



Research article

Two anti-microtubular drugs for two differential responses: A rice cell line resistant to EPC remains susceptible to oryzalin

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ABSTRACT

Sensitivity to the two anti-microtubular drugs oryzalin and EPC (ethyl-*N*-phenylcarbamate) is shown to be uncoupled in the rice EPC-resistant ER31d cell line, derived from the corresponding ER31 mutant. The ER31d cell line grows in the presence of EPC but it remains susceptible to oryzalin. In the presence of concentrations of EPC up to 0.4 mM, ER31d cells remain viable maintaining cell anisotropy and detectable cortical microtubule array. The amount of α - and β -tubulin is also maintained high through a regulatory mechanism that operates at post-transcriptional level. In contrast, all these cellular and molecular parameters are heavily affected by the addition of 1 μ M oryzalin. Also, the pattern of post-translationally modified α -tubulins changes in the ER31d cells compared to that of their Nihon-Masari wild type line of reference. The different response elicited by the two herbicides is discussed in relation to a possible differential sensitivity of the cortical MT array, that may in turn relate to their different tubulin-binding specificities and chemical structure.

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1. Introduction

Plant microtubules (MTs) are essential players for many different cellular events such as growth, division, cell motility, production of the ER body, vesicular sorting, signal transduction, viral movement, cell wall deposition and response to environmental stress [1,2]. MTs are organized in different arrays depending on the phase of the cell cycle. Cortical arrays, that control cell expansion and influence helical growth [3], are present during interphase, while the cell division cycle is characterized by a sequence of three different arrays: the preprophase band, the spindle apparatus and the phragmoplast at the end of mitosis [4]. Because MTs are the main components of the animal and the plant mitotic spindle, they represent ideal targets for compounds with

anti-mitotic activities that are largely used in medicine (anti-tumor drugs) and in agriculture (herbicides).

Dinitroanilines (oryzalin, trifluralin and pendimethalin) are anti-mitotic herbicides that bind to the α -tubulin moiety of the tubulin α/β heterodimer, the main constituent of the MTs [5,6]. Despite the identification of few, shared amino acid residues, efforts to precisely map the oryzalin binding site have produced distinct results in two different model systems [7,8]. In one case, binding of oryzalin is mapped at the dimer interface [7] whereas in the other at the loop that stabilizes the lateral contacts between protofilaments [8]. At the whole-plant level, oryzalin treatment produces root-tip swelling into club-shaped structures that results from the loss of individual cell anisotropy [9–12]. Oryzalin eliminates the cortical MT array thus interfering with the MT-dependent guided deposition of cellulose. Due to the geometry of the cylindrical plant cells, the alteration and the eventual loss of cell wall architecture that is caused by oryzalin, eliminate the biophysical reinforcement upon the isotropic turgor, such that the cells round up [13]. At low concentrations (0.1 μ M), oryzalin decreases the degree of right-handed helical growth of seedlings of the *spr1* and *spr2 Arabidopsis thaliana* L. mutants [3]. A subsidiary effect of oryzalin on the morphology of the endoplasmic reticulum has also been reported [14].

EPC (ethyl-*N*-phenylcarbamate, phenyl urethane) is an antimicrotubular herbicide of the phenylcarbamate group of chemical compounds, with a binding site that was identified at the

Abbreviations: EPC, ethyl-*N*-phenylcarbamate (phenyl urethane); ER31, EPC-resistant mutant 31; ER31d, a rice cell line derived from the ER31 mutant; MAP, microtubule associated protein; MT, microtubule; MTOC, microtubule organizing center; NM, *Oryza sativa* L. cv. 'Nihon-Masari' (wild type background for ER31); Oryzalin, 3,5-dinitro-N4,N4-dipropylsulfanilamide; PCV, packed cell volume; PTM, post-translational modification.

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C-terminal end (C-ter) of plant α -tubulins [15]. The C-ter acidic domain loops out from the main structure of the microtubules thus providing the molecular tag for a series of interactions of regulatory significance [16] that can be modulated by different post-translational modifications (PTMs) [17].

Until recently, antimicrotubular herbicides were all assumed to share a common mode of action, whereby the tubulin heterodimer–herbicide complex sequesters the microtubule subunits from integration into the growing end of the microtubules [18]. As a consequence, the equilibrium of dynamic MTs growth is shifted toward disassembly, and this eventually leads to MTs catastrophe. Such a mechanism has been evoked for microtubule disrupter herbicides of the cyanoacrylate and dinitroaniline types (as oryzalin) that elicit a complete loss of microtubule structures, including cortical, preprophase, spindle and phragmoplast arrays [19,20]. According to the proposed model, *in vitro* experiments have shown that pendimethalin completely inhibits tubulin polymerization [20]. No clear experimental evidences that carbamates can also elicit a similar spectrum of responses have ever been produced. On the contrary, with concern to the mechanism of action, a direct interaction of IPC (isopropyl-*N*-phenylcarbamate) with MTs was shown in *in vitro* polymerization studies on mung bean tubulin [21] and yet plant microtubules may not be the principal target for carbamates because it has been reported, in lines of *Nicotiana sylvestris* L., that IPC primarily damages MTOCs (Microtubule Organizing Centers) possibly interacting with one protein of the complex [22].

Differential responses of the different MT arrays to different drugs and a different dosage of the same herbicide have been recently brought to light. As already mentioned, oryzalin, if used at low concentrations, can determine helical growth with no effect on division. Citral, a monoterpene, has been recently reported to primarily affect mitotic MTs, with respect to cortical MTs, in both wheat roots and BY2 cells [23]. Flamprop-M-methyl is an antimicrotubular herbicide that selectively affects spindle and phragmoplast formation with only minor consequences on the cortical MT array [20].

