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Resistance to Herbicides in the Model Organisms *Saccharomyces cerevisiae* and *Arabidopsis thaliana*: the Involvement of Multidrug Resistance Transporters

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1. Introduction

Herbicides are agrochemicals that control the growth of undesired weeds, bringing about a significant overall increase in crop productivity. Herbicide resistance, the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type, is a weed physiological characteristic (Twonson, 1997). This trait has the potential to cause not only large economic losses in agricultural production, forestry and landscaping, but also deleterious effects on the environment and human health as a result of rising herbicide application rates (Hayes & Wolf, 1997). On the other hand, crops of agronomic relevance exhibiting chemical stress resistance are highly desirable and can be obtained through genetic manipulation, based on the knowledge gathered on the underlying mechanisms.

Emergence of multidrug resistance (MDR), that is, the simultaneous acquisition of resistance to a wide range of structurally and functionally unrelated cytotoxic chemicals, is found in a wide variety of organisms, from bacteria to mammals. The widespread use of herbicides has led, since the early 1970s, to a growing number of resistant weed species and biotypes that possess multiple resistance to various classes of herbicides (Hayes & Wolf, 1997). Indeed, herbicide application is one of the most important selective forces acting on a weed community in an agroecosystem (Owen & Zelaya, 2005).

To date, and particularly during the past two decades, numerous cases of herbicide resistant weed species have been described, in at least 60 different countries (www.weedscience.org). Table 1 shows the number of cases of single and multiple herbicide resistance development in weed species registered in the last 30 years. Among them there are several weed strains that have acquired simultaneous resistance to herbicides with different modes of action (Table 1). For example, in 1982 in South Australia a strain of *Lolium rigidum* (rigid ryegrass), a monocot weed that infests barley and wheat fields, was registered as having developed resistance to multiple herbicides, exhibiting seven diverse modes of action (from the

following HRAC[1]/WSSA[2] groups: ACCase inhibitors - A/1, ALS inhibitors - B/2, Triazoles, ureas, isoxazolidiones - F3/11, Dinitroanilines and others - K1/3, Mitosis inhibitors - K2/23, Chloroacetamides and others - K3/15, Thiocarbamates and others - N/8). Owing to its capacity to rapidly acquire multiple herbicide resistance, *L. rigidum* bursts exert the highest negative economic impact in Australian crops (Owen et al., 2007). Another case of multiple herbicide resistance is *Alopecurus myosuroides* (blackgrass), a monocot weed that infests winter wheat plantations. In 1996, multiple herbicide resistant biotypes of this weed were found in Belgium, exhibiting simultaneous resistance to atrazine, chlorotoluron, clodinafop-propargyl, fenoxaprop-P-ethyl, flupyrsulfuron-methyl-Na, pendimethalin, and propaquizafop (from the following HRAC groups: ACCase inhibitors - A/1, ALS inhibitors - B/2, Photosystem II inhibitors - C1/5, Ureas and amides - C2/7, and Dinitroanilines and others - K1/3)(www.weedscience.org; Eelen et al., 1997).

Mode of action (HRAC/WSSA group)	Herbicide example	Number of resistant weed species	Number of MDR weed species*
ACCase inhibitors (A/1)	Diclofop-methyl	38	16
ALS inhibitors (B/2)	Chlorsulfuron	107	33
Photosystem II inhibitors (C1/5)	Atrazine	68	9
Ureas and amides (C2/7)	Chlorotoluron	21	11
Nitriles and others (C3/6)	Bromoxynil	3	0
Bipyridiliums (D/22)	Paraquat	24	4
PPO inhibitors (E/14)	Oxyfluorfen	4	3
Carotenoid biosynthesis inhibitors (F1/12)	Flurtamone	2	1
Triazoles, ureas, isoxazolidiones (F3/11)	Amitrole	4	1
Glycines (G/9)	Glyphosate	19	10
Dinitroanilines and others (K1/13)	Trifluralin	10	3
Mitosis inhibitors (K2/23)	Propham	1	1
Chloroacetamides and others (K3/15)	Butachlor	4	4
Cellulose inhibitors (L/27)	Dichlobenil	1	1
Thiocarbamates and others (N/8)	Triallate	8	4
Synthetic auxins (O/4)	2,4-D	28	8
Arylaminopropionic acids (Z/25)	Flamprop-methyl	2	1
Organoarsenicals (Z/17)	MSMA	1	0
Unknown (Z/27)	(chloro) - flurenol	1	1

* Number of weed species that have biotypes described as resistant to herbicides with different modes of action.

Table 1. Number of resistant weed species and MDR weed species according to the herbicide mode of action (adapted from www.weedscience.com).

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Some herbicide families seem to be particularly prone to induce herbicide resistance in their target weeds. Indeed, approximately 70% of the registered herbicide resistant weeds exhibit tolerance to only four out of 19 herbicide families. These four herbicide families include ALS inhibitors, photosystem II (PSII) inhibitors, ACCase inhibitors and synthetic auxins.

