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Research article

Comparative analyses of glutathione system of vacuoles and leucoplasts isolated from the storage parenchyma cells of dormant red beetroots (*Beta vulgaris* L.)

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ABSTRACT

The role of glutathione in the plant vacuole is still being debated. In the present paper, the redox state of glutathione and the activity of glutathione S-transferase (GST, E 2.5.1.18) in the vacuole compared to those in leucoplast have been studied. Organelles were isolated from dormant red beet (Beta vulgaris L.) taproots. Two generally used approaches have been applied to quantitatively assess the content of glutathione. Initially, levels of glutathione were measured in isolated organelles after labeling with monochlorobimane (MCB) and imaging with the use of confocal laser scanning microscopy. However, there are factors limiting the specificity of this method, because of which the resulting concentrations of vacuolar GSH have been underestimated. Another approach used was HPLC, which allows to simultaneously quantify the reduced glutathione (GSH) and glutathione disulfide (GSSG). The concentration of the total glutathione (GSHt) and GSSG in vacuoles determined with the aid of HPLC-UV was higher in comparison to that in the leucoplasts. The reduction potential (E_h) for the glutathione couple in the vacuoles was more positive (−163 mV), than that in plastids (−282 mV). The relatively rapid increase in fluorescence in the isolated vacuoles and plastids during MCB-labeling has indicated to the contribution of GSTs, since the conjugation of GSH to bimane is catalysed by these enzymes. The GST activity in the vacuoles has been assessed to be quite high compared to that of leucoplasts. The number of isoforms of GSTs also differed markedly in vacuoles and plastids. Collectively, our findings suggest the idea that the glutathione accumulated by central vacuole seems to contribute to the redox processes and to the detoxification, which can take place in this compartment.

1. Introduction

The role of glutathione (γ-Glu-Cys-Gly) has been studied almost since the time of its discovery (Foyer and Noctor, 2011). Glutathione acting in combination with its dependent enzymes, known as the glutathione system, are responsible for the detoxification of reactive oxygen and nitrogen species and xenobiotic electrophiles (Bleuel et al., 2011). Additionally, glutathione protects the proteins from oxidation via glutathionylation. In recent years, the participation of glutathione in signal transmission has become a topic of interest. An accumulated evidence suggests that GSH is required for the operation of a diverse range of processes including growth, stress tolerance and cell death programs (Diaz-Vivancos et al., 2015; Kumar and Chattopadhyay, 2018).

To date, glutathione and glutathione-dependent enzymes have been identified in many plant cell compartments. For example, glutathione has been detected in cytosol, mitochondria, plastids, peroxisomes, nucleus and apoplastic space (Diaz-Vivancos et al., 2015). Most of the investigations, which analyzed the compartmentalization of the glutathione, have focused on mitochondria, chloroplast and the nucleus (Noctor et al., 2012). At the same time, there is little information on vacuolar glutathione. The presence of glutathione in the vacuolar compartment has so far been considered to be tissue- and species-specific, because the experimental results have shown very low concentrations of glutathione in cell vacuoles of some tissues of some plants (Krueger et al., 2009; Queval et al., 2011; Koffler et al., 2013).

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EA, Ethacrynic acid; GSB, The glutathione-bimane conjugate; GSH, Reduced glutathione; GSSG, Glutathione disulfide; GSHt, Total glutathione; GST, Glutathione S-transferase; NAD-MDH, NAD-dependent malate dehydrogenase; NADP-MDH, NADP-dependent malate dehydrogenase; MBB, Monobromobimane; MCB, Monochlorobimane; TAX, Taxifolin; VIN, Vacuolar invertase

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However, the authors have focused on the results demonstrating that the glutathione S-conjugates of exogenous and endogenous compounds are deposited in vacuoles with the aid of proteins, which belong to subclass C of the ATP-binding cassette (ABC) translocators (members of the multidrug resistance-associated protein family). The corresponding transporters are localized in the tonoplast (Coleman et al., 1997; Carter et al., 2004; Noctor et al., 2013). The same carriers are capable of transporting oxidized glutathione (GSSG). Structurally, GSSG can be considered to be a glutathione S-autoconjugate (Noctor et al., 2013).

It should be noted, there are different assumptions about the physiological role of this process. According to the first assumption, GSSG is excreted from the cytosol to maintain redox homeostasis (Foyer et al., 2001; Zechmann and Muller, 2010). According to the second assumption, the GSSG molecule is not so reactive as the molecule of reduced glutathione (GSH) and therefore it is more suitable for transporting into a vacuole (Noctor et al., 2012).

Some authors have discussed the degradation of GSSG deposited in vacuoles with the participation of vacuolar γ-glutamyl transpeptidase or dipeptidase (Wolf et al., 1996; Ohkamu-Ohtsu et al., 2007; Bleuel et al., 2011). However, it may be assumed that glutathione transported to vacuoles may also contribute to the redox processes as a donor of reducing equivalents or as a substrate for glutathione-dependent enzymes. Noteworthy, several glutathione S-transferases (GSTs) have been detected in seedling vacuoles of *Triticum tauschii* and *Arabidopsis thaliana* (Riechers et al., 2003; Carter et al., 2004). Presently, only two facts indicating the presence of GSTs in the plant vacuole have been registered. However, in the plant cell, GSTs have been identified mainly in the cytosol and in plastids, as well as in mitochondria and in nuclei (Edwards and Dixon, 2005). GSTs are attributed to the superfamily of multifunctional enzymes, which catalyze the conjugation of GSH to electrophilic compounds, and also reduce organic hydroperoxides by using the reducing equivalents of glutathione (Dixon et al., 1998; Riechers et al., 2003; Öztetik, 2008). Furthermore, some members of this superfamily are involved in the anthocyanin-transport (Kou et al., 2019).

If GSTs are indeed localized in the central vacuole, then these will perform the functions that are attributed to GSTs, thereby diminishing the glutathione pool accumulated by the vacuole and altering the ratio of reduced to oxidized glutathione (GSH/GSSG). The oxidative shift of GSH/GSSG balance is known to directly or indirectly influence both the functioning of glutathione-dependent enzymes, and it also influences many redox-regulated proteins (transport, catalytic, structural and signaling proteins) (Kemp et al., 2008). Therefore, the maintenance of GSH/GSSG balance within a proper range in the compartments is essential for functioning of the cell in general. In many cell compartments, the average redox balance of the glutathione couple is maintained at a reduced level (Koffler et al., 2013; Noctor et al., 2013). At the same time, glutathione can be more oxidized in some compartments (such as lysosomes or peroxisomes) and more reduced in others (mitochondria, nuclei and chloroplasts), i.e. subcellular compartments have different redox equilibria of glutathione (Noctor et al., 2013). The GSH/GSSG balance is known to be considered as an indicator of the glutathione redox state, which is often used as the marker for the compartment redox state (Potters et al., 2010). Side by side with the redox ratio GSH/GSSG, the reducing power of glutathione, which is expressed by its reduction potential (Eh), is also often used as an accepted measure for redox conditions in an individual cell compartment (Kirlin et al., 1999; Go and Jones, 2008; Schwarzlander et al., 2008). The redox ratio GSH/GSSG and Eh for the glutathione couple have been evaluated for many plant cell compartments (Noctor et al., 2013), and this is not the case for the central vacuole.

It should be noted that there is still no common opinion on both the content and the redox state of glutathione in the central vacuole of plant cells. There is little information about vacuolar glutathione-dependent enzymes because these have been studied insufficiently. In this connection, the issue of presence of GSTs in the vacuole is still under debate. On the other hand, the available facts allow one to assume the glutathione system may function inside the vacuole. The confirmation of the presence of this system inside the vacuole and the assessment of its efficiency is possible in the case of comparison of the vacuolar glutathione system to the glutathione systems of other cellular compartments, for which the system under scrutiny was investigated in detail. In this connection, in the present investigation, we had (1) to assess the quantity of glutathione accumulated in vacuoles; (2) to assess the values characterizing the redox states of glutathione (ratio GSH/GSSG and Eh for a couple of glutathione) in vacuoles; (3) to assess the activity of GSTs in cell vacuoles; (4) to compare the glutathione system of the vacuoles with that of other cellular structures in order to understand its efficiency. The investigation was planned to be conducted on isolated vacuoles and leucoplasts of red beetroots (*Beta vulgaris* L.) under the conditions of physiological dormancy of taproots.

2. Materials and methods

2.1. Plant material

Organelles were isolated from roots of red beet (*Beta vulgaris* L., cv. Bordo) on the period of their physiological dormancy. The taproots were cooling placed in the chamber with the storage temperature of +4 °C for three months.

