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Vacuolar Sorting Mechanisms Are Differently Influenced by Detoxification Processes

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Abstract: Glyphosate is a non-selective herbicide that inhibits the shikimate pathway's enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) preventing the production of aromatic amino acids. This herbicide is largely used and appreciated because it controls a wide range of annual and perennial weeds but it has a minimal environmental impact when compared with other herbicides. Initially, it was thought that resistance to glyphosate was not easy to evolve but the continuous applications, as it happened for other herbicides, induced the development of several glyphosate-resistant weeds. Glyphosate resistance can be developed as target-site or non-target-site mechanisms. In the target-site mechanism of resistance, either a mutation on the EPSPS enzyme (enzyme modification) or the overexpression of the EPSPS enzyme was found to confer resistance. In the non-target-site mechanism of glyphosate resistance, the translocation and the neutralization of the herbicide is observed. Pumping glyphosate into vacuoles via membrane transporters has been suggested as a possible process involved in the restricted glyphosate translocation. As a consequence, a different vacuolar organization or plasticity could be an interesting character or marker to correlate to glyphosate resistance. Vacuolar markers AleuGFP (Sar1 dependent sorting) or GFPChi (Sar1 independent sorting) respectively can be used to monitor independent vacuolar sorting mechanisms during glyphosate-induced stress. We observed that the adaptive reaction of tobacco protoplasts vacuolar complex to the treatment with glyphosate could be mimicked by the overexpression of a *Triticum durum TdGST* gene. Previous analysis evidenced that the herbicide glyphosate increased *TdGST* expression, confirming the role of GST in the protection against xenobiotics. Non-target-site glyphosate resistance mechanisms may correlate with an independent regulation of cell

compartmentalization and herbicide-induced genes may have a direct effect on it.

1. Introduction

Glutathione *S*-transferases (GSTs) are soluble proteins that catalyze the chemical modification of xenobiotic by covalent linkage to endogenous glutathione and transfer of the resulting conjugates from the cytosol to the vacuole. This is an important detoxification mechanism. This pathway needs to be supported by transporters on the tonoplast and by an adequate vacuolar compartmentalization; in fact it shares many features and problematics with the pathway for vacuolar secondary metabolites accumulation. As for secondary metabolites, such as anthocyanins [1,2], specific compartmentalization adaptations could be observed and the sequence of traffic events remains unclear [3]. Here we investigated the alteration induced by GST overexpression and/or glyphosate treatment on the vacuolar sorting pathway using two known vacuolar markers: AleuGFP (Sar1 dependent sorting) and GFPChi (Sar1 independent sorting) [4]. These reporter proteins follow two different and independent routes to the vacuole [4–6] and can be used to monitor independent mechanisms during glyphosate induced stress. We observed an adaptive reaction of tobacco protoplasts evidenced by alterations in the fluorescent labeling of the vacuolar complex. The reaction was triggered by the treatment with glyphosate but also by the overexpression of a *Triticum durum TdGST* gene [7]. The correlation of non-target-site glyphosate resistance mechanisms [8] with an independent regulation of cell compartmentalization will be discussed.

2. Results and Discussion

2.1. GFPChi and AleuGFP Fluorescence Is Altered by Glyphosate and GST Overexpression

GFPChi and AleuGFP were previously expressed by our group in different plant species and tissues [9–11]. Several studies proved that the two reporters are sorted to vacuoles by different sorting machineries [4,6,12]. GFPChi contains the ctVSD of tobacco chitinase A [13] while AleuGFP harbors the N-terminal ssVSD from barley aleurain, containing the specific conserved sequence NPIR [6]. The reporters were transiently expressed in tobacco protoplasts and their fluorescence pattern was identical to the pattern previously described. For the purpose of this preliminary study, 2 independent experiments were performed to generate from 4 to 6 replicas. Between 50 and 100 cells belonging to the photosynthetic mesophyll subpopulation were counted for each replica and classified in the different pattern categories. Very large or very small cells with less than ten chloroplasts derived from the epidermis or other specialized cell types and less frequent in the population were not considered. Among cells with more than ten chloroplasts, GFPChi labeled the Endoplasmic Reticulum (ER) and the Lytic Vacuole (LV) (Figure 1A) or the ER only (77% of cells; Figure 1B). AleuGFP labeled the Pre-Vacuolar-Compartments (PVCs) and the LV (Figure 1C) or the PVCs only (27%; Figure 1D). These cells were treated with 12 mM glyphosate and/or co-transformed with the cDNA of TdGST and their fluorescent patterns distribution was counted in 3–4 independent experiments (Figure 1E).