The level of MTs sensitivity to different antimicrotubular herbicides can also depend on the binding affinity and on the ratio between the amount of cytosolic tubulin dimers and the rate of MT turnover. PTMs of tubulin may influence the process by altering both MT intrinsic stability and the specificity of drug binding. More specifically, de-tyrosination and re-tyrosination of a highly conserved tyrosine residue at the C-ter of the α -tubulins have been consistently associated with changes of MT dynamic [24,25] while the presence of tyrosine as the last amino acid was shown to reduce affinity for EPC [15]. The binding site of EPC, mapped by affinity chromatography at the last thirteen amino acids on the C-ter of maize isotype2 α -tubulin, was shown to be negatively influenced by the presence of tyrosine [15]. Conversely, efficient binding is restored by de-tyrosination that is a signature for more stable microtubules. This specificity of binding offered an explanation for EPC sensitivity that relies on the status of microtubule turnover. The eventual effect of EPC is therefore dependent on different factors that may include the actual level of α -tubulin tyrosination, the rate of dimers exchange at the growing end of the microtubule and the actual binding status of the C-ter, i.e. to MAPs.

An EPC-resistant 31 (*ER31*) mutation was originally isolated from a rice population exposed to gamma ray irradiation. The *ER31* mutation, found to be lethal at the homozygous state, was shown to affect microtubule reorientation in response to auxin [26]. Viable heterozygous *ER31* rice plants exhibit a lowered rate of MT assembly and disassembly that renders them less susceptible to the EPC treatment. The *ER31* resistant phenotype was explained either as the result of changes in the MTs composition of tubulin isotypes

or as a consequence of alterations in the pattern of microtubule-associated proteins [26].

If the mode of action of carbamates is different compared to dinitroanilines, then it should be possible to find mutants where sensitivity to one of these two types of drugs becomes uncoupled from the other. In this study, a rice (*Oryza sativa* L.) cell line derived from the *ER31* mutant has been instrumentally used as a tool to investigate differences in the mode of action of EPC compared to oryzalin. We report that the *ER31d* cell line is sensitive to oryzalin but resistant to EPC. This differential response associates with several changes occurring at both cellular and molecular levels involving rice root morphology, cell anisotropy, microtubule integrity, tubulin abundance and pattern of post-translational modifications.

2. Results

2.1. Differential effects of EPC and oryzalin at cellular level

The differential effect of EPC on seedling growth of the rice cultivar Nihon-Masari (NM) cv and the isogenic *ER31* mutant was preliminarily evaluated (Supplementary Fig. 1). As expected, 1 mM EPC strongly reduced coleoptile and root growth in wild type NM seedlings while it only moderately affected growth of both organs in the *ER31* seedlings. Significantly, neither NM nor *ER31* primary roots showed relevant changes in shape and diameter when treated with EPC, which was in contrast to the effect obtained with oryzalin. In fact, roots of seedlings germinated in the presence of 1 μ M oryzalin just emerged from the seed assuming a club-like form (Supplementary Fig. 1), a response that is consistent with loss of cell anisotropy.

The differential effect of the two drugs on seedling roots was further substantiated by the data obtained from two cell lines, derived from the NM wild type and the *ER31* mutant, respectively. The effect of different concentrations of oryzalin and EPC on cell viability and growth was followed over a period of seven days of continuous treatment (Figs. 1 and 2). Oryzalin reduced viability of both cell lines progressively, in a dose-dependent manner. Already 1 μ M oryzalin reduced cell viability by 20% within two to three days of treatment. Higher concentrations were even more effective, whereas 0.1 μ M had a limited effect with 20% reduction scored after seven days of treatment in both the NM and *ER31d* cell lines. In contrast to oryzalin, the response of the two cell lines to EPC was different. While NM cells lost viability progressively at each of the different EPC concentrations, the *ER31* cell line remained perfectly viable (Fig. 1). After 48 h of treatment with 0.3–0.4 mM EPC the EPC-sensitive NM line shows the same degree of reduction in cell viability as that observed with 1 μ M oryzalin.

The response of cell viability only partially correlated with the response of Packed Cell Volume (PCV; Fig. 2). Here, oryzalin treatments increased the PCV value well over that of the control untreated NM and *ER31d* cells, and this effect increased with increasing oryzalin concentrations. In contrast, the PCV for EPC-treatment decreased progressively with increasing EPC-concentration for the NM wild type and remained essentially unaffected for the *ER31d* line (Fig. 2). Since the PCV value of the resistant *ER31d* line increased linearly with time independently from the presence of EPC (Fig. 2), the reduced PCV value observed in the sensitive NM cell line is likely to reflect the loss of viability previously documented (Fig. 1).

To understand the apparent discrepancy between PCV and viability responses observed for oryzalin, cell morphology was scrutinized under the microscope (Fig. 3). After oryzalin treatment, cells were swollen and round in both the EPC resistant and EPC sensitive lines, accounting for the increase in PCV. In contrast, normal anisodiametric morphology and cell size were maintained in both lines after EPC treatment. To test, whether the different