2.2. Isolation of organelles

2.2.1. Isolated vacuoles

The red beetroot tissues were cut with a special apparatus (which was made according to Leigh and Branton (1976) specifications) in the cool (+4 °C) isolation medium (0.8 M KCl, 20 mM EDTA, 1 mM β-mercaptoethanol (β-ME), 50 mM NaH_2PO_4–KOH pH 8.0). The filtrate was then centrifuged at 250 g for 10 min at +4 °C. The pellets were washed with cool (+4 °C) buffer (1 M KCl, 1 mM $MgCl_2$, 1 mM β-ME, 6.5 mM Tris–HCl pH 7.4) and centrifuged at 50 g for 15 min at +4 °C. Isolated vacuoles were purified in the step gradient with the specific densities (1.050–1.080–1.145–1.180 g cm^{-3}) prepared from mixtures of mother solutions containing 1 M KCl or 1.8 M sucrose, and also 5 mM EDTA, 1 mM β-ME, 20 mM Tris–HCl pH 7.4. The gradient was centrifuged for 10 min at 125 g. The organelles were collected and suspended with the buffer (1 M KCl, 1 mM $MgCl_2$, 6.5 mM Tris–HCl pH 7.4) (Kuzevanov et al., 1981).

2.2.2. Isolated plastids

Leucoplasts were prepared as described by Asada and Badger (1984), with modifications. Root tissues were homogenized in the cool (+4 °C) isolation medium (330 mM sorbitol, 2 mM EDTA, 5 mM $MgCl_2$, 3 mM cysteine, 5 mM dithiothreitol (DTT), 10 mM $Na_4P_2O_7$–HCl pH 7.8). The filtrates were centrifuged at 3500 g for 5 min at +4 °C. The supernatant was re-centrifuged at 6000 g for 10 min. The pellets were washed with cool (+4 °C) medium (330 mM sorbitol, 2 mM EDTA, 10 mM NaCl, 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, 5 mM DTT, 50 mM HEPES-KOH pH 7.6) and loaded onto 10-22-35-60% Percoll step gradient, next, centrifuged at 9200 g for 5 min at +4 °C (Boyle et al., 1986). The purest plastid fraction was collected at the interface between 22 and 35% Percoll layers.

2.3. Obtaining aqueous extracts

Isolated organelles were placed in a cool medium (1 mM EDTA, 2 mM phenylmethane sulfonyl fluoride, 1% polyvinylpyrrolidone insoluble, 1 mM DTT, 100 mM Na_2HPO_4–KH_2PO_4 pH 7.5). The mixture of plastids was then frozen–thawed twice (using liquid N_2). All extracts were centrifuged at 13,500 g for 20 min at +4 °C.

Tissue extracts were prepared by root tissue homogenization in the same medium. The tissue homogenates were not treated with liquid N_2;

therefore, the extracts obtained from them did not contain proteins of the plastidal stroma and the mitochondrial matrix but contained mainly the water-soluble substances (including proteins) of single-membrane-bounded organelles (vacuole, lysosome, ER, etc.).

2.4. Glutathione determination

2.4.1. The method of confocal microscopy

In order to define the glutathione, the isolated vacuoles and plastids were observed with the confocal luminescent scanning laser microscope MicroTime 200 (PicoQuant Gmbh, Berlin, Germany), monochlorobimane (MCB) (Sigma-Aldrich) being used as the fluorescent dye. The reaction with MCB gave a fluorescent glutathione S-bimane conjugate (GSB), which was excited at 400 nm. The fluorescence emission for GSB was registered at 470–520 nm (Fricker et al., 2000). The isolated organelles were incubated at +20°C during 1–40 min and the fluorescence intensity was recorded with 10 min intervals. The vacuoles were placed into the medium containing 500 mM KCl, 150 mM sucrose, 1 mM EDTA and 10 mM Tris–HCl pH 7.4; the medium for plastids contained 25 mM KCl, 250 mM sucrose, 1 mM EDTA and 50 mM Tris–HCl pH 7.2. In all the treatments, except for control conditions, 0.1 mM MCB was added to the incubation media. In one supplementary treatment, the vacuoles were incubated in the presence of both 0.1 mM MCB and 10 mM Na_3VO_4 (an inhibitor of tonoplast ABC transporters) (Martinoia et al., 2002). Glutathione concentrations were calculated from the calibration curves plotted for each experiment using chemically pure GSH and 0.1 mM monobromobimane (MBB).

2.4.2. MBB/MCB approach

Glutathione was determined in extracts of organelles and tissue. In the case of MCB, the protein-containing extracts were analyzed. These extracts were obtained as described above, and 0.1 mM NADPH and 2 units/mL glutathione reductase from baker's yeast (Sigma) were added before the analysis, so that the GSSG was recycled to GSH. In the case of MBB, deproteinized extracts were used. The extracts were deproteinized with an equal volume of 5% HPO_3 containing 0.1% HCOOH and 1 mM EDTA. The macromolecules were pelleted by centrifugation at 13,000 g for 15 min. The reaction mixture contained 50 mM Tris–HCl buffer (pH 8.9) and 0.1 mM MCB (or MBB) (Kamencic et al., 2000). The GSB adduct was measured in a spectrofluorometer with excitation at 405 nm and emission at 475 nm immediately after the addition of MCB/MBB (1 min) and then after incubation for 5 min (or 10 min) at room temperature.

2.4.3. The method of high-performance liquid chromatography (HPLC)

HPLC with UV-detection for glutathione determination was applied. The deproteinization of samples was conducted as described by Rellan-Alvarez et al. (2006). The determination of GSH and GSSG was carried out with the use of a Milikhrom A-02 liquid microcolumn chromatograph (Russia). Their separation was achieved on a ProntoSILC18AQ (2 × 75 mm, 5 μm) column. The analysis was conducted as described by Lipsa et al. (2015) with minor modifications.

2.5. The redox potential of glutathione

The half-cell reduction potential (E_h) of glutathione was computed using the equations:

$$E_h = E_{pH} - (59.1/2)\ \log_{10}([GSH]^2/[GSSG]) \quad (1)$$

$$E_{pH} = E^{o\prime} - [(pH - 7.0)(\Delta E/\Delta pH)] \quad (2)$$

where, $E^{o\prime}$, the standard potential of glutathione (−240 mV) at pH 7.0 and 25 °C; E_{pH}, $E^{o\prime}$ were adjusted to the value at the pH of interest; $\Delta E/\Delta pH$, the difference in E_h when pH changes by 1 (for the glutathione couple this was 59.1 mV at 25 °C) (Kirlin et al., 1999; Schafer and Buettner, 2001; Jozefczak et al., 2012).

2.6. Determination of pH in aqueous extracts

The dense pellets of vacuoles were washed with a buffer (150 mM KCl, 450 mM sucrose, 1.5 mM Tris–HCl pH 6.0), next, centrifuged at 13,500 g for 3 min. In addition, dense pellets of the plastid were washed with a buffer (25 mM KCl, 250 mM sucrose, 1.5 mM Tris–HCl pH 7.0) and centrifuged at 13,500 g for 15 min. The supernatant was carefully removed by filter paper tampons from the pellet surfaces. The pellets were transferred into clean tubes with the bidistilled water. The vacuoles were disrupted immediately, and the leucoplasts were disrupted by freeze–thawing using liquid N_2. The tissue extract was obtained by homogenizing the tissues in cool (+4 °C) bidistilled water. Next, the samples were centrifuged at 13,500 g for 20 min. The pH of the obtained extracts was measured using a pH-meter.

2.7. Determination of amino acids

In order to quantify free amino acids, an Automatic Amino Acid analyzer (AAA-400, Russia) was used. The procedure was executed according to the manufacturer's protocol.

2.8. Determination of enzymatic activity

The GST (glutathione transferase: EC 2.5.1.18) activity was determined via the two ways. Firstly, total GST activity was measured spectrophotometrically as described by Habig et al. (1974). 1-chloro-2.4-dinitrobenzene (CDNB) was used as the model substrate. The specificity of enzyme reactions was evaluated with the use of a competitive inhibitor – ethacrynic acid (EA) (Kilili et al., 2004). Simultaneously, the enzymatic activity with 0.75 mM EA was determined, since this compound acts as a substrate for GST (Habig et al., 1974). Some GST are known to be inhibited by flavonoids (Mueller et al., 2000). Taxifolin (dihydroquercetin) was used as a potential flavonoid inhibitor. The amount of protein was determined by Bradford's method (1976).