The great majority of herbicides act by inhibiting a specific plant enzyme essential for metabolism, whereas the remainder, including auxinic herbicides, act as general inhibitors. Two types of resistance can be distinguished: target-site and non-target-site herbicide resistance (Powles & Yu, 2010). Target-site herbicide resistance is sustained by modifications of the target site that impair the action of the herbicide. One well described case of such a mechanism involves resistance to the PSII-inhibiting triazine herbicide, shown to be due in most cases to a point mutation in the chloroplastic psbA gene, modifying the site at which triazine competes with plastoquinone within the PSII. Interestingly, this single resistance mutation has evolved independently worldwide (Arntzen et al., 1982). In contrast, non-target-site herbicide resistance implies usually a reduction in the concentration of active herbicide reaching the target site (Powles & Yu, 2010), including often the action of membrane pump-catalyzed active efflux or compartmentalization of the herbicide in plant cells (Windsor et al., 2003).

The present chapter aims to summarize current knowledge on the function of plant plasma membrane transporters in the efflux of herbicides during non-target-site herbicide resistance. Particular emphasis will be given to data obtained through the exploitation of gene-by-gene and genome-wide approaches applied to the single cell eukaryotic model, *Saccharomyces cerevisiae*, and the plant model, *Arabidopsis thaliana* (Cabrito et al., 2009a; Teixeira et al., 2007), and extrapolated, when possible, to plants with relevance in agriculture. Finally, a combined approach using the two model organisms aimed at deciphering the role of membrane transporters in auxinic herbicide resistance will be presented. The integration of the current knowledge on plant herbicide resistance in delineating rationales to design crops with increased herbicide tolerance, leading to the development of important strategies to overcome multiple herbicide resistance, is discussed.

2. *S. cerevisiae* and *A. thaliana* as model organisms to study herbicide resistance

Saccharomyces cerevisiae is widely accepted as a single cell eukaryotic model, being non-pathogenic, easy to manipulate genetically, well characterized, and exhibiting fast and inexpensive growth. The paradigm of research using *S. cerevisiae* has changed following the publication of its genome sequence more than a decade ago (Goffeau et al., 1996). Research on *S. cerevisiae* pioneered the development of several post-genomic experimental approaches and computational tools, which has allowed the gathering of large amounts of biological information easily accessible through public databases (SGD, Saccharomyces Genome Database – www.yeastgenome.com; and YEASTRACT - http://www.yeastract.com, among others). Large-scale biological material has also been produced, including a collection of mutants in which each yeast gene was individually deleted (EUROSCARF – http://web.uni-frankfurt.de/fb15/mikro/euroscarf) (Kelly et al., 2001), facilitating quick, easy and high-throughput search for genes involved in the resistance or susceptibility to any given environmental stressor. Genome-wide analyses in yeast have been successfully used to identify the genes responsible for yeast response and resistance to environmental stress,

in particular those induced by xenobiotic compounds of agricultural interest, such as the herbicide sulfometuron methyl (Jia et al., 2000), the dithiocarbamate fungicides mancozeb (Dias et al., 2010; Santos et al., 2009), thiuram, zineb and maneb (Kitagawa et al., 2003), the benzimidazole fungicide benomyl (Lucau-Danila et al., 2005), the pesticide lindane (Parveen et al., 2003), and the herbicide 2,4-D (Teixeira et al., 2007; Teixeira et al., 2006; Teixeira et al., 2005). Even though many cytotoxic compounds used in agriculture may act in their target organisms via physiological mechanisms that do not exist in yeast, many of the basic mechanisms underlying resistance and adaptation to chemical and environmental stresses are apparently conserved among phylogenetically distant organisms (Landis & Yu, 1999). Therefore, the characterization of mechanisms of resistance to herbicides in yeast cells is expected to contribute to the understanding of these mechanisms in more complex and less easily accessible eukaryotes, such as weeds and crops.

The small flowering plant *Arabidopsis thaliana* is extensively used as a model organism in plant biology. *Arabidopsis* has been the focus of intense genetic, biochemical and physiological study for more than 40 years because of its rapid life cycle, small size and prolific seed production, offering important advantages for laboratory manipulations and cultivation. The complete *Arabidopsis* genome sequence (e.g. http://www.arabidopsis.org, http://www.tigr.org/tdb/e2k1/ath1) has been available since 2000 (The Arabidopsis Genome, 2000), and genome-wide expression approaches have been applied to it with success. Transformation protocols using *Agrobacterium tumefaciens* have also been optimized and large sets of mutant lines are available (e.g. T-DNA Express database - http://signal.salk.edu), making *Arabidopsis* the system of choice for molecular and system-wide studies of plant responses to chemical stress.

3. Multiple herbicide resistance in yeast and plants

3.1 Non-target site mechanisms of resistance to herbicides in plants

The understanding of the molecular mechanisms underlying acquired herbicide resistance is crucial to deal with the emergence of resistant weeds. As stated above, there are two mechanisms involved in weed herbicide resistance: target-site and non-target-site herbicide resistance (Powles & Yu, 2010). The mechanisms underlying non-target-site herbicide resistance are usually the cause for unexpected multiple herbicide resistance (Yuan et al., 2007; Preston, 2004; Preston et al., 1996).

