Overexpression of the Arabidopsis glutathione peroxidase-like 5 gene (*AtGPXL5*) resulted in altered redox status, plant development and salt tolerance

Ph.D. Dissertation

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1. List of abbreviations

ABA: abscisic acid

APX: ascorbate peroxidase

AsA: reduced ascorbate

CaMV35S: cauliflower mosaic virus 35S promoter

CAT: catalase

CDNB: 1-chloro-2,4-dinitrobenzene

CHP: cumene hydroperoxide

CO_2: carbon dioxide

DHA: dehydroascorbate

DHAR: dehydroascorbate reductase

DHE: dihydroethidium

DNA: deoxyribonucleic acid

DTT: dithiothreitol

E_{hc}: half-cell reduction potential

ETC: electron transport chains

FAO: Food and Agriculture Organization

FDA: fluorescein diacetate

FW: fresh weight

GPOX: glutathione peroxidase

GPX: mammalian glutathione peroxidase

GPXL: glutathione peroxidase-like

GR: glutathione reductase

GSH: reduced glutathione

GSSG: glutathione disulfide, oxidized glutathione

GST: glutathione transferase

H_2DCFDA: 2′,7′- dichlorodihydrofluorescein diacetate

H_2O_2: hydrogen peroxide

LP: lipid peroxides

MDA: malondialdehyde

MDHA: monodehydroascorbate

MDHAR: monodehydroascorbate reductase

MES/KCl: 2-(N-Morpholino) ethanesulfonic acid/ potassium chloride buffer

MS: Murashige-Skoog medium

NTR: NADPH-dependent thioredoxin reductase

$^1O_2$: singlet oxygen

$O_2^{\bullet-}$: superoxide radical anion

$OH^{\bullet}$: hydroxyl radical

PCD: plant cell death

POX: guaiacol peroxidase

PSI: photosystem I

PSII: photosystem II

PUFAs: polyunsaturated fatty acids

PVPP: polyvinyl-polypyrrolidone

qRT-PCR: quantitative real-time polymerase chain reaction

ROS: reactive oxygen species

SOD: superoxide dismutase

$t_{1/2}$: half-life

TBA: thiobarbituric acid

TBARS: thiobarbituric acid reactive substances

TCA: trichloroacetic acid

TPOX: thioredoxin peroxidase

TRIS: Tris(hydroxymethyl)aminomethane

TRX: thioredoxin

2. Introduction

2.1 The main environmental stresses and the effect of excess salt on plants

Being sessile organisms, plants always live in a very dynamic environment that are often adverse or stressful for their growth and development. These disadvantageous environmental circumstances include biotic stresses, such as attack by the pathogens and herbivores; abiotic stresses, such as drought, heat, cold, nutrient deficiency and surplus of salt or toxic metals in the soil. More than 50 % reduction in the average yield of large-scale crops has been attributed to the abiotic stresses (Wang et al., 2001; Fahad et al., 2017). Drought, salt and high or low temperature are the major environmental abiotic stress factors that affect the geographical distribution of plants in nature, limit plant productivity in agriculture and chief threats to food security. A published Food and Agriculture Organization (FAO) of the United Nations report in 2013 reported that 37.6% of the 13,003 million hectares (Mha) of the global land area is devoted to agriculture (Polle and Chen, 2015). According to Wicke et al. (2011), 1128 Mha, including 20% of irrigated lands, are estimated to be affected by soil salinity (http://www.fao.org/water/en/), with the largest area of 189 Mha being place in the Middle East (Fig. 1). Worldwide, soil salinity has adversely affected about 30 % of the irrigated land and 6% of total land area (Chaves et al., 2009) with a resultant monetary loss of 27.3 billion US$ in agricultural production (Qadir et al., 2014). Approximately 13% of Hungary or one third of the soils on the Great Hungarian Plain (N 46–48.5° and E 19–22.5°) are mainly affected by salinity problem and loose a significant amount of crop (Tóth, 2011).

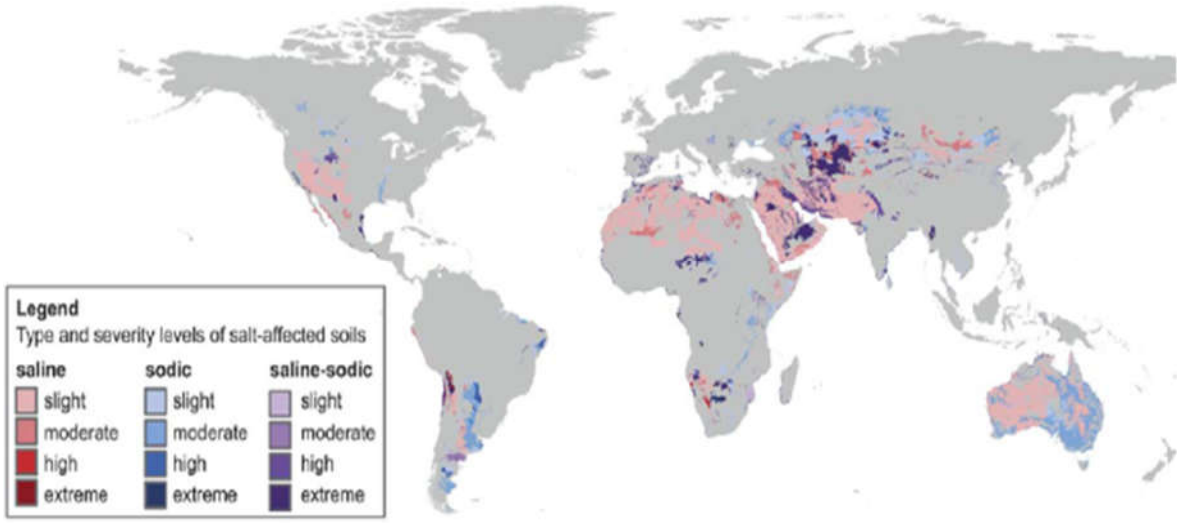

Fig. 1. Salt-affected soils around the world (Wicke et al., 2011).

Among the abiotic stresses, salt stress is considered one of the most adverse factors influencing the plant growth and productivity in the field. However, saline soils are rich in the form of most soluble and abundant sodium chloride salt that affect plant growth and productivity due to the gathering of salts over long periods of time in arid and semiarid zones or due to weak drainage and absence of impermeability in soils and accumulation of salt particles in the superficial horizons (Imadi et al., 2016; Hachicha et al., 2018). There are mainly two causes of soil salinity which are the followings:

- Primary cause of salinity happens due to natural processes such as wind, rain, flooding of the land by seawater or seepage of seawater. The increment in soil salinity also occurs with the aggravation of freshwater shortage (https://www.salineagricultureworldwide.com/salinization).

- Secondary cause of salinity is due to anthropogenic activities (salinity due to irrational land use and inappropriate agricultural practices). In a non-sustainable manner, the continuous use of groundwater is reducing the water tables in a rather concerning way notably because 20% of the water used in irrigation supplied from groundwater. Beside reducing its potential for future use, it generates multiple negative externalities, including salinity, stream depletion, or land subsidence that directly affect agricultural productivity, water users and the environmental deposition of oceanic salt (Munns, 2005; Manchanda and Garg, 2008; Hasanuzzaman et al., 2013).

Plant's responses to salinity occur in two phases: a rapid phase and another one is slower phase. Rapid phase is an osmotic phase which inhibits the growth of leaves by decreasing in the soil the water potential, the slower one is an ionic phase that accelerates the senescence of mature leaves due to increase of salt in the cell wall or cytoplasm of plants (Munns et al., 1995, Munns and Tester, 2008). An early phase manifest itself as an osmotic stress due to high salt concentrations outside of the root cells and mostly overlaps with drought stress, whereas the later phase is a specific ionic stress caused by accumulation of Na^+ and Cl^- ions to toxic levels that inhibits key enzymatic reactions in plant cells, mostly affecting the photosynthesis and shoot growth. Long-distance signals must regulate the reduction in the growth of leaves in the form of hormones or their precursors because the reduced leaf growth rate is independent of carbohydrate supply (Munns et al., 2000) and water status (Munns et al., 2000; Fricke et al., 2002). In the response of different environmental conditions, DELLA proteins are negative growth regulators that mediate the growth-promoting effects of gibberellins in several species and integrate signals from a range of hormones and abiotic stress conditions, including salinity

(Achard et al., 2006; Zhang and Zhang, 2016). The increased salt concentrations outside the roots create osmotic stress and induce stomatal closures which restrict CO_2 uptake, resulting in reduced carbon fixation and assimilation in leaf tissues (Fricke, 2004; James et al., 2008). Carbohydrate production during photosynthesis is therefore reduced, which impacts on plant growth and crop yield. Another consequence of stomatal closures can be the interruption of evapotranspiration of water, water potential and aggregation of reactive oxygen species (ROS) (Møller et al., 2007).

To acclimatize in the saline habitat, halophyte plants emerged an efficient mechanism to sustain their growth and yields (Orsini et al., 2010). According to Taji et al. (2004), *Thellungiella salsuginea* (also named as *T. halophila* or salt cress) due to the incorporation of efficient physiological and genetic processes proved to be highly tolerant than *Arabidopsis thaliana* (glycophyes) not only to high salinity but also to oxidative stress. The level of lipid hydroperoxide in the leaves of *Thellungiella* was remarkably lower than in Arabidopsis under salt stress (M'rah et al., 2007).

Plants are subjected to both abiotic (salinity, drought, high/low temperature, nutrient deficiency and high light) and biotic (pathogen attack) environmental factors throughout the entire life and the generation of ROS takes place in different organelles of the plants. Inequality between the rate of ROS generation and detoxification can create oxidative stress. Accumulation of ROS in excessive amounts is deleterious to the plants (Tripathy and Oelmüller, 2012; Huang et al., 2019) and can lead to uncontrolled oxidation of membranes, proteins and DNA, causing oxidative stress and cells death (Fichman et al., 2019). ROS are playing a pivotal role in various cellular, subcellular processes and act as a systemic signal in plants and other organisms. ROS also regulate many different hormonal, physiological pathways and various other fundamental biological processes in plants, including development, proliferation, defence, recycling, death pathways and acclimation to different abiotic and biotic conditions (Fichman et al., 2019).

2.2 Reactive oxygen species (ROS), oxidative stress, and their challenges

In plants, ROS exist either in ionic and/or molecular states (Huang et al., 2019) and formed as a by-product of the aerobic cell metabolic processes. The healthy metabolism of oxygen (O_2) or reduction of oxygen leads to the generation of ROS, which are naturally and constantly produced (about 1–2% of the total consumed O_2 molecules) (Mittler, 2017). ROS consists of free radicals like superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical (•OH), along with nonradical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In plants, ROS formations

are an unavoidable leakage of electrons onto O_2 from the various normal metabolic activities which occurred in different kind of multiple cellular compartments such as in chloroplasts, mitochondria, and plasma membranes (Heyno et al., 2011; Sharma et al., 2012; Mhamdi and Breusegem, 2018). ROS are the example of a metabolic product that has a dual role in signaling as well as in the governance of plant growth and development (Foyer and Noctor, 2009; Mittler, 2017; Noctor et al., 2018). Every type of ROS has unique and distinct chemical properties; at low levels they can act as intracellular signaling agents, inducing a decesive response in the antioxidant defence system (in both enzymatic and non-enzymatic); however, beyond the threshold level of excessive ROS it become deadly noxious and capable of interacting with all types of organic molecules, such oxidize lipids, cellular proteins and nucleic acids leading to cell death (Sharma et al., 2012; Foyer, 2018).

2.2.1 Superoxide radical anion ($O_2^{\bullet-}$)

With a 1-4 μs half-life, a highly reactive nucleophilic $O_2^{\bullet-}$ formation happen in the plant cells (Mittler, 2017), which begins a chains of reactions to produce other "secondary" ROS, namely hydrogen peroxide with either dependently or independently through enzyme- or metal-catalysed processes depending on the cell type or cellular compartment (Kimura et al., 2017; Janků et al., 2019). A significant source of $O_2^{\bullet-}$ production in a plant cell is the primary organelle such as mitochondria and chloroplast in complexes I, III and photosystems (PSI, PSII), respectively, and it is mainly associated with electron transport chains (ETC) (Noctor et al., 2006; Sharma et al., 2012). However, its production also takes place in other organelles, such as peroxisomes, glyoxysomes, as well as in the cell wall (Gill and Tuteja, 2010). $O_2^{\bullet-}$ radical anions can be diffuse for a few up to micrometers from the site of the generation and react with two molecules of H^+ and dismutate into H_2O_2 and O_2 (Demidchik, 2015). Superoxide radical anion preferentially reacts with other radical compounds, including nitric oxide derivatives and diverse protein components in both containing hem and non-hem iron centers (Halliwell, 2006; Janků et al., 2019). Being a moderate reactive radical relatively to other ROS molecules, it can not directly bind and chemically modify biological macromolecules. Accumulation of $O_2^{\bullet-}$ concentration become highly toxic due to their reducing ability, which can donate an electron to Fe^{3+} to become reduced Fe^{2+} moleculece. Fe^{2+} directly interact with H_2O_2 and accelerates the production of $OH^{\bullet}$, which is one of the lethal ROS due to its nature of highly reactiveness and it may cause peroxidation to membrane lipids and cellular weakening of different organelles (Ahmad et al., 2008; Demidchik, 2015; Mittler, 2017).

2.2.2 Hydrogen peroxide (H_2O_2)

Peroxisomes are one of the foremost leading production sites of H_2O_2 (Foyer and Noctor, 2003; Corpas, 2015), which is the most stable ROS with essential physiological functions (Demidchik, 2015). Several enzymatic systems are responsible for the direct production of H_2O_2 within these organelles (Corpas et al., 2015). Due to activities of catalase and peroxidases, the lifetime of H_2O_2 in the plant cell is not tremendously long. However, H_2O_2 with 1ms longer half-time is relatively stable molecules and can migrate from the subcellular synthesis sites to adjacent compartments and even neighboring cells (Corpas et al., 2015; Soares et al., 2019). However, the high toxicity of H_2O_2 can be easily interpreted by its oxidizing nature. H_2O_2 can react to enzymes and make them inactive by oxidizing their thiol groups. Oxidizing power of H_2O_2 makes them potentially fatal candidates for the surrounding cellular environment. Although, H_2O_2 also play a very important role as a signaling molecule to regulate the various physiological process, growth and development of plants (Gechev et al., 2006).

2.2.3 Hydroxyl radical ($^{\bullet}OH$)

The hydroxyl radical ($^{\bullet}OH$) formed as an outcome of the Haber-Weiss reaction, due to the interplay between $O_2^{\bullet-}$ and H_2O_2 in the existence of redox-active metals such as Cu and Fe (Cuypers et al., 2016) is the most highly reactive among all ROS with a half-life of 1 μs. Despite its low migration capacity (around 1 nm), However due to high constant reaction rate it rapidly interacts with all cellular molecules and causes of oxidative damages to proteins and nucleic acids, as well as lipids. It is directly involved in oxidative stress signaling and plant cell death (PCD) (Demidchik et al., 2010; Demidchik, 2015). Since there is no any potent enzymatic defence system to scavenging this noxious radical, its overproduction and excess accumulation in cells lead to triggers programmed cell death (Das and Roychoudhury 2014; Czarnockaa et al., 2018). Hydroxyl radicals are one of the most prevalent ROS, which cause lipid peroxidation.

2.3 Lipid peroxides (LP)

In general, plant lipid peroxidation is mainly due to ROS activity, where the dominant target of the ROS attack on lipids is the 1,4- pentadiene structure of polyunsaturated fatty acids (PUFAs) (Wagner et al., 1994; Porter et al., 1995), which are either free or esterified to cholesterol or glycerol (Browne and Armstrong 2002). Peroxidation of PUFAs, lacated in membrane phospholipids are notably susceptible to aggression by ROS, can lead to chain deterioration of membrane and, thereby, increase in membrane fluidity and permeability (Sharma et al., 2012). LP aggravates the oxidative stress through the production of lipid-derived free radicals that

themselves can react with other macromolecules like proteins and DNA (Sharma et al., 2012, Anjum et al., 2015) and triggering cell death (Foyer and Noctor 2005a). Over the years, with the expansion of molecular and biochemical studies on plant stress responses, secondary product of LPs, reactive aldehydes such as malondialdehyde (MDA) has been extensively used as a widely accepted warning signal of the occurrence of oxidative damage.

Most of the terrestrial plants including agricultural crops are glycophytic that can not withstand high concentration of salt and eventually die. Plant growth and development are hampered due to high salinity stress through (1) low osmotic potential of the soil solution (water stress), (2) nutritional imbalance, (3) specific ion effect (salt stress) or (4) a combination of these factors. Increased soil salinity can negatively influence the germination rates, growth and reproducibility of plants, physiological processes including photosynthesis, respiration, transpiration, membranes properties, nutrient homeostasis, and hormone regulation can lead to the production of higher ROS levels and their accumulation may lead to plant cell death (Mahajan and Tuteja, 2005; Hasanuzzaman et al., 2012). The effect of abiotic stresses have been investigated by using a number of approaches to elucidate the plant's responses including genetics (Snape et al., 2007; Bressan et al., 2009), genomics (Vij and Tyagi, 2007; Collins et al., 2008; Hu et al., 2009), transcriptomics (Rostoks et al., 2005; Mohammadi et al., 2007; Zeller et al., 2009), proteomics (Qureshi et al., 2007; Caruso et al., 2009), metabolomics (Shulaev et al., 2008) and ionomics (Salt et al., 2008; Vanhoudt et al., 2008; Jeong & Guerinot, 2009), as well as physiological measurements and morphological traits (Izanloo et al., 2008; Bowne et al., 2018).

Crop plants elicit a complex and unique cellular and molecular response in response to various stresses in order to prevent the damage and ensure survival (Fahad et al., 2015). In order to maintain the cellular homeostasis under both abiotic and biotic stresses (Mittler, 2017), plants equipped with a powerful and multifaceted antioxidant system that is consist of enzymatic and non-enzymatic components (Fig. 2), with various kind of biochemical properties and distinct subcellular localization (Foyer and Noctor 2003, 2005a, b), which are involved in sensing, detoxification, elimination and/or neutralization of ROS overproduction (Gratão et al., 2005; Liebthal et al., 2018; Soares et al., 2019).