Secondly, the gel-based experiments were conducted as follows. In order to visualize the GST activity, the gel was first incubated in a buffer (100 mM Na_2HPO_4–KH_2PO_4 pH 6.5) for 10 min and, next, transferred to the reaction mixture containing 4.5 mM GSH, 1 mM CDNB (or 0.5 mM glyphosate or 0.5 mM clopyralid and/or 1 mM EA), 1 mM nitroblue tetrazolium, 100 mM K_2HPO_4–KH_2PO_4 pH 6.5, for 10 min. After that, the gel was incubated in a buffer (100 mM Tris–HCl pH 9.6; 3 mM phenazine methosulfate) (Gupta and Rathaur, 2005).

2.9. The method of electrophoretic separation of proteins

The standard procedure of protein electrophoresis under non-denaturing conditions (CN-PAGE or native PAGE) was used (Gaal et al., 1980). The protein samples were separated with the aid of 10% (w/v) acrylamide gels.

2.10. Statistics

All the experiments were conducted at least in three replicates in three to five independent series of experiments (the data are presented as the mean ± SD). Analysis of Variance (one-way ANOVA or Kruskal-Willis ANOVA, as appropriate) was applied. The differences between means were evaluated using Tukey HSD tests. Statistical significance was considered at $P < 0.05$.

3. Results and discussion

3.1. Evaluation of purity of isolated vacuole fractions

Unlike vacuoles, plastids are stable cellular structures. There are accepted methods to purify the fractions of plastids from contaminants. Since isolated vacuoles are quite fragile structures, presently no

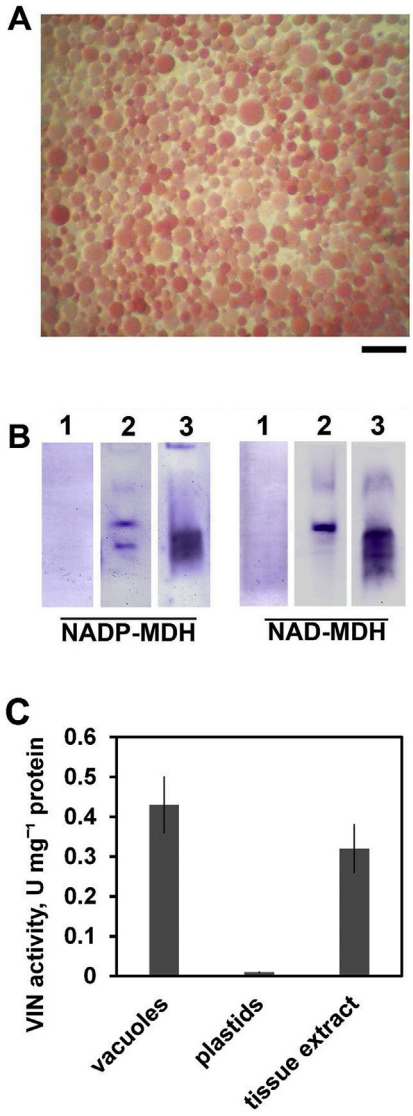

Fig. 1. Evaluation of purity of vacuolar fractions. **A** – Analysis of the isolated vacuoles in the light microscope. Bar = 100 μm. **B** – Malate dehydrogenase (MDH) has been considered as marker enzyme. NADP-MDH is specific for plastids, and NAD-MDH is found in many cellular compartments (peroxisomes, glyoxysomes, mitochondria, plastids, microsomes and cytosol). The activities of NAD-MDH and NADP-MDH have been detected in the plate of gel (CN-PAGE): 1, vacuolar fraction; 2, leucoplast's fraction; 3, tissue extract. This activity was not registered in the vacuolar fractions indicating the absence of contamination of isolated vacuoles by other organelles. **D** – Activities of vacuolar invertase in the isolated vacuoles and plastids, and also in tissue extract: (U), equals 1 μmol of sucrose hydrolyzed per minute.

universally accepted procedure for isolation of vacuoles has been proposed. In each case, an individual approach is needed and used.

The fraction of isolated vacuoles was obtained as described by Leigh and Branton (1976) with modifications. The main modification was represented by a chemical composition of the solutions for the isolation of organelles (as described in 'Materials and methods'). These solutions with high ionic power (0.8–1 M KCl) were used to purify the vacuoles from cytosolic proteins (Kuzevanov et al., 1981). Under these conditions, both cytosolic proteins and a substantial part of the weakly associated out-of-the-vacuole proteins were washed off, so the surface of the vacuoles was relatively 'clean'. The purity of the isolated vacuoles was assessed with the use of light microscopy (Fig. 1A); it was also evaluated by the marker enzymatic activity of plastids, nuclei, mitochondria and other organelles which could be the potential contaminants.

In the present paper, we consider malate dehydrogenase (MDH) as a marker enzyme for the following several cell compartments: NADP-MDH (L-malate:NADP-oxidoreductase; EC1.1.1.82), which is known to be specific to plastids; NAD-MDH (L-malate:NAD-oxidoreductase; EC 1.1.1.37), which has been found in many cellular compartments (peroxisomes, glyoxysomes, mitochondria, plastids, microsomes and cytosol) (Musrati et al., 1998). NAD-MDH and NADP-MDH activities were determined in gels (CN-PAGE) as described by Beeler at al. (2014). This activity was not detected in the vacuolar fractions indicating that there was no contamination of isolated vacuoles with other organelles (Fig. 1B).

We also analyzed the activity of acid invertase (ß-D-fructofuranosidase; EC 3.2.1.26), the latter being often considered as a marker enzyme of the central vacuole. The activity of vacuolar invertase (VIN) was determined as described by Tomlinson et al. (2004) and by Andersen et al. (2002). The level of the activity of VIN was relatively high in the fractions of isolated vacuoles, as shown in Fig. 1C.

In isolated organelles, we planned to determine the content of such a low-molecular weight compound as glutathione. However, it is believed that during the isolation procedure, the organelles partially lose their low-molecular weight compounds, what prevents from complete understanding of content of the latter compounds (Krueger et al., 2009). Since plant vacuoles were known to accumulate various free amino acids, we estimated their concentration in isolated vacuoles. Simultaneously the amino acid composition in leucoplasts and tissue extracts was analyzed. Eighteen free amino acids have been found in the vacuoles of red beetroots. Table 1 shows only the concentrations of abundantly present amino acids, as well as the concentration of tyrosine, which will be discussed below. Noteworthy, the concentrations of some abundantly present amino acids in tissue extracts were lower than in vacuoles. These high concentrations indicated to the fact of accumulation of amino acids in vacuoles. On the other hand, these high concentrations indicated to the relative preservation of low-molecular weight compound pools during the vacuole isolation procedure.

It should be noted that the tissue extracts contain mainly water-soluble substances of the vacuolar sap (because vacuoles occupy up to 90% of the cell volume of storage parenchyma of beetroots) and also contain water-soluble substances of other single-membrane-bounded organelles and of cytosol (as described in 'Materials and methods').

3.2. On the content of glutathione

The redox systems of cell compartments were formed according to their functions (Go and Jones, 2008). However, the redox system of glutathione was found in many compartments. Glutathione was detected in the nucleus, cytosol, mitochondria, leucoplasts and other compartments. Glutathione was found also in the central vacuole of some plants (Noctor et al., 2012).

Table 1
Free amino acid content.

Concentration, μM/L			
Amino acids	Vacuoles	Tissue extract	Plastids
Ala	0.82 ± 0.25	0.56 ± 0.18	0.13 ± 0.03
Asp	0.93 ± 0.18	1.15 ± 0.27	0.01 ± 0.00
Asn	0.64 ± 0.19	0.39 ± 0.06	0.22 ± 0.04
Arg	1.72 ± 0.23	1.62 ± 0.19	0.03 ± 0.01
Glu	1.38 ± 0.26	1.21 ± 0.28	0.16 ± 0.03
Gln	11.14 ± 1.53	4.76 ± 1.27	0.12 ± 0.02
Gly	0.27 ± 0.04	0.11 ± 0.03	0.09 ± 0.02
Ser	0.93 ± 0.13	0.75 ± 0.15	0.13 ± 0.04
Tyr	**0.12** ± 0.01	**0.09** ± 0.01	**0.01** ± 0.00

Here, concentrations of abundantly present amino acids and tyrosine are shown. Data are represented by means ± SD, n = 5.

There are different approaches, which have given a more detailed insight into the subcellular distribution of glutathione. Glutathione has been measured by biochemical methods after isolation of the compartments and has been studied in cells with the use of microscopy (the method of immunogold-cytochemistry and the fluorescence probe method). These methods have allowed the researchers to register glutathione and measure its concentrations: in chloroplasts – 0.5 and 5 mM; in the cytosol – 1 and 15 mM; in the vacuoles of certain plants – 0.03 and 0.13 mM, etc (Noctor et al., 2002; Krueger et al., 2009).