Non-target-site herbicide resistance has been proposed to be caused by a plant detoxification process with three phases (Martinoia et al., 1993; Bartholomew et al., 2002; Sandermann, 2004; Yuan et al., 2007). Phase I consists in metabolic changes of the herbicide within the plant, typically oxidation, peroxydation, or reduction mainly by P450 monooxygenases, resulting in the formation of less toxic metabolites. During phase II, herbicides or their metabolites are directly conjugated with glutathione, sugars or amino acids to produce hydrophilic molecules that are easily handled by the plant cell. Phase III consists in the translocation of the resulting conjugated metabolites into the vacuole or the extracellular space through the action of transporters. Further degradation of the resulting molecules may then occur in the vacuole or the extracellular space. Non-target-site herbicide resistance has been described to involve mainly P450 monooxygenases (phase I), glutathione S-transferases and glycosyltransferases (phase II) and ABC (ATP-binding cassette) transporters (phase III) (Reade et al., 2004; Yun et al., 2005; Bowles et al., 2005; Klein et al., 2006).

Although this three-step mechanism of non-target-site herbicide resistance is largely recognised, it is more than probable that it is not sustaining the resistance to some classes of herbicides. Such is the case of glyphosate and paraquat, two herbicides for which mutation of the target site or phase I metabolism cannot explain entirely the multiple herbicide resistant phenotype observed (Coupland, 1985; Feng et al., 1999; Lorraine-Colwill et al., 2002). In these cases, decreased herbicide influx into the plant, decreased level of translocation within the plant, and/or increased rate of sequestration/efflux could be the mechanisms underlying the reduction of the amount of herbicide reaching the target site. All of these three mechanisms involve the action of membrane transport systems. Therefore, and owing to their high relevance in herbicide resistance, this chapter will focus on the action of membrane pumps, mainly those putatively involved in efflux during MDR.

3.2 Multidrug efflux pumps involved in herbicide resistance

Several membrane transport systems have been shown to be involved in drug resistance, both in prokaryotes and eukaryotes. One of the first systems of this nature to be described was the mammalian P-glycoprotein efflux pump (Kartner et al., 1983); cell lines overexpressing this transporter were shown to display multiple resistance to drugs. As a matter of fact, many of these membrane transporters play an important role in conferring MDR, presumably due to the catalysis of energy-dependent extrusion of a large number of structurally and functionally unrelated compounds out of the cells or within cell compartments, against their concentration gradient (Balzi & Goffeau 1995; Bolhuis et al., 1997; Hayes & Wolf 1997; Roepe et al., 1996).

On average, the predicted multidrug pumps represent more than 10% of all transporters in a cell. The increasing number of multidrug transporters identified so far is a clear indication that MDR by efflux pumps is not an exceptional phenomenon, but a highly conserved defence mechanism. Significantly, most multidrug transporters share significant homology with transporters with more specific substrates (Paulsen, 2003). Based on bioenergetic and structural criteria, drug transporters are classified as ATP-dependent or secondary transporters (Bolhuis et al., 1997). The latter can be driven by the electrochemical proton gradient or proton motive force (Δp), composed of an electrical potential ($\Delta\psi$; negative interior) and a chemical proton gradient (ΔpH; higher interior). Currently, two large ubiquitous superfamilies, the major facilitator (MFS) and the ATP binding cassette (ABC) superfamilies (Bolhuis et al., 1997; Jack et al., 2001; Sá-Correia et al., 2009; Crouzet et al., 2006), are recognized to include multidrug efflux pumps in eukaryotes. Structures have now been obtained for multidrug transporters of each of these families (see Higgins, 2007). Further discussion will be focused on these two types of transporters and their involvement in MDR and herbicide resistance.

3.2.1 ABC transporters

ABC transporters constitute one of the largest gene families in all living organisms, using the free energy of ATP hydrolysis to drive substrate transport across cell membranes (Bolhuis et al., 1997). These proteins mediate diverse cellular transport processes, having been reported to transport a wide range of substances, including ions, carbohydrates, lipids, xenobiotics, antibiotics, drugs and heavy-metals. They can also work as ion channels or display antigen activity (Martinoia et al., 2002). The mammalian P-glycoprotein is probably the best characterised of all ABC transporters, but many others have been described in

bacteria and fungi. The minimal functional unit of all ABC transporters consists of four domains: two hydrophobic transmembranar domains, each with six transmembrane α-helical segments (TMS), plus two cytoplasmic, nucleotide-binding domains containing the ATP-binding cassette. The four can be fused into a single polypeptide, as in the mammalian P-glycoprotein. This typical 'two times two' domain organisation (Bolhuis et al., 1997) probably arose from an internal gene duplication event. On the other hand, most bacterial ABC transporters comprise only one TMS and one nucleotide-binding domain.