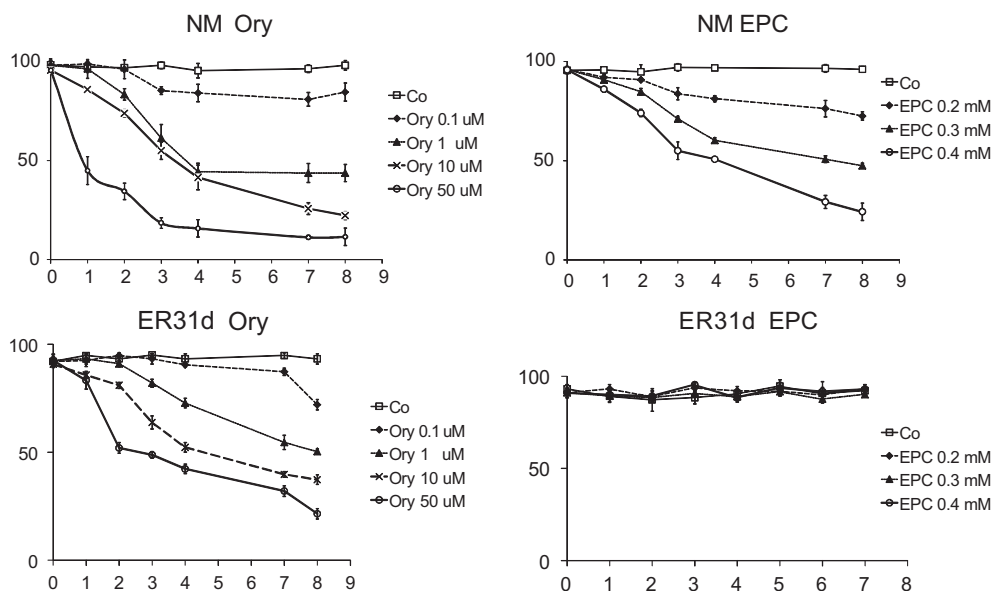


Fig. 1. Effect of different concentration of oryzalin and EPC on NM and ER31d cells viability monitored over a minimum of seven days of treatment. In the y axis, viability is expressed in % value over the total number of cells.

effect of the two herbicides correlated with a differential effect on the cortical MT array, MTs were visualized by immunofluorescence in both the NM and the ER31d cell lines (Fig. 3). While cortical MT arrays could not be detected after treatment with oryzalin, they could be found in the anisodiametric ER31d EPC-treated cells (Fig. 3). With reference to untreated ER31d cells, MTs were still fairly intact although often interspersed with fluorescent speckles characteristic for remainders of disassembled MTs. A similar pattern was sporadically found in the transiently viable NM cells, treated with EPC (Fig. 3 and data not shown).

2.2. Differential effects of EPC and oryzalin at molecular level

To test, whether EPC-resistance of the ER31d line correlated with different responses at the molecular level, we measured the abundance of α - and β -tubulin protein in response to oryzalin and

EPC (Figs. 4 and 5). Oryzalins drastically reduced the α - and β -tubulin protein content in both the NM (data not shown) and the ER31d (Fig. 4) cell lines. In contrast, the amount of α -tubulin remained high in the ER31d line at both 0.3 and 0.4 mM EPC concentration, ranging from 90 to 65% of the amount present in the untreated controls, depending primarily on drug concentration rather than on the length of treatment (Figs. 5 and 6). In the NM line, the amount of α -tubulin was drastically reduced by EPC (Fig. 6). The reduction of β -tubulin in the ER31d line was more relevant compared to α -tubulin, i.e. after 48 h at 0.4 mM EPC could be as low as 50% of the total, but still the residual level of β -tubulin remained significantly higher as compared to the negligible level of β -tubulin detectable in response to oryzalin or in the wild type NM line (Figs. 5 and 6). To test, whether the reduction in tubulin levels was caused by inhibition of transcription, the tubulin mRNA levels were measured in all the experimental conditions described (Figs. 4

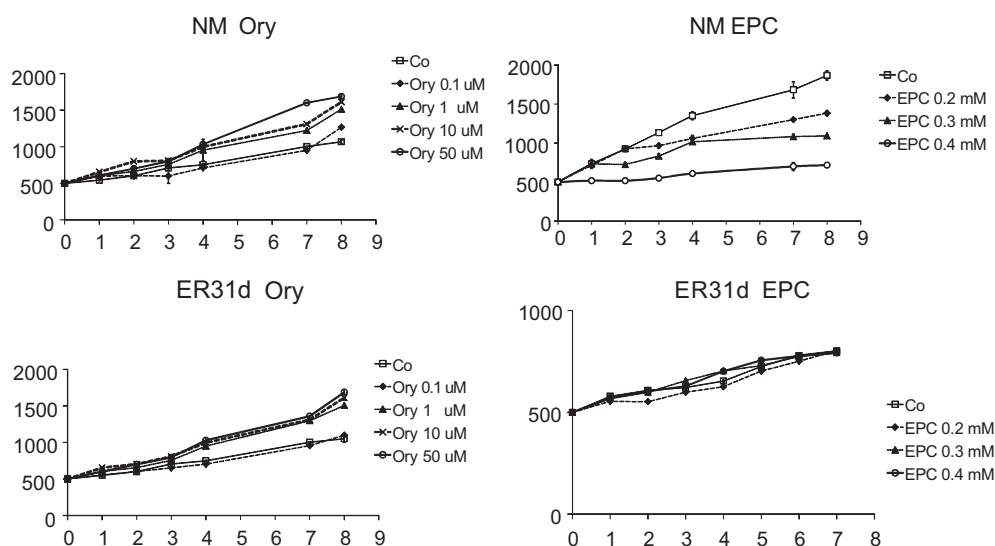


Fig. 2. Effect of different concentration of oryzalin and EPC on NM and ER31d packed cell volume (PCV), monitored over a minimum of seven days of treatment. In the y axis, the PCV value is expressed in μ l of packed cells.