Previous analysis evidenced that the herbicide glyphosate increased *TdGST* expression and the goal of the experiment was to verify if herbicide treatment and GST overexpression both had independent effects on cell compartmentalization.
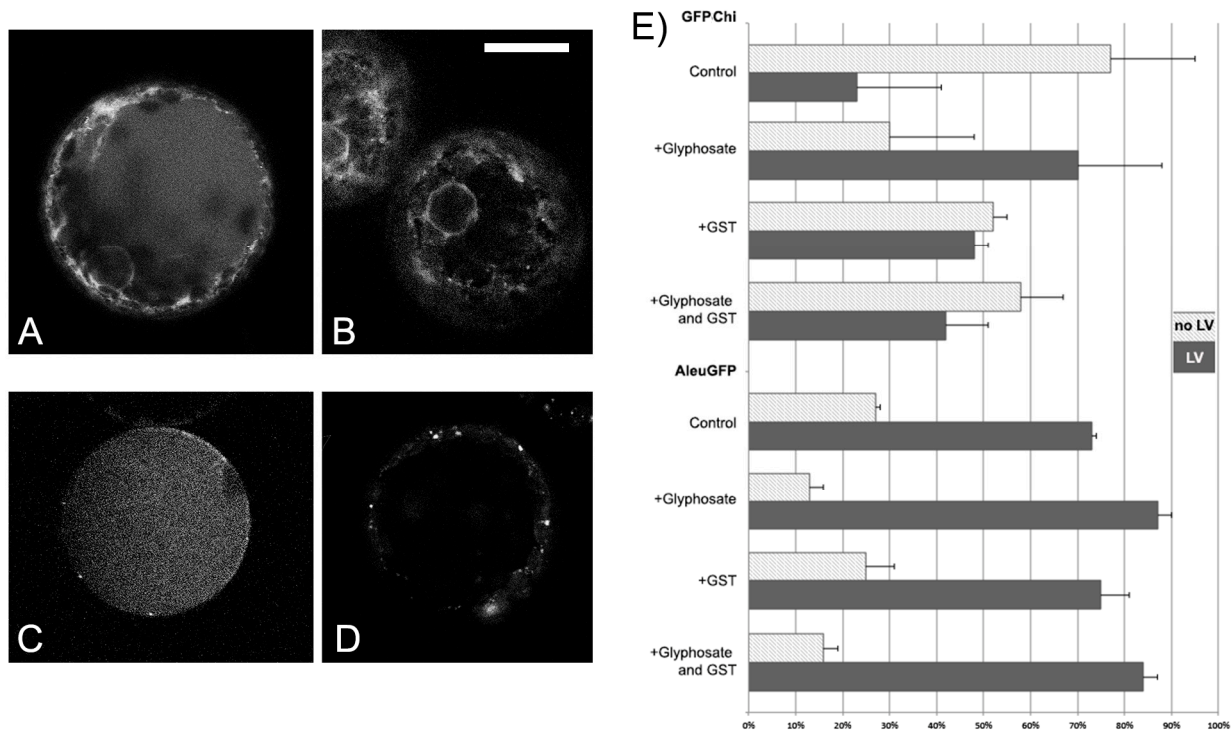

Figure 1. Fluorescent patterns of protoplasts transformed with GFPChi or AleuGFP and graphic representation of fluorescence distribution. (**A**) Cell with GFPChi fluorescence in the vacuole (LV); (**B**) cell with no GFPChi fluorescence in the vacuole (no LV); (**C**) cell with AleuGFP fluorescence in the vacuole (LV); (**D**) cell with no AleuGFP fluorescence in the vacuole (no LV); and (**E**) graphic representation of the fluorescence observed in vacuoles of cells treated with 12 mM Glyphosate and/or co-transformed with *TdGST*. GFPChi or AleuGFP were transformed in parallel and compared in the different conditions. LV = clearly fluorescent central lytic vacuole, alone or in addition to other fluorescent structures; no-LV = no fluorescence observed in the central lytic vacuole but limited to other smaller compartments. Scale bar = 20 μm.