The *S. cerevisiae* genome encodes a total of 22 ABC proteins (Paumi et al., 2009). Among these, 16 are about twice as long as the remainder, the former having 12 TMS (indicative of a putative duplication of the half-size ABC transporters). The 22 identified yeast ABC transporters belong to four families, recently re-designated as ABCB, ABCC, ABCD and ABCG (formerly named MDR (Multi Drug Resistance), MRP (Multidrug Related Protein), ALDP (AdrenoLeukoDystrophy protein) and PDR (Pleiotropic Drug Resistance)) (Paumi et al., 2009). The ABCB family includes four members, three of which are half transporters that dimerize to form full transporters localized to the mitochondrial inner membrane (Mdl1p, Mdl2p and Atm1p) and Ste6p, the plasma membrane a-factor pheromone exporter. The ABCC family includes five vacuolar membrane transporters, two of which, Ycf1p and Bpt1p, are glutathione conjugate transporters involved in cytosolic detoxification from metal ions, and Yor1, a plasma membrane multidrug transporter. The ABCD family is composed of Pxa1p and Pxa2p that transport acyl-CoA across the peroxisomal membrane. Finally, the PDR family in yeast comprises nine transporters (Pdr5p, Pdr10p, Pdr11p, Pdr12p, Pdr15p, Pdr18p, Snq2p, Aus1, Adp1 and Yol075c), which can be seen as the cell's primary line of defence: PDR function not only decreases the cell's sensitivity to many unrelated chemical stresses, but also protects it against endogenous toxic metabolites (Paumi et al., 2009; Jungwirth & Kuchler 2006). Interestingly, PDR genes are only found in plants and fungi.

In *S. cerevisiae* only one ABC transporter, Pdr5p, was described as a determinant of resistance to herbicides (Teixeira & Sá-Correia, 2002). Transient activation (two fold) of *PDR5* transcription takes place during the adaptation period preceding cell division under stress induced by the auxin-like herbicides 2-methyl-4-chlorophenoxiacetic acid (MCPA) or 2,4-dichlorophenoxiacetic acid (2,4-D). *PDR5* induction is mediated by both Pdr1p and Pdr3p, two transcription factors controlling MDR in yeast and, as soon as adapted cells start duplication under herbicide stress, mRNA levels are drastically reduced to basal values (Teixeira & Sá-Correia, 2002). Differently from what has been seen for fungicides and drugs, resistance to herbicides has not been extensively studied in *S. cerevisiae*. Thus, it is expectable that many other of the yeast ABC multidrug transporters may also render the cell herbicide resistant..

The *Arabidopsis* genome was found to encode about 600 predicted membrane transporters, a large number of which belong to the ABC superfamily. In fact, this superfamily alone comprises 131 members, with 53 encoding full-size and the remainder half-size transporters. *Arabidopsis* genes encoding full-size ABC transporters are significantly more numerous than those reported in other eukaryotes such as yeast and humans, but the reasons for such diversification in the plant kingdom are unknown. The great majority of plant ABC genes remains uncharacterised, with those encoding full-size transporters being the best studied to date. These can be subdivided into three main groups: the MDR or PGP (P-glycoproteins), the MRP and the PDR gene families (Martinoia et al., 2002).

The MDR-PGP family is the largest, consisting of 22 *Arabidopsis* genes coding for full-size ABC transporters. The first demonstration of an MDR-like mechanism in plants, able to

confer tolerance to different herbicides, stemmed from functional studies on *AtPGP1*. Overexpression of this P-glycoprotein in *A. thaliana* conferred increased resistance to herbicides from different chemical classes, such as dicamba, pendimethalin, oryzalin or monosodium acid methanearsonate (MSMA), suggesting a resistance mechanism related to decreased retention or increased active toxin efflux from plant cells (Windsor et al., 2003). Loss of function approaches later showed that both *AtPGP1* and the closely related *AtMDR1* participate in auxin efflux required for polar auxin transport in *Arabidopsis* (Lin & Wang, 2005). In fact, all plant PGP transporters characterized so far appear to be involved in cellular and long-distance auxin transport (Terasaka et al., 2005; Geisler & Murphy 2006; Lewis & Muday 2009), pointing to a role of these plant transporters in growth and development in addition to detoxification processes.

The best characterized plant ABC transporters to date belong to the MRP gene family. The *Arabidopsis* genome includes a small redundant family of 15 *AtMRP* isogenes, which encode vacuolar sequesters of glutathione conjugates potentially involved in phase III of the detoxification process during non-target-site herbicide resistance. Five of the *Arabidopsis* MRPs (*AtMRP1-5*) have been biochemically characterized and shown to encode *bona fide* functional transporters of model glutathione conjugates after heterologous expression in *S. cerevisiae*. In addition, several are able to transport other amphipatic organic anions, such as linear tetrapyrroles derived from chlorophyll catabolism and folates (Lu et al., 1998; Raichaudhuri et al., 2009). Functional analysis of four *Arabidopsis* MRP isogenes (*AtMRP1*, *2*, *11* and *12*) has shown that their contribution to detoxification is marginal, as the corresponding loss-of-function mutants showed no drastic changes in sensitivity towards different herbicides. Only a knockout allele for *AtMRP2* exhibited reduced sensitivity to 1-chloro-2,4-dinitrobenzene, a classical glutathione *S*-transferase substrate (Frelet-Barrand et al., 2008). These results may be explained by putative high levels of functional redundancy among MRP *Arabidopsis* transporters. On the other hand, it is also likely that the contribution of vacuolar sequestration during phase III of the detoxification process has been largely overestimated, with plasma membrane efflux pumps potentially playing a more preponderant role than recognised so far.