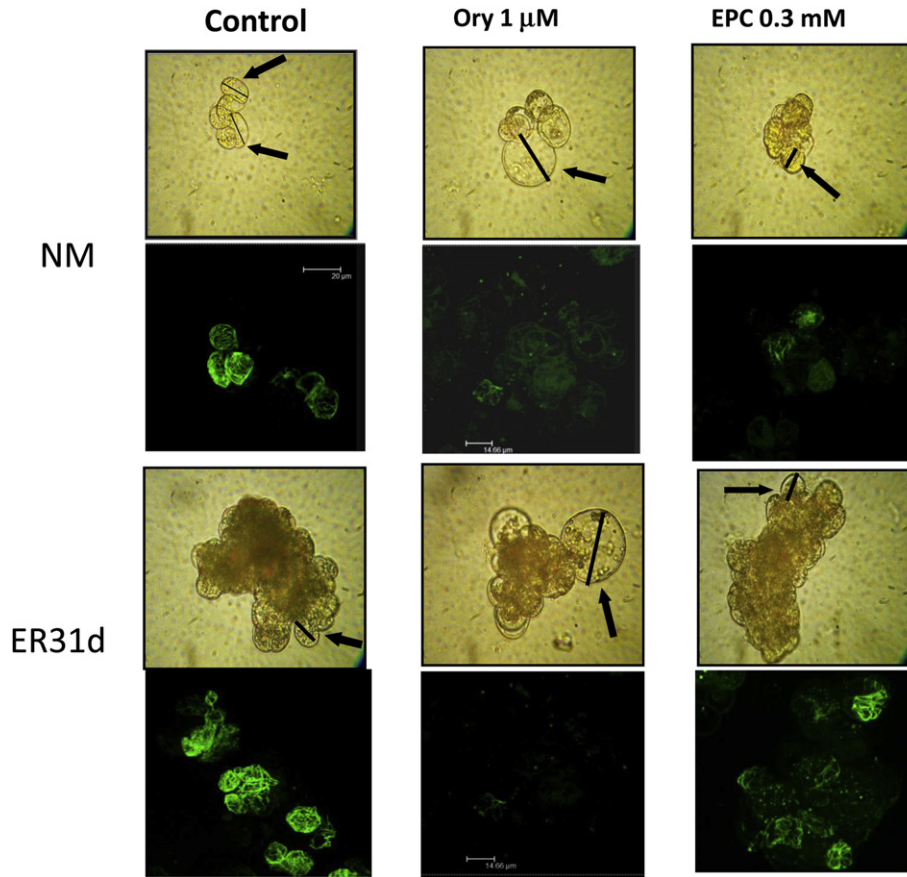


Fig. 3. Effect of 0.3 mM EPC and 1 μM oryzalin on cell morphology and cortical MT array of the NM and ER31d lines, after 48 h of treatment. Arrows point to single cell diameters. Cortical MT arrays are labeled with fluorescein.

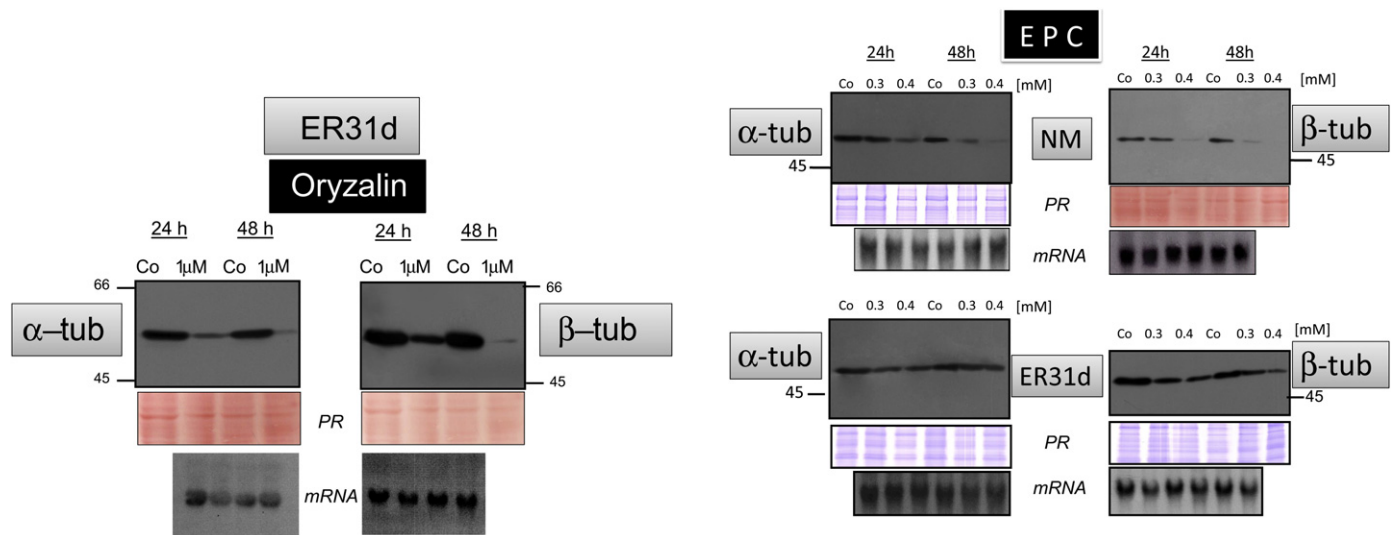


Fig. 4. Western blot analysis reporting the effect of 1 μM oryzalin on α- and β-tubulin protein and mRNA levels in ER31d cells. Co stays for control untreated cells. Tubulin protein levels were assayed after 24 h and 48 h of treatment. 45 indicates a 45 kDa marker band. The Northern blot data shown in the insert (mRNA) refer to the same treatments and experimental points as those of the Western blot analyses shown above. Equal loading of protein samples (PR) was ascertained by red ponceau staining of the immunoblot filters prior the treatment with the specific antibodies. Equal loading of total RNA samples was checked by ethidium bromide (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Western blot analysis reporting the effect of 0.3 and 0.4 mM EPC on α- and β-tubulin protein and mRNA levels in NM and ER31d cells. Co stays for control untreated cells. Tubulin protein levels were assayed after 24 h and 48 h of treatment. 45 indicates a 45 kDa marker band. The Northern blot data shown in the insert (mRNA) refer to the same treatments and experimental points as those of the Western blot analyses shown. Equal loading of protein samples (PR) was ascertained by coomassie blue staining of a parallel gel or by red ponceau staining of the immunoblot filters prior the treatment with the specific antibodies. Equal loading of total RNA samples was checked by ethidium bromide (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