The vacuolar glutathione pool may be evaluated by comparing it with the glutathione pool of some other cell compartments. Therefore, the glutathione concentration has been measured not only in isolated vacuoles, but also in leucoplasts.

3.2.1. The method of bimane labeling

We have applied two generally accepted approaches, one of which was the fluorescent dye method. A widely used fluorescence method for determination of GSH in the living cells presumes addition of membrane-permeant MCB to the incubation medium and participation of the intracellular GSTs to form GCB adducts, which can be measured fluorometrically (Coleman et al., 1997). Therefore, MCB labeling not only highlights the GSH levels in cells, but can also

Provide for the information on activity of GSTs. Unlike other bimanes such as MBB, bimane MCB forms an adduct mainly with GSH (Kamencic et al., 2000). Often, the MCB labeling was used to determine the glutathione compartmentalization in situ (Meyer et al., 2001). However, many known investigations were limited due to inability of the probe to infiltrate definite organelles. So, it was not possible to obtain more information about the localization of GSH in peroxisomes, dictyosomes and endoplasmic reticulum. It was not also possible to quantify specific differences of the compartment by this method (Schwarzlander et al., 2008). Besides, it was previously shown that MCB-label could bind mainly to the cytosolic GSH due to the high activity of GSTs in the cytosol (Noctor et al., 2012). As for the vacuoles, the determination of GSH level in vacuoles in situ was hampered by the vacuolar sequestration of the GSB conjugates, which were formed in the cytosol and carried across the tonoplast by the ABC-transporters.

The fact is that accumulation of cytosolic GSB in the vacuoles of whole cells prevents from visualization of GSH in the vacuolar compartments (Coleman et al., 1997). Therefore, it is not possible to determine whether MCB permeates the tonoplast and reacts with GSH inside the vacuole, or not. The effect described above, which has been observed on whole cells, has induced us to conduct an investigation on isolated vacuoles.

In the very beginning of our investigation, we were unable to conduct precise measurement of glutathione content in the leucoplasts with the use of confocal microscopy because the isolated leucoplasts were mobile in the incubation medium. Meanwhile there were no such problems with the detection of GSH in vacuoles, because the characteristic of isolated vacuoles implied their attachment to the glass slide, what limited their mobility. However, isolated vacuoles are unstable structures, which cannot withstand prolonged manipulations during the experiment. A slight modification of this method has allowed us to limit the mobility of the isolated plastids during the incubation time, and we have reached evident success.

The optical images of isolated vacuoles and leucoplasts were obtained in the automatic mode, for identical times of signal acquisition, and at equal settings for the same microscope used. Fig. 2 shows the result of a representative experiment. There was an increase in fluorescence for 10 min, when the isolated plastids were labeled with MCB (Fig. 2F), and the progress curve for the reaction ended up at a plateau in about the same time (Fig. 3B).

In the case of vacuoles, MCB labeling was slower (Fig. 2B and C) and it still increased after 30 min without reaching a plateau (Fig. 3A). There was a noticeable increase in the fluorescence intensity during 40 min. Nevertheless, this observation revealed the presence of GSH in the vacuoles (Fig. 2B and C).

Obviously, to obtain objective results, long-term incubation of the organelles is needed, but such incubation is impossible because of disruption of isolated vacuoles. What is noticeable is that fluorescence of isolated vacuoles incubated with MCB substantially differs for the stable vacuoles. The fluorescence of small vacuoles was substantially higher than that of large vacuoles (Fig. 2B and C). Probably one of the causes was bound up with the diffusion distance, which was substantially shorter in the small organelles. The results of this experiment shown in Table 2 made us sure that the average GSH concentration was lower in the vacuoles (89 μM) than in the leucoplasts (366 μM).

Since some vacuoles disrupted during the analysis, the vacuolar sap of the disrupted vacuoles containing glutathione and GSTs was released into the reaction medium. It may, therefore, be assumed that GSB could be formed in the incubation medium and transported by ABC-transporters into stabilized vacuoles. However, the latter seemed unlikely because of the insufficiency of the required ATP concentration in the reaction medium, which was necessary for the functioning of above carriers in vitro (Coleman et al., 1997). Nevertheless, the experiment (as the control variant) with the inhibitor of ABC-transporters (10 mM Na_3VO_4) was conducted. The inhibitor had no effect on the concentration of GSH in the vacuoles (Table 2), although it substantially destabilized the vacuolar membrane. By the end of the experiment, only 20–30% of all initially observed organelles remained in the sample (Fig. 2D). In general, we may speak that GSBs are formed inside vacuoles and are not transported through the membrane from the environment.

The results obtained in our experiments have demonstrated that the MCB permeates into the vacuoles and reacts with GSH at low pH of the vacuolar sap, while the increase in the fluorescence is slow. As we assume, the slow fluorescence dynamics in vacuoles is a result of a larger size of the organelles and the longer transfer distance of the dye molecules in the vacuolar environment. However, when the MBB/MCB approach was applied, a different cause was revealed. As we made sure, the vacuolar contents influenced the probe's fluorescence. In the cases of samples with vacuolar and tissue extracts, a noticeable decrease in the fluorescence with MCB and especially with MBB was observed in relation to control samples (Fig. 4A and B). The decline in fluorescence was especially visible, when the measurements were conducted immediately after the moment, when MCB was added into the reaction medium (within 1 min). However, the latter was not true in the case of leucoplast samples. On the contrary, there was a noticeable increase in fluorescence after 1 min (Fig. 4A). Subsequent increases in the fluorescence were observed in 5 min (with MBB) and in 10 min (with MCB) in all the scrutinized samples.

As noted earlier, all detectable GSH had formed a GCB adduct within 10 min after the moment, when homogenate (extract) was incubated in the presence 0.1 mM MCB (Meyer et al., 2001). Therefore, we limited the incubation time to 10 min. Furthermore, the incubation time with MBB had to be reduced to 5 min, because after exposure to light, there was an obvious increase in the fluorescence of MBB by itself (without any interaction with glutathione) in the control samples (Fig. 4B).

In the case of MCB, proteinized samples were used, as described in 'Materials and methods'. This was due to the fact that the MCB labeling was accelerated by 10 times with GSTs (Coleman et al., 1997; Eklund et al., 2002). In the vacuolar and tissue extracts, the relatively rapid increase in fluorescence for 10 min compared with respect to the first minute of incubation gives evidence of the contribution of enzymes to this process. It should be noted that the fluorescence intensity in the deproteinized samples during MCB labeling virtually did not differ in control and experimental variants (data not shown).

In the case of MBB, we used deproteinized samples because this fluorescent dye reacted with protein thiols (Coleman et al., 1997). Furthermore, the bimane fluorescence was found to be quenched by tryptophan and tyrosine residues chains of proteins (Islas and Zagotta,