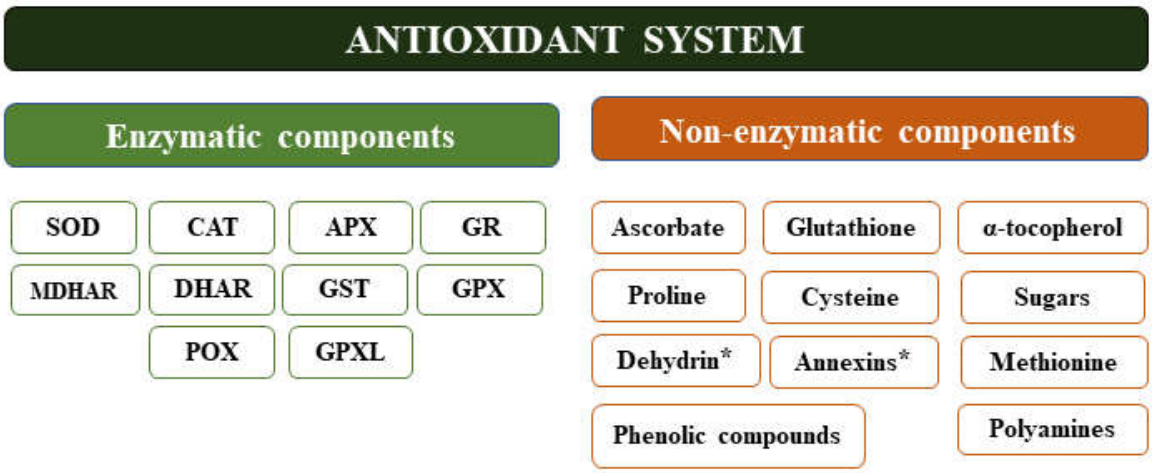

Fig. 2. Enzymatic and non-enzymatic antioxidant players in a typical plant cell. Words marked with a * represent new emerging components of the plant antioxidant system. SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GST, glutathione transferase; GPX, glutathione peroxidase; GPXL, glutathione peroxidase like; POX, guaiacol peroxidase (Soares et al., 2019).

2.4 Non-enzymatic antioxidant defense system

The components of non-enzymatic antioxidant defense system include the primary cellular redox buffers ascorbate (AsA) and tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as tocopherol, carotenoids, proline and phenolic compounds. These components of antioxidant system can neutralize, remove and transform ROS, allowing the management and sensing of ROS homeostasis in order to achieve the cellular redox balance in plants under stresses (Gratão et al., 2005; Mittler, 2017; Carvalho et al., 2018).

2.4.1 Ascorbic acid (AsA)

Ascorbic acid, commonly known as vitamin C, is an essential and most abundant antioxidant among non-enzymatic antioxidants in plant cells (Smirnoff, 2008) and acts to prevent or in minimizing the damage caused by ROS in plants (Smirnoff, 2005; Khan et al., 2008). AsA is a most potent ROS scavenger compound because of its nature to provide electrons in many enzymatic and non-enzymatic reactions. It is a water-soluble metabolite, which can reach up to 300 mM concentration in different organelles of the plant cells (Smirnoff, 2008), and present in distinct subcellular compartments, about 30 to 40% of the total ascorbate is available in the chloroplast (Gill and Tuteja, 2010).

The ascorbate redox system consists of L-ascorbic acid, monodehydroascorbate (MDHA) and dehydroascorbate (DHA). Smirnoff (2000b) has been reported that AsA mostly remain available in reduced form in leaves and chloroplasts under normal physiological conditions.

The reduced pool of AsA is maintained due to the activity of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and glutathione (Gill and Tuteja, 2010). Evidence to support the actual role of DHAR, GSH and GR in maintaining the foliar AsA pool have been observed in transformed plants overexpressing GR (Foyer et al., 1995). Furthermore, it can protect membranes by directly scavenge the $O_2^{\bullet-}$ and $OH^{\bullet}$ and by regenerate α -tocopherol from tocopheroxyl radical. In plants, mitochondria played a central role in metabolism and biosynthesis of AsA by L-galactono-γ-lactone dehydrogenase (EC 1.3.2.3), being posteriorly transported to other organelles via active transport or facilitated diffusion (Sharma et al., 2012). Ascorbic acid has essential function in AsA-GSH cycle, as well as maintained the activities of enzymes that contain prosthetic transition metal ions (Noctor and Foyer, 1998).

2.4.2 Glutathione (GSH)

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is a crucial low molecular weight non-protein thiol. GSH is a potential scavenger that can react chemically with $O_2^{\bullet-}$, $^{\bullet}OH$ and H_2O_2 and therefore functions as a master regulator of intracellular redox homeostasis (Sharma et al., 2012). It is localized in all cell compartments like cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes as well as in apoplast (Mittler and Zilinskas, 1992, 1993; Jimenez et al., 1997, 1998). GSH plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotic compounds (Xiang et al., 2001), expression of stress-responsive genes (Mullineaux et al., 2005) and serving as an electron-donating cofactor in biochemical reactions (Cobbett and Goldsbrough, 2002; Noctor et al., 2011). GSH plays a fundamental role in the antioxidative defense mechanism by regenerating another antioxidant component, like AsA via the AsA-GSH cycle (Foyer and Halliwell 1976). Under normal conditions, total tissue glutathione pool is mostly reduced; ratios of the reduced and oxidized forms (GSH/GSSG) in leaves are usually no less than 20:1 (Noctor et al., 2012). Many scientific reports indicate that GSH is one of the most important scavengers of ROS, and its ratio with oxidised glutathione (GSSG) may be used as a marker of oxidative stress.

2.4.3 Glutathione half-cell reduction potential

The most important components which can determine the redox state of the cell are the pyridine nucleotides ($NADPH/NADP^+$; $NADH/NAD^+$), glutathione (2GSH/GSSG), ascorbate (AsA/DHA), thioredoxins (TRX_{red}/TRX_{ox}), tocopherols, glutaredoxins, peroxiredoxins and

thiol proteins (Dietz, 2003; Kocsy et al., 2013; Halliwell and Gutteridge, 2015). Many reports indicate that the level of stress tolerance in the plants were highly correlated with the redox change (H_2O_2 level, AsA and GSH concentration and the ratio of their reduced to oxidized form) and the reduction potential of the GSH/GSSG couple (Soltesz et al., 2011). Glutathione reduction potential (E_{GSH}) depends on the absolute glutathione concentration and the ratio of GSH to GSSG (Meyer and Hell 2005). The reduction potential of the GSH/GSSG couple (half-cell reduction potential; E_{hc}) can be calculated from concentrations of GSH and GSSG by using the Nernst equation applying the formula of Schafer and Buettner (2001). Aller et al. (2013), reported that under optimal conditions, cytosolic GSH buffer is immensely reduced and hence more negative than −310 mV and changes in the E_{GSH} can drastically affect the plant development. Increase of E_{GSH} to −260 mV (e.g., in the roots of *root meristemless1* (*rml1)* mutants) is sufficient to prevent the cell cycle G_1/S progression and induced large changes in the transcript profiles of roots and shoots and an increase to −170 mV instigate the apoptosis in Arabidopsis (Aller et al., 2013; Schnaubelt et al., 2015).

2.5 Enzymatic antioxidant defense system

The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), MDHAR, DHAR, GR, glutathione peroxidase (GPX) and guaiacol peroxidase (POX) (Fig. 2.).

2.5.1 Superoxide dismutase (SOD; EC 1.15.1.1.)

The first line of defence provided by the SOD isoforms against the ROS-induced damages in the plants (Gratão et al., 2005; Soares et al., 2019). The antioxidant SOD enzyme belongs to metalloenzymes group can catalyze the transformation of $O_2^{\bullet-}$ by dismutation into H_2O_2 and O_2 (Giannopolitis and Ries, 1977). SOD antioxidants enzyme prevents the possibility of $OH^{\bullet}$ formation by the Haber-Weiss reaction which is extremely reactive and may cause serious damage to membrane lipids, proteins and DNA macromolecules (Ahmad et al. 2010; del Río et al., 2018; Luis et al., 2018). SOD is a part of antioxidant defense system plays a key role in maintaining cellular defense against ROS and providing help to the plants against combating the environmental stresses. Based on the metal cofactors, SODs are classified into three known types: the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD), which are localized in different cellular compartments (Mittler 2002). SOD activity is increased by abiotic stress conditions (Boguszewska et al., 2010). Moreover, the improvement of stress

tolerance in plants over-expressing *SOD* genes underlines the vital role of these enzymes in counteracting the potential adverse effects of ROS (Gill et al., 2015).

2.5.2 Catalase (CAT; EC 1.11.1.6)

Catalases are tetrameric heme-containing proteins with pivotal enzymatic function; able to remove of peroxisome-generated H_2O_2 by directly catalyzing the dismutation of H_2O_2 into H_2O and O_2 (Gill and Tuteja, 2010) without the need of reducing cofactors, was the first antioxidant enzyme to be discovered and functionally characterized (Sharma et al., 2012; Soares et al., 2019). Among all the antioxidant enzyme, CAT has one of the highest turnover rates and able to reduce 6 million molecules of H_2O_2 to H_2O and O_2 per minute (Gill and Tuteja, 2010). However, plant catalases have relatively low affinity for H_2O_2, meaning that catalase activities rise linearly as H_2O_2 levels increase (Mhamdi et al., 2012; Soares et al., 2019). There are three catalase genes (*CAT1*, *CAT2* and *CAT3*) in the *Arabidopsis thaliana* (Frugoli et al., 1996; Hu et al., 2010) and their expression patterns indicate that these genes help in the elimination of H_2O_2 in the metabolic processes such as β-oxidation of fatty acids, photorespiration and senescence, respectively (Mhamdi et al., 2012). At the subcellular level, it is not only present in the peroxisome, but in other cell compartments like cytosol, chloroplasts, and mitochondria (Mhamdi et al., 2010).

2.5.3 Enzymes of the ascorbate-glutathione (AsA-GSH) cycle

The Foyer-Halliwell-Asada pathway (other name is ascorbate–glutathione cycle; AsA-GSH) is an important pathway to scavenge H_2O_2 in which ascorbic acid (AsA) is used as the electron donor (Gill and Tuteja, 2010; Polle, 2001). The AsA-GSH cycle mainly involves in the consecutive oxidation and reduction of AsA, GSH, and NADPH catalyzed by these following enzymes: APX, MDHAR, DHAR, and GR.

2.5.3.1 Ascorbate peroxidase (APX; EC 1.11.1.11)

APX is a central component of AsA-GSH cycle and involved in scavenging of H_2O_2 in water-water and AsA-GSH cycles (Foyer and Noctor, 2003, 2005a, b). APX utilized two molecules of AsA to reduce H_2O_2 to water molecule with a concomitant generation of two molecules of MDHA (Sharma et al., 2012) and plays a crucial function in the regulation of intracellular ROS levels (Mittler et al., 2004; Sharma et al., 2012). APX isoenzymes have much higher affinity for H_2O_2 than CAT (Wang et al., 1999; Soares et al., 2019) and can also exert its functions even with low amount of this ROS, while CAT is mainly participated in preventing H_2O_2-induced

cellular damage by eleminating excessive ROS production (Mittler, 2002). APX not only responsible for modulation of H_2O_2 levels necessary in signaling events but also act as an efficient scavenger of H_2O_2 under stressful conditions.

2.5.3.2 Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4.) and dehydroascorbate reductase (DHAR; EC 1.8.5.1.)

The scavenging of H_2O_2 by APX leads to the formation of a very unstable MDHA radical, and if not rapidly reduced, it nonenzymatically disproportionates to AsA and DHA (Ushimaru et al., 1997; Smirnoff et al., 2000a). MDHAR and DHAR are essential participants of the AsA-GSH cycle as they both help ascorbate to get reduced from either monodehydro ascorbate or dehydroascorbate (Inze´ and Van Montagu, 1995; Asada, 2006). So far, MDHA can also be reduced by MDHAR, a flavin adenine dinucleotide (FAD) containing enzyme using reducing power from NAD(P)H (Park et al., 2016). Due to the nonenzymatic disproportion of MDHA to AsA and DHA, the very short-lived DHA can either be hydrolyzed irreversibly to 2,3-diketogulonic acid or recycled to AsA by DHAR. DHAR classified as a monomeric thiol protein, is a necessarily present in seed tissues, roots and green organs (Eltayeb et al., 2007). DHAR enzyme is a crucial element of the AsA-GSH cycle, allowing the regeneration of AsA from its oxidized form DHA using GSH as the electron donor; consequently, it also contributes to the regulation of redox balance (Hossain et al., 1984; Mittler, 2002; Jaleel et al., 2009; Gill and Tuteja, 2010; Soares et al., 2019).

2.5.3.3 Glutathione reductase (GR, EC 1.6.4.2.)

GR is a flavo-protein oxidoreductase and potential enzyme of the AsA-GSH cycle. GR is mainly present in chloroplasts, but it can be located in mitochondria, cytosol and peroxisome too (Gill and Tuteja, 2010). It catalyzes the reduction of GSSG to GSH, allowing the maintenance of GSH/GSSG ratio (Yannarelli et al., 2007), therefore it is regarded as playing an important role in defense system against ROS by maintaining the GSH pool of cells (Arora et al., 2002; Gill and Tuteja, 2010).

2.5.4 Guaiacol peroxidase (POX; EC: 1.11.1.7)

POX, a heme-containing protein can regulate H_2O_2 intracellular levels using different organic aromatic electron donor compounds such as guaiacol or pyrogallol (Gill and Tuteja, 2010). Guaiacol peroxidase is a ubiquitous protein; structurally composed by monomers of around 40–50 kDa and possesses four conserved disulfide bridges and two structural Ca^{2+} ions (Gill and

Tuteja, 2010; Das and Roychoudhury, 2014). POX isoenzymes can be found explicitly in distinct plant organs and organelles, especially in vacuoles, cytosol and cell wall (Sharma et al., 2012). POXs are not only widely accepted as a stress enzyme but also participates in many critical biosynthetic processes, contributing to cell wall's lignification, wound healing, catabolism of IAA, and biosynthesis of ethylene (Sharma et al., 2012).

2.5.5 Glutathione transferases (GSTs; EC 2.5.1.18)

GST represents a very ancient class of enzymes that participate in a broad network of catalytic and regulatory functions and their existence in different types of organisms, including animals and plants (Basantani and Srivastava, 2007; Ghelfi et al., 2011). Plant GSTs are grouped into ten different classes, and generally they are cytoplasmic, but some isoenzymes were also been present in chloroplasts, apoplasts and microsomes (Gill and Tuteja, 2010), among them the tau (GSTU), phi (GSTF), lambda (GSTL) and dehydroascorbate reductase (DHAR) are specific to plants. The tau and phi classes are mostly responsible for catalyzing the conjugation of GSH with a wide range of electrophilic substrates (Marrs, 1996; Gill and Tuteja, 2010; Cummins et al., 2011). Their most known function is the detoxification of exogenous and endogenous harmful toxic compounds, including herbicides, xenobiotics and endogenous stress metabolites. Besides their function as necessary antioxidant enzymes, they have also been involved in numerous redox, hormone, and stress responses. Glutathione-dependent peroxidase activity can be associated with GST isoenzymes and can convert lipid peroxides and other peroxides to less harmful compounds (Edwards et al., 2000; Horváth et al., 2019).

2.5.6 Thiol-based peroxidases

Peroxidases oxidize various substrates utilizing H_2O_2 or organic hydroperoxides, hence they are involved in scavenging of ROS (Bela et al., 2015). Peroxidases can be divided in heme-based and thiol-based peroxidases. CAT, APX and POX (already described above) are heme co-factor-containing peroxidases, while into the non-heme-containing peroxidases belong glutathione peroxidases (GPX; EC 1.11.1.9) and thioredoxin peroxidases, which possess redox active cysteine or selenocysteine residues in their active site (Bela et al., 2015; Dietz, 2016). Both GPXs and GSTs reduce H_2O_2 and hydroperoxides by thiol-mediated pathways (Dietz et al., 2002; Chang et al., 2009; Bela et al., 2015). According to some authors, enzymes like GSTs can also be considered as thiol-based peroxidases; however, strictly, only thioredoxin peroxidases and GPXs are thiol peroxidases, due to their high affinity to peroxides (Dietz, 2016).

The plant thiol peroxidases can be classified into five subgroups, which include the 2-Cys Prx, 1-Cys Prx, type II Prx, Prx Q and GPX type peroxidases (Rouhier and Jacquot, 2005). In *Arabidopsis thaliana* 18 thiol peroxidases were identified: one 1-Cys Prx, two 2-Cys Prxs, six type II Prxs, one type Q Prx and eight GPXs (http://peroxibase.toulouse.inra.fr; Koua et al., 2009, Bela et al., 2015

2.6 Glutathione peroxidases (EC 1.11.1.9, EC 1.11.1.12 and EC 1.11.1.15)

GPX enzymes are non-heme thiol peroxidases that catalyse the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione or thioredoxin (TRX) (Arthur, 2000; Battin and Brumaghim, 2009; Yang et al., 2016; Bela et al., 2018). The first time, Mills observed the reaction with H_2O_2 in enzyme preparations from mammalian red blood cells and coined the term glutathione peroxidase; mostly accepted abbreviation of them is GPXs (Mills, 1957; Bela et al., 2015). The mammalian GPXs are central components of processing ROS and lipid peroxides, thus they participate in the maintenance of the membrane integrity (Islam et al., 2015). GPXs act as intermediate signaling molecules that can pass the redox signals via the oxidation of cysteine-containing proteins involved in the signaling, such as phosphatases, kinases, and transcription factors can be induced or regulate the different pathways (Luo et al., 2005; Marinho et al., 2014). In the sperm of mammals, after oxidization of GPX4 by hydroperoxide act as signal transducer and reacts with sperm mitochondria-associated cysteine rich proteins and involved in the sperm motility (Maiorino et al., 2005).

GPX1 regulates the insulin signaling by affecting of the hydrogen peroxide homeostasis, it also helps to prevent oxidative DNA damage and inhibit the initiation of carcinogenesis (Baliga et al., 2007; Brigelius-Flohé and Kipp, 2009; Bela et al., 2015). Moreover, GPX2 and GPX3 are not only involved in the oxidative stress, but also connected to many types of inflammation and cancer even to obesity (Lee et al., 2005, 2008; Dittrich et al., 2010; Burk et al., 2011; Brigelius-Flohé and Kipp, 2012). Some reports indicate that, GPX7 and GPX8 supposed to be involved in the re-oxidation of protein disulphide isomerase during the protein folding in the endoplasmic reticulum (Brigelius-Flohé and Maiorino, 2013). In *Saccharomyces cerevisiae*, the GPX-like enzyme Orp1 (GPX3) has been reported to act as hydroperoxide sensor (receptor and redox transducer) that promotes the oxidation of Yap1 transcription factor to its intra-molecular disulfide bond (Delaunay et al., 2002). This relay mechanism has been exploited for the development of genetically encoded H_2O_2-sensors (Gutscher et al., 2009). The role of GPXs in signaling occurs in a wide range of organisms.

In contrast to the animal enzymes, the plant enzymes contain cysteine in their active site instead of selenocysteine, and most of them prefer TRX as electron donor rather than GSH (Figs. 3 and 4) (Herbette et al., 2002; Iqbal et al., 2006; Navrot et al., 2006; Lubos et al., 2011, Bela et al., 2018). Attacha et al. (2017) suggested using the GPX-like (GPXL) nomenclature for the *Arabidopsis thaliana* isoforms to avoid any confusion resulting from protein names, and now this abbreviation is applied in all referees to the earlier published information concerning plant glutathione peroxidases. The plant glutathione peroxidase-like genes are closely related to animal phospholipid hydroperoxide glutathione peroxidases (Margis et al., 2008), and it was reported that plant isoenzymes reduce more efficiently peroxides different from H_2O_2 such as lipid peroxides (Milla et al., 2003). They are commonly considered as one of the key players in the enzymatic defence system of plants.