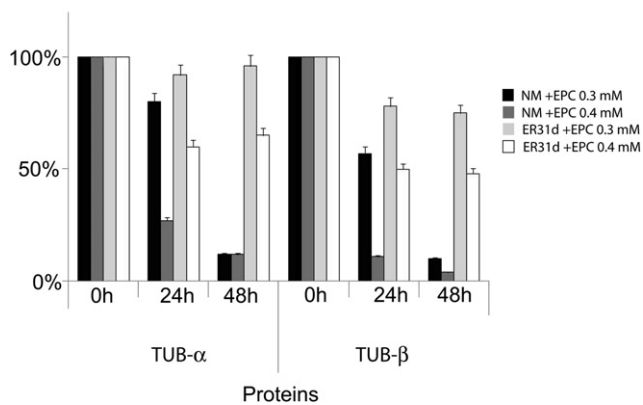


Fig. 6. Molecular quantitative response to EPC treatment measured at protein level in EPC-sensitive Nihon-Masari and EPC-resistant ER31d cell lines. Data come from five independent experiments. Tubulin protein levels were assayed after 24 h and 48 h of treatment. Percentage values refer to the amount of α - and β -tubulin protein present at time = 0.

and 5 and Supplementary Fig. S2). Since these were found fairly constant at all experimental points, it is inferred that the control on the amount of intracellular tubulin is largely exerted at protein level. The data on tubulin protein amount in the NM wild type and the ER31d cell line clearly indicate that EPC-resistance associated with the presence of a significant level of both α - and β -tubulin proteins.

EPC was reported to bind the C-ter domain of α -tubulin in *in vitro* binding-affinity experiments. The C-ter domain of assembled α -tubulins loops out from the main structure of the MT thus providing a target for a series of post-translational modifications. EPC binding could then be hindered by changes occurring at its putative binding domain that may result from specific mutations or alterations, both capable of masking the site of herbicide interaction. To understand whether the differential sensitivity to EPC is related to qualitative modifications of tubulins, we analyzed the pattern of α -tubulin isoforms present in protein extract preparations made from either EPC-sensitive NM or the ER31d cell line. These experiments, performed through high resolution two-dimensional protein gel electrophoresis followed by immunostaining with anti α -tubulin specific antibodies, revealed few but significant differences in the pattern and level of expression of the different α -tubulin isoforms (Fig. 7). The intensity of three protein spots that immunolocalize in correspondence with the putative pI(s) calculated for the three rice α -tubulin isotypes (4.88 for $\alpha 1$, 4.85 for $\alpha 2$ and 4.95 for $\alpha 3$ respectively) diminished in the extracts made from the EPC-resistant ER31d cell line (arrows in Fig. 7). This would suggest that MTs of the ER31 resistant line are enriched in α -tubulin isoforms with higher pI(s), the likely consequence of a more extensive post-translational modification. Due to its reported correlation with increased MT stability, the level of α -tubulin tyrosination was also controlled (Fig. 7). Protein extracts obtained from the EPC-resistant ER31 and the EPC-sensitive NM cell lines were probed with an antibody raised against a tyrosinated C-ter peptide of mammalian α -tubulin. Minor differences in the pattern of α -tubulin tyrosination were found by comparing the two cell lines (Fig. 7).

3. Discussion

This work shows that the sensitivity to oryzalin and EPC can be uncoupled in the rice ER31d cell line, originally derived from a rice mutant selected for resistance to EPC [26]. The ER31d cells are resistant to EPC over a long period of cultivation, while remaining

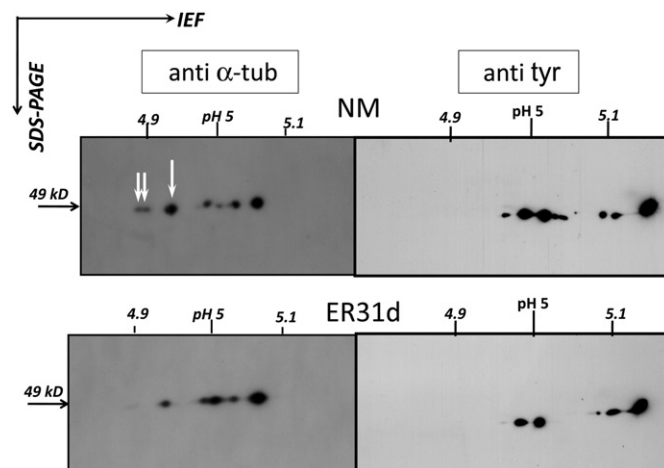


Fig. 7. Immunoblotting from two-dimensional gel electrophoresis. Left panels: the patterns and levels of expression of the different α -tubulin isoforms in EPC-sensitive Nihon-Masari (NM) and EPC-resistant ER31d cell lines are shown. α -Tubulin isoforms have been detected with the use of a generic anti α -tubulin antibody (Amersham). Arrows point to the position of native α -tubulin isotypes in accordance with their respective pI value. Right panels: the pattern and level of expression of tyrosinated α -tubulin isoforms in EPC-sensitive Nihon-Masari (NM) and EPC-resistant ER31d cell lines are shown.

highly sensitive to oryzalin. This coexistence of oryzalin sensitivity and EPC resistance is manifest in a cascade of differential responses that we have monitored at cellular, sub-cellular and molecular levels. At the cellular level, while ER31d cells treated with oryzalin loose viability and anisotropy (very similar to the control NM cell line), they steadily grow, although at a low rate, in the presence of different amounts of EPC with no observable effects at the level of cell shape and morphology. A differential effect of the two herbicides on cell morphology was already observable in control rice cell lines or rice seedlings. With regard to seedlings, EPC treatments severely impaired seed germination allowing the emergence of just a thin and short primary root (Supplementary Fig. 1). On the other hand, the root emerging from seeds treated with oryzalin is shaped like a club (Supplementary Fig. 1) resulting from loss of cell wall anisotropy, a consequence of massive elimination of cortical MTs as it has been already documented by several laboratories including ours [27].