It was observed that glyphosate visibly increased the fluorescence of GFPChi in the LV of cells (Figure 1E) while such increase was minor in the case of GST overexpression with or without glyphosate treatment. AleuGFP expressing cells did not show a comparable alteration of fluorescence in the central vacuole (Figure 1E) but evidenced a different kind of alteration. AleuGFP was more evident in the intermediate compartments, the PVCs, also in those cells with a clearly fluorescent central vacuole.

2.2. Not GFPChi's but AleuGFP's Fluorescent Pattern Is Altered by Treatments

To a more accurate observation it was evident that, despite fluorescent intensity change, GFPChi distribution within the cells did not appear altered. The increase of fluorescent vacuoles did not imply any morphological visible alteration. On the contrary the apparent stability of fluorescence distribution for AleuGFP (Figure 1E) did not take into account a great increase of number, visibility and size of PVCs also in those cells showing a fluorescent LV.

Dividing the population of transformed cells into three subgroups, cells showing PVCs only, PVCs in presence of a LV and LV only, the effect of glyphosate and of GST overexpression become evident. First, both glyphosate and GST overexpression caused an altered aspect of PVCs. These small punctate compartments (Figure 1D) appeared to be larger and more irregular in shape both in cells in which the LV was not fluorescent (Figure 2A) and in cells with a visibly fluorescent LV (Figure 2B). Second, glyphosate greatly induced an increase of PVCs quantity reducing from 42% to 9% the cells showing fluorescence only in the LV. GST overexpression caused a more moderate effect reducing cells showing fluorescence only in the LV to 27%. GST overexpression in presence of glyphosate re-established conditions close to normal even if the alteration of size and number of PVC, when observed, persisted (Figure 2C).
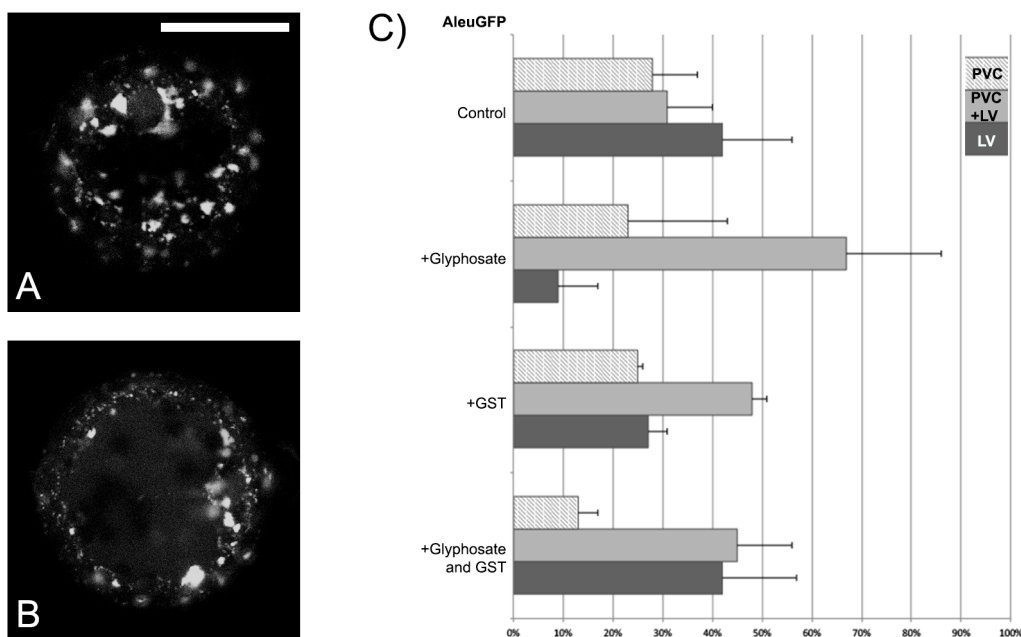

Figure 2. Distribution of three fluorescent patterns observed in protoplasts transformed with AleuGFP and treated with 12 mM Glyphosate and/or co-transformed with *TdGST*. When visible, PVCs appear enlarged and irregular both because of the effect of glyphosate or GST overexpression (**A**) no visible LV; (**B**) with fluorescent LV; and (**C**) Graphic representation of patterns distribution. Experiments are partly independent from Figure 1. LV = fluorescent vacuole with no other labeled structures; LV + PVC = fluorescent vacuole with several small punctate structures known as pre-vacuolar compartments; PVC = labeled pre-vacuolar compartments with no fluorescence observed in the central lytic vacuole. Scale bar = 20 μm.

