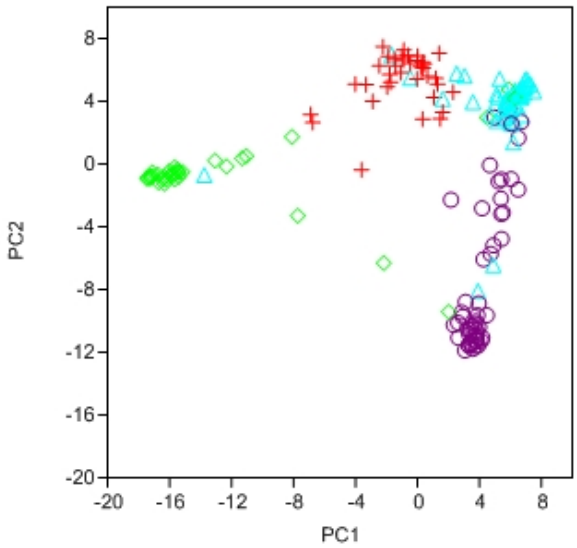

Figure 2 Scatterplot of Principal Component Analysis scores of components 1 and 2 based on 1587 DArT and SSR markers used in this study. A. Represented are the genotypes belonging to cluster 1(red), cluster 2 (green), cluster 3 (violet) and cluster 4 (blue).

Cluster relationships

As previously stated PCA showed a separation between clusters but also a clear pair-wise relationship between clusters. Quantitative results for genetic distance (according to Nei´s parameter) between clusters are shown in Table 1. Cluster 2 comprising the red oats was by far the most distant from all other clusters, with an average distance of 180 whereas the two white oat landraces clusters were the most closely related groups with an average distance of 66. Clusters 1 and 4 corresponding to the white oat landraces adapted to low altitude and the commercial varieties, respectively were also closely related with a distance of 75 (Table 1).

Table 1 . Population average pairwise genetic distance according to Nei's parameter of pairwise difference

	C1	C2	C3
C2	150		
C3	125	205	
C4	75	185	66

These relationships between clusters were in agreement with those depicted by the PCA scatter plot (Fig. 2). These results suggest that clustering was also efficient in separating the oat types for the germplasm used in this study.

Linkage disequilibrium

Identification of disequilibrium between markers is highly useful since it may condition the strength of the association study. Since physical map distances between markers were not available, LD was represented by the disequilibrium matrix visualizing the linear arrangement of LD between polymorphic sites, represented by r^2, and the probability (Flint-Garcia et al., 2003; Gaut and Long, 2003) (Fig.3). A total of 507,042 pairs of markers showed a significant LD value with an average p=0.004. From these, 277,920 pairs of markers showed an r^2<0.1 chosen here as nominal level, according to the studies performed by (Newell et al., 2011) in oat.

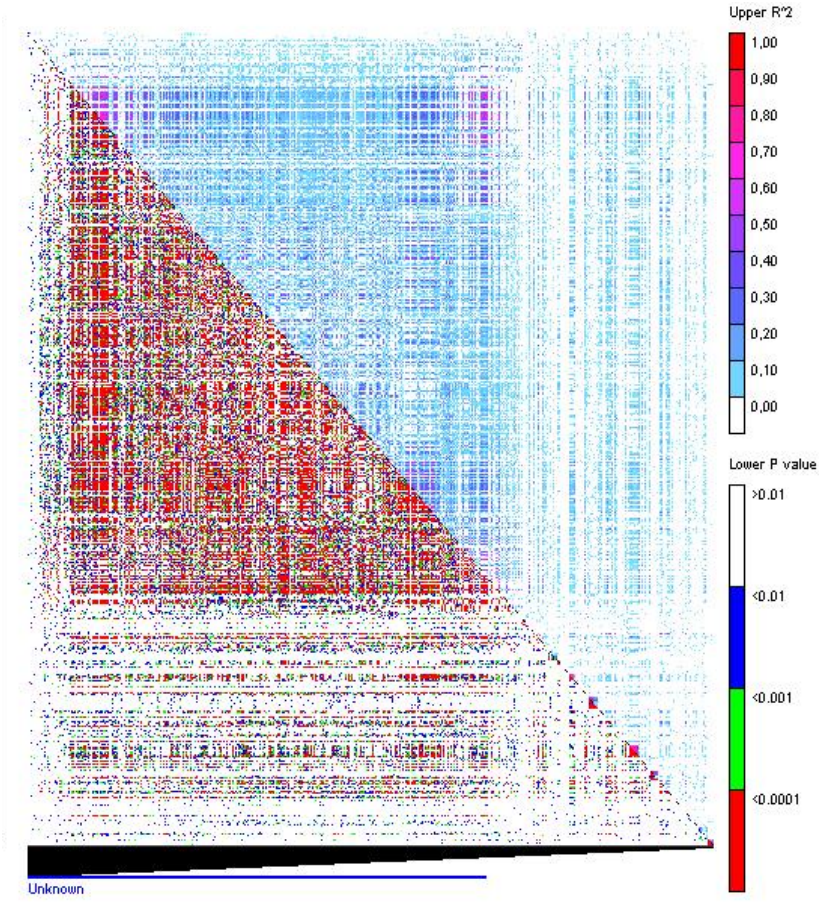

Figure 3. Linkage disequilibrium matrix. Pair-wise LD values of polymorphic sites displaying r^2 above the diagonal and the corresponding p-values from rapid 1000 shuffle permutation test below the diagonal. Each cell represent the comparison of two pairs of marker sites with the color codes for the presence of significant LD. Colored bar code for the significance threshold levels in both diagonals is shown.

In this study with a world-wide oat collection of more than 1,200 accessions, the 14,122 unlinked markers pairs assessed showed an $r^2 < 0.1$ except for one point with a value of 0.15. The average linkage disequilibrium represented by r^2, was 0.055. When assessing the LD of each cluster, similar values were observed ranging between 0.03 and 0.04 with the exception of cluster 2 that showed a slightly higher LD of 0.08, probably reflecting the low number of individuals of this cluster of red oats.

Phenotypic data

Both traits followed a normal distribution with accessions ranging from highly resistant to highly susceptible (Fig. 4). From these data a selection of resistant genotypes (c.a. 15 for each trait) has been evaluated to unravel the resistance mechanisms underlying the resistant response in separate studies (Sánchez-Martín et al., 2011a; Sánchez-Martín et al., 2011b).