This differential response on cell sizes and shape is accompanied by differences in the response of the cortical MTs. In fact, while the cortical MTs of ER31d cells treated with oryzalin appear largely destroyed, EPC-treated ER31d cells maintain a net of cortical MTs, found often interspersed with dot-like structures. As reported by several laboratories under different experimental conditions that ultimately lead to disruption or elimination of microtubules, interspersed punctuated structures are characteristic for cortical microtubule-nucleation sites that remain after MT shrinkage. In the EPC-resistant *ATER* mutants of tobacco [28,29] cortical microtubules emanated from such sites, when the factor that caused disassembly was released [28].

Cell anisotropy is strictly associated to the presence of a functional, well organized cortical MT array. This has been shown by many laboratories either by studying the effect of oryzalin addition to cell cultures and seedling roots or by using oryzalin as a tool for characterizing the effect on cell growth of different mutations occurring in MAP or CeSA encoding genes [30,31]. Therefore complete MT depolymerization triggered by dinitroanilines restores the plant cell to its isotropic, default pattern of growth. On the contrary, low doses of propyzamide and taxol cause a change in the arrangement of the cortical MT array, from transverse to

oblique, that associates to a reduced anisotropic expansion of the cells in the inner cortex of *A. thaliana* seedling roots. Ultimately, this different arrangement produces helical growth with no effect on cell division [3].

Here we show that EPC can arrest growth and determine loss of cell viability with no relevant influence on cell anisotropy. This suggests that EPC does not drastically alter the functionality and the structure of the cortical MT array. Because cell growth is inhibited by EPC in both rice seedlings and the NM rice cell line, the possibility that EPC can be more effective on those MT arrays that are more strictly associated with cell division (preprophase band, spindle and phragmoplast) must be seriously considered.

The likelihood that different anti-microtubular drugs may have a different efficacy toward diverse MT arrays finds the following suggestive evidences. First, it has been reported that IPC, a close relative to EPC, does not depolymerize plant MTs of *N. sylvestris* cell lines, but causes MTOC fragmentation and spindle abnormalities [22]. These data are only partially consistent with ours because we show that EPC does have an effect on the cortical MTs although their alteration is not enough to release the physical constraint that preserve cell anisotropy. Also, we often observed dot-like structures in cells surviving to the EPC treatment that could represent microtubule reorganization centers (data not shown). At present we cannot say if they are fragments of pre-existing MTOC or nucleation sites resulting from MT reorganization. Further evidences suggesting that cortical MT nucleation may be under a specific control come from recent results obtained by manipulating the expression of the TOONEAU2/FASS (TON2) gene in *A. thaliana* [32].

Second, different forms of modified tubulin were found in association to various microtubule structures in *Nicotiana tabacum* L. cells [33]. They may contribute to assign specific features to different MT arrays and this may result in the formation of specific fractal patterns. Features of cortical MTs may indeed be different from those of mitotic MTs as suggested by the dual response to citral [23].

Third, additional evidence is based on the chemical structure of EPC that appears to be much more similar to flamprop-M-methyl than to dinitroanilines (Supplementary Fig. 3). Flamprop-M-methyl, a derivative of the arylaminoproponic acid, is structurally defined as an amide. In particular, EPC and flamprop-M-methyl share a common central core made up by an arylamine linked to a carbonyl group (brackets in Supplementary Fig. 3). Such a structural feature, absent in oryzalin, may be important for supporting an alternative mode of action on microtubules. This would be consistent with data reporting that flamprop-M-methyl has almost no effect on cortical microtubules but it seriously damages spindle and phragmoplast formation causing cell division arrest in meristematic maize root cells [19]. More importantly, cell division arrest does not correlate with isodiametric cell growth or root clubbing. On the contrary of pendimethalin, that is a dinitroaniline, flamprop-M-methyl doesn't inhibit *in vitro* soybean MT polymerization. This suggests that flamprop-M-methyl disrupts mitosis and cytokinesis through a different mechanism of action that is not based on the classical model involving binding of the tubulin heterodimers and subsequent MT disassembly. Flamprop-M-methyl is more likely to interact with the polymerized MTs promoting their disassembly from the minus-end.

Differential sensitivity to drugs may also depend on the cell type. When challenged with low concentrations of taxol or propyzamide, ground tissue of *A. thaliana* seedling roots look more sensitive to the drugs than epidermis with regard to the integrity of MT organization and anisotropic growth [3]. Because these two drugs, both causing helical growth, do not alter the gross organization of cortical MTs, it is inferred that they may be influencing

a MT function other than the basic process of MT polymerization/depolymerization.

Although a clear evidence has not been demonstrated yet, the site of EPC binding, that is the C-ter of the α -tubulin monomers, is compatible with an effect that could be exerted on polymerized MTs. In fact, the C-ter of the α -tubulin that is incorporated into the MTs loops out from the main structure, free to interact with different MAPs. The efficacy of such interactions may also depend on the presence of specific PTMs such as tyrosination. In agreement with this view, the α -tubulin C-ter peptide bound to a column, that was used to demonstrate specific binding with EPC, may be assumed to be equivalent to the C-ter looping out from protofilaments. In accordance, the possibility that EPC may poison MTs by acting directly on them must be considered in view of the data of this paper. This may also explain the molecular basis of the ER31 mutation. This mutation, if heterozygous, is still compatible with the growth of rice seedlings and plants despite that some morphological changes (modified tiller formation) or alteration to stimuli (i.e. slower reorientation after auxin addition or higher resistance to chilling) are observed. These are likely to occur because of a less dynamic population of MTs that may depend on the fact that the normal intracellular traffic that involved interactions with the C-ter is slowed down in the mutant.