The third group of plant ABC transporters was defined based on similarity to the yeast *PDR5* gene and, in *Arabidopsis*, includes 15 members encoding putative efflux transporters for cytotoxic compounds. Expression of *SpTUR2* from the water plant *Spirodella polyrhiza,* the first plant PDR gene identified, is induced by abscisic acid, cold and salt stresses, suggesting a role for the encoded transporter in the response to abiotic stress (Smart & Fleming, 1996). Interestingly, the herbicide 2,4-D also has an inductive effect on the transcription of *SpTUR2*, and expression of *SpTUR2* in *Arabidopsis* confers increased resistance to the antifungic sclareol and to lead (van den Brule & Smart, 2002). Expression of another member of the PDR gene family, *NpABC1* from wild tobacco, is also strongly induced by sclareol and has been suggested to be involved in its excretion from the tobacco cell (Jasinski et al., 2001). In *Arabidopsis*, three PDR transporters have been characterized at the functional level. These include *AtPDR8*, which is a key factor controlling the extent of cell death upon pathogen infection (Kobae et al., 2006) and contributes to non-host resistance (Stein et al., 2006), having also been identified as a plasma membrane cadmium extrusion pump (Kim et al., 2007). *AtPDR12* is regulated by multiple plant defence signalling pathways and, as its homologue *SpTUR2*, confers resistance to the diterpenoid sclareol (Campbell et al., 2003) and lead (Lee et al., 2005). In addition, *AtPDR12* was recently reported to be a plasma membrane ABA uptake transporter (Kang et al., 2010). Importantly,

another *Arabidopsis* PDR member, *AtPDR9*, a homologue of *S. cerevisiae PDR5*, has recently been shown to encode a 2,4-D efflux facilitator localized in the plasma membrane; while overexpression of *AtPDR9* leads to 2,4-D resistance and hypoaccumulation, a loss-of-function mutant for this gene displays increased sensitivity and hyperaccumulation of the herbicide (Ito & Gray, 2006). Results from a more recent study suggest that *AtPDR9* also facilitates the efflux of the auxin precursor IBA from root cells (Strader & Bartel, 2008). Although none of the 23 *PDR* genes identified in *Oryza sativa* has been functionally analysed so far, expression of many of these genes is induced in response to diverse abiotic stresses (Moons, 2008), suggesting that these transporters may also play a role in conferring resistance to chemical stress agents in rice.

3.2.2 MFS-MDR transporters

The MFS is a very large and ancient superfamily found throughout nature, from bacteria to mammals, and is energised by the electrochemical gradient across membranes. Diverse substrates show affinity to these transporters in a proton motive force dependent mechanism of symport, antiport or uniport. The MFS proteins are involved in drug resistance, among other cellular functions (Paulsen et al., 1996). Among the eleven known families of MFS transporters in yeast (www.tcdb.org), MFS multidrug resistance (MFS-MDR) efflux pumps are proposed to be drug:H^+ antiporters, belonging to the DHA1 family (12 predicted spanners) or the DHA2 family (14 predicted spanners). These transporters comprise at least 23 proteins that have largely escaped characterisation by classical approaches, as most of the present knowledge on these putative drug:H+-antiporters was driven by the disclosure of the *S. cerevisiae* genome sequence in April 1996 (Goffeau et al., 1996; Sá-Correia et al., 2009).

Among the MFS-MDR, Tpo1p, which belongs to the DHA1 family, is the transporter with the broadest described range of substrates. In addition to polyamine excretion, *TPO1* is involved in resistance to cytotoxic compounds ranging from antimalarials to herbicides and fungicides (Alenquer et al., 2006; Markovich et al., 2004; Sá-Correia et al., 2009; Tomitori et al., 2001; Tomitori et al., 1999; Tucker & Fields, 2001; Kennedy & Bard, 2001). This transporter is a yeast determinant of resistance to the auxin-like herbicides 2,4-D and MCPA (Teixeira & Sá-Correia 2002). In addition, the DHA1 transporters Qdr1 (Vargas et al., 2004), Qdr2 (Vargas et al., 2004) and Qdr3 (Tenreiro et al., 2005) were found to be determinants of yeast resistance to the herbicide barban.

Although several *S. cerevisiae* MFS transporters have been linked to MDR, this is not the case in plants. The *Arabidopsis* genome encodes a wide array of metabolite transporters, around 100 within the MFS family alone. Available sequence data indicate that a similar situation exists in other plant species as well (Williams et al., 2000). The best characterised subfamily of MFS transporters in plants is the monosaccharide-like transporters one, whose several members are indeed involved in sugar transport (Reinders et al., 2006; Buttner 2007). Another subfamily has been implicated in phosphate transport (Stefanovic et al., 2007; Mudge et al., 2002). To date, only two plant MFS transporters have been related to chemical stress resistance: *A. thaliana ZIF1* is a tonoplast-localized protein that influences zinc tolerance and accumulation (Haydon & Cobbett, 2007) and, in maize, Zm-mfs1 was identified as a transcript induced by a range of defence-related conditions, including fungal pathogenic infection (Simmons et al., 2003). By direct *in silico* methods few MFS-MDR family members have been predicted in *A. thaliana* or in any higher eukaryote, including humans.