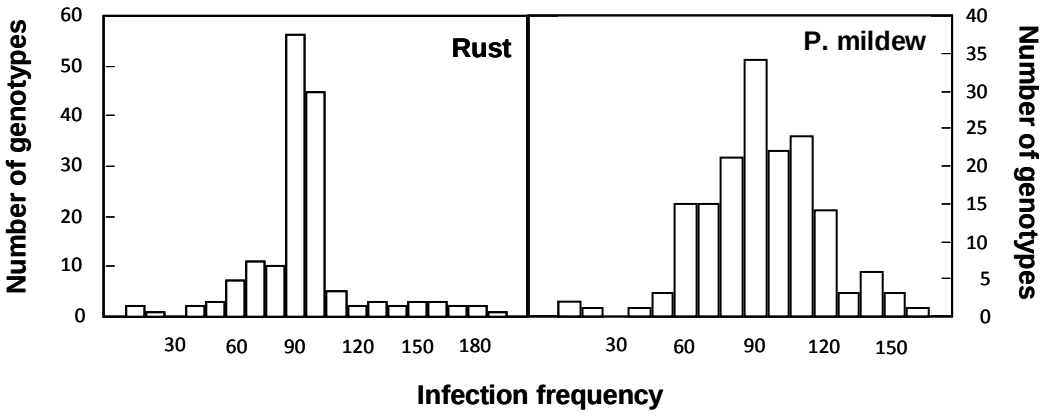

Figure 4. Distribution of the infection frequency of the rust and powdery mildew infection in the oat collection.

We compared the means of the 177 accessions assigned to the 4 subpopulations for the different traits, excluding the admixed accessions (Table 2). We observed significant differences between subpopulations for all traits. Thus, subpopulation 4 had a significantly lower relative infection frequency (RIF) after rust inoculation than the others ($p<0.005$) and showed a high resistant response. Subpopulation 2 had lower relative disease severity to powdery mildew than the others ($p<0.001$) (Table 2). Powdery mildew was the trait most affected by population structure although the proportion of variance explained by population structure remained under 4% (Table 3).

Table 2. Mean comparison between subpopulations for the accessions assigned to a subpopulation (>80% membership in the subpopulation).

Subpopulation	Rust[a]	PM[b]
1	90.1 ab	88.6 a
2	108.8 a	72.9 b
3	95.5 ab	90.8 a
4	86.0 b	86.1 a

[a]Relative infection frequency (number of pustules cm^{-2}) 8 days after rust inoculation

[b] PM (powdery mildew) resistance expressed as relative disease severity (percentage of the leaf covered by mycelium) 8 days after powdery mildew inoculation

Table 3: Statistic for stress resistance and percentage of variation of these traits explained by population structure (K=4) through multiple linear regression.

TraitTrait	Mean	MinM	MaxMa	SDSDS	CV (%)CV	%Var%
Rust[a]Rus	89.578	2.052.	196.40	28.51	31.8331.	1.5^{ns}1.5
PM[b]PM[b]	85.518	0.000.	150150	27.44	32.0932.	3.7*3.7

[a]Relative infection frequency (number of pustules cm^{-2}) 8 days after rust inoculation

[b] PM (powdery mildew) resistance expressed as relative disease severity (percentage of the leaf covered by mycelium) 8 days after powdery mildew inoculation

Association analysis

We first ran association tests using four variations of general linear models, a simple GLM model, a model using the percentages of admixture (Q matrix) obtained for $k = 4$ as cofactors (GLM-Q), a model using the PCAs axes (from 1 to 4) as covariates (GLM-PCA) and finally a model using both, Q matrix and PCAs (GLM-Q-PCAs) in order to limit false positives. In parallel, we used a mixed linear model that combine both the Q matrix and the kinship coeficients (K matrix) in a stringent method to limit false positive. Table 4 and 5 shows the markers considered to be significantly associated with a rust or powdery mildew resistance trait according to the threshold of 0.25 for Q-value in the FDR test (Newell et al., 2012) in any of the models corrected for population structure.

As expected considerably fewer markers showed a significant association with rust resistance when applying a correction accounting for the population structure than when using GLM

alone. Thus, we observed a decrease of significant markers of c.a 95% for any of the models accounting for structure, GLM-Q, GLM-PCA and GLM-Q-PCA with respect to GLM alone. The high differences in the number of significant tests between GLM and GLM-Q, GLM-PCA or GLM-Q-PCA showed that the structure effect was strong and needed to be taken into account to avoid false positives. We found 5 markers significantly associated with rust. Markers oPt-11795 and MAMA5-163 were the two more significantly associated showing significant association in all models tested including the MLM models accounted for population structure and kinship and explaining 20 and 10% respectively of the variation observed for this trait (Table 4). Three additional markers, AM30-178, AME176-3 and oPt-15665 were significantly associated in all GLM models including those accounting for population structure (GLM-Q, GLM-PCA and GLM Q+PCA) but no in the MLM models (Table 4).

A significant reduction of associated markers with powdery mildew resistance was also observed when applying a correction accounting for the population structure than when using GLM alone. We observed a decrease of c.a 98% for the models corrected with STRUCTURE coefficients. Since the significance of the marker associated according to GLM-Q model was in the limit of 0.25 (Table 5) and it was neither highlighted by the MLM models this marker, oPt-14317, related to powdery mildew resistance in seedlings was not considered strongly associated.

In order to find markers associated with powdery mildew resistance we took advantage of a previous detailed evaluation for powdery mildew adult plant resistance performed in a subpopulation of the collection (Sánchez-Martín et al., 2011a). In this, following a preliminary field assessment, 54 genotypes representing the different clusters were evaluated under controlled conditions for adult plant resistance. This population covered a continuous range for powdery mildew resistance between 0 and 100%, showed a similar structure than the oat collection and showed 414,311 significant marker pairs in linkage disequilibrium with 70,657 of them showing a $r^2 < 0.1$ (Supplemental Fig 1 A,B,C). Association analysis for adult plant resistance yielded a marker, oPt-5014, highly significant in all models tested including the GLM accounting for population structure and the MLM accounting for both structure and kinship. This marker would explain approximately 30% of the observed variation according to r^2. Other two markers, oPt-3306 and oPt-793335 were strongly associated in all GLM performed accounting for population structure but not in the MLM models.

Table 4 : Markers associated with rust resistance according to different of association analysis models: General Lineal Model, GLM corrected for population structure according to (percentage of admixture coefficients, Q, principal component covariates , PCA, or both respectively, and Mixed Lineal Model, MLM, corrected with kinship and structure matrix. Markers were considered to be associated with the traits if the markers were significant (FDR≤0.25) in GLM models corrected for population structure or MLM models.