Reduced MT dynamics has been proposed to associate with increased levels in tubulin PTMs [34]. This may find some consistency with our findings reporting a general shift toward less acidic pI(s) isoforms of the α -tubulin population present in the ER31d cell line. A more pronounced shift toward higher pI(s) and a strongly reduced amount of those tubulin spots that correspond to the native proteins suggest an enrichment in post-translational modified α -tubulins that may effectively interfere with EPC poisoning, not with oryzalin, since the former binds at the C-terminal tail, the target of all but one (acetylation) tubulin PTMs. Changes in pI of expressed tubulin isoforms occurring in relation to different pathways of bud development, has been recently observed in grapevine [35]. Transition of buds to leaves, for instance, is characterized by the increase in more acidic α -tubulin isoforms. Authors argue that this can be put in relation with the assembly of specific MT structures more appropriated for a tissue with low mitotic activity such as leaves. When compared to leaves, tendrils and flowers associated with more basic α -tubulin isoforms. In principle the maintenance of cell division, hence mitotic activity, of ER31d cells may as well relate to the presence of a more basic population of α -tubulin isoforms.

MT stability can also depend on the actual concentration of the unpolymerized tubulin monomers, as shown by studying the effects of mutations occurring in prefoldin, a protein involved in tubulin biogenesis [36]. It is shown that disruption of genes encoding subunits of prefoldin lead to a reduced concentration of the intracellular tubulin pool that, in turn, increases MT stability. These data may provide a further explanation of our findings. In fact, oryzalin and EPC determine a different level of reduction in α - and β -tubulin in the ER31d cells. The amount of the α - and β -tubulin peptides remains well over 50% of that present in untreated cells when EPC is added to the medium but it drops to less than 10% in the presence of oryzalin. It is therefore conceivable that while oryzalin determines a critical reduction in tubulin amount leading to MTs fragmentation and disassembly, EPC may primarily affect MTs dynamicity. The fact that the level of α -tubulin tyrosination remains substantially similar in both the sensitive and the resistant cell line, may just indicate that tyrosination is one of the mechanisms by which MT dynamicity can be controlled. Tyrosination may not be that relevant for MT stability in the presence of suboptimal amount of tubulin. Also, because accumulation of α - and β -tubulin in EPC-treated ER31d cells associates

with the maintenance of detectable MT structures, a cross-talk between the status of assembly/disassembly of the MTs and the amount of intracellular tubulin (monomers and dimers) must be operating in such a way that tubulin can be replenished when required. A fast and *ad-hoc* replenishment seems to be efficiently ensured by tubulin mRNA that, remaining substantially unaltered by the different treatments, provides an immediate exploitable platform for tubulin *de novo* production through translation. Tubulin mRNA translation rather than tubulin monomer degradation represents a bidirectional ductile step of a more complex regulatory circuit that control tubulin synthesis, accumulation and assembly in plants [37].

In conclusion, the availability and the use of the ER31d cell line has allowed us to uncover differences in the response to treatments with two drugs, oryzalin and EPC, for which a common mechanism of action has been postulated until now. These differences indicate that the cortical MT array is not a preferential target for herbicides of the carbamate family.

4. Materials and methods

4.1. Cell cultures and treatments

The ER31d rice cell line was derived from mature embryos of a heterozygous *ER31* mutant, selected for growth in the presence of EPC. Caryopses were de-husked and surface-sterilized for 3 min with 70% ethanol, then treated with 1% commercial bleach (ACE, Milano, Italy) for 20 min and eventually rinsed several times with distilled water. For callus induction, seeds were placed on Murashige and Skoog medium made solid with 0.8% microagar (Micropoli, Legnano, Italy, supplemented with 30% (w/v) sucrose and 2 mg/l 2,4-dichlorophenoxyacetic acid (MS-2,4D). After induction, an appropriate sized callus was selected to make the cell culture. In brief, a callus was macerated in R2 medium [38] and single cells detaching from the callus were selected for the subsequent subcultures. Rice (*O. sativa* L. cv. 'Nihon-Masari' WT (NM) and ER31 obtained in the NM wild type background) suspension cell cultures were serially sub-cultured in R2 medium in a cycle of two weeks [38], and kept in darkness at 25 °C on a horizontal shaker at 120 rotations per min. Under these conditions, the cells grew in microcalli of approximately one hundred cells each. Cells in the exponential phase of growth (4–7 days) were sub-cultured into 20 ml of fresh R2 medium for 24 h and then treated with different concentrations of oryzalin (0.1, 1, 10, 50 µM) or EPC (0.2, 0.3, 0.4 mM) and for different times of incubation (from one to eight days). Oryzalin and EPC dilutions were prepared from 10 mM stock solutions in dimethylsulfoxide, or 500 mM in ethanolic stock solutions, respectively. The viability of oryzalin- and EPC-treated cells was monitored by the fluorescein diacetate assay, based on the ability to metabolize the non-fluorescent fluorescein diacetate into diacetate and fluorescein [39] that is fluorescent after excitation by the blue light at a wavelength of 450–490 nm. Percentage of fluorescent cells over the total was taken under the microscope. Packed cell volume (PCV) was determined each day through seven days of culture by measuring the volume of cellular sediment collected within the graduate tube of a rotating tissue culture flask. Reported data on both cell growth and packed cell volume originate from three independent experiments. Mean and standard deviation values were calculated using the Microsoft Excel application.