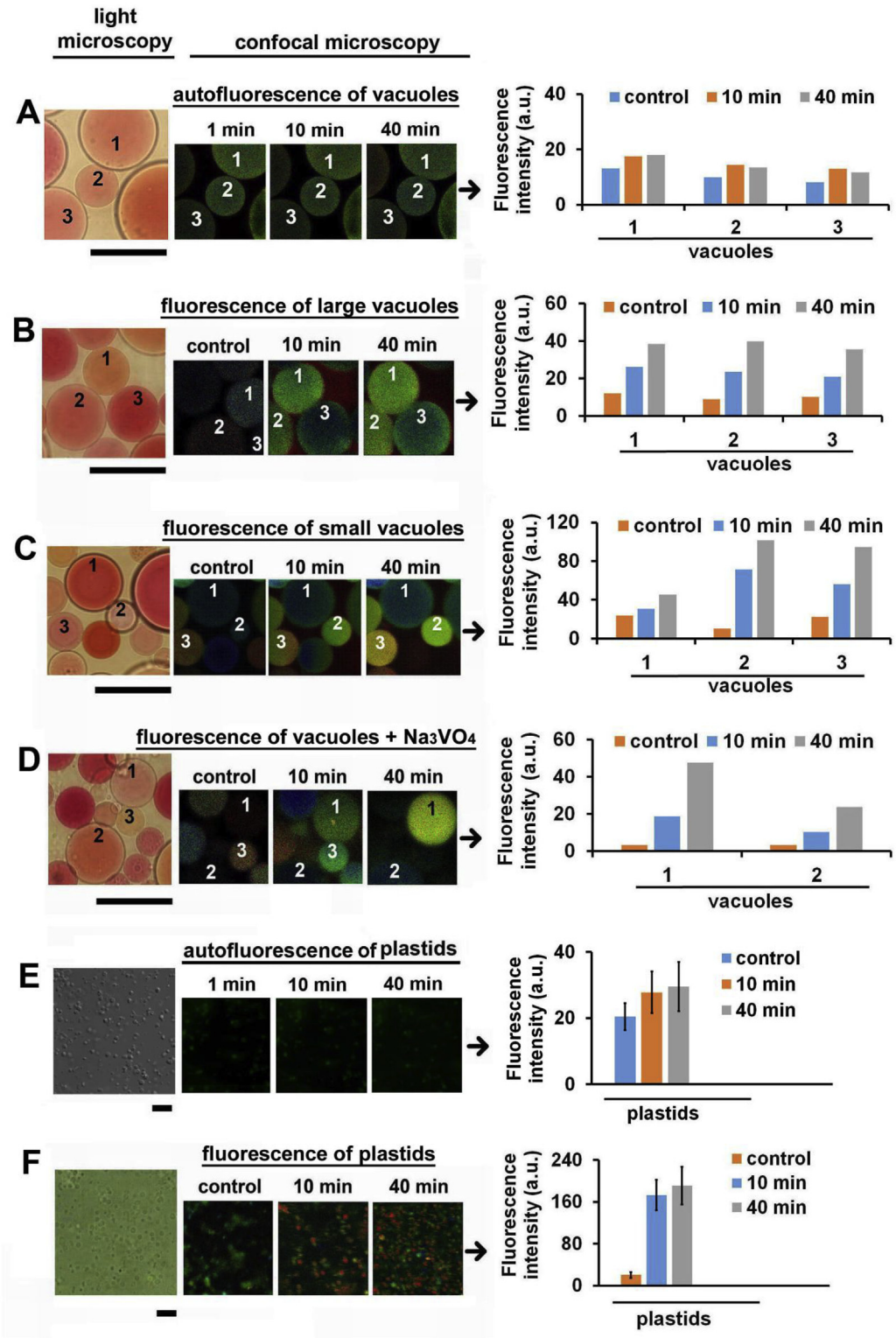

Fig. 2. The confocal microscopy images of isolated vacuoles and plastids from beetroot cells. Monochlorobimane (MCB) (0.1 mM) has been used to measure the relative contents of GSH. **A–F –** Confocal microscopy images are designated as 'confocal microscopy'. The isolated vacuoles and plastids have been also visualized in the light fields. The corresponding images are designated as 'light microscopy'. The set of images designated as 'autofluorescence of organelles' is represented by (i) images obtained after 1 min; (ii) images obtained after 10 min and (iii) images obtained after 40 min of incubation. The set of images designated as 'fluorescence of organelles' is represented by (i) 'control' is images of autofluorescence of organelles; (ii) images obtained after 10 min and (iii) images obtained after 40 min of incubation. **A –** The autofluorescence of vacuoles remained almost unchanged during the incubation for 40 min. **B, C –** The increase in the fluorescence of GSB in isolated vacuoles after 40 min was depended on the size of organelles; GSH was lower for larger vacuoles compared to smaller ones. Some of the vacuoles disrupted during 40 min of incubation. **D –** The stability of vacuoles decreased obviously, when 10 mM Na_3VO_4 (inhibitor of ABC transporters) was added into the incubation medium. After adding Na_3VO_4, only 30% of all vacuoles retained their integrity. **B –** However, the fluorescence of GSB in the 'surviving' vacuoles incubated with Na_3VO_4 practically did not differ from the variants (**B** and **C**). **E –**The autofluorescence of leucoplasts remained almost unchanged during 40 min of incubation. The average size of slightly colored leucoplasts was 3 μm. In order to make the light microscopy image more clear, a contrast image is displayed in pattern(**E**). **F** – The fluorescence of GSB in the isolated plastids. The histograms pointed by arrows show the vases of increase of GSB fluorescence in each of the numbered (1–3) vacuoles (**B-D**) and in most of plastids (arithmetic means) (**F**). In the vacuoles, an increase in fluorescence proceeded slowly, within 40 min, whereas in the plastids, the fluorescence intensity almost reached its maximum value within 10 min. Bar = 50 μm (for vacuoles). Bar = 10 μm (for plastids).

2006).

As it was shown, on the first minute of bimane labeling the fluorescence intensity in the experimental samples (of vacuoles and tissue extracts) was lower, so the obtained values of glutathione concentrations may be quite misleading. Nevertheless, we have presented these values herein (Table 3) because we think that it is expedient to give some explanations of the slower dynamics of fluorescence in isolated vacuoles.

Side by side with the tryptophan and tyrosine residues of protein chains, free amino acids also quench the bimane fluorescence (Sato et al., 1988). Vacuoles accumulate free amino acids including tyrosine (Tohge et al., 2011). As noted above, the red beetroot vacuoles also accumulate various free amino acids including tyrosine (Table 1). Tyrosine is known to be synthesized in plastids (Chapman and Leech, 1979; Rippert et al., 2009), but its concentration in leucoplasts of red beetroots is much smaller than in the vacuoles. The tyrosine appears to be accumulated mainly in vacuoles. Perhaps this amino acid is one of the factors of partial quenching of bimane fluorescence observed in

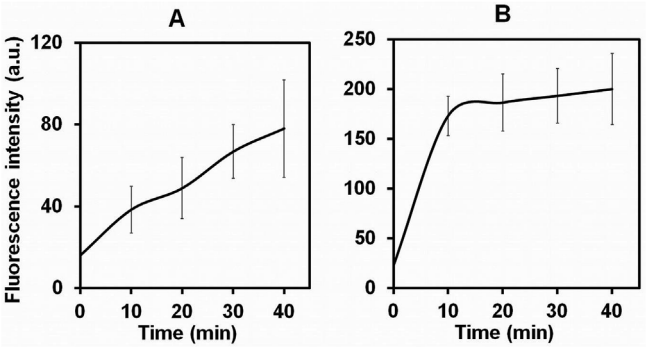

Fig. 3. Progress curves for the development of fluorescence, when the isolated vacuoles (**A**) and the leucoplasts (**B**) were incubated with 0.1 mM MCB for 1, 5, 10, 20 and 40 min. Observed was a slow increase in fluorescence in the vacuoles (within 40 min), and a rapid increase in fluorescence in the leucoplasts (within 10 min).

Table 2
The content of glutathione determined by confocal microscopy.

Organelles	Concentration GSH, μM
large vacuoles	59.2 ± 14.7*
small vacuoles	118.9 ± 29.6*
leucoplasts	366.1 ± 56.9*
all vacuoles	88.7 ± 24.9
all vacuoles + Na_3VO_4	79.6 ± 23.7

All vacuoles, the arithmetic mean of glutathione concentration in all the vacuoles (large and small vacuoles). Data are represented by the means ± SD, n = 5. *Asterisks indicate to the values, which are statistically differ from each other. Differences between the values are significant at $P < 0.05$.

Table 3
The content of glutathione determined by MBB/MCB approach (rough estimates).

Organelles	Concentration GSH, μM	
	with MCB (10 min)	with MBB (5 min)
vacuoles	91.8 ± 10.7	131.4 ± 28.1
plastids	249.7 ± 51.2	336.9 ± 33.9
tissue extract	197.9 ± 26.5	315.7 ± 46.8

5 and 10 min, the incubation time. Data are represented by the means ± SD, n = 3.

vacuoles. It may also be assumed that betacyanins localized in red beet root vacuoles can somehow influence bimane fluorescence, because the betalains (including betacianin) are synthesized from tyrosine (Sepulveda-Jimenez et al., 2004).

Summing up the results, we can conclude that the GSH content in MCB-labeled vacuoles might be understated because of: (i) large sizes of vacuoles with respect to plastids (i.e. larger diffusion distances) and low stability of isolated vacuoles; (ii) chemical composition of the intraorganelle fluid, which quenches the fluorescence of GSB (the presence betacianin and tyrosine in vacuoles); (iii) relatively low GST activity under acidic conditions of the vacuolar compartment (while the weak alkali conditions in leucoplasts are more optimal for the GST activity). Despite all the limitations of the bimane method, our results have convincingly demonstrated the presence of both GSH and GSTs in isolated vacuoles of red beetroots. On the other hand, we have not found any factors limiting the possibility of this method in the case of MCB labeling of glutathione in isolated leucoplasts.