Marker	GLM			GLM+Q			GLM+PCA			GLM+Q+PCA			MLM+Q+Kinship		
	p	FDR	r^{2a}	*p*	FDR	r^2	*p*	FDR	r^2	*p*	FDR	r^2	*p*	FDR	r^2
oPt-11795	$8.1\cdot 10^{-8}$	**$7.8\cdot 10^{-5}$**	0.19	$1.6\cdot 10^{-7}$	**$2.6\cdot 10^{-4}$**	0.18	$3.7\cdot 10^{-7}$	**$6.0\cdot 10^{-4}$**	0.16	$2.3\cdot 10^{-7}$	**$3.8\cdot 10^{-4}$**	0.16	$1.6\cdot 10^{-7}$	**$2.7\cdot 10^{-4}$**	0.22
MAMA5-163	$7.0\cdot 10^{-5}$	**0.02**	0.10	$3.2\cdot 10^{-4}$	**0.11**	0.08	$1.9\cdot 10^{-4}$	**0.10**	0.08	$1.1\cdot 10^{-4}$	**0.06**	0.08	$1.3\cdot 10^{-4}$	**0.11**	0.10
AM30-178	$8.2\cdot 10^{-5}$	**0.02**	0.09	$3.5\cdot 10^{-5}$	**0.02**	0.10	$9.4\cdot 10^{-5}$	**0.07**	0.09	$1.7\cdot 10^{-4}$	**0.06**	0.08	$1.0\cdot 10^{-3}$	0.49	0.07
AME176-3	$2.0\cdot 10^{-4}$	**0.04**	0.10	$4.8\cdot 10^{-4}$	**0.11**	0.09	$8.0\cdot 10^{-4}$	**0.25**	0.08	$4.2\cdot 10^{-3}$	**0.63**	0.06	$1.2\cdot 10^{-3}$	0.49	0.09
oPt-15665	$1.0\cdot 10^{-3}$	**0.08**	0.08	$7.0\cdot 10^{-3}$	0.42	0.06	$9.8\cdot 10^{-4}$	**0.20**	0.08	$7.\cdot 10^{-5}$	**0.05**	0.10	$2.9\cdot 10^{-3}$	0.72	0.08

[a] Percentage of phenotypic variance (partial R^2x100%) of the total variation explained by the marker after fitting the other model effects

Table 5 : Markers associated with powdery mildew resistance according to different of association analysis models: General Lineal Model, GLM corrected for population structure according to (percentage of admixture coefficients, Q, principal component covariates , PCA, or both respectively, and Mixed Lineal Model, MLM, corrected with kinship and structure matrix. Markers were considered to be associated with the traits if the markers were significant (FDR≤0.25) in GLM models corrected for population structure or MLM models. Data in bold indicates values statistically significant according to the False Discovery Rate (FDR) test.

Marker	GLM			GLM+Q			GLM+PCA			GLM+Q+PCA			MLM		
	p	FDR	r^{2a}	*p*	FDR	r^2	*p*	FDR	r^2	*p*	FDR	r^2	*p*	FDR	r^2
Seedling Stage															
oPt-14317	$1.1\cdot 10^{-5}$	**0.01**	0.13	$1.5\cdot 10^{-4}$	**0.25**	0.10	$3.8\cdot 10^{-4}$	0.45	0.09	$4.0\cdot 10^{-4}$	0.4	0.09	$8.0\cdot 10^{-4}$	0.90	0.09
Adult Plant Stage															
oPt-5014	$5.0\cdot 10^{-7}$	**$7.7\cdot 10^{-4}$**	0.45	$7.1\cdot 10^{-6}$	**0.01**	0.34	$6.7\cdot 10^{-6}$	**$5.4\cdot 10^{-3}$**	0.36	$4.6\cdot 10^{-6}$	**$7.4\cdot 10^{-3}$**	0.344	$3.2\cdot 10^{-4}$	**0.19**	0.35
oPt-3306	$2.0\cdot 10^{-4}$	**0.01**	0.29	$5.7\cdot 10^{-5}$	**0.04**	0.29	$7.5\cdot 10^{-5}$	**0.04**	0.30	$2.1\cdot 10^{-5}$	**0.01**	0.30	$7.7\cdot 10^{-4}$	0.62	0.30
oPt-793335	0.8	0.94	0.05	$3.5\cdot 10^{-4}$	**0.01**	0.24	$5.0\cdot 10^{-6}$	**$5.4\cdot 10^{-3}$**	0.36	$5.0\cdot 10^{-5}$	**0.02**	0.28	$2.1\cdot 10^{-3}$	0.99	0.26

[a] Percentage of phenotypic variance (partial R^2x100%) of the total variation explained by the marker after fitting the other model effects

Sequence homology

Since many of the DArT markers evaluated have been previously sequenced (Tinker et al., 2009) we took advantage of this information to characterize further the significantly associated markers and identify homologous sequences in oat or related species by BLAST approaches. The marker oPt-11795 showed homology (38% identity, E-value of 10^{-12}) with an autophagy-related protein 2 of *Triticum urartu* (Table 6). A hypothetical protein of *Oriza sativa* containing the same functional domains as the autophagy-related 2 of *T. urartu* also shared homology with this marker. Interestingly, marker oPt-15665 showed homology (56% identity) with an anthocyanin 5-aromatic acyltranferase of *Aegilops tauschii* with e-value of 10^{-23} and two other hypothetical proteins of sorghum.

Marker	Blastx	Species	E-value	Cov	Ident
oPt-11795	Autophagy-related protein 2	Triticum urartu	1,00E-12	85%	38%
	Hypothetical protein	Oriza sativa	2,00E-12	96%	38%
oPt-15665	Anthocyanin 5-aromatic acyltransferase	Aegilops tauschii	1,00E-23	54%	56%
	Hypothetical protein	Sorghum bicolor	1,00E-22	65%	41%
	hypothetical protein	Sorghum bicolor	3,00E-17	50%	45%
oPt-5014	hypothetical protein	Sorghum bicolor	6,00E-30	94%	61%
	hypothetical protein	Sorghum bicolor	2,00E-26	90%	56%
	hypothetical protein	Sorghum bicolor	5,00E-26	93%	58%
	hypothetical protein	Sorghum bicolor	7,00E-17	79%	48%
	hypothetical protein	Sorghum bicolor	2,00E-16	78%	48%
	hypothetical protein	Sorghum bicolor	2,00E-13	90%	48%
	hypothetical protein	Sorghum bicolor	2,00E-13	90%	48%
	hypothetical protein	Triticum urartu	5,00E-13	90%	44%
	hypothetical protein	Oriza sativa	1,00E-12	65%	43%

Table 6: Potential homologous sequence of significant markers using the function BlastX of the BLAST algorithm (Altschul et al., 1990) implemented in the ncbi webserver (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Following the blast for markers associated with powdery mildew resistance, oPt-5014 showed homology with hypothetical proteins of *Sorghum bicolor, Triticum urartu* and *Oriza sativa* with E-values ranging from 10^{-12} to $6{\cdot}10^{-30}$. All these potential homologs contained a Zinc Knuckle domain (Table 6). Marker oPt-14317, that only shows low association with powdery mildew resistance, did not show any homology with sequences of the database. For the two remaining significant markers associated with powdery mildew adult plant resistance,

sequences were not available and work is in process to determine the possible nature of the associated locus.