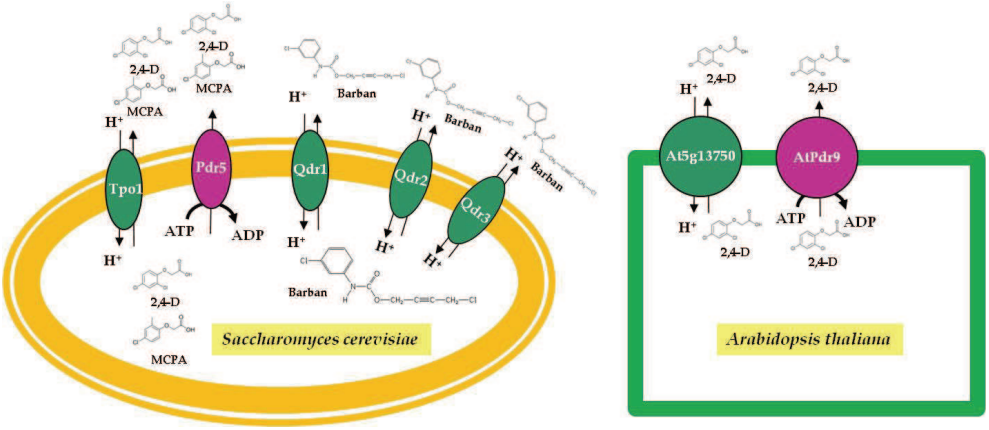

Fig. 1. Proposed model for the role of the so far described *S. cerevisiae* and *A. thaliana* ABC efflux pumps (purple) and MFS-MDR transporters (green) involved in herbicide stress resistance. The role of At5g13750 in 2,4-D stress resistance in plants, proposed based on the results obtained through heterologous expression in yeast, remains to be clarified.

Mima *et al.* (2007) identified a human orthologue of the yeast MFS-MDR gene *TPO1*, *TETRAN*. Although sequence homology was relatively low, the authors were able to show that both *TPO1* and *TETRAN* are capable of conferring NSAIDs (non-steroidal anti-inflammatory drug) resistance. There are also reports that plant pathogenic fungi utilise MFS drug:H^+ antiporters to export their own toxins, thus rendering themselves resistant, while delivering toxins to the plant (Del Sorbo et al., 2000).

The data linking MFS transporters and MDR in higher eukaryotes are less numerous and conclusive than those available for ABC transporters, and the role of the plant MFS is just beginning to be unravelled. This could reflect increased functional redundancy among the MFS transporter family, but also perhaps decreased interest in analysing their involvement in MDR. It is consequently of utmost importance to further investigate the potential role of MFS transporters in the MDR phenomenon in higher plants. Results stemming from the combined study described below strongly strengthen the hypothesis that a subfamily of plant MFS transporters may be involved in MDR. Furthermore, it is expected that genome-wide expression analysis applied to the study of plant response to stress induced by herbicides may bring additional clues to the functional analysis of MDR transporters in plants. For example, the observed up-regulation of the putative MFS encoding ORF *At1g79410* in *A. thaliana* exposed to herbicidal concentrations of 2,4-D suggests that this transporter may play a role in the adaptation to this stress (Raghavan et al., 2005).

4. Mechanisms underlying auxin-like herbicide resistance in *S. cerevisiae* and *A. thaliana*: a case study

2,4-dichlorophenoxyacetic acid (2,4-D) is the most commonly used member of the auxin-like herbicide family and, having been introduced in 1946, is still one of the most widely used

herbicides in the world. The effects of 2,4-D in weeds include epinastic bending and growth abnormalities, its toxicity depending mainly of the acid form (Twonson, 1997). Recent genome-wide gene expression studies focused on the *Arabidopsis thaliana* response to herbicidal concentrations of 2,4-D report the remodelling of its transcriptional repertoire at the level of genes involved in the auxin response, ethylene signalling and ABA biosysthesis, signalling and response (Raghavan et al., 2005; Raghavan et al., 2006). *S. cerevisiae* has been intensively used as a model to investigate the mechanisms underlying herbicide resistance, focusing on 2,4-D. In low pH environments, the highly lipophilic weak acid 2,4-D exists in its undissociated lipophilic toxic form (RCOOH), which can readily cross the plasma membrane by passive diffusion. In the neutral cytosol, the 2,4-D molecule dissociates leading to internal acidification (Fernandes et al., 2003; Simões et al., 2003) and to accumulation of the toxic counter-ion ($RCOO^-$) that cannot easily cross the plasma membrane lipid bilayer. Therefore, at low pH (e.g. acidic soils, the alimentary canal of animals) the toxic potential of the herbicide increases dramatically (Cabral et al., 2003). In acidified growth medium, suitable for fungal growth, yeast cells challenged with the herbicide 2,4-D suffer a strong reduction in their cytosolic and vacuolar pH (Fernandes et al., 2003; Simões et al., 2003), which is counteracted by the activation of the plasma and vacuolar membrane H^+-ATPases (Fernandes et al., 2003; Teixeira et al., 2005). Significantly, auxins were also shown to induce the activity of the *Arabidopsis* plasma membrane H^+-ATPase, contributing to maintain the intracellular pH in plant roots (Shen et al., 2006).