Rice seedlings were cultured on R2 medium, solidified by the addition 4.5 g/l of agar. Sterile solutions of EPC and oryzalin at a final concentration of 1 mM and 1 µM respectively were added to the agar before pouring the plates. Root and coleoptile lengths as function of drug concentration were measured with a caliper after eight days of cultivation in the dark at 25 °C.

4.2. Protein extraction, precipitation, electrophoresis and immunoblotting

Rice cell cultures were cultivated in the presence of different concentrations of oryzalin and EPC and in the absence of drugs as described above. Detailed procedures for protein extraction, electrophoresis and Western blotting for detection of α - and β -tubulin polypeptides have been previously reported [27]. Mouse monoclonal primary anti- α (Amersham N. 356) and anti- β -tubulin antibodies and horse-radish peroxidase-conjugated secondary antibody from Amersham were used at 1:3000 dilution. Primary monoclonal anti-tyrosinated α -tubulin antibody (mouse IgG3 isotype clone TUB-1A2) from Sigma was used at 1:1000 dilution.

Quantification of the α - and β -tubulin specific signals was performed by scanning with a TLC-scanner II (Camag, Berlin, Germany) the exposed films resulting from five independent experiments. Mean and standard deviation values were calculated using the Microsoft Excel application.

For two-dimensional gel electrophoresis, trichloroacetic acid (TCA)/acetone protein precipitation was performed in a solution of 80% ice-cold acetone and 20% TCA, allowing the proteins to precipitate at –20 °C for 1 h. The solution was then spun at 13,000 rpm (18,000 × g) for 15 min at 4 °C in a microcentrifuge. The sediment was washed with 1 ml ice-cold acetone and spun again at 13,000 rpm for 15 min at 4 °C. The precipitates were then stored at –80 °C, ready for use.

4.3. Extraction of total RNA and Northern blot analyses

Total RNA was isolated from 1600 µl of freeze-dried sedimented cell cultures using Triazol (Invitrogen, Carlsbad, CA, USA). Protocols for total RNA extraction, Northern blot analysis and detection of total α - and β -tubulin mRNAs were performed as previously described [9].

4.4. Two-dimensional poly-acrylamide gel electrophoresis: high resolution iso-electrofocusing

The first dimension of isoelectric focusing (IEF) was performed on ready-to-use Immobiline DryStrips (Amersham). Prior to IEF, the dry strips were rehydrated and placed onto the cooling plate of an electrofocusing chamber. The sample was applied by in-gel rehydration. The standard protocol described here is valid for broad gradients in the pH range between 3 and 12 as well as narrow (pH 4.5–5.5) gradients. 100 µg of total protein extract were precipitated by TCA/acetone precipitation and the sediment resuspended in 340 µl of DeStreak Rehydration solution (Amersham). Rehydration and IEF were performed using the IPGphor device (Amersham) with the following running protocol: (50 V – 12 h, 200 V – 1.5 h, 500 V – 1.5 h, 1000 V – 1.5 h, from 1000 to 8000 V in 2 h and 8000 V – 4 h). The strips were either stored at –80 °C until used or immediately loaded on a 12.5% standard poly-acrylamide gel for the second dimension and Western blot. The second dimension was run for 15 min at 25 mA/gel and then for 5 h at 4 °C at 35 mA/gel.

4.5. Visualization of microtubules and nuclei

200 µl aliquots of sedimented rice cells (*O. sativa* cv. 'Nihon-Masari' wild type or ER31d) either treated for 48 h with 1 µM oryzalin or 0.3 mM EPC were washed in microtubule stabilizing buffer (MSB) containing 50 mM piperazine-diethanesulfonic acid pH 6.9, 5 mM ethylene glycoltetracetic acid (EGTA), and 1 mM MgCl₂. The cell wall was partially digested for 10 min at RT with a solution containing 0.25 M mannitol, 5 mM EGTA, 1% Macer-ozyme R-10, 0.2% (w/v) pectinase and protease inhibitors (20 µg/

ml leupeptin hemisulphate and 1 mM phenylmethyl-sulfonyl fluoride). After washings in MSB the digested cells were fixed for 30 min at RT in 3.7% (w/v) para-formaldehyde in MSB including protease inhibitors, 0.2 mM guanosine triphosphate and 0.05% Nonidet-P40 and then washed three times in MSB buffer. Fixed cells were washed twice with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 and twice in PBS, then incubated for 1 h at RT with a monoclonal mouse antibody (1:100 diluted in PBS) raised against a conserved epitope of α -tubulin (Amersham). After two washes in PBS/Tween and two in PBS without detergent, a fluorescein-linked antimouse IgG antibody made in sheep (Amersham) was added as the secondary antibody at 1:25 dilution. Specimens were incubated overnight at 4 °C followed by two washes in PBS. All antibody dilutions were done with 3% (w/v) bovine serum albumin in PBS. Nuclei of immunolabeled cells were stained with 1 ml of a 0.1 μ g/ml solution of 4,6-diamino-2-phenylindole (DAPI) for 10 min. After a brief wash with PBS to remove unbound DAPI, cells were mounted on a slide with a drop of diluted anti-fading solution (Prolong Gold, Invitrogen), sealed with nailpolish. All samples were viewed under a TCS SR2 AOBs confocal laser scanning microscope (Leica Microsystem, Heidelberg, Germany) equipped with laser Argon/Krypton and a PLAPO 63 oil immersion objective. Fluorescein isothiocyanate was excited with a laser line of $\lambda = 488$ nm, and the fluorescence was collected between 500 and 560 nm. DAPI was excited in the UV at $\lambda = 364$ nm and fluorescence was collected in the range of 410–470 nm.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2012.11.017>.

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