DISCUSSION

As the first step for the association study, structure of the population was inferred since it has great implications on the design and analysis of GWAs. The different approaches used here indicated subtle population structure within the germplasm collection evaluated. Thus, four oat groups could be detected albeit they presented a certain degree of admixture according to STRUCTURE software with up to 30% of accessions with less than 80% membership to a determinate group. This was also observed following PCAs with several accessions covering "gaps" between clusters. Interestingly the group comprising the commercial varieties showed the highest degree of admixture, likely due to the high exchange of germplasm within this group in which most accessions shared common ancestors in its genealogy as reported by Montilla-Bascón et al., (2013). One concern in respect to oat is the establishment of population structure arising from the different oat types, winter or spring, or interbreeding species such as the white and red (*A. byzantina*) oats. Indeed in a study by Newell et al., (2012) a small cluster of red oat differentiated from the rest of the collection. Taking into account that our collection was consciously formed with diverse oat types to achieve high genetic diversity, its population structure was considered relatively weak compared with that found in other cereals such as barley (Hamblin et al., 2010) or wheat (Stich et al., 2008).

Despite the relative weak structure of this oat population, it contains a high genetic diversity which is an important feature in order to find markers significantly associated with a trait (Ingvarsson and Street, 2011). Estimation of genetic distances between accessions and clusters revealed inter- and intra-group genetic diversity which is confirmed by the phenotypic evaluation for rust and powdery mildew resistance that showed a wide variability ranging from resistant to susceptible. As expected the less distant groups were the white oat landraces adapted to high and low altitude followed by commercial cultivars while the most distant group was the red oats.

The extent of linkage disequilibrium in a species influences the strength and resolution of GWAS. The study of linkage disequilibrium in the oat collection showed a very high number of marker pairs in significant LD. In particular we found more than 275,000 marker-pairs with r^2 <

0.1, selected here as nominal level for effective linkage according to previous work in oat (Newell et al., 2012; Newell et al., 2011). This indicated high genome coverage with non associated markers. In oat it has been proposed that a marker every cM (2,000 marker in total) would explain, on average 20% of QTL variance, since r^2 between a marker and a QTL is equal to the percentage of phenotypic variation of a QTL that can be explained by a marker, and LD decay was found on average 0.2 for DArTs separated by 1.0 cM (Newell et al., 2011). Our work performed on 1,715 markers would cover an important part of the genome although obviously increasing the number of markers would increase the probability of identifying additional markers in high LD with a QTL.

The molecular marker data set in combination with the phenotype evaluation was used to examine linkage-related marker-trait associations. Separating the role of population structure and genetic linkage as causes for marker-trait association remains the greatest challenge in association analysis (Achleitner et al., 2008). The five models used in this study accounted for "Q" (population structure from subpopulations) and/or "K" (genetic similarity in the background from shared kinship) which may be important to identify marker-phenotype associations not related to genetic linkage between markers and QTL. In addition we tested models containing PCA covariates, which may account for some proportion of both "Q" and "K". We performed a tentative comparison between the GLM and the MLM models, since MLM models that accounts for kinship relationships, such as that described by (Yu et al., 2006), might remove more of the structure effect. This point was demonstrated by (Brown et al., 2008) in sorghum and (Cockram et al., 2008) in barley. Co-examination of different models and traits can provide an informative summary of the major trends affecting the analysis.

As expected, a reduction of significant markers associated with both traits was observed when correcting the models for population structure and/or kinship. Five markers, two of them highly significant in all models tested were associated with rust resistance. No significant similarity was identified by blast or blastx against NCBI databases other than with putative repetitive elements or retrotrasposons. However, two of these DArT sequences were somehow related to an autophagy-related protein 2 and an anthocyanin 5-aromatic acyltransferase that have been related to the plant immune defense reaction. Recently an autophagy-related protein 2 Arabidopsis mutant, atg2-2, has been reported to have enhanced resistance to powdery mildew (Wang et al., 2011). In our work this marker was found to be present in 23 out of the 31 red oats, showing this subpopulation the highest rust relative infection frequency. This would be in agreement with the positive correlation between *atg2-2*

presence and powdery mildew susceptibility found in Arabidopsis (Wang et al., 2011). An anthocyanin 5-aromatic acyltransferase gene has been located within the anthracnose resistance locus *Co-4* of common bean (Melotto et al., 2004) and its expression have also been found to be altered in resistant *A. thaliana* ecotypes infected with cucumber mosaic virus (Ishihara et al., 2004). In agreement with the negative effect of expression of the anthocyanin acylation gene in Arabidopsis, the oPt-15665 marker was observed in 28 out of 31 red oat with high RIF and in 9 white oats with a mean RIF of 123 indicating highly susceptibility. However, further work would be needed to ascertain the relationship between the DArTs markers and these genes.

Despite the wide distribution of powdery mildew resistance in our collection, we did not identify strong association between any marker and seedling resistance. It may be that the combination of marker density and the phenotypic variation were insufficient. Polymorphisms causing variation for this trait may have been in linkage equilibrium with our markers, and higher marker densities could have uncovered more QTLs. Alternatively, a high number of rare alleles causing variation in seedling powdery mildew resistance in our collection might cause less variation in the data and therefore be not detected. Indeed, rare alleles are a leading hypothesis for the "missing heritability observation" in human association studies (Yang et al., 2010). Alternatively, the low association for this trait could be due to the development of markers from a genetically narrow set of germplasm in relation to the lines used in this study. However this is highly unlikely since DArT markers were developed from a panel of 60 accessions of global representation. Interestingly one DArT sequence, oPt-5014, was strongly associated with powdery mildew rust resistance in adult plants. The strong association observed taking into account the relative low number of accessions evaluated for this trait, suggest that a careful selection of accessions covering a complete range of phenotypic and genotypic variation may be adequate in some cases to find significant associations. Marker oPt-5014 was associated with hypothetical proteins of sorghum, wheat and rice containing a Zinc knuckle domain (pfam14392) which has been detected in several plants transcription factors and might therefore be involved in the regulation of gene expression.