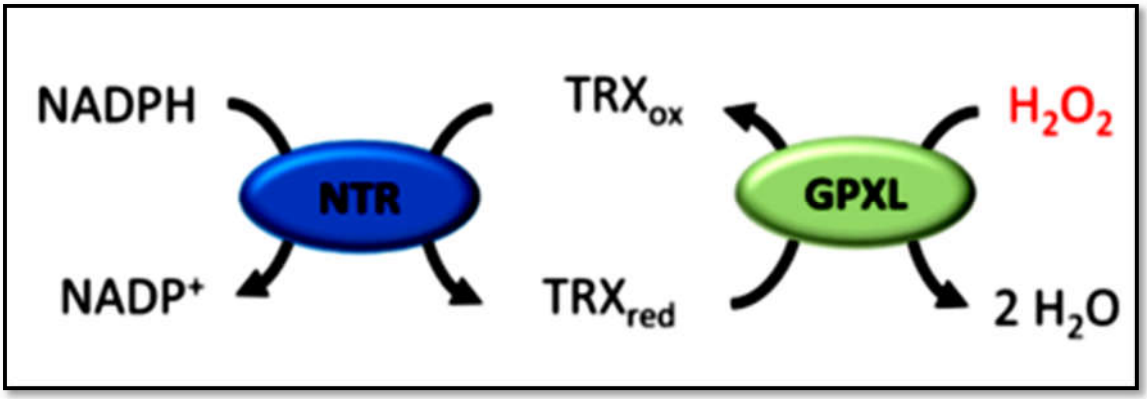

Fig. 3. Detoxification of H_2O_2 by plant glutathione peroxidase-like (GPXL) enzymes. GPXLs converts hydrogen peroxide into water using reducing equivalents from thioredoxin (TRX_{red}). The oxidized TRX (TRX_{OX}) is again converted into reduced form by NADPH-dependent thioredoxin reductase (NTR) (https://images.app.goo.gl/x3uJqGPrbGP8Q3b68).

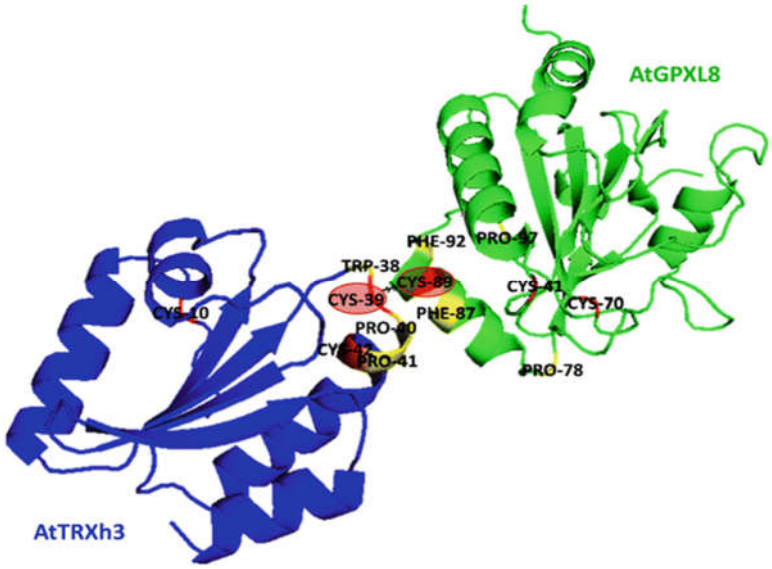

Fig. 4. Model of the *Arabidopsis* GPXL8 and TRXh3 complex interaction. During the GPX regeneration, the CYS-39 of TRXh3 forms a disulfide bond with the CYS-89 of AtGPXL8, while aromatic residues could involve in the protein-protein interaction (Koh et al., 2007).

A conserved structure of GPXs protein consists of a central β-sheets surrounded by α-helices (Koh et al., 2007), some mammalian GPXs forms the tetramers due to facilitated by their oligomerization loop between the α3-helix and β6-strand (Toppo et al., 2008). Interestingly, oligomerization loops do not exist in the plants GPXLs are reside in the monomeric forms (Maiorino et al., 1995, Navrot et al., 2006) except for the poplar (*Populus trichocarpa*) GPXL5, which showed a unique dimerization pattern mainly depending on hydrophobic contacts and was able to interact with Cd^{2+} ions (Fig. 5, Koh et al., 2007).

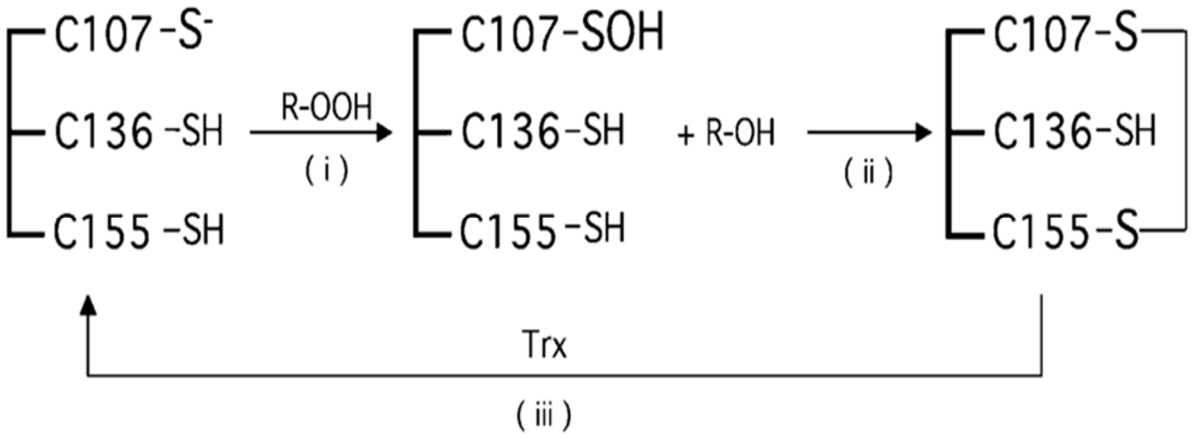

Fig. 5. Proposed catalytic and TRX-dependent recycling mechanisms for poplar GPXL: (i) Nucleophilic attack of Cys-107 on peroxide (ROOH) leading to the formation of a sulfenic acid and the concomitant release of an alcohol; (ii) Formation of an intramolecular disulphide bridge between Cys-107 and Cys-155; and (iii) Reduction of the intramolecular disulfide bridge by TRX leading to a reduced enzyme and an oxidized TRX (Navrot et al., 2006).

However, *AtGPXL* genes encode a protein of 167-236 amino acid residue long, 18.9-26.0 kDa molecular weight with 5.11-9.53 pI value and contained only six exons in the transcripts. Despite the highly conserved structures of *Arabidopsis* GPXLs members, some minor variations were also present. It seems that these divergences in GPXLs may not change the protein-3D structure, but they could attribute the new functional roles to catalytic activities (Fig. 6).

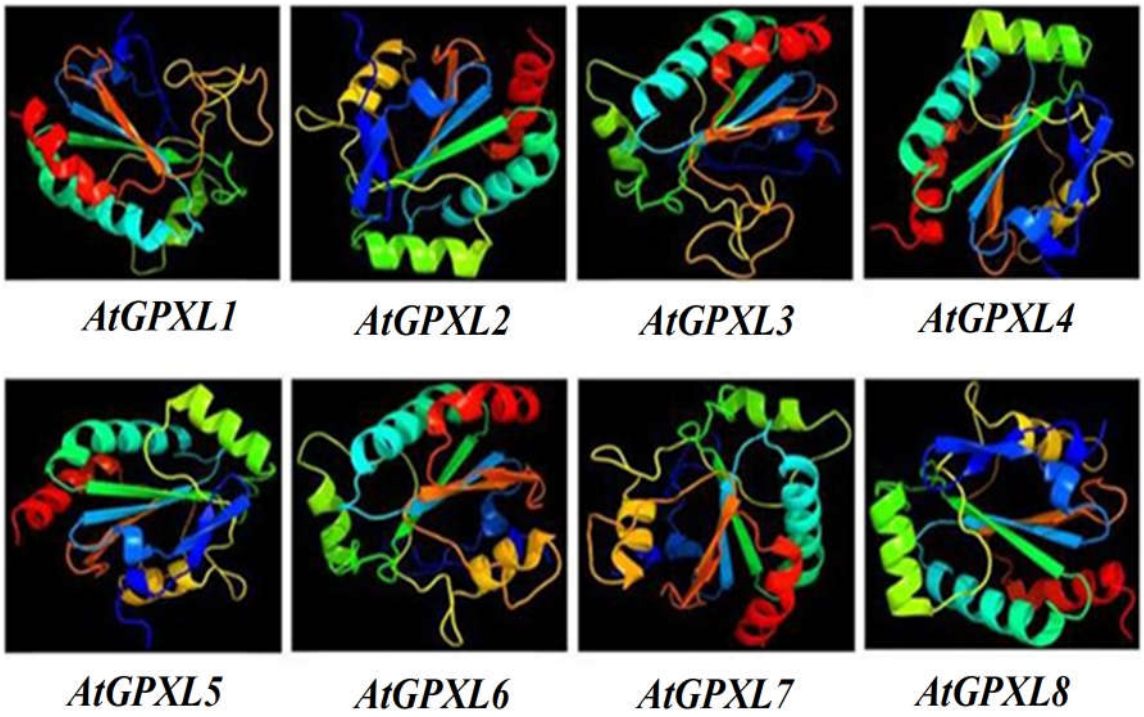

Fig. 6. 3D models of predicted *Arabidopsis* glutathione peroxidase GPXL1-8 sequences. Models were constructed by using Phyre[2] server for AT2G25080.1 (GPXL1), AT2G31570.1 (GPXL2), AT2G43350.1 (GPXL3), AT2G48150.1 (GPXL4), AT3G63080.1 (GPXL5), AT4G11600.1 (GPXL6), AT4G31870.1 (GPXL7), and AT1G63460.1 (GPXL8) sequences, and colored by rainbow from N- to C-terminus (Ozyigit et al., 2016).

Plant GPXLs contains three conserved cysteines in their catalytic sites, but only two of them, the so-called peroxidatic (CysP-S$^-$) and the third one, the resolving cysteines (CysR-SH) take part in the catalytic activity (Navrot et al., 2006; Koh et al., 2007). During the reduction of peroxide, firstly peroxidatic cysteine transformed into the sulfenic acid (CysP-SOH), and after the concomitant release of an alcohol molecule, resolving cysteine form an intramolecular disulphide bridge with the peroxidatic cysteine, protecting CysP-SOH against overoxidation (Waszczak et al., 2014). However, in the Chinese cabbage the second and third cysteines might be having an involvement in the formation of disulphide bridge, while mostly in other plants the third cysteine is the resolving type (Jung et al., 2002; Navrot et al., 2006). The 2-Cys disulfide is reduced by thioredoxin – a low-molecular-weight protein with two vicinal Cys

residue (Fig. 5) – or by glutathione (Jung et al., 2002; Iqbal et al., 2006; Koh et al., 2007; Toppo et al., 2009).

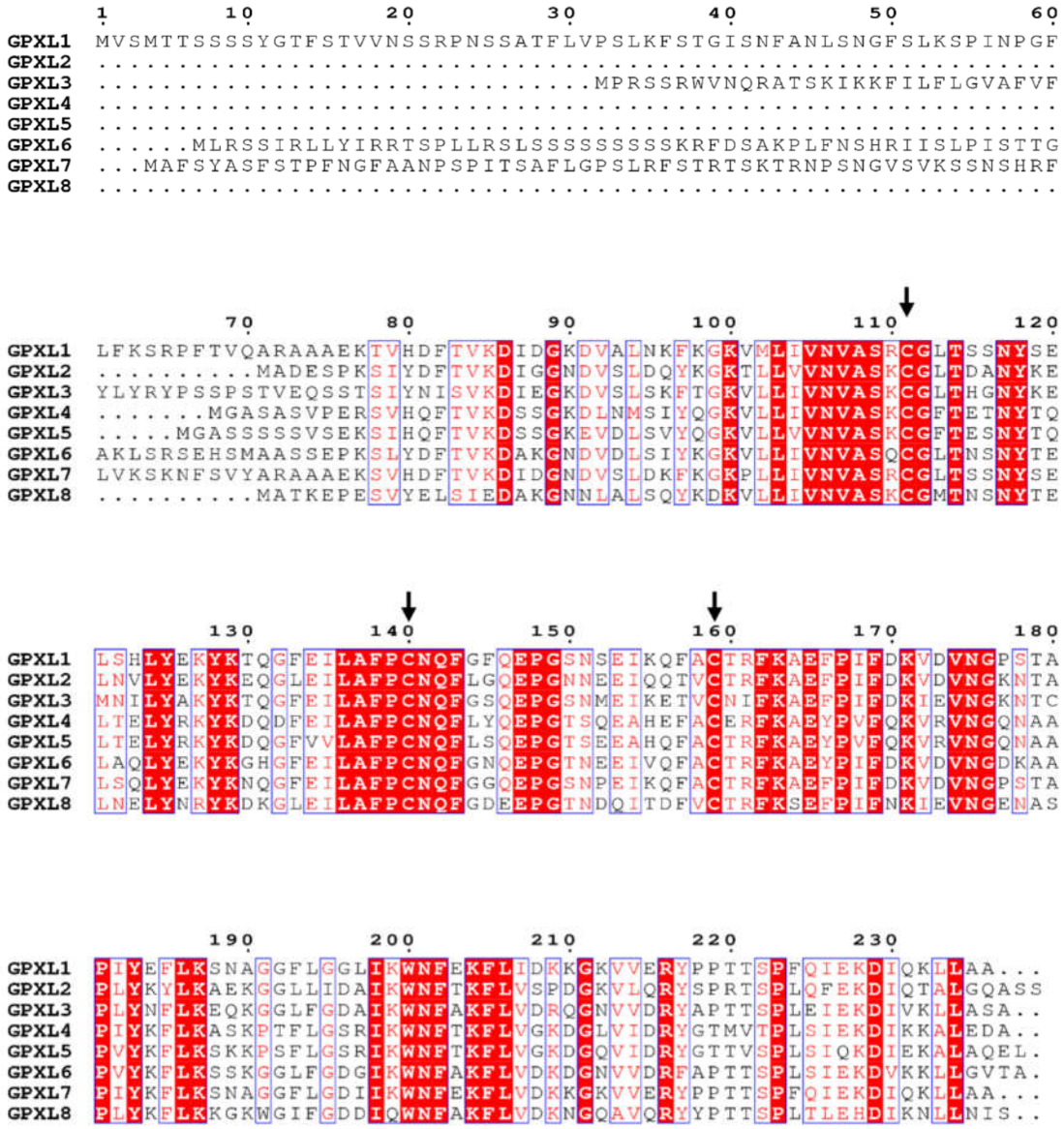

Fig. 7. Amino acid sequence alignment of GPXLs from *Arabidopsis thaliana.* Numbering is according to GPXL1. Arrows mark the three conserved cysteines present in *Arabidopsis* GPXLs. Sequences were aligned by Mafft with default settings using JalView. Gaps within the signal peptides until position 70 were removed manually. Highly similar residues (Score >0.8) are framed and coloured in red. Identical residues are marked in white letters on red background (Attacha et al., 2017).

Glutathione and thioredoxin are the main components of cellular redox homeostasis and critically involved in healthy plant growth, development, successful organogenesis and regeneration of cultured cells (Marty et al., 2009; Bashandy et al., 2010; Lu and Holmgren 2014; Bela et al., 2017). Reduced thiols are important for the processes that determine the root architecture (Benitez-Alfonso et al., 2009). Characterization of the Arabidopsis *root*

meristemless1 (*rml1*) mutant, which has damaged GSH biosynthesis (Vernoux et al., 2000), displayed altered expression of several hundred genes (Schnaubelt et al., 2015). GSH deficient mutants *rml1* are unable to maintain the root apical meristem, however, the shoot apical meristem of *rml1* mutants is not much affected, might be due to the thioredoxin-dependent control (Diaz Vivancos et al., 2010). Among the genes regulated by low GSH, numerous encode redox-related proteins, such as glutaredoxins (GRXs), h-type thioredoxins (TRXhs) and GPXLs. During the acute shortage of glutathione, more TRX is used as electron donor compared to the GSH, suggesting having a possible connection between the reduced GSH- and TRX systems (Schnaubelt et al., 2015).

The plant GPXLs are present in different plant tissues, compartments and developmental stages (Bela et al., 2015). The plant glutathione peroxidase gene family has been studied and characterized in many plant species including *Arabidopsis thaliana* (Rodriguez Milla et al., 2003), *Lotus japonicus* (Ramos et. al., 2009), *Thellungiella salsuginea* (Gao et al., 2014), *Oryza sativa* (Islam et al., 2015), *Gossypium hirsutum* (Chen et al., 2017), *Cucumis sativus* (Zhou et al., 2018), *Sorghum bicolor* (Akbudak et al., 2018) and *Triticum aestivum* (Tyagi et al., 2018).

The Arabidopsis genome encodes 8 GPXL isoforms, which have been predicted to be localized in different subcellular compartments. AtGPXL1 and AtGPXL7 are chloroplastic proteins, AtGPXL2 and AtGPXL8 are localised in cytosol as well as in nucleus, AtGPXL6 can be found in cytosol and mitochondria, AtGPXL3 is a transmembrane protein of the secretory pathway, while AtGPXL4 and AtGPXL5 are associated to the inner side of the plasma membrane (Attacha et al., 2017).

The chloroplastic isoenzymes AtGPXL1 and AtGPXL7 showed 82% amino acid similarity and are considered to be specific for chloroplasts. Milla et al. (2003) assumed their overlapping function because of the high sequence homology; however, both genes have different expression patterns under different stimuli (Chang et al., 2009). Chang et al. (2009) reported their role in photooxidative stress and immune response, and AtGPXL1 might be involved in the defence against the virulent pathogen infection. In the leaves of single insertional mutant *Atgpxl7* or antisense double mutants (*Atgpxl1* and *Atgpxl7*) showed higher H_2O_2 levels so that reduced the tolerance against photooxidative damage but enhanced the cell death and improved the basal resistance to virulent *Pseudomonas* bacteria. Additionally, a mutation can alter the leaves and chloroplast morphology: transgenic lines had irregular spongy mesophyll cells and larger intercellular air spaces and more abundant starch grains in the chloroplast compared to the wild type. Their essential role was also reported in the chloroplastic ROS homeostasis and

redox signaling between cellular compartments that may coordinate acclimatory and defense responses (Chang et al., 2009). The important role of AtGPXL7 has been verified in the regulation of the shoot and root development (Passaia et al., 2014).

The least investigated isoenzyme AtGPXL2 was found in the cytoplasm as well in the nucleus. Xu et al. (2010) reported as a part of an antioxidant defense system and involved in the stress defence by converting H_2O_2 into H_2O. *AtGPXL2* gene exhibited the highest expression level among *AtGPXLs* in the shoot and roots of 10-day-old wild type seedlings (Passaia et al. 2014). However, the transcript level of *AtGPXL2* interestingly decreased under osmotic stress caused by mannitol (Milla et al., 2003), but salt treatment increased the transcript level of *AtGPXL2* (Gao et al., 2014). Based on the results of Bela et al. (2018), the important role of AtGPXL2 in the stress response and development can be assumed.