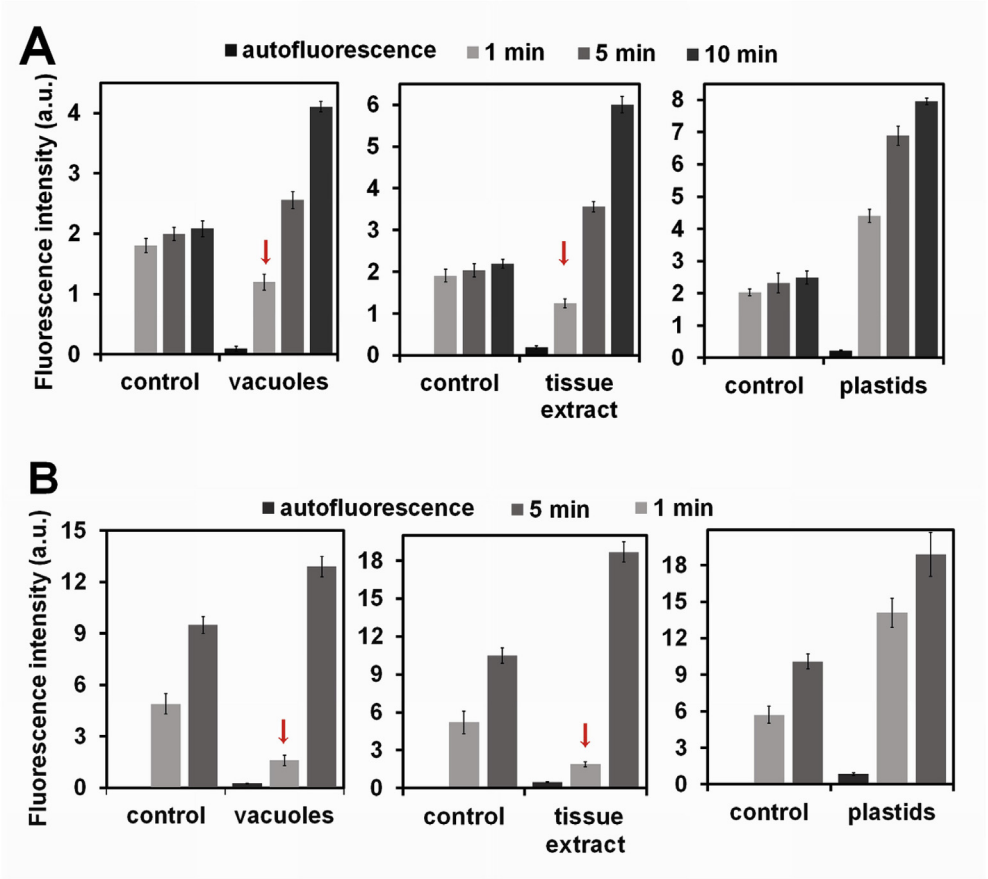

Fig. 4. Quantitative measurement of GSH in the vacuoles, plastids and in the tissue extracts. 0.1 mM MCB (**A**) or 0.1 mM MBB (**B**) was added into the reaction medium containing one of the extracts. The measurements were conducted with the aid of a fluorometer immediately after adding the sensor to the reaction medium and after 10 min of incubation with MCB (**A**) and 5 min of incubation with MBB (**B**).'Control', a sample, which does not contain extracts; 'Vacuoles', 'tissue extract', 'plastids', samples containing the corresponding extracts.
Arrows indicate a decrease in fluorescence immediately after adding MCB or MBB into the reaction medium. All the values are represented the means ± SD (n = 3).

3.2.2. Determination of glutathione by HPLC

It should be mentioned that all currently available fluorescent sensors (small molecule probes and genetically encoded fluorescent proteins) for detecting the compartmentalization of the glutathione couple and another redox couple have, to some extent, limitations from the viewpoint of selectivity and sensitivity (Kaludercic et al., 2014). In this connection, redox couples, including glutathione, have to be additionally detected by HPLC or mass spectrometry to confirm the results obtained with the aid of the fluorescent probe method (Kaludercic et al., 2014; Aon and Camara, 2015). Furthermore, HPLC is often used to quantify the redox potentials in definite compartments (Go and Jones, 2008). This method is characterized high specificity for revealing the quantity of oxidized and reduced forms of redox couples (Aon and Camara, 2015).

We also used the HPLC in order to determine glutathione in vitro in vacuolar extracts. Furthermore, it was important for us to determine the molar concentrations of GSH and GSSG in order to estimate Eh for the glutathione couples.

The results obtained by this method were different from those obtained with the use of fluorescent probes. The concentrations of GSH (341 μM) and GSHt (438 μM) in the vacuoles, which were calculated for all the degrees of dilution, turned out to be slightly higher than in the isolated leucoplasts (289 and 313 μM, respectively) (Table 4). Side by side with this fact, the concentration of GSSG in vacuoles (22% of the vacuolar GSHt) was higher than in the plastids (8% of plastidal GSHt). In this connection, the values of the GSH/GSSG ratio for the plastids (23) markedly exceeded those for the vacuoles (7). A relatively high value of GSH/GSSG was found for the tissue extracts (15), which were characterized by high GSHt contents (1 mM) in comparison to the vacuoles and leucoplasts.

So, the glutathione concentrations assessed by us in vacuoles isolated from beetroots (on the period of their physiological dormancy) turned out to be smaller than that in the tissue extracts, but these concentrations were slightly higher than those in isolated leucoplasts. These results differed from the data obtained for other plants. For example, wild-type *A. thaliana* cells had the highest concentration of GSH in cytosol and in chloroplast, and the lowest concentration of GSH in the vacuole. In wild-type *Nicotiana tabacum* cells, the plastids and mitochondria were characterized by the highest GSH content, whereas the content of GSH in vacuoles was the lowest. The abovementioned cellular compartments also differed with respect to the redox state of glutathione expressed as GSH/GSSG ratio (Noctor et al., 2012). The GSH/GSSG values for the chloroplasts and mitochondria in individual studies were higher than the GSH/GSSG values for the vacuoles (Dietz et al., 1992; Noctor et al., 2012). In our investigation, the plastidal GSH/GSSG values were quite high compared to those of vacuoles.

3.3. The reduction potential for the glutathione couple

The GSH/GSSG ratio may be used as a marker of the oxidative stress (Kemp et al., 2008). However, variations in this ratio are usually small under normal conditions, and the ratio indicator is not effective in assessing the redox state of cell compartments under normal conditions. Under normal conditions, Eh for the glutathione couple can be a more valuable indicator in assessing the intensity of redox processes (Go and Jones, 2008). Eh, also known as electromotive force, is a quantitative measure of the molecule's ability to donate or receive electrons (Kemp et al., 2008). Sometimes Eh is used as a way to quantify the ability of reduction (Kirlin et al., 1999).

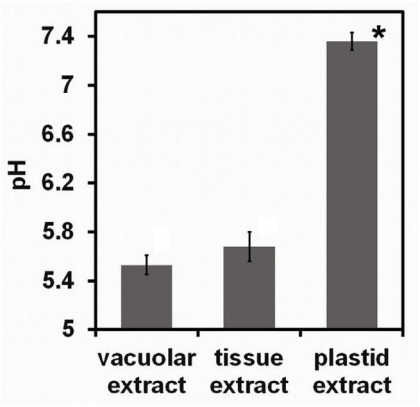

Fig. 5. Differences between the pH values of aqueous extracts of vacuoles (**a**), tissues (**b**) and plastids (**c**) under the conditions of physiological dormancy of red beetroots. Data are represented by the means ± SD, n = 5. *The asterisk indicates to the value, which is statistically different from the other two values ($P < 0.05$).

Having fixed the molar concentrations of GSH and GSSG, we estimated the E_h for the glutathione couple. To this end, we had to determine the corresponding E_{pH} of glutathione for vacuoles and leucoplasts. Our approach to determination of pH in the aqueous extracts gave approximate pH values; nevertheless, it allowed us to obtain reproducible results during several years. We made sure that the pH values of aqueous extracts of the isolated vacuoles and tissue extracts were almost similar (5.5 and 5.7, respectively) (Fig. 5). Side by side with that, the pH of leucoplast's aqueous extracts was 7.3. Our resulting pH values for vacuoles and leucoplasts of red beetroots corresponded to the pH values for the similar organelles of other plants (Otegui et al., 2005; Su and Lai, 2017).

On account of these pH values we computed the E_{pH} values of glutathione for the vacuoles and the leucoplasts, and these corresponded to −157 and −251 mV, respectively. As we expected, the value of E_h for the vacuolar glutathione (−163 mV) was substantially higher than the value of E_h for plastidal glutathione (−282 mV) (Table 4). It was previously shown (Schwarzlander et al., 2008) that Eh for the glutathione couple was lower in mitochondria (−360 mV) of *A. thaliana*; the redox potential for a chloroplastic glutathione redox couple was −230 mV (Noctor et al., 2002).