Recent oat maps sharing common markers allow us to more specifically locate the DArTs markers within the oat genome. Thus, according to (Tinker et al., 2009), oPt-11795 marker maps onto KO32 which is equivalent to chromosome 4C in the first physically anchored consensus oat map (Oliver et al., 2011) where there is no previously reported crown rust resistance genes. Recent studies showed synteny between this chromosome and regions of

Brachypodium dystachion chromosome 4 or *Oryza sativa* chromosome 9. Interestingly QTLs for resistance to the rust fungus *Puccinia Brachypodii* have been reported in chromosome 4 of *B. dystachion* (Barbieri et al., 2012). In addition the powdery mildew resistance gene *PmAS846* mapped in wheat chromosome 5BL is collinear with genomic regions on *Brachypodium* chromosome 4 and rice chromosome 9 (Xue et al., 2012) and a locus associated with broad-spectrum resistance to rice blast, *Pi5(t)*, also mapped onto rice chromosome 9. MAMA5 is reported in Wight et al., (2003) to map near to cdo53 on KO17 equivalent to chromosome 9D (Oliver et al., 2013). Interestingly, in this position the partial crown rust resistance Pc38 maps (Wight et al., 2004). In addition the major QTL for partial rust resistance, *Prq1b*, close to the markers cdo608x and cdo1467 also map (Portyanko et al., 2005), leading to authors to suggest and association between this QTL, *Pc38*, and other resistance genes including Pc62 and *Pc63* that cluster with *Pc38(Harder et al., 1980)* Harder et al., 1980). AME176 maps onto 15A (unpublished Buffalo x Tardis results) which shows homology with 9D where according to Oliver et al., (2013) a number of other resistance genes maps. According to Tinker et al., (2009), oPt-14317 maps onto KO22_44_18 within the same framework marker than AM102 now annotated as chromosome 19A. This is a similar position to where the dominant powdery mildew resistance gene *Eg5* has been mapped (Yu and Herrmann, 2006)). Finally, has been mapped in a number of populations (eg. (He et al., 2013; Hizbai et al., 2012)) onto chromosome 21D. This chromosome is also known to contain a number of crown rust resistance genes such as Pc54, Pc59 and Pc68. However, lack of common markers makes it difficult relate how close oPt-5014 is to these genes.

Overall, the markers showing the strongest association in this study provide ideal candidates for further studies and future inclusion in strategies of marker assisted selection.

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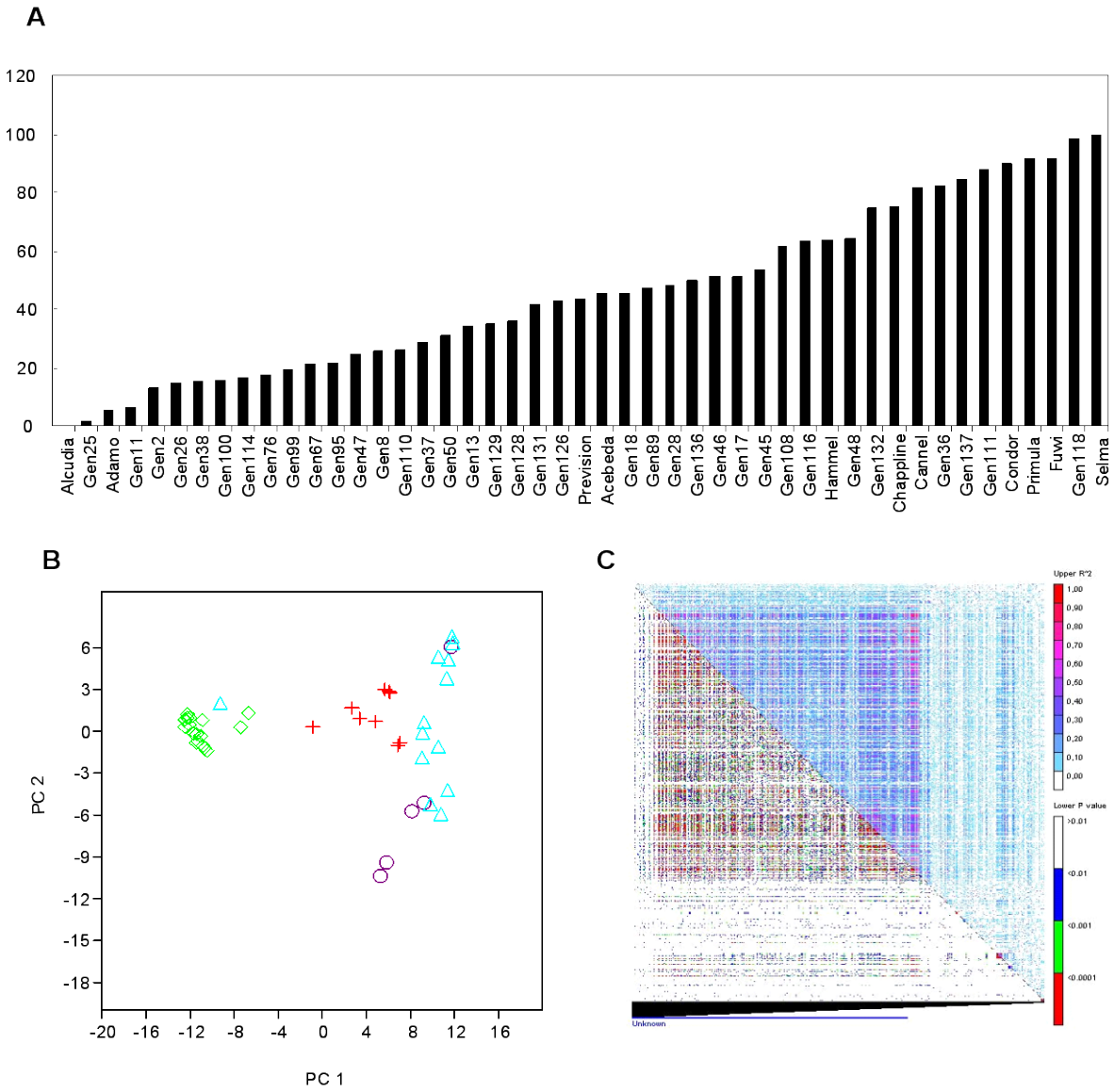
Supplemental Figures