AtGPXL8 was also found in the cytosol and in the nucleus (Gaber et al., 2012), and the localization of AtGPXL8 was proved later by the Attacha et al. (2017). The most important role of AtGPXL8 is not only the protection of nucleic acid from the oxidative stress by scavenging the harmful peroxidases, but also the protection of nuclear cellular components (nucleus). *AtGPXL8* knockout and overexpressing Arabidopsis transgenic plants showed decreased and increased tolerance against paraquat, respectively, that was correlated with enlarged and reduced growth inhibition (Gaber et al., 2012). According to the transcriptional data, the gene expression level of *AtGPXL8* was upregulated by hypoxia, germination, callus formation, short term treatment of ABA and SA and long term of cold treatment; however, its expression level was down regulated by long term effect of drought stress and ABA treatment (Bela et al., 2015). The induction of *AtGPXL8* gene in Arabidopsis shoot by plant hormones indicated that phytohormones regulate the *AtGPXL8* gene expression (Gaber, 2011; Bela et al., 2015).

The putative localization of AtGPXL6 in mitochondria and the cytosol has been reported and explained by an assumption that it may encode mitochondrial and cytosolic enzymes by alternative initiation (Milla et al., 2003). The mitochondrial localization of AtGPXL6 is also in agreement with the identification of AtGPXL6 in the proteome of mitochondria in *Arabidopsis* (Yoshida et al., 2013). Beside AsA-GSH cycle, PRX-IIF and APX, the most suitable candidate for direct detoxification of H_2O_2 or preventing lipid peroxidation in mitochondria would be AtGPXL6 using TRX as an electron donor system. The mitochondrial potato homolog of AtGPXL6 has been found to be a potential target of TRX (Balmer et al., 2004). In Arabidopsis, two TRX proteins (TRXo1 and TRXo2) have been reported in mitochondria (Laloi et al., 2001) that can potentially act as a physiological electron donor for AtGPXL6. Sugimoto and

Sakamoto (1997) proved that the *AtGPXL6* expression level elevated in salt and osmotic stress as well as under the influence of aluminum and iron treatments (Noctor et al., 2011). Other studies have also shown that the expression level of *AtGPXL6* is mostly affected by abiotic stress and higher expression level were measured in the protoplast and guard cells (Milla et al., 2003).

AtGPXL3 is a monomeric transmembrane protein of the secretory pathway with enzymatically active portions of the Golgi lumen (Attacha et al., 2017). *Atgpxl3* mutants have been shown to be more sensitive to drought stress and AtGPXL3 overexpressed lines as drought tolerant, and higher H_2O_2 content than wild-type plants (Miao et al., 2006, 2007). According to the results of Miao et al. (2006), AtGPXL3 functions as both a cytosolic redox transducer and a scavenger of H_2O_2 in abscisic acid (ABA) and drought stress responses in the guard cell. It was demonstrated that AtGPXL3 does not only have a scavenging function but can also interact with 2C-type protein phosphatase ABA INSENSITIVE2 (ABI2), therefore it functions as an oxidative signal transducer in ABA and drought stress signaling. To support the above hypothesis, Miao et al., (2006) provided data indicating physical interaction of AtGPXL3 with the ABI1 and ABI2 proteins in both yeast two-hybrid and pull-down assays as well as bimolecular fluorescence complementation for GPXL3 and ABI2 fused with yellow fluorescent protein (YFP)-fragments complementing each other in the cytosol. The *Atgpxl3* mutation disrupted the ABA activation of calcium channels and the expression of ABA- and stress-responsive genes (Miao et al., 2006). Passaia et al. (2014) reported the role of AtGPXL3 also in the root development in ABA-independent manner.

The AtGPXL4 and AtGPXL5 are poorly investigated GPXL isoenzymes of Arabidopsis. They are anchored to the plasma membrane, but they do not have a transmembrane domain (Attacha et al., 2017). In i*n vitro* studies, biochemically these recombinant proteins are capable of scavenging H_2O_2 and organic hydroperoxide using thioredoxin as an electron donor but, interestingly, AtGPXL5 was unable to reduce the cumene hydroperoxide substrate by either TRX or GSH (Iqbal et al., 2006). Iqbal et al. (2006) reported their essential roles in the redox homeostasis by the equilibrium of thiol/disulfide or NADPH/$NADP^+$ ratio. The transcript level of *AtGPXL4* and *AtGPXL5* were significantly notable in the pollen, stamen and phloem, but the expression level of *AtGPXL4* in the shoot and root was below the detection level (Passaia et al., 2014). According to data found in the Genevestigator database, *AtGPXL5* is expressed in all developmental stages of Arabidopsis plants, both in shoots and roots (Bela et al., 2015). The phenotype of the *Atgpxl5* mutants with severely decreased *AtGPXL5* expression showed a rather

small alteration (Bela et al., 2018). Our earlier experiments conducted on 6-week-old hydroponically grown plants revealed relatively low *AtGPXL5* transcript amounts compared to the other *AtGPXLs* both in control conditions and after applying salt or osmotic stress (Bela et al., 2018). *In silico* promoter analysis of the *AtGPXL5* gene revealed the presence of two *cis*-regulatory sequences connected to seed development (AAGAA-motif and Skn-1_motif), and several abiotic stress-related, such as anaerobic responsive element (ARE), heat shock elements (HSE), MYB binding sites (MBS) and biotic (Box-W1) stress-related *cis*-acting elements in the 5' regulatory region (Bruce et al., 1991; Washida et al., 1999; Bela et al., 2015).

Detailed investigation of the *GPXL* genes and proteins were performed in Arabidopsis and in its extreme abiotic stress tolerant relative, *Thellungiella salsuginea* by Gao et al. (2014). It was hypothesized that differences in salt tolerance mechanisms between salt-sensitive glycophytes, such as *A. thaliana*, and salt-tolerant halophytes, such as *T. salsuginea,* are resulted from changes in the regulation of the same basic set of genes involved in salt tolerance (Zhu, 2001). Comparing the protein and gene expression patterns of glutathione peroxidases in Arabidopsis and *Thellungiella*, Gao and his co-workers found that more *GPXL* genes were induced under salt and osmotic stress conditions in *Thellungiella* than in Arabidopsis (Gao et al., 2014). According to their results, expression of *TsGPXL2*, *TsGPXL3*, *TsGPXL4/TsGPXL5*, and *TsGPXL7* genes were induced under short-term osmotic treatment in both leaves and roots, and four of the encoded proteins (TsGPXL3, TsGPXL5, TsGPXL7 and TsGPXL8) were shown to be important for salt and osmotic stress response. Investigating the *AtGPXL* gene expression data found in AtGenExpress, they reported that *AtGPXL2*, *AtGPXL6*, and *AtGPXL8* were significantly up-regulated due to short-term (6-24 h) salt treatments in leaves, and *AtGPXL1*, *AtGPXL2*, *AtGPXL4*, *AtGPXL6*, and *AtGPXL7* in roots. GPXL5 was responsive to salt stress in *Thellungiella*, but not in Arabidopsis. It was concluded that the salt stress inducible TsGPXL5 can be implicated in the enhanced stress tolerance of *Thellungiella*, moreover, they suggested that TsGPXL5 is essential in the effective salt tolerance of *Thellungiella* (Gao et al., 2014).

3. Aims

Our aims were to investigate the role of AtGPXL5 isoenzyme in the salt stress response of Arabidopsis plants and to check the involvement of the enzyme in the oxidative stress responses. First the ROS levels and vitality of the *Arabidopsis thaliana* ecotype Columbia (Col-0) and a glutathione peroxidase-like 5 T-DNA insertional mutant (*Atgpxl5*) seedlings were compared after applying NaCl stress, then the *AtGPXL5* gene was overexpressed in Arabidopsis Col-0. Using the Col-0 wild type, the *Atgpxl5* mutant and two *AtGPXL5*-overexpressing lines (OX-AtGPXL5-1 and OX-AtGPXL5-2), we were looking for the answers on main questions as follows:

1) Has the AtGPXL5 role in the regulation of ROS and redox homeostasis and in the maintenance of the cell's vitality?
2) What is the effect of the decreased or increased *AtGPXL5* expression on the activity of antioxidant mechanisms in control conditions and in the short-term salt stress response? Have the *AtGPXL5*-overexpressing or *Atgpxl5* mutant plants altered glutathione redox potential?
3) Has the AtGPXL5 any kind of function in the growth and development of Arabidopsis seedlings under control conditions and in the presence of 100 mM NaCl?

4. Materials and methods

4.1 Plant material

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col-0) as a wild type control, a glutathione peroxidase-like 5 [AT3G63080] T-DNA insertional knockdown mutant (*Atgpxl5*, SALK_076628C) (Bela et al., 2018) and AtGPXL5-overexpressing lines (OX-AtGPXL5-1, OX-AtGPXL5-2) were investigated. The relative expression levels of the *AtGPXL5* gene in these knockdown mutants were 0.27 and 0.16 in shoot and root, respectively, compared to the wild type (Bela et al., 2018). The T-DNA insertional line was obtained from the Nottingham Arabidopsis Stock Centre (NASC) (Scholl et al., 2000), and a homozygous mutant was used (Riyazuddin et al., 2019).

4.1.1 Growth conditions and stress treatments

In the present study, two different experimental systems were applied. Firstly, the growth and development of wild type, *Atgpxl5* mutant, and two overexpressing lines, OX -AtGPXL5-1 and OX-AtGPXL5-2 seedlings were compared *in vitro* under control conditions and in the presence of NaCl. Seeds were surface sterilized (Bela et al., 2018) and, after incubation overnight on 8–10 °C, germinated on half-strength Murashige and Skoog medium (½ MS, Duchefa Biochemie; (Murashige and Skoog, 1962) with 0.5% sucrose. Plants were grown in controlled growth chambers (Fitoclima S 600 PLH, Aralab, Rio de Mouro, Portugal) at 21 $^{\circ}$C under 100 μmol m^{-2} s^{-1} photon flux density with a 10/ 14 h light/dark photoperiod and the relative humidity was 65%. For stress treatments, seeds were germinated in the presence of 100 mM NaCl (germination assay), or 5-day-old seedlings were transferred to square Petri dishes containing ½ MS medium supplemented with 100 mM NaCl (Fig. 8). The growth of roots was monitored on vertical culture plates containing 0.8% agar after 10 days (Passaia et al., 2014).

Fig. 8. 5-day-old seedlings were transferred to the ½ MS media containing the 100 mM of salt concentration and were grown vertically.

Because the Arabidopsis AtGPXL5 showed a relatively low expression level and it was not induced by salt stress, but the homologous Thellungiella TsGPXL5 proved to be essential in the salt tolerance of plants (Gao et al., 2014), we aimed to overexpress *AtGPXL5* gene to estimate the involvement of AtGPXL5 in the salt stress response of plants.

4.2 Cloning of the *AtGPXL5* gene and the used vector constructions

Cloning of the *AtGPXL5* gene was performed using the Gateway cloning system. A full length of cDNA was inserted into the pDONR201 vector by BP Clonase™ and by LR Clonase™ II enzymes (Thermo Fischer Scientific, Vilnius, Lithuania) into the pTCO27235S vector (Rigó et al., 2016), following the manufacturer's instructions. Recovered clones were tested by sequencing using the pTCO35S new 5' oligonucleotide (pTCO35SNEW5': GCAGGACGATCCGTATTTTTACAAC) (Rigó et al., 2016, Supplementary Table 1). The verified pTCO27235SAtGPXL5 binary vector construction (Fig. 9A, B) was transformed into GV3101/pMP90 *Agrobacterium* strain with tri-parental mating (Koncz et al., 1994). The presence of the proper sequence was confirmed using the pTCO35SNEW5' forward and the T35S/RD29rc reverse primers (T35S/RD29rc3': GGACTCTAGCATGGCCGCGGG) (Supplementary Table 1). To generate constitutively overexpressing *AtGPXL5* lines, pTCO27235SAtGPXL5 construct was introduced into Arabidopsis Col-0 plants by *Agrobacterium*-mediated transformation as follows: *Agrobacterium tumefaciens* GV3101 (pMP90) carrying the pTCO27235SAtGPXL5 construct were inoculated in liquid YEB media (5 g/L beef-extract, 1 g/L yeast extract, 5 g/L peptone (casein-hydrolysate), 5 g/L sucrose and 2 mM $MgSO_4$) containing 50 mg/L spectinomycin, 100 mg/L rifampicin and 25 mg/L gentamicin and grown overnight at 27°C. Cells were collected by centrifugation and resuspended in distilled water supplemented with 5% sucrose and 0.01% SILWET L-77 (Arysta

Lifescience; Hungary). Arabidopsis inflorescences were dipped into the *Agrobacterium* solution and covered with a foil paper for 24 hours. Plant transformation was repeated two times allowing 5-6 days between each infiltration. The primary transformants (T1) were selected in the greenhouse on the bases of survivorship after spraying with Basta herbicide (300 mg/L glufosinate-ammonium; Bayer Cropsience, Hungary) according to Rigó et al. (2012) (Fig. 10). The *AtGPXL5* gene expression level was measured in 10 transformant plants and two of them with the highest *AtGPXL5* transcript amounts (OX-AtGPXL5-1 and OX-AtGPXL5-2) were introduced into our experiments. The seeds of the T3 generation of the genetically stable transformants were used in our experiments.

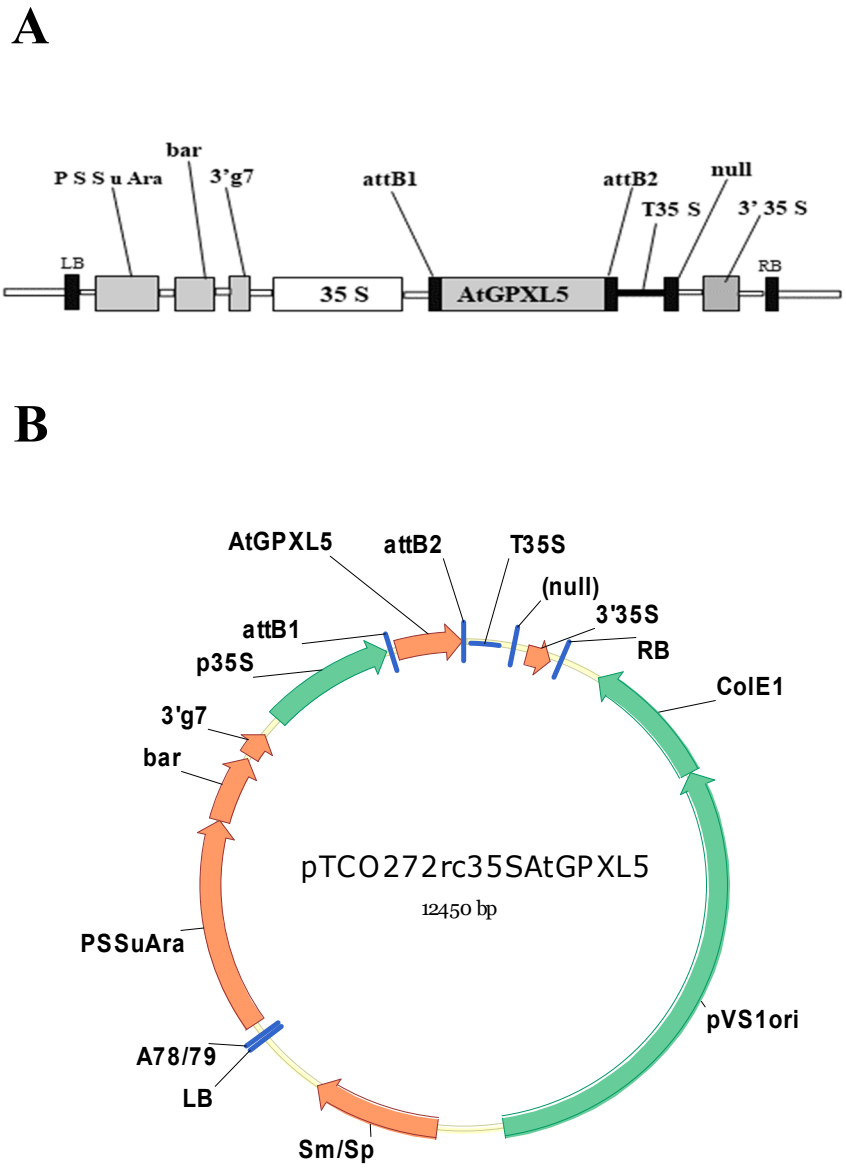

Fig. 9. Overexpression of *Arabidopsis* glutathione peroxidase-like5 gene. Schematic maps of the pCaMV35S-AtGPXL5 gene construct (A) and the pTCO27235SAtGPXL5 binary vector (B).

Fig. 10. Selection of primary transformants (T1) after spraying with Basta herbicide (300 mg/L glufosinate-ammonium). The base of the selection was the robust growth of green transformed seedlings, while the untransformed plants were died.

4.3 RNA extraction, expression analyses with quantitative real-time PCR (qRT-PCR)

The expression rate of AtGPXL5 gene was determined by RT-qPCR after the purification of RNA from 100 mg plant material according to Chomczynski and Sacchi (1987), as was described in Bela et al. (2018). Plant material was collected from root and shoots of 6-week-old hydroponically grown Col-0, *Atgpxl5* and OX-AtGPXL5 plants. The concentration of the isolated RNA samples was checked on a Biophotometer plus microvolume spectrophotometer (Eppendorf; Germany). cDNA synthesis of 1 µg of total RNA was carried out in a total volume of 20 µL using reverse transcriptase (Thermo Fisher Scientific; USA) and random hexamer primers. To check the purity of cDNA synthesis, control transcripts without reverse transcriptase were also constructed and then PCR samples were run on the gel to check the purity. Diluted cDNA was used for the qRT-PCR reaction as a template, Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific; USA), high rhodamine-X (ROX) reaction mixture, and primers in accordance with the manufacturer's instructions in a final volume of 10 µl. The used oligonucleotide sequences are: F: 5'AATGGAAAATTGACCGGAATGT3'; R: 5'CGGTGAGATCAACAACTGAGACA3' (Supplementarry Table 1). Quantitative real time PCR (qRT-PCR) was carried out applying the SYBR Green master mix (Thermo Fisher Scientific) with the Fast Real Time System (qTOWER Real-Time qPCR System, Analytik Jena, Jena, Germany) following the next protocol: denaturation at 95 °C for 7 minutes, followed by 40 cycles of denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. To analyse the qRT-PCR data, we used qTOWER Software 2.2 (Analytik Jena, Jena, Germany) software. Data from the RT-qPCR analysis were calculated using the $2^{-(\Delta\Delta Ct)}$ formula (Livak and Schmittgen, 2001). The glyceraldehyde-3-phosphate dehydrogenase-2 (*GAPDH2*) gene

[At1G16300] was used as internal control for data normalization. To demonstrate the differences in the expression level of *AtGPXL5*, the transcript amount in the *Arabidopsis thaliana* Col-0 control shoot and root samples were considered to be one.

4.4 Detection of ROS levels and vitality

5-day-old *Arabidopsis thaliana* Col-0 (wild type) and *Atgpxl5* T-DNA insertional mutant seedlings were transferred to ½ MS media containing different concentration of NaCl (50 mM NaCl and 100 mM NaCl) and were grown further for 7 days. Besides the investigation of the superoxide anion level; total ROS level and the cell vitality was detected of by fluorescent microscope (Zeiss Axiowert 200 M microscope, Carl Zeiss, Jena, Germany).
To measure the level of superoxide radical anion, 12-day-old seedlings after applying 7 days of salt stress were incubated in 2 ml of 10 µM dihydroethidium (DHE) dye for 30 min at 37°C and then sample were washed more than 2 times with the Tris-HCl buffer. DHE dye prepared in Tris-HCl buffer (10 mM, pH 7.4) was used to visualise superoxide radical anions in seedlings roots according to Horváth et al. (2019).