Based on the participation of the MDR transporters Tpo1p and Pdr5p (belonging to the MFS and ABC superfamily, respectively) in yeast resistance to 2,4-D, the active export of the 2,4-D counter-ion catalysed by these plasma membrane transporters was postulated (Teixeira & Sá-Correia, 2002). Interestingly, the *Arabidopsis PDR5* homologue *AtPDR9* was recently shown to encode a 2,4-D plasma membrane efflux facilitator contributing to 2,4-D resistance in this plant (Ito & Gray, 2006). The expression of the *S. cerevisiae TPO1* gene was shown to decrease the accumulation of the herbicide 2,4-D counter-ion in yeast cells, indicating that this gene is also, directly or indirectly, involved in 2,4-D export (Cabrito et al., 2009a). Other details on the yeast adaptive response to the mechanisms underlying 2,4-D toxic effects were reviewed by Teixeira *et al.* (2007) and are summarized in Figure 2.

Since the *TPO1* gene was previously found to confer resistance to 2,4-D in yeast (Teixeira & Sá-Correia, 2002), to be transcriptionally activated in response to the herbicide (Teixeira & Sá-Correia, 2002; Teixeira et al., 2006), and required to reduce the intracellular concentration of the 2,4-D counter-ion (Cabrito et al., 2009a), ScTpo1p homologs encoding putative plasma membrane MFS transporters from the plant model *A. thaliana* were analysed by Cabrito *et al.* (2009) for a possible role in 2,4-D resistance. *At5g13750* transcript levels were found to increase in 2,4-D stressed plants. The functional heterologous expression of this plant ORF in yeast was found to confer increased resistance to the herbicide in wild-type and *Δtpo1* cells, through the reduction of the intracellular concentration of 2,4-D counter-ion. Heterologous expression of *At5g13750* in yeast also leads to increased resistance to Al^{3+} and Tl^{3+}. Hence, *At5g13750* gene-encoded protein is the first plant putative MFS transporter to be suggested as possibly involved in MDR (Cabrito et al., 2009a). These new insights suggesting a role for higher eukaryotic MFS transporters in multidrug resistance may open an entirely new field of research with promising repercussions not only in agriculture but also in medicine and biotechnology.

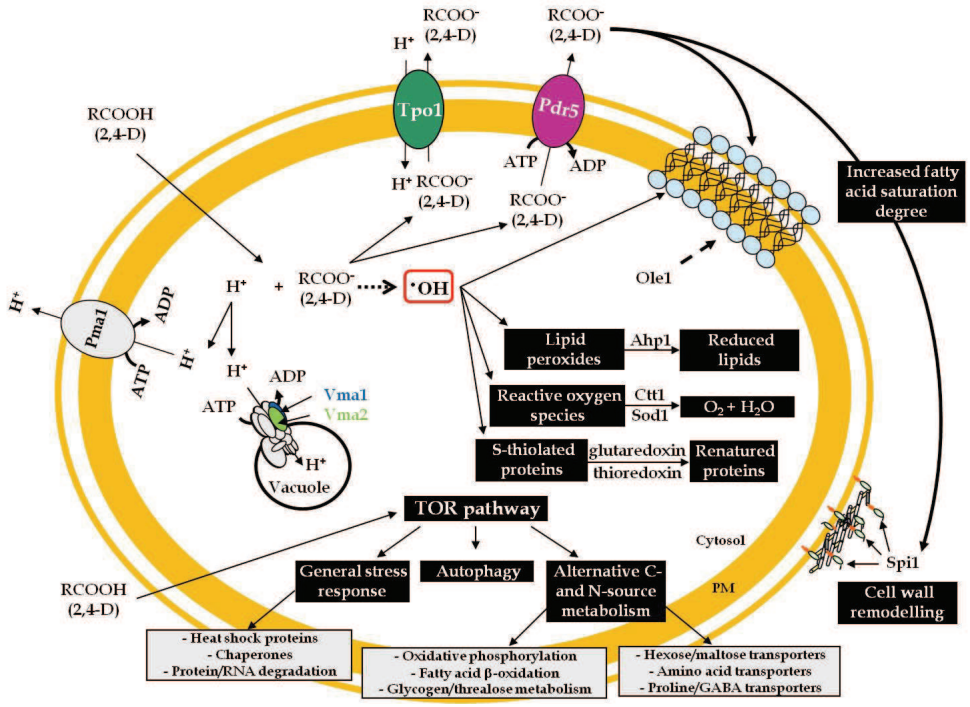

Fig. 2. Model for the adaptive yeast response to 2,4-D.