Supplemental Figure 1

Supplemental Figure 1. **A.** Phenotypic evaluation of a subcollection of oat adult plants for powdery mildew resistance. B. Scatterplot of Principal Component Analysis scores of components 1 and 2 based on 1587 DArT and SSR markers of the oat subcollection. Represented are the genotypes belonging to cluster 1(red), cluster 2 (green), cluster 3 (violet) and cluster 4 (blue). **C.** Linkage disequilibrium matrix of the oat subcollection. Pair-wise LD values of polymorphic sites displaying r^2 above the diagonal and the corresponding *p*-values from rapid 1000 shuffle permutation test below the diagonal. Each cell represent the comparison of two pairs of marker sites with the color codes for the presence of significant LD. Colored bar code for the significance threshold levels in both diagonals is shown.

Discussion

GENERAL DISCUSSION

Oat (Avena sativa L.) is an important cereal crop cultivated for grain, feed, fodder and straw over more than 9 million hectares worlwide (FAO). Due to its good adaptation to a wide range of soil types and because oats can perform better than other small-grain cereals on marginal soils, there is an increasing interest to expand oat cultivation to southern countries (Buerstmayr et al., 2007; Forsberg and Reeves, 1995; Løes et al., 2007; Ren et al., 2007; Stevens et al., 2004). In fact, autumn sown oat is increasing in Australia (Armstrong et al., 2004), south of Japan (Katsura, 2004), south China (Wang, 2004) and temperate areas of South America (Federezzi and Mundstock, 2004) where winter sowing of spring crops is a common practice. Thus, oats may be well suited to Mediterranean climates and farming systems. Indeed in Spain around half million hectares of oat crop produce more than 1,2 M Tons annually (MAPA). In addition, the current PAC policies and the reduction of subsidies is leading to a greater cereal crop diversification reducing the area of durum wheat and increasing oat crop (RAEA). However, in the rainfed Mediterranean environments oats will predictably encounter water limitations as well as disease incidence, such as the crown rust (*Puccinia coronata* f.sp. *avenae*) adapted to the warmest regions and the powdery mildew (*Blumeria graminis* f. sp. *avenae*) adapted to cooler regions, that provoke the instability of the yields and limits its cultivation. Thus, it is necessary to reduce these limitations to increase the profitability of the crop for the farmer.

Water limitation is an important feature of the Mediterranean environments. Thus, adaptation to Mediteranean conditions implies the development of drought tolerance, particularly for rainfed crops in marginal areas such as oat. Furthermore, the Intergovernmental Panel on Climate Change predictions suggest that many more areas would exhibit increased temperatures and severe droughts (Kumar, 2007). Crop breeders are responding to the challenge of developing new drought tolerant lines. As in most crops this is achieved by selection of appropriate progeny. However, for selection of complex traits such as stress tolerance, breeding programmes must be based on a sound understanding of innate tolerance mechanisms (Blum, 1999; Dita et al., 2006). Focussing only in plant survival has lead to yield penalties in drought tolerant lines (Passioura, 2002; Turner, 1979) and selection only on the basis of yield is not appropriate due to the low heritability of this trait and a high genotype x environment (GE) interaction (Araus et al., 2002). This is supported by our previous work on GE interaction in oat around the Mediterranean basin (Sánchez-Martín et al., 2013) performed in our ressearch group. Consequently, modern breeding strategies attempt to include

assessments of physiological, biochemical and molecular characteristics which provide a better understanding of the intricate processes underlying the tolerance response and may proportion adecuate markers for selection (Araus, 1996; Richards, 1996; Sanchez-Martin et al., 2012)

Biotic stresses such as the crown rust and the powdery mildew are also among the main oat constraints. Crown rust causes high losses in oat yield and grain quality worldwide (Simons, 1985) but particularly in the Mediterranean basin where rust populations are more virulent than in the centre and north of Europe (Herrmann and Roderick, 1996). Powdery mildew becomes more intense after mild winters and warm springs (Priestley and Bayles, 1982) causing looses up to 30% in western Europe. Both pathogens can be controlled with fungicides but this is relatively expensive and harmful due to its negative effects on human health and environment. Consequently, host resistance is being explored as the most effective, economical and environmentally friendly control method (Stevens et al., 2004). However, resistance obtained is often overcome by emerging pathogenic races. This is mainly due to the inappropriate use of resistance sources, of monogenic nature. Thus, it is necessary to identify the bases of resistance mechanisms including those involved in durable resistance (Niks and Rubiales, 2002).

This scenario is usually even more complex since in the field, crops usually have to face more than one stress at the same time. This is particularly true for certain stresses such as drought and rust that are both favoured under high temperatures. Then, in this work we tackled molecular nodes that may influence both biotic and abiotic stress responses. Until recently, interaction between biotic and abiotic resistance mechanisms have been poorly addressed. However, recent studies (Fujita et al., 2006; Schenke et al., 2011) revealed that certain transcription factors, MAP kinases, abscisic, salicylic and jasmonic acid, ethylene, reactives oxygen and nitrogen species are key molecules for both stresses, sometimes as synergistic but also as antagonistic factors (Fujita et al., 2006; Glazebrook, 2005). This is a good opportunity for breeding since synergistic effects could protect against different threats whereas antagonistic effects should be avoided. We focussed mainly in the role of polyamines in different aspects of the resistance to biotic and abiotic stresses including crosstalk with other signalling molecules. Polyamines, are known to stabilize macromolecular structures, and to act as regulatory molecules in many fundamental cellular processes including cell division, embryogenesis, senescence and in response to stress (Martin-Tanguy, 1997), but, in addition,

recent studies indicate that polyamines may act as cellular signals in intricate crosstalk with hormonal pathways, such as abscisic acid, ethylene, hydrogen peroxide and nitric oxide (An et al., 2008; Toumi et al., 2010; Yamasaki and Cohen, 2006). Traditionally, changes in polyamines were associated with the response to abiotic stresses including salinity, drought, chilling, heat, hypoxia, ozone, UV, heavy metals or wounding (Alcazar et al., 2010) and few few was known about their implication in biotic stress responses. However, recent studies show polyamine accumulation during compatible and incompatible plant-pathogen interactions (Walters, 2003). In the present work we have highlighted the importance of polyamines for the resistance to powdery mildew in oat during host and non-host resistance and unraveled the role of the polyamines during the resistance responses of oat to rust highlithing their relevance particularly during the pre- and penetration resistance mechanisms which are highly desirable since they may provide more durable resistance. Further, we explored its role during drought and the crosstalk with other hormones also involved in biotic and abiotic stresses such as nitric oxide and salicylic acid and showed that polyamines constitute a key node during the tolerance responses to drought in intimate crosstalk with other molecules traditionally associated to biotic stress resistance.