For ROS level detection, 2′,7′- dicholorodihydrofluorescein diacetate (H_2DCFDA) fluorescent dye was used. Seedlings were incubated in 10 µM H_2DCFDA (prepared in the 10 mM MES/ 50 mM KCl, pH 6.15) for 15 min at 37°C and washed 3 times with MES/KCl buffer as it was published in Horváth et al. (2019).

To detect the cell vitality, 10 µM fluorescein diacetate (FDA) (prepared in 10 mM MES/50 mM KCl buffer, pH 6.15) was used. 12 days old Arabidopsis seedlings were incubated in 2 mL of 10 µM fluorescein diacetate (FDA) fluorescent dye for 30 min at 37°C and washed with the same buffer at least 3 times as was described in the Horváth et al. (2019).

The intensity of fluorescence was quantified on digital images using Axiovision Rel. 4.8.2 software (https://carl-zeiss-vision-axiovision-viewer.software.informer.com/4.8/) in the leaves, as well as in the proximal meristem of the roots in a circle with 50 µm radius, or in the middle of the leaves in a circle with 150 µm radius. The measurements were performed in three independent experiments ($n \geq 15$) with the same microscopic settings.

4.5 Investigation of the growth and development of seedlings

The germination assay was carried out by counting the number of individual seeds using a small stereo microscope (Carl Zeiss Jena 402339) from three independent replicates of 50 seeds that

had a protruded radicle. The rate of germination in the presence of 100 mM NaCl or without salt was monitored daily for 7 days according to Zsigmond et al. (2012).

The root length and number of lateral roots were analysed on seedlings grown vertically with or without the 10-day-long NaCl treatment. Square Petri plates containing 4 plants from each lines were scanned and root lengths were measured using ImageJ software (Schindelin et al., 2012). Lateral roots were counted and lateral root density (LRD) was calculated by dividing the number of visible lateral roots by the primary root length for each root analysed (Passaia et al., 2014). To measure the fresh weight (FW) the roots belonging to one genotype were pooled from 5 plates.

Morphological parameters (rosette size, convex area, convex percentage) and pigment contents (chlorophyll and anthocyanin contents) of *in vitro* grown 15-day-old Col-0 wild type, *Atgpxl5* mutant and OX-AtGPXL5 seedlings were investigated using PlantSize (http://www.brc.hu/pub/psize/index.html) software according to Faragó et al. (2018). Convex area shows how large is the area within convex hull that encompasses the image and convex % is the ratio of the detected leaf area divided by the convex hull area in pixel unit. The convex hull is the smallest convex set of pixels that contains all other pixels in the system. To analyse the growth and phenotype of 15-day-old seedling's shoots color images were taken after 10 days of treatment with 100 mM NaCl by photographing the plates with white, transmission illumination of a transilluminator (biosetup LED) (Fig. 11). Canon 700D digital camera (18.0 mega pixel APS-C (22.3 x 14.9 mm) sized CMOS sensor, maximum resolution of 5184 x 3456 pixels with aspect ratios of 1:1, 4:3, 3:2 and 16:9, made in Taiwan) was used without any filter to take 2592x1728-pixel images. The following settings were used: ISO: 100, Integration time: 5.0 millisecond (ms), Aperture: F/5.6, Manual focus, Exposure mode: macro.

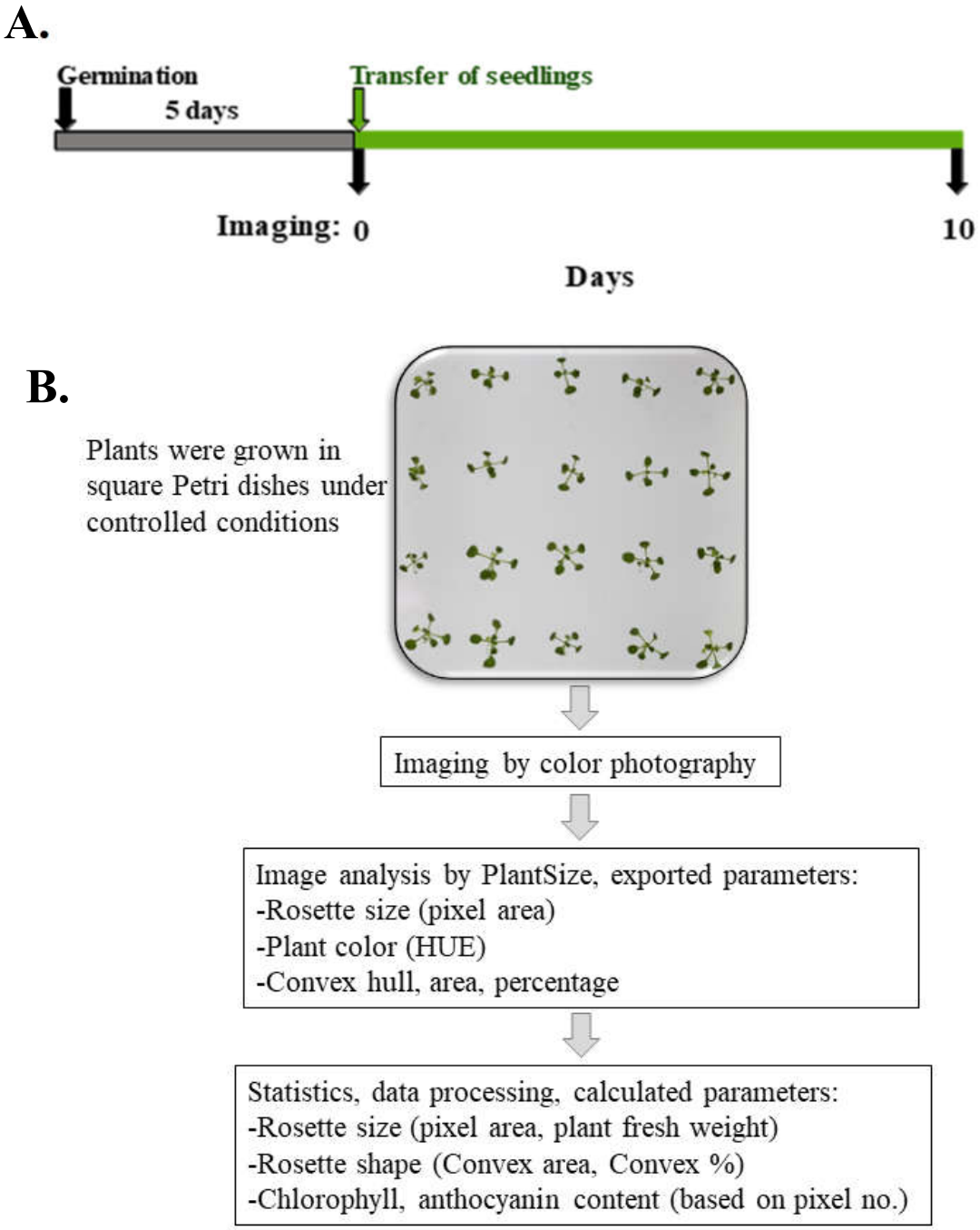

Fig. 11. An experimental outline for non-destructive image analysis method to monitor the growth and physiological parameters. (A) 5 days old seedlings were transferred to ½ MS media supplemented with 100 mM NaCl and photograph the pictures after 10 days. (B) Workflow of image analysis by PlantSize software and subsequent data processing (Modified from Faragó et al., 2018).

In the second set of experiments, plants were grown in Hoagland nutrient solution. A Hoagland nutrients solution is the mixture of the following compounds: 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 1 µM Fe-EDTA, 0.0475 µM H_2BO_3, 14.48 µM $MnCl_2$, 0.8148 µM $ZnCl_2$, 0.3731 µM $CuCl_2$, 0.001213 µM Na_2MoO_4 (pH 5.8). Seeds were surface sterilized (Bela et al., 2018) and after incubation overnight on 8–10 °C, the sterilized seeds were placed

on the top of end cutted Eppendorf filled with 0.7% agar and were grown for 6 weeks in a 1.5 L pots filled with Hoagland solutions. Plants were grown in the growth chamber with similar parameters as was described earlier (at 21°C, 100 mmol $m^{-2}s^{-1}$ photon flux density, short-daylight photoperiod), the relative humidity was 65%. 6-week-old plants were treated with 100 mM NaCl for 24 hours, then samples were collected from fully expanded leaves and roots (Fig. 12).

Fig. 12 Six-week-old Arabidopsis Col-0 wild type, *Atgpxl5* T-DNA insertional mutants and OX-AtGPXL5-1 plants grown in the hydroponic system.

4.6 Analysis of some stress markers

Hydrogen peroxide (H_2O_2) content and the thiobarbituric acid (TBA)-reactive lipid peroxidation products were determined in 6-week-old hydroponically grown Col-0, *Atgpxl5* and OX-AtGPXL5 plants supplemented with or without 100 mM NaCl for 24 hours. H_2O_2 level was measured from the 200 mg fresh weight of shoot or root ground with 375 µl 0.1% trichloroacetic acid (TCA) on pre-chilled pestle and mortar as was described earlier in Bela et al. (2018), and the homogenate was centrifuged at 13,000×*g* for 20 min. In the reaction mixtures, 250 µL supernatant was added to 250 µL phosphate buffer followed by 500 µL potassium iodide (KI). After 10 minutes of incubation in dark, the absorbance of the samples was recorded at 390 nm (Uvikon 930 spectrophotometer, Kontron AG, Eching, Germany), using 10 mM PH 7.0 phosphate buffer as a blank. The amount of H_2O_2 was calculated using a standard curve prepared with 0.1-5 mM H_2O_2 concentrations.

Malondialdehyde (MDA) formation was measured with a TBA-reactive substances assay, based on the formation of TBA-MDA conjugate (Heath and Packer, 1968). Lipid peroxidation was measured from 50 mg frozen shoot or root tissues grind with liquid nitrogen and homogenized on ice with 0.5 mL 0.1% TCA and 50 µL of 4% butylhydroxytoluene (BHT) was added to avoid further lipid peroxidation (Bela et al., 2018) and centrifuged at 4°C, 13000×*g* for 20 minutes. In the reaction, 250 µL supernatant was added to the reaction mixture (20% TCA + 0.5% TBA) and boiled at 98°C for 30 minutes. After incubation, it was immediately transfered into an Eppendorf tube on ice and then refilled up to 1.5 ml. The absorbance of the samples was measured at 600 nm and 532 nm using 0.1% TCA as a blank. MDA concentrations were calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. Malondialdehyde is expressed by nmol MDA g^{-1} FW.

4.7 Evaluation of the AsA and GSH contents

The leaves or roots (300 mg) of Arabidopsis plants was homogenized with 1.2 mL of 5% TCA. The homogenate was centrifuged at 13000×*g* for 20 min at 4°C and the supernatant was collected for the further assay of AsA and GSH pool. To measure the total ascorbate content 100 µL 10 mM dithiothreitol (DTT; Sigma-Aldrich, Germany) was added to 100 µL of the supernatant. After 10 min of incubation at room temperature, the excess DTT was removed by 100 µL 0.5% N-ethylmaleimide (NEM; Sigma-Aldrich, Germany). The ascorbate (AsA contents were measured spectrophotometrically as was published originally by Law et al. (1983). AsA concentrations were determined by measurement of the optical density (OD) at 525 nm against the 5% TCA reference. Oxidized dehydroascorbate (DHA) content was calculated as a difference between the concentration of total and reduced ascorbate. A standard curve was obtained from reduced AsA (AsA; Sigma-Aldrich, Germany) within the 0-10 mM range.

Total glutathione and oxidized glutathione (GSSG) concentrations were measured spectrophotometrically (Griffith, 1980). To measure the GSSG contents in shoot or root, the reduced glutathione was masked by adding 2-vinylpyridine and incubated at room temperature for 60 minutes then 2 µL triethanolamine (TEA; Sigma-Aldrich, Germany) was added to the reaction. Again, centrifuged at 13000×*g* at room temperature for 20 minutes and then 20 µl supernatant was used for measurement. GSH and GSSG concentrations were determined by measuring OD at 405 nm, using a glutathione reductase (GR) enzymatic assay (Carlberg and Mannervik, 1985). The reaction mixture contained 0.2 mM NADPH (Sigma-Aldrich, Darmstadt, Germany), 0.25 mM 5,5'-dithiobis-2-nitrobenzoic-acid (DTNB; Sigma- Aldrich,

Germany), 20 µL tissue extract and 1 U of GR (from baker's yeast, Sigma-Aldrich) in a phosphate buffer (0.1 M, pH 7.5) in a total volume of 1 mL. Reduced GSH content was calculated from the difference between the concentration of total GSH and GSSG. Standard curves were obtained for GSH (Sigma-Aldrich, Germany) and GSSG (Sigma-Aldrich, Germany) within the 0-2 µM range.

4.8 Calculation of the glutathione half-cell reduction potential

The reduction potential of the GSH/GSSG couple (half-cell reduction potential; E_{hc}) was determined with the Nernst equation using the formula of Schafer and Buettner (2001): E_{hc}= -240 - (59.1/2) log([GSH]^2/[GSSG]) mV; where –240 mV is the standard reduction potential of glutathione on 25°C, pH = 7.0.

4.9 Antioxidant enzyme activity measurements

The enzyme activities were determined as published in Bela et al. (2018). 250 mg fresh shoot or root tissue was homogenized on ice in 1 mL cold extraction buffer (100 mM phosphate buffer pH 7.0, containing 1 mM phenylmethylsulfonyl fluoride and 1% polyvinylpolypyrrolidone). The homogenate was centrifuged for 20 min at 13000×*g* at 4°C. The supernatant of the enzyme extract was collected in new Eppendorf tube and filled the volume up to 1 mL with extraction buffer and used for enzyme activity assays.

The thioredoxin peroxidase (TPOX; EC 1.11.1.15) enzyme activity was measured with cumene hydroperoxide (CHP; Sigma-Aldrich) substrate. The reaction mixture contained 0.2 mM NADPH, 5 µM TRXh3, 0.1 µM NADPH-dependent thioredoxin reductase (NTRa) recombinant protein produced by *E. coli* according to Marty et al. (2009), 50 µL of enzyme extract and 0.25 mM substrate in a Tris-HCl buffer (0.1 M, pH 7.4) in a total volume of 1 mL. The NADPH consumption was followed by measuring the absorbance at 340 nm. The nonspecific NADPH decrease was corrected by using additional measurements without the CHP substrate. One U was equal to nmol converted NADPH in 1 min, ε_{340} = 6.22 mM^{-1} cm^{-1}.

The glutathione peroxidase (GPOX; EC 1.11.1.9) activity was also measured spectrophotometrically with CHP substrate using a protocol of Horváth et al. (2015a). The reaction mixture contained 4 mM GSH, 0.2 mM NADPH, 0.05 U of GR (from baker's yeast, Sigma-Aldrich, Germany), 100 µL enzyme extract and 0.5 mM substrate in a phosphate buffer (0.1 M, pH 7.0) in a total volume of 1 mL. One U was equal to nmol converted NADPH in 1 min, ε_{340} = 6.22 mM^{-1} cm^{-1}.

The glutathione transferase (GST; EC 2.5.1.18) enzyme activity was measured spectrophotometrically using the artificial 1-chloro-2,4-dinitrobenzene (CDNB; Sigma-Aldrich, Germany) substrate, as published earlier (Horváth et al., 2015a). The reaction mixture contained 1 mM GSH, 100 µL enzyme extract and 1 mM substrate in a phosphate buffer (0.1 mM, pH 6.5) in a total volume of 1 mL. One U is the amount of the enzyme producing 1 nmol conjugated product in 1 min, ε_{340} = 9.6 mM^{-1} cm^{-1}.

The guaiacol peroxidase (POX; EC 1.11.1.7) activity was determined by monitoring the increase in A_{470} during the oxidation of the guaiacol substrate (Sigma-Aldrich), according to Horváth et al. (2015b). The reaction mixture contained 30 mM H_2O_2 (Merck Millipore, Darmstadt, Germany), 10 µL enzyme extract and 20 mM substrate in a phosphate buffer (50 mM, pH 7.0) in a total volume of 1.5 mL. The amount of enzyme producing 1 µmol of oxidized guaiacol in 1 min was defined as 1 U, ε_{470} = 26.6 mM^{-1} cm^{-1}.

The ascorbate peroxidase (APX; EC 1.11.1.11) activity was assayed according to the protocol of Tari et al. (2015). For the APX assay, 1 mM ascorbate (Sigma-Aldrich) was added to the extraction buffer. The H_2O_2-dependent oxidation of ascorbate was followed by a decrease in A_{290}. The reaction mixture contained 1 mM H_2O_2, 100 µL enzyme extract and 50 µM ascorbate in a potassium phosphate buffer (50 mM, pH 7.0) in a total volume of 1 mL. One U was equal to nmol oxidized ascorbate in 1 min, ε_{290} = 2.8 mM^{-1} cm^{-1}.

The catalase (CAT; EC 1.11.1.6) activity was determined by the decomposition of H_2O_2, measured spectrophotometrically by following the decrease in A_{240} (Horváth et al., 2015b). The reaction mixture contained 100 µL enzyme extract and 20 mM H_2O_2 in a phosphate buffer (50 mM, pH 7.0) in a total volume of 1.5 mL. One U is the amount of decomposed H_2O_2 (µmol) in 1 min, ε_{240} = 43.6 M^{-1} cm^{-1}.

GR (EC 1.8.1.7) activity was determined by measuring the absorbance increment at 412 nm when DTNB was reduced by GSH, generated from GSSG (Csiszár et al., 2018). The activity was calculated as the amount of reduced DTNB in nmol $min^{-1}g^{-1}$ FW, ε_{420}=13.6 mM-1cm^{-1}.
Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined spectrophotometrically by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT; Sigma-Aldrich, Germany) in the presence of riboflavin in light (Csiszár et al., 2007). One U of SOD was the amount that causes a 50% inhibition of NBT reduction in light.

4.10 Statistical analysis

Experiments were carried out at least two times. In the case of fluorescent microscopic analysis values presented here represent mean with standard error (±SE), $n \geq 15$. Statistical analysis was carried out with SigmaPlot 12.0 software (SigmaPlot, Milano, Italy). After analysis of variance (ANOVA), Duncan's multiple comparisons were performed. Means were considered to be significantly different if $p \leq 0.05$. In the second set of experiments (to analyse growth parameters of seedlings) means and standard error (±SE) were calculated, n=20, unless indicated otherwise. For seed germination assay, Student's t-test was used, and asterisks indicate the significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). In the third set of experiments (to analyse ROS processing systems and redox state of plants) measurements were performed in three independent replicates, means and standard deviation (±SD) were calculated from the data of at least 3 measurements (n=3).