On the bases of these detailed observations, it was evident that glyphosate strongly retarded AleuGFP arriving in the LV while GST had a more complex effect altering the intermediate compartments but having a general positive effect on protein sorting. Indeed, overexpression of GST during glyphosate treatment greatly reduced the herbicide effect.

3. Experimental Section

3.1. Transient Expression and Glyphosate Treatment in N. tabacum Protoplasts

Tobacco protoplasts preparation and transient transformation was performed as previously described [14] digesting leaves from 3 to 4 weeks old plants grown at 24 °C with a light/dark cycle 12/12. Leaves were surface sterilized in a 1:5 bleach bath for 40 s and rinsed in abundant distilled water. The transformation efficiency of the reported experiments was always above 40%.

Protoplasts were also incubated with different glyphosate concentrations: 4, 8, 12 mM to determine the dose to be used in the experiment replicates. Within 18 h only the highest dose cause sensitive alteration of fluorescent patterns.

3.2. Microscopy and Observation

Protoplasts transiently expressing the various GFPs were observed by fluorescence microscopy in their culture medium at different times after transformation, using a confocal laser-scanning microscope LSM Zeiss 710, Observer 0.1. Fluorophore excitation was obtained with laser at 488 nm. GFP emission was detected between 505 and 530 nm chlorophyll fluorescence was detected above 650 nm but not reported in the figures. Fluorescent pattern counting was performed directly observing the cell population. Intensity of fluorescence in the LV is visually evaluated with the non-quantitative support of ZEN software "profiles" function. Images were mounted using Adobe Photoshop 7.0 software (Mountain View, CA, USA).

4. Conclusions

To investigate the early effects of glyphosate and *TdGST* overexpression we performed transient transformation experiments in *N. tabacum* protoplasts using AleuGFP [6] or GFPChi [5] co-transformed with *TdGST* [7] or incubated with glyphosate.

We observed that *TdGST* overexpression is able, alone, to alter PVC-mediated traffic and increase vacuolar sorting. The sorting machinery activated by GST change the LV environment changing the cargo molecule transported. The more evident change in the vacuolar sap is the increased stability of GFP, revealed by the increased fluorescence of GFPChi. If it is due to the reduction of vacuolar proteases or to pH increase remains to be investigated. In this preliminary study we show how non-target-site glyphosate resistance mechanisms may correlate with the regulation of cell compartmentalization and how *TdGST*, a gene induced by the herbicide, may have a direct effect on it, independently from detoxification requirements.

More experiments to enlarge the statistical evaluation based on cell counting and using different approaches will be planned to understand if GST has a relevant role in the protection against xenobiotics acting as a regulatory of vacuolar traffic.

These observations anticipate the possibility to alter the cell compartmentalization mimicking naturally occurring processes such as detoxification. The real alteration of vacuolar sap characteristics remains to be investigated in more details.

Author Contributions

This work is the result of the collaboration between the groups of Botany/Plant Cell Biology (Gian-Pietro Di Sansebastiano, Gabriella Piro, Fabrizio Barozzi) and Plant Physiology (Luigi De Bellis, Eliana Nutricati, Alessio Aprile, Erika Sabella) at DISTEBA. Gian-Pietro Di Sansebastiano and Eliana Nutricati defined the research theme. Gian-Pietro Di Sansebastiano designed methods and experiments. Fabrizio Barozzi and Erika Sabella performed the experiments. Eliana Nutricati and Gian-Pietro Di Sansebastiano analysed and discussed the data, interpreted the results and wrote the manuscript. Gabriella Piro, Alessio Aprile and Gian-Pietro Di Sansebastiano critically revised data and text.

Conflicts of Interest

The authors declare no conflict of interest.

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