We have found that Eh for the glutathione couple in beetroot cells may be lower in the plastids and not so low in the vacuoles. Likewise, other authors, we have obtained approximate values of Eh for the glutathione couple because all the Eh values determined are operational, i.e., these represent functions of extraction, fractionation and assay methods (Kemp et al., 2008).

According to our results, the vacuoles of the storage parenchyma of the beetroot (storage part of the plant) during dormancy can accumulate some more glutathione compared to that in leucoplasts. However,

Table 4
The content of glutathione determined by HPLC.

Samples of	Glutathione, μM					
	GSH	GSSG	GSHt	GSH/GSSG	GSSG%	Eh mV
vacuoles	341.4 ± 39.2	48.4 ± 9.4	438.2 ± 57.9	7.1	22.1	−163
leucoplasts	288.5 ± 37.3	12.3 ± 1.5	313.1 ± 40.3	23.5	8.3	−282
tissue extract	879.7 ± 38.9	68.3 ± 17.7	1016.3 ± 74.2	14.9	13.4	

Data are represented by the means ± SD, n = 5. Differences between the values are significant at $P < 0.05$.

high GSSG concentrations and low pH of vacuolar sap are the factors, which contribute to low values of glutathione Eh in vacuoles. In the acidic environment of vacuoles, the reduced glutathione is supposed to be more stable. However, the high content of GSSG can result from both the intensive oxidation and the predominant transport of GSSG by the tonoplast-localized ABC-transporters (Tommasini et al., 1993; Queval et al., 2011). (Tommasini et al., 1993; Queval et al., 2011).

Nevertheless, the GSH level in the vacuoles is obviously higher than the GSSG level. Glutathione transported into vacuoles is likely to be involved in various redox reactions, which take place either spontaneously or with the participation of the corresponding enzymes. However, glutathione dependent enzymes of the central vacuole are still poorly studied (Ohkamu-Ohtsu et al., 2007).

3.4. On the activity and the isozyme composition of GSTs

The technique of MCB labelling was earlier recommended to quickly detect GST activity in the samples under study (Eklund et al., 2002). The relatively rapid increase in fluorescence in isolated vacuoles and leucoplasts during the MCB-labeling indicated to the contribution of GSTs.

In order to determine the GST activities, we used CDNB as the model substrate, since most of GSTs were known to catalyze the conversion of this compound to dinitrophenol-glutathione (Edwards and Dixon, 2005). Some of the corresponding results of the present investigation were discussed previously (Pradedova et al., 2016). High activity of vacuolar and plastidal GSTs was detected in the cells of red beet roots (Fig. 6). At the same time, GST activity was characterized by obvious dependence on pH conditions. Under moderate acidic conditions (pH 5.5) this activity decreased with respect to the activity under neutral conditions (pH 7.0) (Fig. 6A and B).

The vacuolar GST activity considerably exceeded the plastidal GST activity under all the conditions analyzed. However, the GST activity of vacuoles under moderate acidic conditions (pH 5.5) was obviously lower than the GST activity of plastids under neutral conditions (pH 7.0). Relatively low activity of GSTs under acidic conditions of the vacuolar content may be one of the causes of lowering down the fluorescence dynamics in the case of detection of glutathione with MCB. According to opinions of some researchers, the GST activity may be one of the factors limiting the potential of the fluorescence microscopy method with MCB, because this activity differs in various cellular compartments (Meyer et al., 2001).

GSTs of many plants interact with a great variety of substrates (Dixon et al., 2009). Likewise CDNB, EA, which is considered as a structural analogue of endogenous aldehydes, can serve as a substrate for GSTs of vacuoles. But at the same time, EA is not a substrate for plastidal GSTs (Fig. 6A and B). Depending on the properties of GST isoenzymes, EA can serve both as a substrate and as an inhibitor; therefore, it is widely used as a competitive inhibitor (Phillips and Mantle, 1991; Gronwald and Plaisance, 1998). EA (0.75 mM) used in combination with CDNB inhibits GST activity of vacuoles, leucoplasts and extract tissues by more than 80%, what, as we believe, speaks in favor of specificity of the observed enzymatic reaction. Conjugation of CDNB to GSH catalysed by GSTs was suppressed also by taxifolin. This flavonoid noticeably inhibited the GST activity of vacuoles, but not as obviously as in the case of the GST activity of tissue extracts and leucoplasts (Fig. 6C).

Definite enzymes of the glutathione transferase family interact with xenobiotic molecules, for example, with herbicides. The capability of GSTs to deactivate herbicides is usually testified with fluorodifen as the substrate. Conjugation of GSH to fluorodifen, unlike other herbicides, can be revealed spectrophotometrically (Pascal and Scalla, 1999). Since this approach may be used to determine the enzymatic activity with not all the herbicides that are in our interest, we considered the CN-PAGE method as a visual technique to investigate the interaction of GSTs with various substrates. Furthermore, this approach allows one to identify the GST isoforms, which react with a particular substrate. Some results of the representative experiment are shown in Fig. 7. The GST activity in the gel plates was visualized with such substrates as CDNB, EA, glyphosate and clopyralid. As mentioned above, GSH was the second substrate.

The plant cells contained a number of GSH-dependent enzymes, whose activity, in the present experiment, might be interpreted as a nonspecific reaction. In order to detect the non-specific activity, an additional control sample containing only GSH, was analyzed (lane 1). In the case, when GSH was used together with another substrate, the number of enzymatic activity zones increased (lanes 2–5). With substrates such as CDNB, EA, glyphosate and clopyralid, there were two zones appearing in the samples of vacuole and tissue extract (Fig. 7A, C).

Let us note again, the aqueous tissue extracts contained mainly water-soluble substances of the vacuolar sap, because vacuoles

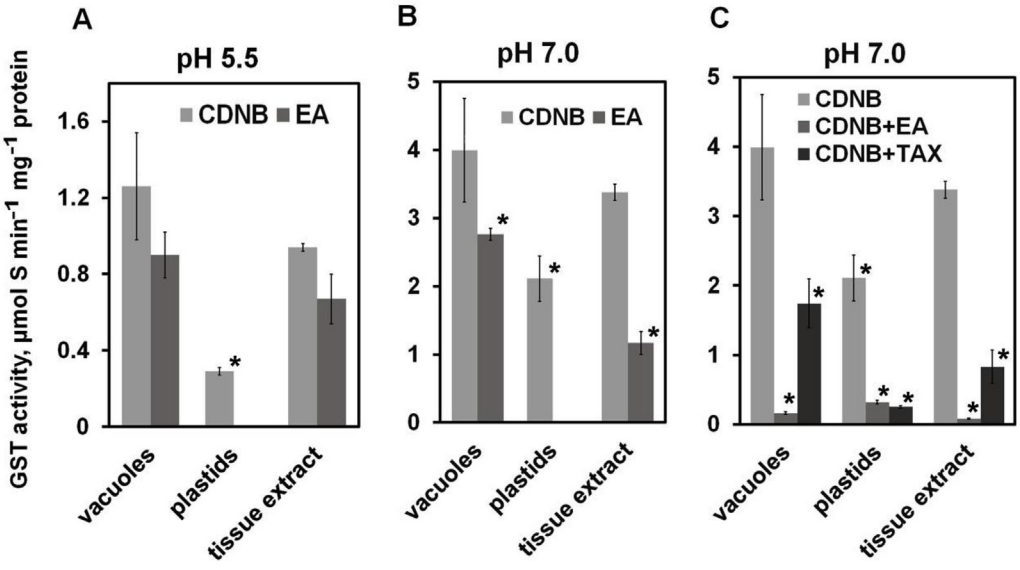

Fig. 6. The activity of glutathione S-transferase (GST). This activity was characterized by an obvious dependence on pH conditions. The reaction media with pH 5.5 as well as with pH 7.0 were used because these pH values corresponded to average pH values of the environment of vacuoles and leucoplasts, respectively. Used were the substrates and inhibitors such as of 1-chloro-2,4-dinitrobenzene (CDNB, model substrate), ethacrynic acid (EA, substrate and competitive inhibitor), taxifolin (TAX, potential inhibitor). **A, B –** The GST activities of the vacuoles, leucoplasts and tissue extracts with CDNB and EA as substrates can be found on these histograms. No activity bound up with EA in the plastidal fractions was detected. **C** – The enzymatic activity was suppressed by EA, what indicated to the specificity of these reactions. Flavonoid (taxifolin) also inhibited the GST of all objects studied.

* Asterisks above the bars indicate significant differences between the respective enzymatic activities with the specific substrate (or substrate + inhibitor) in vacuoles, in plastids and tissue extract ($P < 0.05$).