5. Results

5.1 Investigation of the role of AtGPXL5 in ROS homeostasis using the mutant seedlings

In plants, glutathione peroxidases like (GPXLs) isoenzymes are important ROS scavenger and protect cells against oxidative damage caused by generation of excessive reactive oxygen species (ROS) *in vivo*. These GPXLs are very important to prevent H_2O_2 accumulation, protect membranes against ROS-induced damages and act in cellular signaling (Foyer and Noctor, 2011, Paiva et al., 2019). Plant GPXLs have their role in detoxifying lipid hydroperoxides and other reactive molecules in different species and under several stress conditions (Csiszár et al., 2004; Bela et al., 2015). The ROS levels were investigated in the 12-day-old *Arabidopsis thaliana* Col-0 and *Atgpxl5* insertional mutants after 7 days of treatments with 50 mM and 100 mM NaCl the concentrations.

5.1.1 Effect of the mutation of *AtGPXL5* on the ROS levels and vitality of seedlings

Using dihydroethidium allows to investigate the superoxide ($O_2^{\bullet-}$) levels in roots. In ground stage, superoxide radical anion ($O_2^{\bullet-}$) contents in the root of *Atgpxl5* was higher compared to the wild type Col-0 (Fig. 13). In Col-0, the lower (50 mM) and higher (100 mM) concentration of salt increased significantly the DHE fluorescence in the roots. The already elevated level of $O_2^{\bullet-}$ in the root of *Atgpxl5* mutants increased further due to 100 mM NaCl treatments and became even higher compared to the treated Col-0 roots (Fig. 13A, B).

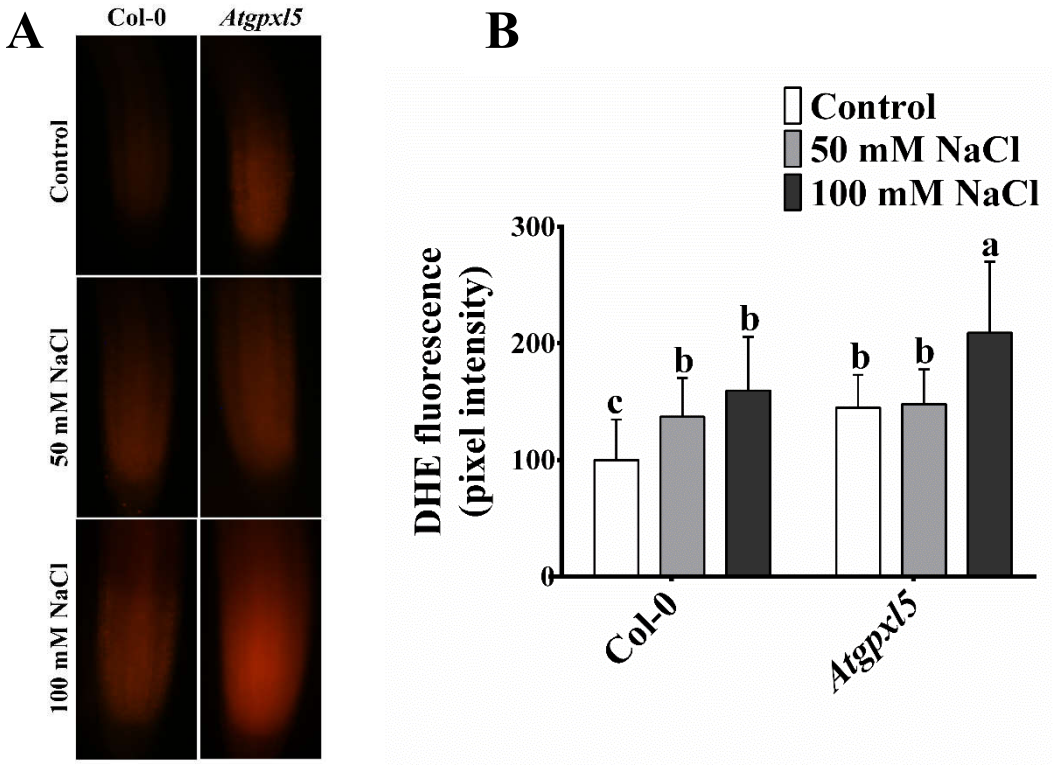

Fig. 13. Superoxide content in the roots of 12-day-old *Arabidopsis thaliana* Col-0 and *Atgpxl5* insertional mutant after applying 50 mM NaCl and 100 mM NaCl concentrations for 7 days. Representative images of the fluorescent analysis of the superoxide radical anion ($O_2^{\bullet-}$) contents (A). Quantification of the $O_2^{\bullet-}$ level in the roots (B) of wild type and *Atgpxl5* plants by dihydroethidium (DHE) fluorescent dye. Data are mean ±SE, n ≥ 15, Different letters represent data considered statistically significant at p≤0.05 (Duncan test). Experiments were repeated 3 times independently.

However, using the DCF fluorescent dyes revealed no significant changes in the total ROS level in the roots of Col-0 and *Atgpxl5* plant under the control or salt treatment (Fig. 14A). Under control conditions, *Atgpxl5* shoot had higher total ROS level compared to the Col-0. However, both low and high concentration of salt elevated the ROS contents in the shoot of Col-0 and *Atgpxl5* plants (Fig. 14A-D).

There were no differences in the vitality of the roots between wild type and mutant lines under the normal growth condition (Fig. 15A, C). Low (50 mM) concentration of salt has not, while high (100 mM) significantly reduced the FDA fluorescence in the root of Col-0 and *Atgpxl5* insertional mutant lines. In contrast, the lack of AtGPXL5 led to decrease in vitality at high concentration of NaCl. The vitality of *Atgpxl5* mutants shoot under control condition were significantly lowered compared to Col-0 (Fig. 15B, D). However, applying both 50 mM and 100 mM salt concentrations significantly decreased the shoot vitality of Col-0 and *Atgpxl5* mutant plants (Fig. 15A-D).

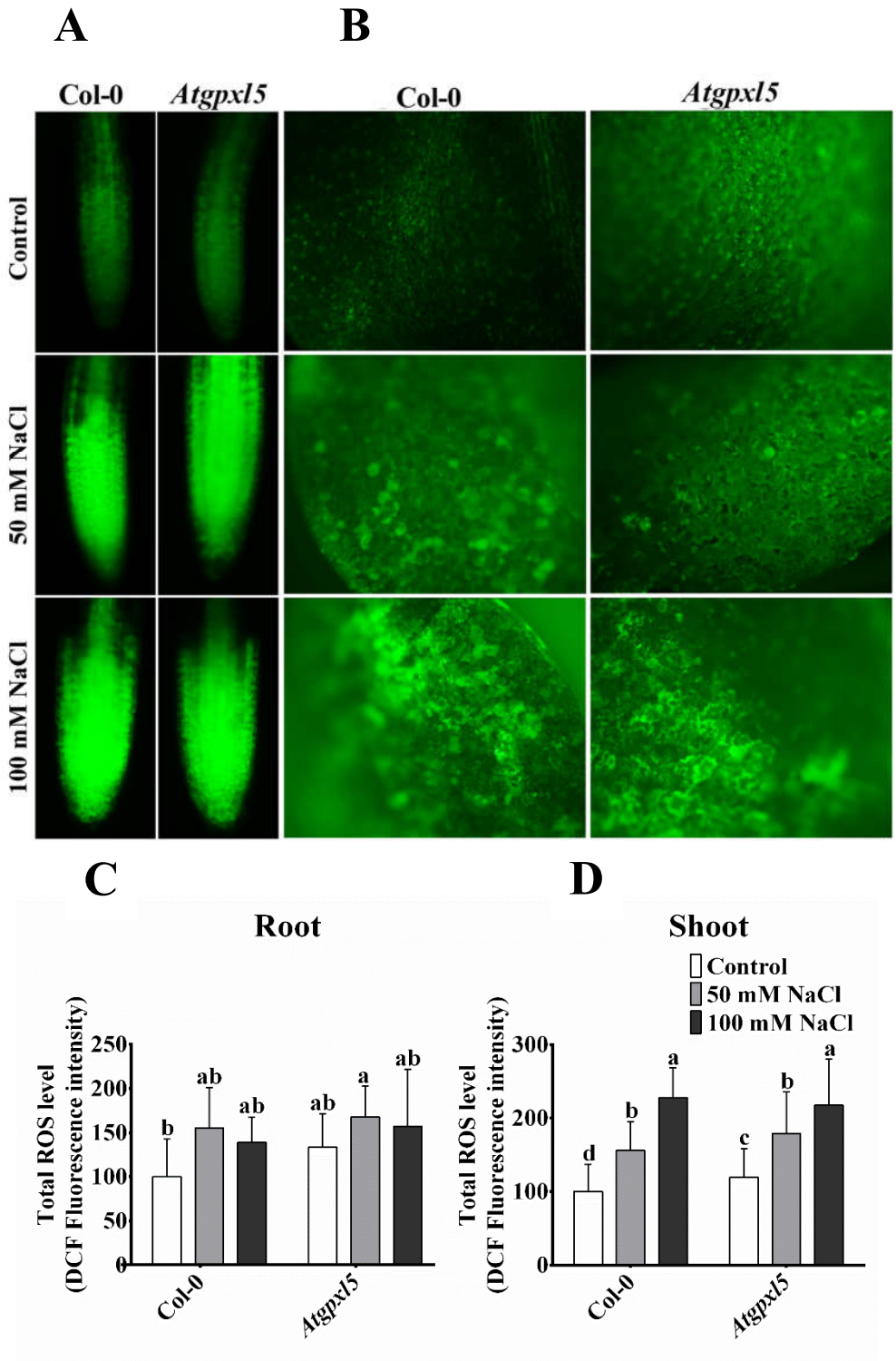

Fig. 14. Effect of 50 mM and 100 mM NaCl on the level of total ROS in the root and shoot of 12-day-old Col-0 and *Atgpxl5* plants after 7 days of treatment. Representative images of the fluorescent analysis of the total ROS in the root (A) and shoot (B). The level of total ROS was analysed in the roots (C) and shoots (D) of wild type and *Atgpxl5* plants by H_2DCFDA fluorescent dye. Data are the mean ±SE, n ≥ 15. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at $p \leq 0.05$. Scale bars =100 μm. Experiments were repeated 3 times independently.

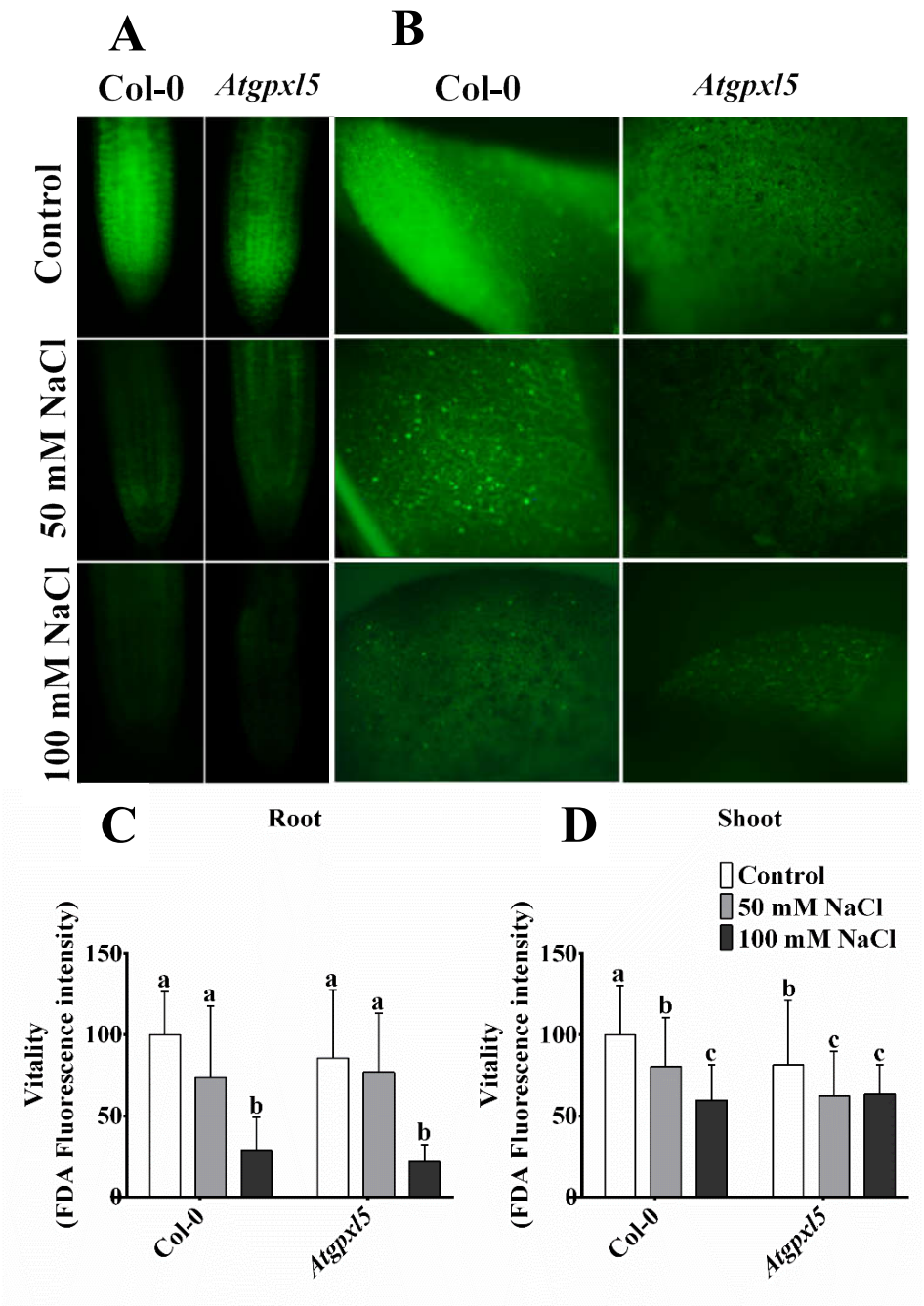

Fig. 15. Representative images of the fluorescent analysis of the vitality in the 12-day-old *Arabidopsis thaliana* Col-0 and *Atgpxl5* insertional mutants after applying 7 days treatment with the concentration of 50 mM and 100 mM NaCl. The vitality in the roots (A, C) and shoot (B, D) were investigated by the FDA dye. Data are the mean ±SE, n ≥ 15. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at p≤0.05. Scale bars =100 μm. Experiments were repeated 3 times independently.

5.2 Overexpression of *AtGPXL5* gene

Full length of *AtGPXL5* cDNA was overexpressed under control of CaMV35S constitutive promoter in the *Arabidopsis thaliana* wild type Col-0. To investigate the involvement of the AtGPXL5 in development, the rate of germination and growth of two overexpressing lines with 35 to 40-fold enhanced *AtGPXL5* expression level (OX-AtGPXL5-1 and OX-AtGPXL5-2, Fig. 16), the *Atgpxl5* knockdown T-DNA insertional mutant and the Col-0 wild type seedlings were compared in the presence of NaCl and without the salt.

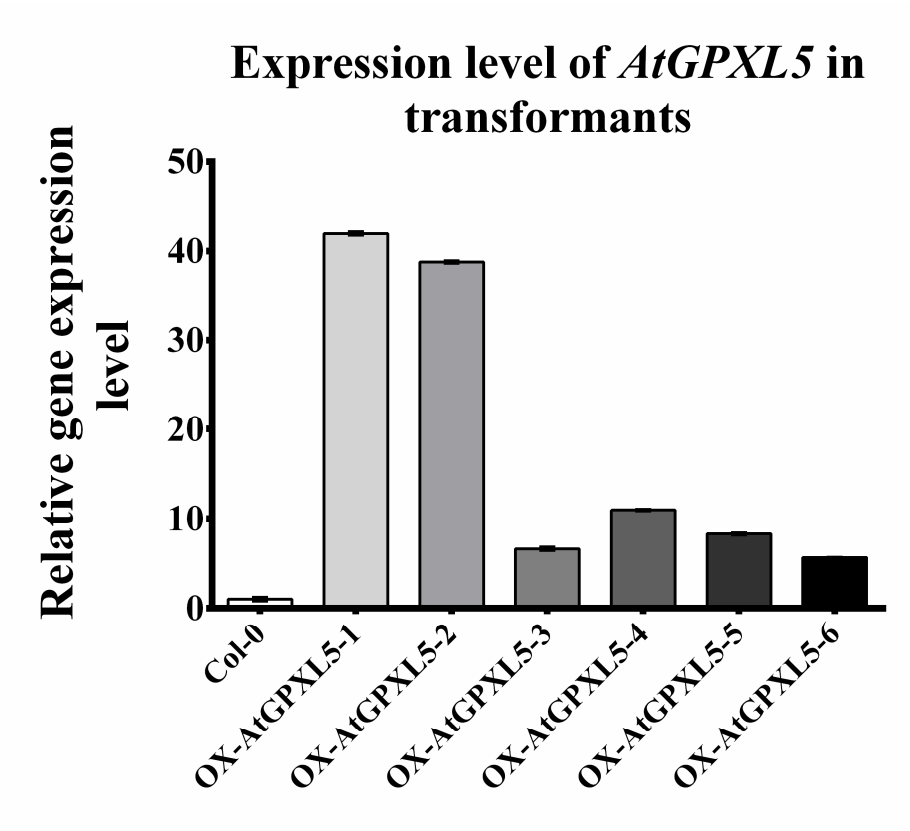

Fig. 16. Expression level of *AtGPXL5* gene in the shoots of selected OX-AtGPXL5 transformants (T1 generation).

5.2.1 The germination rate of the *AtGPXL5*-overexpressing seeds was less inhibited under salt stress

The germination rate of OX-AtGPXL5-1 and OX-AtGPXL5-2 or *Atgpxl5* mutant's seeds did not differ from that of Col-0 under control conditions (Fig. 17A). However, while the seeds of the wild type and *Atgpxl5* mutant showed delayed germination in the presence of 100 mM NaCl, germination of OX-AtGPXL5-1 and OX-AtGPXL5-2 seeds was less inhibited by the salt on the 2nd day after sowing (Fig. 17B).