The complexity of the resistance responses stated here usually limit the improvement of crop for resistance to biotic and abiotic stresses. Despite this, stress tolerant cultivars have been bred introducing traits from stress-adopted wild relatives or landraces (Bartels and Sunkar, 2005). Landraces have considerable potential for use in improving abiotic stress tolerance since the transfer of beneficial traits is relatively straight-forward in that there is no barrier to crossing. Association of genetic markers with regions of the genome controlling different traits may enable efficient and precise transfer of useful alleles from landraces to modern cultivars. In oat, genetic markers have been used with a number of experimental bi-parental oat populations to identify quantitative trait loci (QTL) associated with resistance to different biotic and abiotic stresses (Jackson et al., 2010; Jackson et al., 2008; Locatelli et al., 2006; Maloney et al., 2011; Zhu et al., 2004). However, genomic studies often turn out to be unique to a specific genetic background limiting their usefulness in marker-assisted selection. Association analysis which exploits the variation in a collection of genetically diverse materials to uncover a significant association between a trait and a gene or molecular marker on the basis of linkage disequilibrium has emerged as a promising and valuable tool to exhaustively identify QTL in plants. Therefore as a first step to take advance of these molecular approaches for the oat breeding for resistance to biotic and abiotic stresses, we established the genetic

diversity and population structure of a oat collection including commercial varieties and landraces. The results showed that the collection contained a high genetic diversity and showed appropriate for further studies. Then taking advantage of the screening for resistance to rust, and powdery mildew performed in our research group, we genotyped the collection with DArT markers which provide the sufficient genome coverage for a solid association study of markers for rust, and powdery mildew. This work indeed identified new regions of the genome involved in the resistance to both biotic and abiotic stresses.

Overall in this work we advanced in the dissection of the molecular and cellular basis of resistance mechanisms of the most important constraints of the oat crop taking into account the potential interactions between them which is a novel aspect that is only recently being considered. This will undoubtedly help in improving the oat crop performance.

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Conclusions

Chapter 1. Role of polyamines in host and non-host oat-powdery mildew interactions.

1. During the host interaction between oat and the powdery mildew (*Blumeria graminis* f. sp. *avenae*) the resistant cultivar Charming increased significantly its content in the polyamine spermidine respect to the susceptible Selma. This was accompanied by an increase in the activity of the arginine decarboxylase (ADC) activity suggesting an involvement of this polyamine in the resistance response of Charming to powdery mildew.
2. Inoculation of oat with the non-host powdery mildew (*Blumeria graminis* f. sp. *hordei*) induced increases in putrescine and agmatine, accompanied by induction of the ADC activity.
3. Induction of resistance to powdery mildew by polyamines was supported by the exogenous application of polyamines to leaf surface before inoculation which leads to an increase of penetration resistance reducing the percentage of established colonies without toxic effect on fungal development.

Chapter 2. Role of polyamines in host and non-host oat-powdery mildew interactions.

1. Following rust (*Puccinia* coronata f. sp. *avenae*) inoculation the resistant oat cultivars Kankan and Saia showed increased levels of polyamine spermidine, spermine and agmatine whereas susceptible cultivar Araceli showed overall higher levels of putrescine.
2. Detailed quantification of excreted polyamines to the leaf surface showed an induction following inoculation in both susceptible and resistant cultivars. However, polyamine induction was by far higher in the resistant cultivars. Particularly, cultivar Saia, with the highest pre/penetration resistance showed also the highest increase in excreted spermidine, spermine and agmatine to the leaf surface.
3. Exogenous application of polyamines particularly, spermidine and spermine, increased the pre/penetration resistance and the percentage of early aborted colonies.
4. Susceptible cultivar Araceli showed a strong down-regulation of the expression level of the ADC gene between 24 and 72 hours following rust inoculation. This down regulation was not as strong in cultivar Kankan and interestingly although ADC expression was dow-regulated at 24 hai in Saia, it significantly increased at 48 hai.
5. Arginine decarboxylase activity showed steady state levels in susceptible cv. Araceli during the whole time course after inoculation with the crown rust whereas resistant cv. Kankan and Saia showed significantly higher ADC activity. In addition, a significant increase in

bound-wall DAO activity was observed in resistant Saia at 48 hai respect to control non-inoculated plants, not observed in the susceptible cultivar.

Chapter 3. Role of polyamines in host and non-host oat-powdery mildew interactions.

1. Assessment of *in vivo* NO production in oat plants revealed approximately, 50% reduction in the levels of NO at mild and high water stress in the drought resistant cv Patones whereas susceptible Flega increased its NO level respect to control, well watered, plants.
2. The HHb barley line overexpressing the hemoglobin HvHb1 gene showed significantly reduced levels of NO generation with respect to the wild type. This confirmed the efficiency of the HHb line in scavenging important amounts of the NO generated by the plant.
3. The HHb barley plants overexpressing the hemoglobin HvHb1 gene showed increased drought tolerance compared with WT plants.
4. The HHb plants showed higher constitutive levels of the polyamines, putrescine, spermidine and spermine than WT plants. After a water stress deficit treatment, the levels of putrescine dramatically increase in the WT plants respect to control level whereas the content of spermidine in HHb plants increased near 2-fold the content of WT plants. This confirmed a role for NO influencing polyamine content in HHb plants in control conditions and also under drought stress.
5. Key aminoacids of the polyamine biosinthesis pathway such as arginine and methionine showed a significant increase in HHb plants under drought stress compared with WT plants. By contrats, WT plants increased GABA, lisine and proline under drought stress with respect to HHb plants. These results support a role for NO influencing the concentration of the aminoacid precursors of polyamines.
6. Nitric oxide regulated the expression of several polyamine biosynthesis genes. In particular, a significant downregulation of AdoMetDC was observed in WT plants subjected to drought stress compared with HHb plants in the same conditions, and a strong up-regulation of ACS gene, (ACS1, ACS2 and ACS5), of up to more than 100-fold was observed in WT plants subjected to water stress, not observed in HHb plants.
7. Different nitration and S-nitrosylation pattern of proteins between WT and HHb plants confirmed differential post-tranlational modification. Lack of protein identification does not allow to confirm these post-translational modification in proteins related to polyamine biosynthesis.