5. Conclusions and perspectives

Multiple herbicide resistance is a widespread phenomenon among weed species and poses severe agro-economical and environmental problems (Hayes & Wolf, 1997). At the same time, herbicide-resistant crop strains have been developed during the past decades, altering profoundly agricultural practices as this approach allows the use of specific herbicides in previously sensitive crops. The use of genetic engineering to express multidrug efflux pumps, identified as specific for a given herbicide, has been suggested as a method to increase crop resistance to that particular herbicide (Windsor et al., 2003). Thus, the identification of new MDR plant transporters playing a role in herbicide resistance may prove an invaluable asset in modern and future agricultural practices. Such studies are facilitated by the use of *S. cerevisiae* as a eukaryotic model system in the search for herbicide resistance determinants. Similar extrapolations have been made for agricultural fungicide resistance in phytopathogenic fungi (Cabrito et al., 2009b). The use of yeast as a host for the heterologous expression of plant transporters has also been considered. Although a number of reports have found this task extremely difficult mainly due to the inhibitory effect that the overexpression of foreign membrane proteins appears to have on yeast viability (Crouzet et al., 2006; Ito & Gray, 2006; van den Brule & Smart, 2002), Cabrito *et al*. (2009a) managed to optimize heterologous expression conditions in order to functionally express the plant gene *At5g13750* in the yeast cell. These results reinforce the notion that *S. cerevisiae* is highly suitable not only as a eukaryotic model system but also as an experimental platform for the

study of the MDR phenomenon in higher eukaryotes. Furthermore, approaches combining *S. cerevisiae* and the plant *A. thaliana* as a model for functional and comparative genome-wide studies in weed species can help us understand better the mechanisms underlying herbicide resistance.

Based on the herbicide resistance mechanisms identified so far, different approaches aimed at improving the resistance of crops against various herbicides have been developed in the last decade. Initial practices relied on the type and application rate of the herbicide used in the agrosystem. For instance, herbicide tolerance can be improved by the use of safeners, synthetic compounds that enhance herbicide tolerance in selected monocot crops without impairing herbicide susceptibility in target weeds. They are suggested to reduce toxicity in crops by inducing the expression of glutathione-S-transferases capable of conjugating the herbicide to glutathione. This approach has been successful in different crops, such as maize, sorghum, wheat, rice and barley (DeRidder & Goldsbrough, 2006). The second and most successful contribution of modern agricultural biotechnology was the introduction of herbicide resistance traits in crops. Traditional methods involved plant breeding, but in the last decade the most promising approach consists in the generation of transgenic crops exhibiting cross resistance to various classes of herbicides. In 2005, only three years after the introduction of the first transgenic crop in the field, over 52 million hectares of transgenic crops were planted worldwide, and from these 41 million hectares were planted with herbicide-resistant crops (Owen & Zelaya, 2005). The vast majority of transgenic crops consists of soybean containing a bacterial gene that encodes a glyphosate-insensitive form of the enzyme 5-enolypyruvylshikimate-3-phosphate synthase (EPSPS), catalyzing the penultimate step of the shikimate pathway, the glyphosate target. This transgenic soybean enables glyphosate, a non selective herbicide active on nearly all plant species, to be used selectively after soybean emergence (Dill, 2005). Similar approaches are currently being pursued for further development. For example, expression of the human cytochrome P450 monooxygenase encoded by the *CYP1A1* gene in rice plants has been recently shown to confer broad cross resistance towards various herbicides with different structures and mode of action, via an increased and quicker metabolism of these herbicides (Kawahigashi et al., 2007).

Similarly, the identification of an MDR transporter functioning as a multi-component detoxification system in plants could open the possibility of developing engineered plants, thus achieving useful phenotypes from the agricultural point of view. Plants grow in diverse environments, in which their roots are exposed to toxic or inhibitory chemical substances. Toxins can be pollutants, such as herbicides or the xenobiotic products of neighbouring plants or endemic microorganisms. Phytoremediation — i.e. the use of plants and their associated microbes for the remediation of soils contaminated with organic and inorganic pollutants — can also represent a positive outcome of the MDR phenomenon. The phytoremediation efficiency of plants can also be substantially improved by engineering technologies, including overexpression of genes involved in the transport and sequestration of toxic compounds. The development of transgenic plants in heavy metal and metalloid soil decontamination has been largely prospected and revealed their usefulness and suitability (Kotrba et al., 2009). Again, identification of MDR efflux pumps involved in herbicide detoxification could also have an interest in phytoremediation approaches of herbicide-contaminated soils.

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Herbicides and Environment

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Herbicides are much more than just weed killers. They may exhibit beneficial or adverse effects on other organisms. Given their toxicological, environmental but also agricultural relevance, herbicides are an interesting field of activity not only for scientists working in the field of agriculture. It seems that the investigation of herbicide-induced effects on weeds, crop plants, ecosystems, microorganisms, and higher organism requires a multidisciplinary approach. Some important aspects regarding the multisided impacts of herbicides on the living world are highlighted in this book. I am sure that the readers will find a lot of helpful information, even if they are only slightly interested in the topic.

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