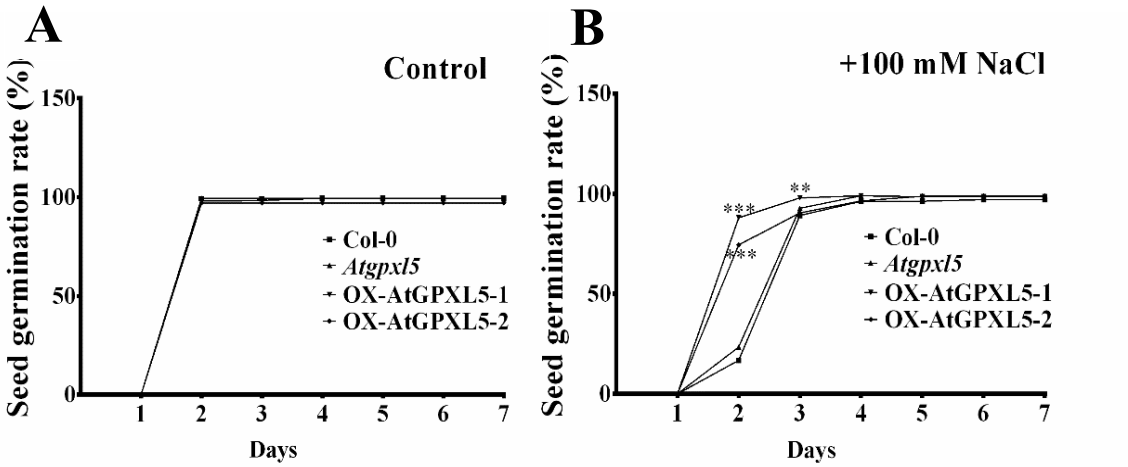

Fig. 17. Analysis the role of AtGPXL5 in seed germination. The rate of germination of two *AtGPXL5*-overexpressing lines (OX-AtGPXL5-1 and AtGPXL5-2), the Col-0 wild type and *Atgpxl5* mutant lines on control ½ MS medium (A) and on the medium supplemented with 100 mM NaCl (B). To compare the mean values of the WT, *Atgpxl5* mutant and the OX-AtGPXL5 plant's germination rate, Student's t-test was used; asterisks indicate the significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

5.2.2 The growth of roots of *AtGPXL5*-overexpressing lines under salt stress condition was more retained

Under control conditions, the length of roots of 15-day-old T-DNA insertional mutant (*Atgpxl5)* seedlings were significantly lower compared to the Col-0. AtGPXL5-overexpressing lines had similar root length but higher fresh weight of roots than the Col-0 (Fig. 18A, C, D).

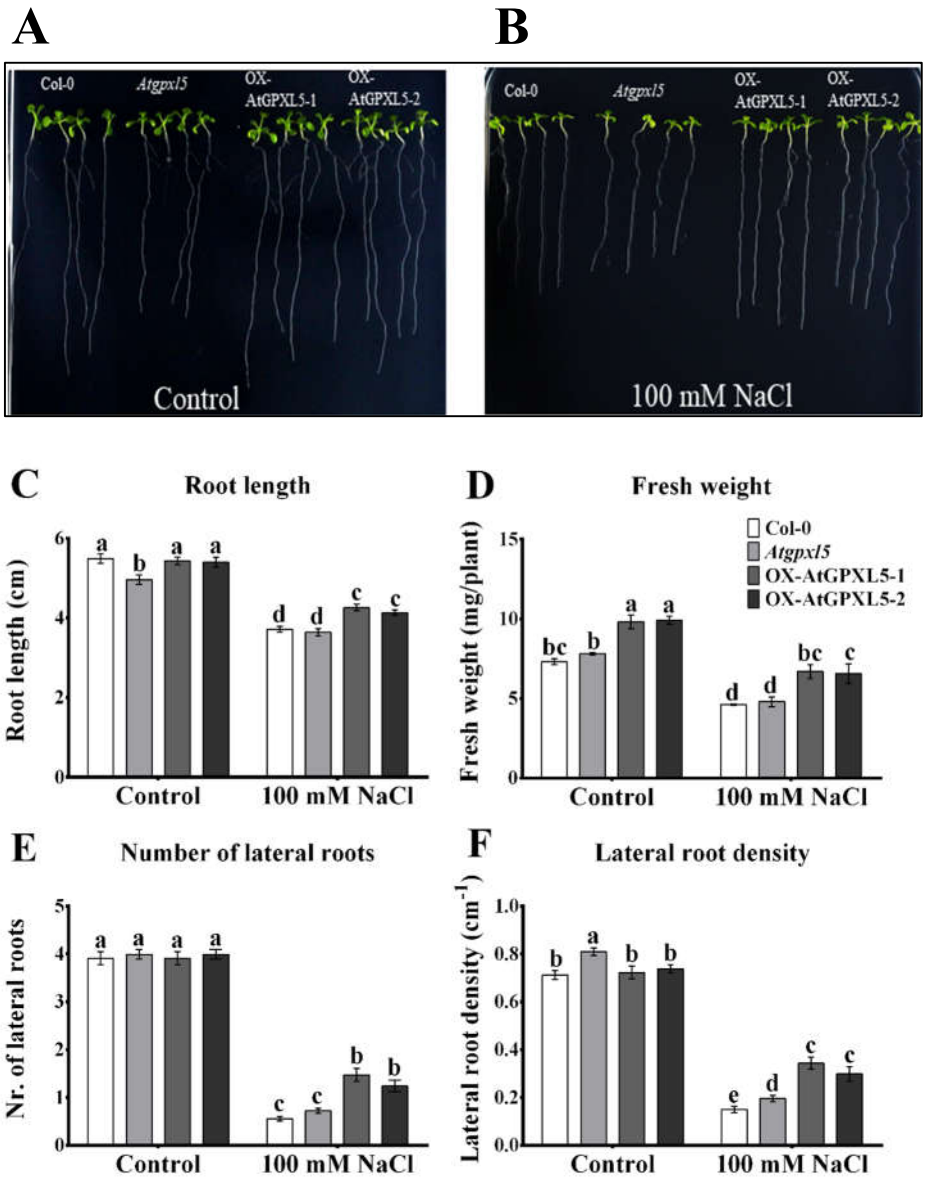

Fig. 18. Root phenotypes of 15-day-old *Arabidopsis thaliana* Col-0, *Atgpxl5* mutant and two AtGPXL5-overexpressing (OX-AtGPXL5) lines after 10 days of salt stress. The growth of the seedlings in vertical plates on control ½ MS medium (A) and on the medium supplemented with 100 mM NaCl (B), length of primary roots (C), the fresh weight of roots (D), the number of lateral roots (E) and the lateral root density (F) are shown. Data are the mean ±SE, n=20. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at $p \leq 0.05$.

Among the salt-treated seedlings, the wild type and *Atgpxl5* T-DNA insertional mutant lines had similarly lowered root length and fresh weight. The length and fresh weight of roots of plants overexpressing the *AtGPXL5* gene were significantly higher compared to the Col-0 and *Atgpxl5* mutants growing in the presence of 100 mM NaCl for 10 days (Fig. 18A-D). While there was no significant difference in the number of lateral roots among the untreated lines, under salt stress the OX-AtGPXL5-1 and OX-AtGPXL5-2 seedlings formed more lateral roots than the other genotypes (Fig. 18E). Under control conditions the LRD value was higher in the *Atgpxl5* mutant compared to the wild type and overexpressing lines, but in the presence of salt the OX-AtGPXL5-1 and OX-AtGPXL5-2 seedlings had the highest lateral root density among the investigated lines (Fig. 18F, Fig. S1).

5.2.3 Developmental traits of shoots under salt stress

Analysis of the development of shoots of plants by the PlantSize imaging software revealed that the total area, weight and convex area of rosettes of the *Atgpxl5* mutant were significantly lower compared to the Col-0, However, convex percentage of the *Atgpxl5* was like wild type. Under control condition, fresh weight, total area, convex area and convex percentage of overexpressing lines were similar to wild type (Fig. 19A-F). Applying 10-day-long treatment of 100 mM NaCl reduced the fresh weight in all investigated lines, rosette size and convex area, but salt stress increased the convex percentage in wild type and transgenic lines compared to the control condition. Interestingly, the overexpressing plants grew better in the presence of 100 mM NaCl: they had larger rosettes, convex area and lower convex percentage values than that of the wild type and *Atgpxl5* plants (Fig. 19A-F).

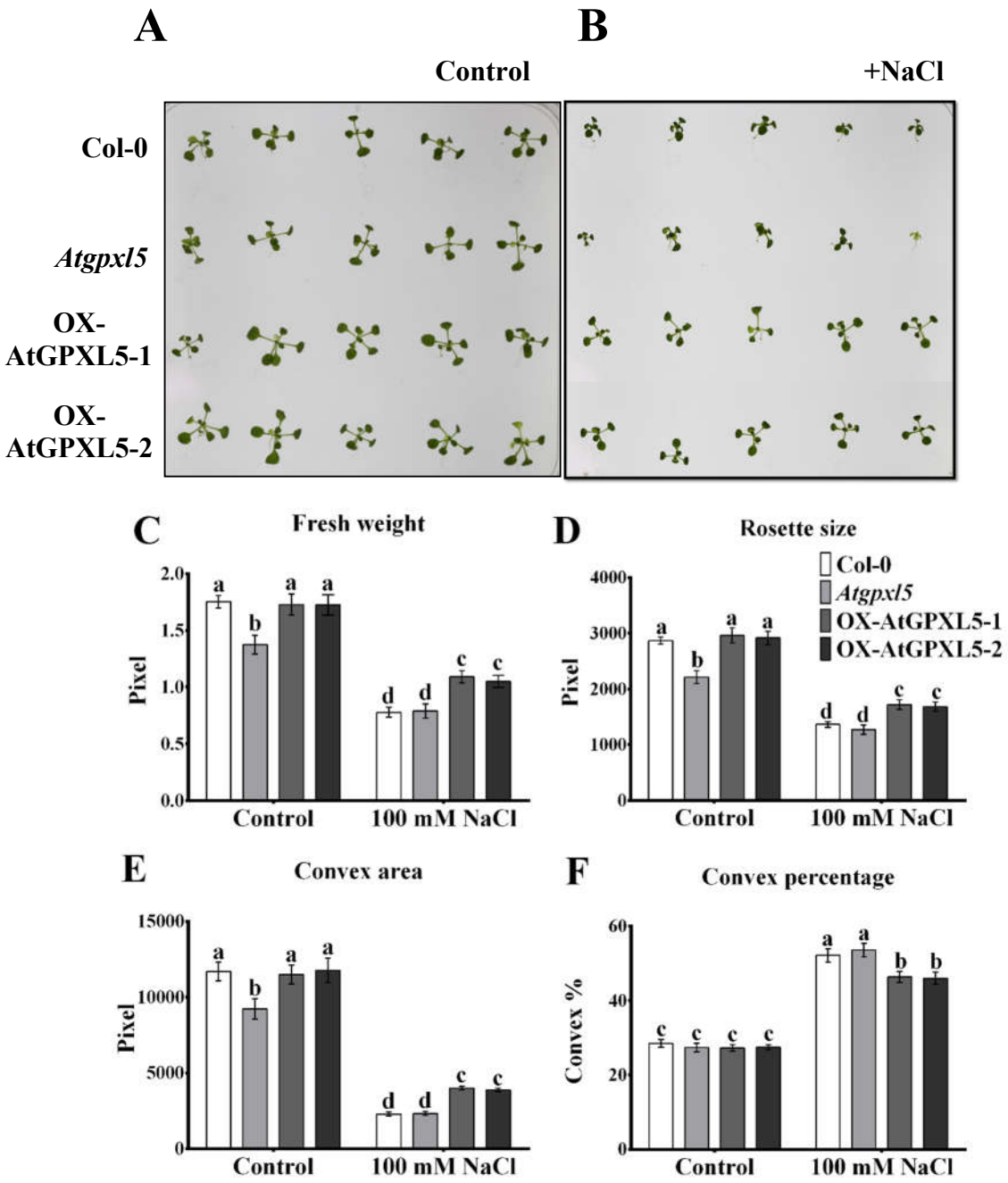

Fig. 19. Growth parameters of 15-day-old *Arabidopsis thaliana* Col-0, *Atgpxl5* mutant and two *AtGPXL5*-overexpressing (OX-AtGPXL5) lines after 10 days of salt stress estimated by PlantSize software after taking photographs. The relative rosette sizes (A, B, D), fresh weight of rosette (C) and the convex size of the rosette (E), and convex ratio (F) are shown. Data are the mean ±SE, n=20. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at $p \leq 0.05$.

5.2.4 Image analysis of shoots indicate less affected chlorophyll and anthocyanin contents in overexpressed plants under salt stress

Estimation of the chlorophyll content by the non-destructive image analysis showed decreased amounts of the photosynthetic pigments in the untreated *Atgpxl5* mutants compared to Col-0 and *AtGPXL5*-overexpressing lines (Fig. 20A, B). Under salt stress, the chlorophyll content of the OX-AtGPXL5-1 and OX-AtGPXL5-2 lines remained higher in comparison to the wild type and mutant plants (Fig. 20A). In addition, the anthocyanin content of plants changed according to a similar pattern: while under control conditions the mutants had lower pigment content than the other genotypes, in the presence of NaCl it decreased in a lower extent in the overexpressing lines (Fig. 20B).

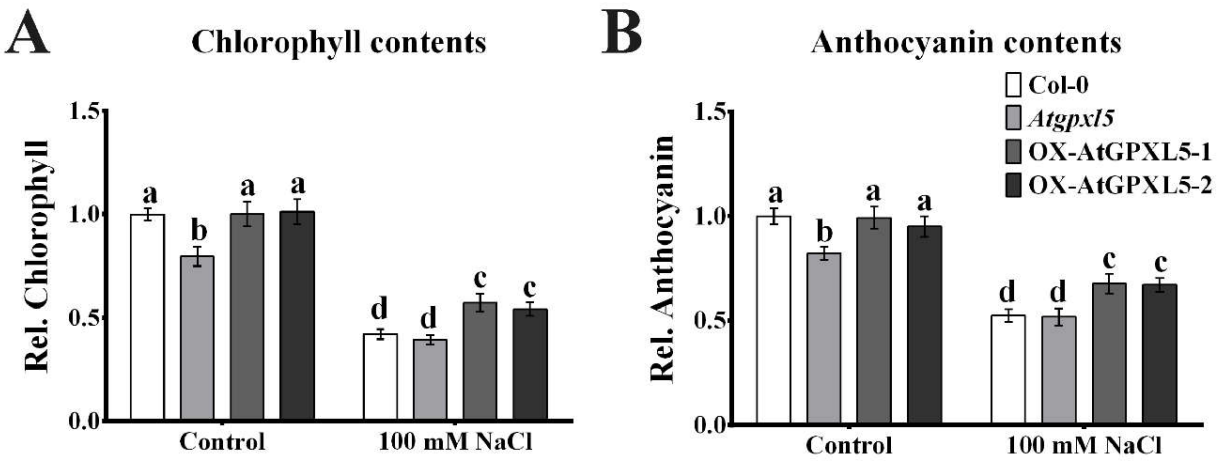

Fig. 20. Effect of salt stress on the chlorophyll (A) and anthocyanin (B) contents of 15-day-old Col-0, *Atgpxl5* mutant and the *AtGPXL5*-overexpressing (OX-AtGPXL5-1) plants after 10 days of salt stress estimated by PlantSize software. Data are the mean ±SE, n= 20. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at $p \leq 0.05$

The two investigated transgenic lines behaved rather similarly, thus further experiments were performed on the OX-AtGXL5-1, the *Atgpxl5* and the Col-0 plants (Fig. 16).

5.3 Overexpression of *AtGPXL5* influences some stress-related parameters in adult plants

To investigate the involvement of AtGPXL5 in salt stress response, hydroponically grown 6-week-old OX-AtGXL5-1 and wild type (Col-0) plants along with *Atgpxl5* mutants were treated with 100 mM NaCl, and several physiological parameters and the *AtGPXL5* expression level were determined after 24 h. The developmental stages of 6-week-old plants are demonstrated in Supplementary material (Fig. S1).

5.3.1 Expression level of *AtGPXL5* in the root and shoot of Arabidopsis plants with or without salt treatment

Under control conditions, the *AtGPXL5* transcript amounts were about 24 and 17-fold higher in the roots and the shoots of OX-AtGPXL5-1, respectively than in the wild type. On the contrary, the expression level of this gene in the *Atgpxl5* mutant was about one-third of the value of Col-0. One-day salt treatment reduced the *AtGPXL5* transcript levels in the shoots and the roots of the Col-0 plants by ca. one-third (Fig. 21A, B).

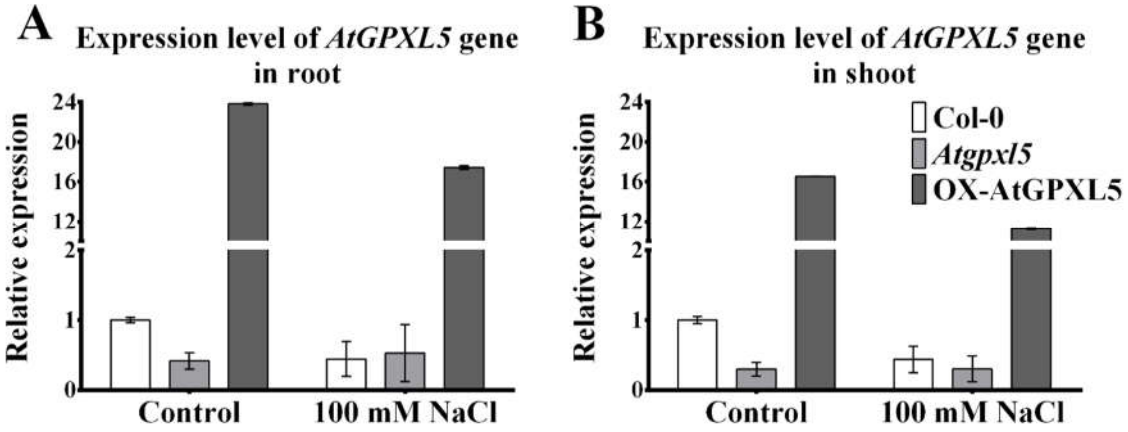

Fig. 21. Effect of one-day 100 mM NaCl treatment on the expression of AtGPXL5 gene (A, B), in roots and shoots, respectively, of 6-week-old *Arabidopsis thaliana* wild type (Col-0), *Atgpxl5* insertional mutant and AtGPXL5-overexpressing (OX-AtGPXL5-1) plants. The data are presented by mean values ± SD, n=3.

5.3.2 Thioredoxin peroxidase and glutathione peroxidase activities of the overexpressed Arabidopsis plants are almost like in the wild type plants

Under normal growth condition, both the total extractable glutathione peroxidase and thioredoxin peroxidase activities of OX-AtGPXL5 or *Atgpxl5* plants were similar to that of wild type, moreover the GPOX activity in the root of *AtGPXL5*-overexpressing plants were even lower than in Col-0 (Fig. 22A).