Chapter 4. Genetic diversity and population structure among oat cultivars and landraces.

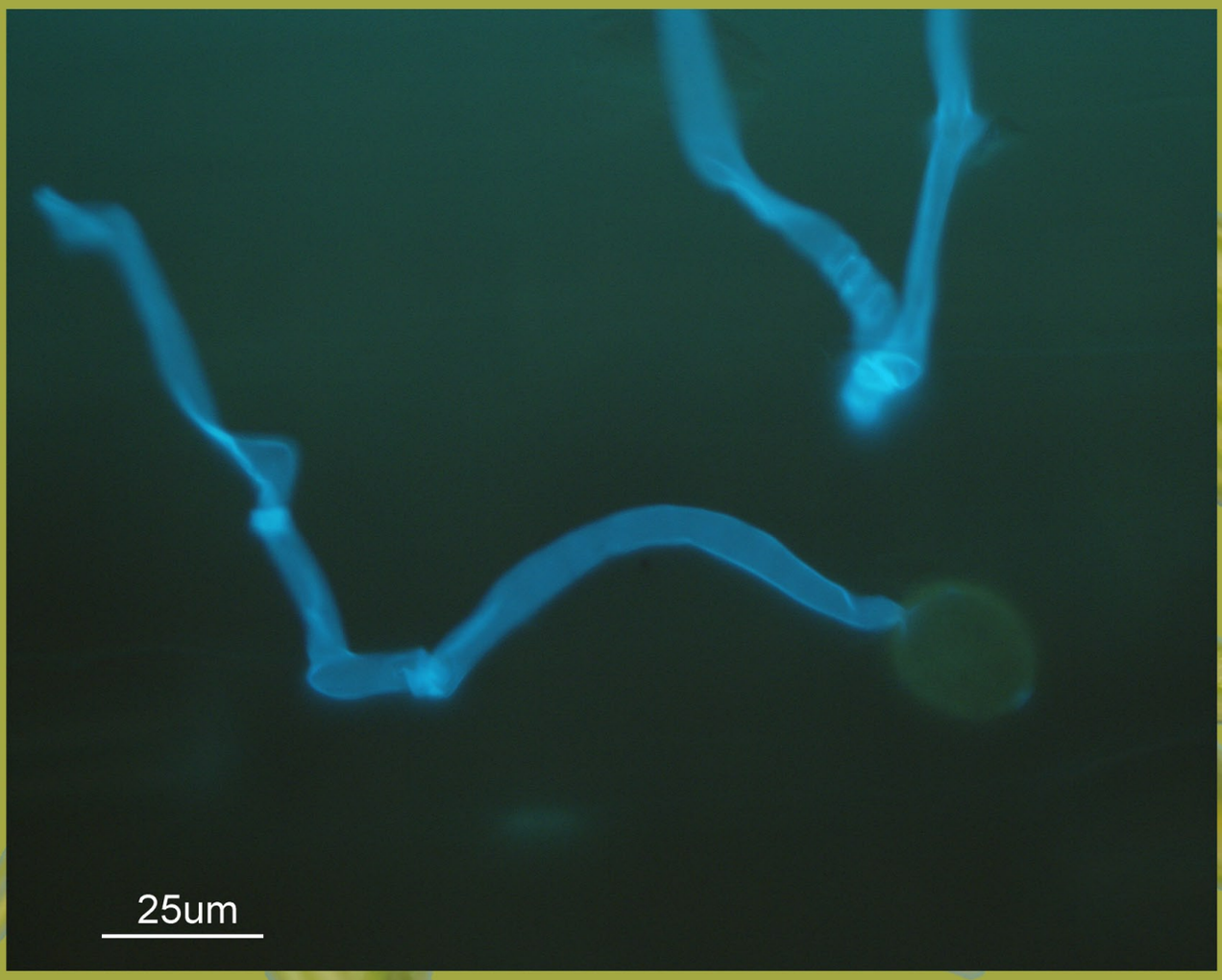
1. The genetic diversity among 177 oat (*Avena sat*iva L.) accessions including both, white and red oats landraces and 36 commercial cultivars, was by average 0.29 indicating considerable genetic variation within the collection.
2. Both, genetic similarity calculated using the Dice coefficient, and population structure using a Bayesian clustering approach identified 4 discrete subpopulations that clearly separated the commercial cultivars, the red oats landraces and two clusters of white oat landraces.
3. The average polymorphic information content of 0.80 for the SSR loci indicated the usefulness of many of the SSR for genotype identification. In particular two markers, MAMA5 and AM04, were sufficient to discriminate among all commercial cultivars studied highlighting their potential use for variety identification.

Chapter 5. Genome-wide association study for crown rust and powdery mildew resistance in an oat collection of commercial varieties and landraces.

1. Genetic diversity of the collection with more than 1500 DArT markers in addition of the SSR markers confirmed 4 subpopulations. However, slight modifications of the genotype-cluster assignation and the corresponding percentage of admixture were observed when increasing the number of markers. Multivariate analysis based on PCAs also revealed a separation of 4 subpopulations which indicate a high consistency of the data.
2. A total of 507,042 pairs of markers showed a significant LD value with an average p=0.004. From these, 277,920 pairs of markers showed an r^2<0.1 chosen here as nominal level indicating a good potential genome coverage of the markers.
3. We found 5 markers significantly associated with rust resistance. Markers oPt-11795 and MAMA5-163 were the two more significantly associated showing significant association in all models tested including the MLM models accounted for population structure and kinship and explaining 20 and 10% respectively of the variation observed for this trait. Three additional markers, AM30-178, AME176-3 and oPt-15665 were significantly associated in all GLM models including those accounting for population structure
4. The marker, oPt-14317, highlighted as related to powdery mildew resistance in seedlings was not considered strongly associated. However, oPt-5014, was highly significant associated with powdery mildew adult plant resistance in all models tested. This marker

would explain approximately 30% of the observed variation. Other two markers, oPt-3306 and oPt-793335 were strongly associated with adult plant resistance in all GLM performed accounting for population structure but not in the MLM models.

5. The marker oPt-11795 showed homology with an utophagy-related protein 2 of *Triticum urartu* and marker oPt-15665 showed homology with an anthocyanin 5-aromatic acyltranferase of *Aegilops tauschii.* However, further work would be needed to ascertain the relationship between the DArTs markers and these genes.



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