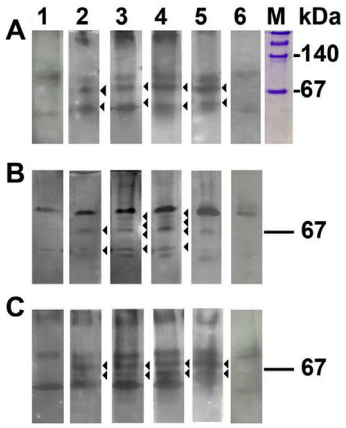

Fig. 7. CN-PAGE analysis of the glutathione S-transferase (GST) activity for the vacuoles (**A**), the leucoplasts (**B**) and the tissue extracts (**C**). On the present figure shown are mainly negative images. The substrates for GSTs have been used, such as: **1**, 1 mM glutathione; **2**, glutathione +1 mM CDNB; **3**, glutathione +0.5 mM glyphosate; **4**, glutathione +0.5 mM clopyralid; **5**, glutathione +0.5 mM ethacrynic acid; **6**, glutathione + CDNB (or herbicides) + 1 mM ethacrynic acid (competitive inhibitor). **M**, molecular markers (the gel was stained with Coomasie blue). Arrows indicate to GST isoforms. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

occupied up to 90% of the total cell volume (as described in 'Materials and methods' above). In the leucoplast samples, there were two zones with GST activity in the plate of gel when CDNB served as the substrate (Fig. 7B). In the case when glyphosate and clopyralid were used as the substrates, four zones appeared in the gel (lanes 3, 4), and weakly detectable GST isoform activity was also observed with EA (lane 5). These data gave evidence that leucoplasts probably contained a number of isoforms, which could catalyze the conjugation of GSH to EA. However, this reaction of conjugation with EA was not detected photometrically because of low rate of this reaction. All the zones with the enzymatic activity disappeared when a competitive EA inhibitor was introduced into the reaction medium with CDNB, glyphosate and clopyralid (lane 6).

In the gel plates, the assumed GST isoforms were observed in approximately the same zones corresponding to the marker protein with a molecular mass of 67 kDa. CN-PAGE analysis allowed us to roughly estimate the molecular mass of the observed isoforms. Nevertheless, these results did not contradict the data, according to which the molecular mass of the plant GST was close, on the average, to 50 kDa (Frova, 2006).

On the whole, one can find a lot of information on GSTs of plants. Meanwhile, there is little information on glutathione transferases found in vacuoles (Dixon et al., 2009). It is known that enzymes from a broad group of glutathione transferases are multifunctional proteins (Dixon et al., 1998; Riechers et al., 2003). The fact of rather wide subcellular distribution of GSTs should be emphasized (Öztetik, 2008). For example, application of proteomic analysis has given an opportunity to reveal specific GSTs of classes tau, phi, and lambda in the case of *A. thaliana* chloroplasts and mitochondria (Dixon et al., 2009). Specific isoenzymes have been found in cytosol, apoplast, and nucleus (Edwards and Dixon, 2005). One can also find the two investigations, in which GSTs in vacuoles have been demonstrated. GST isoforms, presumably of the phi class, and one microsomal isoform have been found in vacuoles of *A. thaliana* with the use of proteomic analysis (Carter et al., 2004). In the vacuoles of wheat seedlings, isoforms of presumably the tau class GSTs have been revealed with the use of immunoenzymatic assay (Riechers et al., 2003). Noteworthy, on the whole, the GST activity in vacuoles has not yet been evaluated.

In our work, enzymatic activity was determined by conventional techniques based on measurements with a model substrate CDNB. We found out that CDNB was not a universal substrate for all isoform GST. Furthermore, it was known earlier that specificity of this substrate (despite its widespread usage) for the enzymes belonging to the same GST class can vary substantially (Edwards and Dixon, 2005; Öztetik, 2008). The activity of two isoforms with this substrate could be observed on the gel plates in tracks with vacuolar and plastidal proteins (Fig. 7). In the process of analysis of the other substrates, significant differences in the isoenzyme composition of plastidal GSTs were revealed. In leucoplasts, the most part of the isoforms interact with herbicides rather than with CDNB. These facts indirectly indicate the localization of the various members of a wide family of glutathione transferases in vacuoles and plastids. As it has been previously shown, the isoenzyme composition of GST varies substantially in various cell structures of a single plant, as well as on different stages of plant development and under stress (Edwards and Dixon, 2005). There also were other differences revealed between GSTs of vacuoles and leucoplasts in the present investigation. For example, on the whole, the activity of vacuolar GSTs with CDNB was higher than that of plastidal GSTs. However, the vacuolar activity at pH 5.5 was noticeably lower than that of plastids at pH 7.0. This suggests the idea that the GST activity in a moderately acidized sap of the vacuoles in vivo will be lower than in a neutral stroma of plastids.

Relying on our own facts and on the information available, we can assume that, in vacuoles, GSTs fulfil the functions, which are attributed to GSTs. On the one hand, vacuolar enzymes react with EA (structural analogue of endogenous aldehydes) and with herbicides, what gives evidence of the fact of their participation in the process of detoxification of endogenous and exogenous toxic compounds. On the other hand, the fact of partial suppression of vacuolar GSTs by taxisphalin allows one to consider GSTs as elements of the flavonoid transport mechanism. Definite members of the glutathione transferase family are known to be involved in the transport of anthocyanins (flavonoid pigments) to vacuoles (Mueller et al., 2000). However, betalains (indole-derived pigments), rather than anthocyanins, are stored in red beetroot vacuoles, although the variety of other flavonoids have been found to accumulate within this compartment (Tohge et al., 2011). Perhaps flavonoids serve as a substrate for definite vacuolar GST. Furthermore, likewise definite glutathione transferases (Noctor et al., 2012), some of the vacuolar GSTs may be assumed to reduce lipid hydroperoxides with the use of the reducing equivalents of GSH. Therefore, in vacuole, the glutathione system may be assumed to be involved in detoxification, as well as in the antioxidant defense, and, possibly, in other various redox processes.

4. Conclusion

In our investigation, we pursued the following main objective: comparison of the glutathione system of vacuoles with that of leucoplasts. Within the frames of this objective the following parameters have been assessed: (i) quantity of glutathione accumulated in vacuoles and leucoplasts of taproot cells of red beet, and also the glutathione redox states (ratio GSH/GSSG and Eh for a couple of glutathione) in these organelles; (ii) activity of GSTs in vacuoles in comparison with that in leucoplasts. The issue of the presence of GSH and GSTs in plant vacuoles is still known to be under debate. Meanwhile, in course of this investigation we have applied the two abovementioned approaches in determination of glutathione. These classical approaches have allowed us to demonstrate the presence of both GSH and GSTs in isolated vacuoles of red beetroots. However, the fluorescent probe approach (with bimane labeling) has been found to be unsuitable for estimating GSH levels in vacuoles because of several limiting factors, which have not been discussed earlier and which, to our opinion, should be taken into consideration. The bimane labeling has clearly shown underestimated concentrations of glutathione. At the same time, the glutathione concentrations, determined with the use of HPLC, have been found to be

relatively high. Glutathione in the vacuoles of dormant red beetroots was more oxidized, and, as a result, the reduction potential (E_h) was not so reducing as in the case of leucoplasts.

On the other hand, MCB labeling has given evidence of possible presence of glutathione transferases in red beetroot vacuoles. In course of our investigation, the GST activity in the red beetroot vacuoles was evaluated with the aid of the ordinary photometric method and CN-PAGE analysis. This has given us an opportunity to obtain a new evidence of the presence of GST inside the central vacuole. Comparison of the GST activity of vacuoles and leucoplasts demonstrated that the vacuolar enzymatic activity might be relatively high. In this connection, we may assume that glutathione transported to the vacuole is needed to ensure functioning of GSTs. These glutathione dependent enzymes, which to some extent diminish the concentration of GSH, can vary the redox state of the glutathione pool in vacuoles, as well as in leucoplasts.

Obviously, the functions of vacuolar glutathione transferases have to be studied in greater detail. In this connection, future work have to be focused on the identification of glutathione-dependent and glutathione-regulated vacuolar proteins and on the elucidation of mechanisms, which facilitate the reduction of glutathione in the vacuoles. Such studies are keep steps to unravelling new aspects of the central vacuole functioning.

Author contributions

Oksana Nimaeva and Elena Pradedova performed most of the experiments. Alexander Rakevich performed the experiments using confocal microscopy. Elena Pradedov planned and designed the overall study, analyzed the data and wrote the manuscript. Rurik Salyayev led the project, analyzed the data and revised the manuscript. All authors discussed the results and commented on the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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