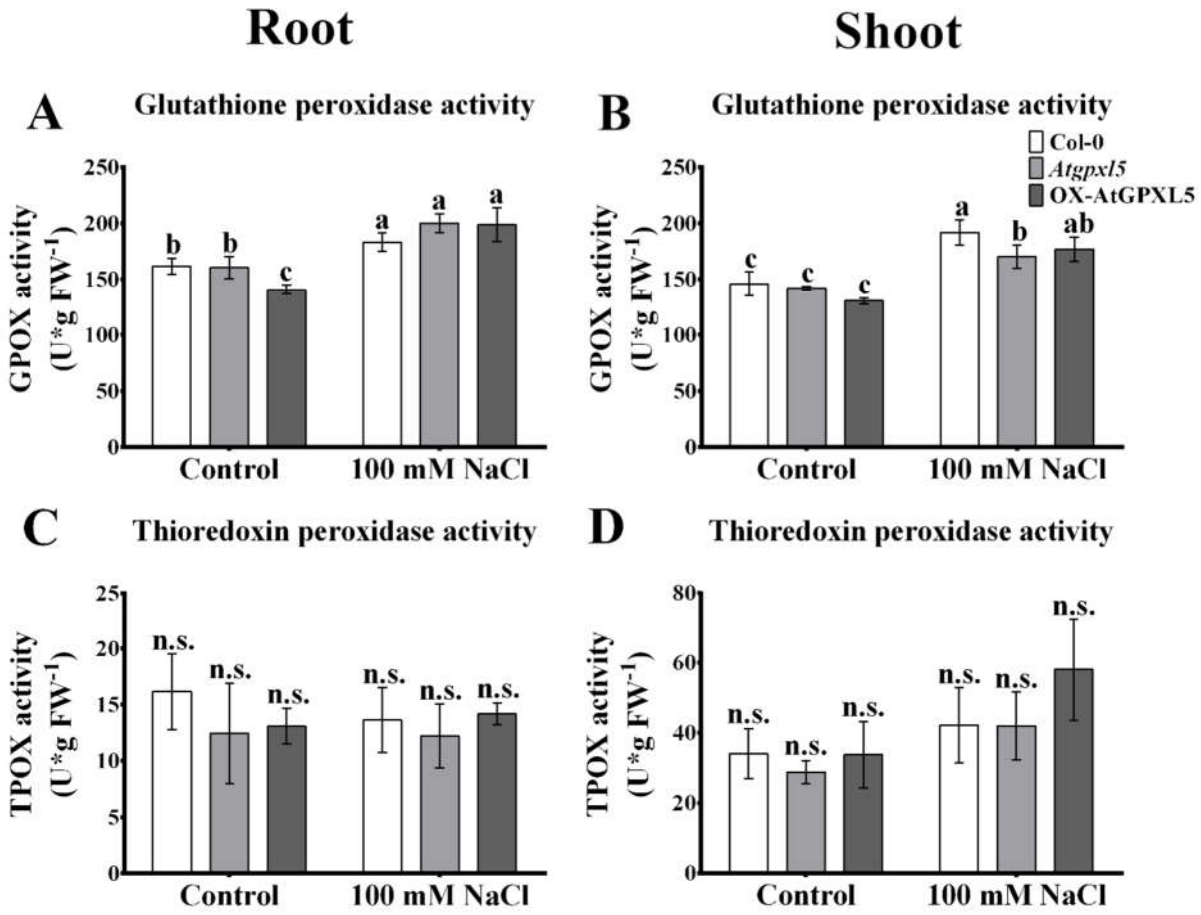

Fig. 22. Effect of one-day 100 mM NaCl treatment on the glutathione peroxidase (GPOX) activity (A, B), thioredoxin peroxidase (TPOX) activity (C, D) in roots and shoots, respectively, of 6-week-old *Arabidopsis thaliana* wild type (Col-0), *Atgpxl5* insertional mutant and *AtGPXL5*-overexpressing (OX-AtGPXL5-1) plants. The data are presented by mean values ± SD, n=3. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at $p \leq 0.05$ and non-significant (n.s.) at $p > 0.05$.

In the root and shoot of wild type and transgenic plants, the salt stress did not cause any significant changes in the TPOX activity but increased the GPOX activity in these genotypes (Fig. 22A-D) compared to the control condition. The GPOX activity in *Atgpxl5* shoot was also induced at a lower extent than that in Col-0 under 100 mM NaCl treatment (Fig. 22B). However,

in the *AtGPXL5*-overexpressing plants neither the GPOX nor the TPOX activities increased significantly compared to the other lines (Fig. 22A-D).

5.3.3 Hydrogen peroxide content and lipid peroxide level of plants

In the wild type shoot, the H_2O_2 level was significantly increased but in its roots the level of H_2O_2 remained unchanged after the 24-hour NaCl exposure. More changes occurred in the *Atgpxl5* mutant shoot after salt stress. The amount of H_2O_2 in the *Atgpxl5* shoot was increased significantly and reached higher level than that in the wild type (Fig. 23A, B).

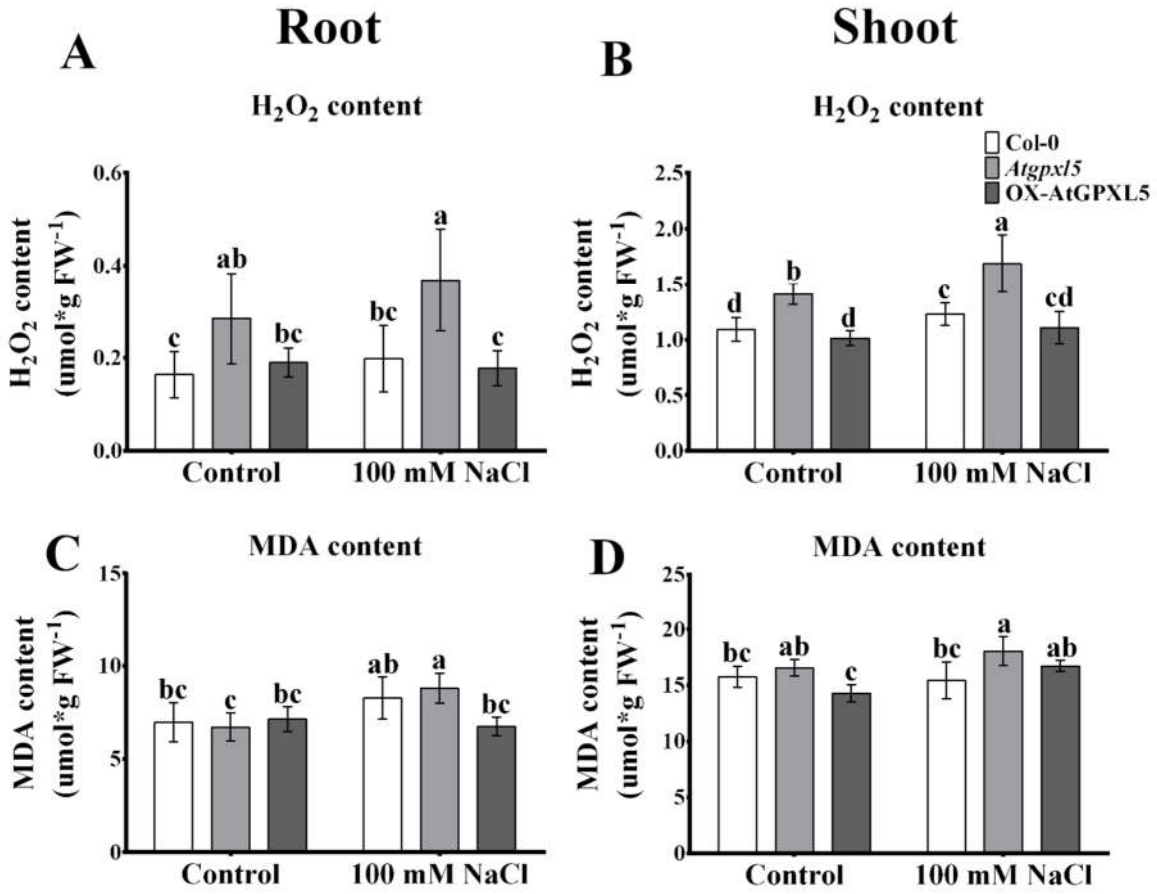

Fig. 23. Effect of one-day 100 mM NaCl treatment on the level of H_2O_2 (A, B) and malondialdehyde (MDA) contents (C, D) in roots and shoots, respectively, of 6-week-old *Arabidopsis thaliana* wild type (Col-0), *Atgpxl5* insertional mutant and AtGPXL5-overexpressing (OX-AtGPXL5-1) plants. The data are presented by mean values ± SD, n=10. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at $p \leq 0.05$ and non-significant (n.s.) at $p > 0.05$.

There was no difference in the lipid peroxidation marker MDA level in roots of the investigated plants, but in shoots the amount of MDA in the OX-AtGPXL5 was significantly lower than that in *Atgpxl5* mutants under normal conditions. In the wild type root and shoot, there were no

significant changes in the MDA level, but in the *Atgpxl5* root the amount of lipid peroxide was elevated significantly under the 100 mM NaCl treatment (Fig. 23C, D). MDA level was increased also in the OX-AtGPXL5 shoots due to salt stress. The lowest H_2O_2 level was found in these transgenic plants after the salt treatment and their roots possessed the least MDA among the treated ones, indicating the presence of efficient ROS-processing mechanisms.

5.3.4 H_2O_2-related enzyme activities in the wild type and transgenic Arabidopsis plants

Investigation of the main antioxidant enzyme activities in 6-week-old plants revealed that although in the *Atgpxl5* mutant some peroxidases worked in an elevated level compared to the Col-0 (POX in control shoot, APX both in the treated and control shoots), other enzymes had lower activity (CAT in untreated shoots and SOD in the NaCl treated root and shoot). In the OX-AtGPXL5 plants the investigated ROS-processing enzymes worked similarly to those in the Col-0 wild type; elevated activity was found in the case of CAT in the control roots. The APX activity in the roots of the overexpressing line was lower than that in the wild type in both with salt stress and without it. In the shoot, the activity of two other enzymes was decreased (CAT in control plants, SOD in the 100 mM NaCl treated shoot) (Fig. 24A-H).

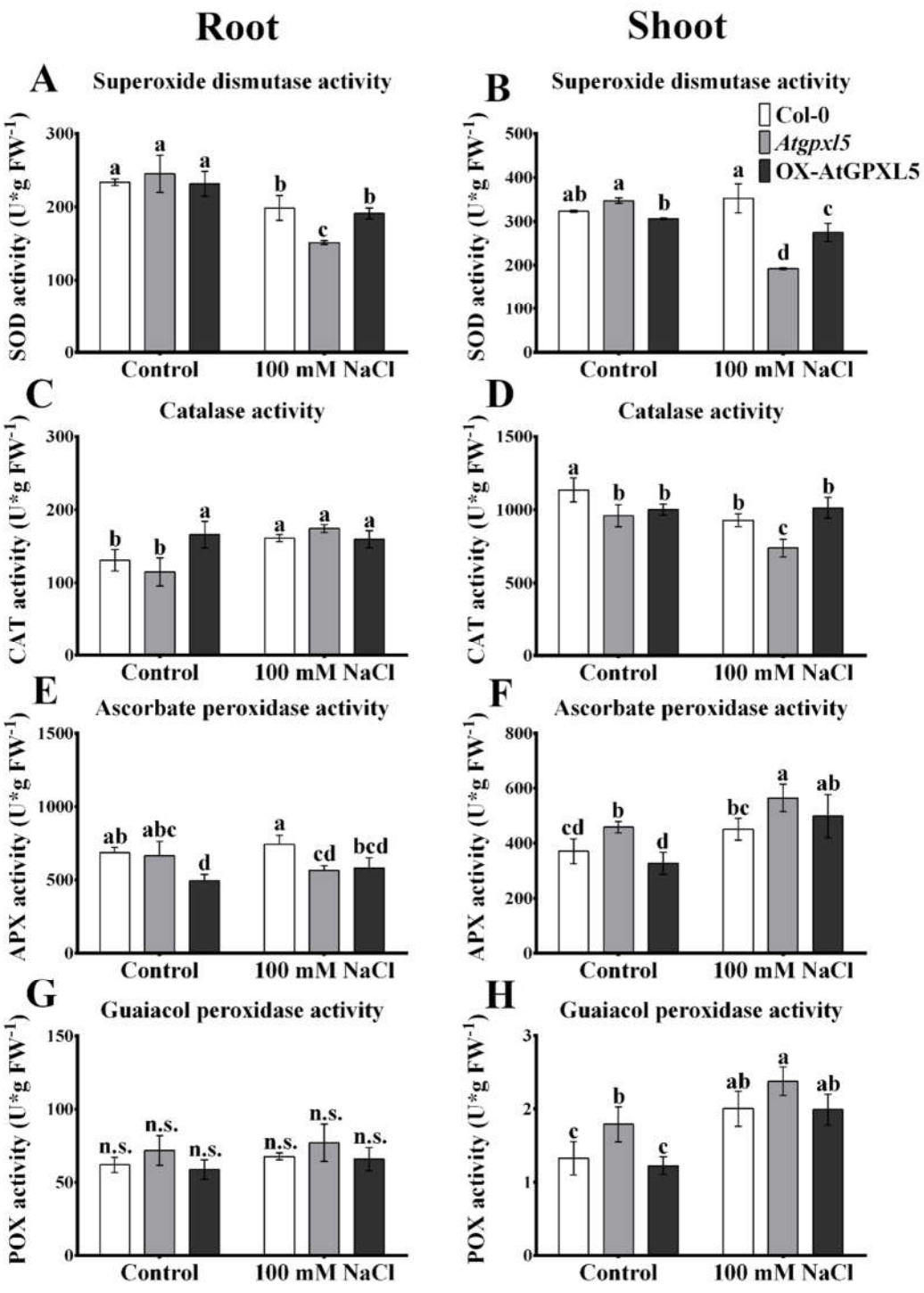

Fig. 24. Effect of one-day 100 mM NaCl treatment on the superoxide dismutase (A, B), catalase (C, D), ascorbate peroxidase (E, F), guaiacol peroxidase (G, H) activities in roots and shoots, respectively, of 6-week-old *Arabidopsis thaliana* wild type (Col-0), *Atgpxl5* mutant and OX-AtGPXL5 plants. The data are presented by mean values ± SD, n=3. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at $p \leq 0.05$. n.s: statistically not significant.

5.3.5 GSH-related antioxidant enzymatic activities of plants

There are other thiol peroxidases, such as GSTs with glutathione peroxidase activities, that may have a similar function to those of GPXLs in the maintenance of the ROS level. Under control condition, GST activity was found higher only in the root of overexpressing OX-AtGPXL5 plants compared to the wild type plants. In Col-0 plants, 24-hour salt treatment elevates the activity of GST enzyme in both root and shoot. However, there were no changes in the activity of GST enzyme in the root and shoot of *Atgpxl5* mutant plants. In the OX-AtGPXL5 plants, GST activity was increased in the shoot after 100 mM NaCl stress (Fig. 25A, B).

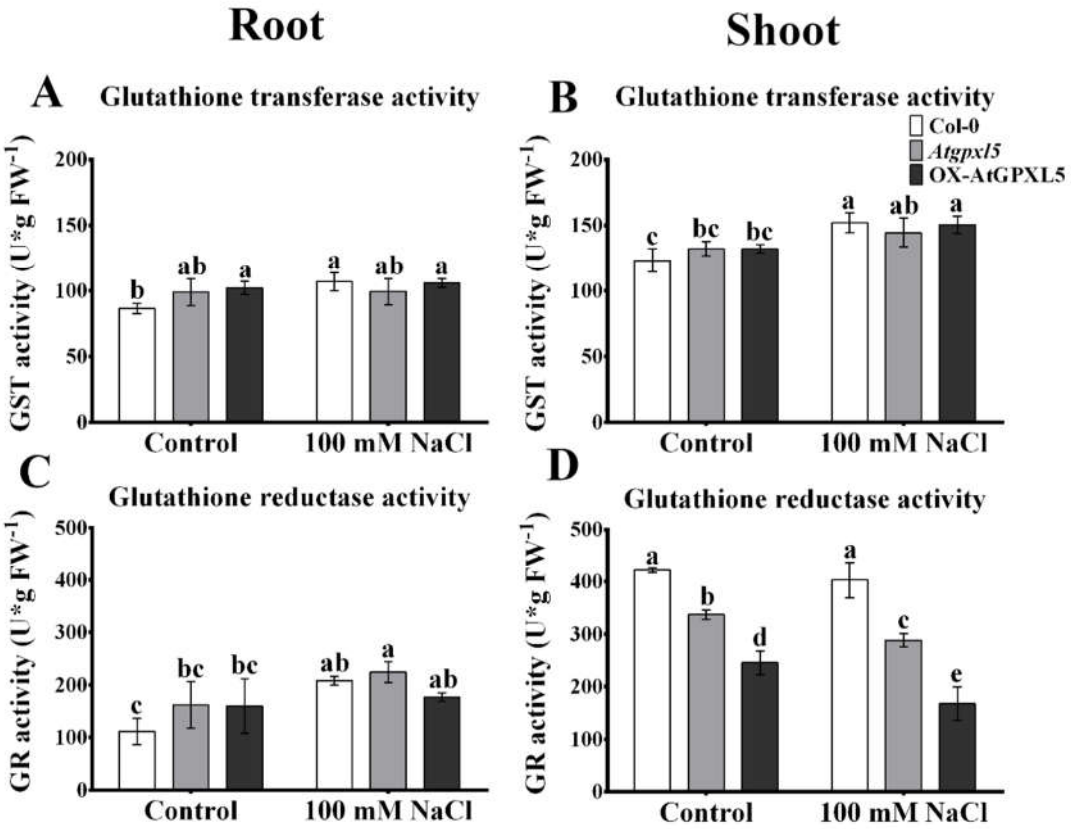

Fig. 25. Effect of one-day 100 mM NaCl treatment on the glutathione transferase (GST) activity (A, B) and glutathione reductase (GR) activity (C, D) in roots and shoots, respectively, of 6-week-old *Arabidopsis thaliana* wild type (Col-0), *Atgpxl5* insertional mutant and *AtGPXL5*-overexpressing (OX-AtGPXL5-1) plants. The data are presented by mean values ± SD, n=3. Data were analysed using one-way ANOVA followed by Duncan's test. Different letters represent data considered statistically significant at $p \leq 0.05$.

In normal condition, GR activity was decreased in the shoot of transgenic lines compared to wild type. In Col-0 plants, 24-hour salt treatment elevates the level of GR enzyme activity only in the root. However, the shoot of *Atgpxl5* mutant plants had lower GR activity in control and treated conditions compared to the Col-0. After applying the salt stress, GR activity in the root of *Atgpxl5* was elevated while in the shoot GR activity was decreased. The GR activity was

lower in the shoots of the overexpressing OX-AtGPXL5 line than that in the wild type under the 100 mM NaCl treatment (Fig. 25C, D) and the lowest GR activity were also found in the treated overexpressing shoots among all investigated lines

5.4 The ascorbate and glutathione levels and their reduced status

Comparison of the amounts and redox status of the main non-enzymatic antioxidants revealed no difference in the AsA or DHA levels among the investigated plants under control conditions, except for higher AsA in OX-AtGPXL5 shoot than in *Atgpxl5* mutant shoot (Fig. 26A, B). Applying 100 mM NaCl increased both the reduced and oxidized ascorbate forms in the Col-0 and *Atgpxl5* mutant roots. However, in shoots the applied salt stress decreased the AsA level of the wild type, while elevated both the DHA and AsA levels in the *Atgpxl5* plants. In OX-AtGPXL5 root salt stress did not cause any changes in the amount of AsA but increased the level of DHA. In shoot of OX-AtGPXL5 plants, the DHA content did not change, but the level of the reduced AsA was elevated and became higher by ca. 30% than in the salt-treated wild type shoot (Fig. 26A, B).

Different alterations were detected in the case of glutathione. Under control conditions, significantly lower GSH was found in the *Atgpxl5* mutant roots while its amount was significantly elevated in the OX-AtGPXL5 shoot. The applied salt stress caused generally moderate changes at all plants, but revealed that the overexpressing plants accumulated the highest amount of reduced glutathione and the less GSSG among the investigated lines even after the treatment, and the oxidized form (GSSG) increased most in the *Atgpxl5* roots (Fig. 26C, D).

In line with these results, the redox potential values calculated from the measured GSH and GSSG contents showed the lowest, –223±6.74 and –222±5.04 mV values in the *Atgpxl5* roots both under control conditions and after applying 100 mM NaCl, respectively, indicating the most oxidized status in mutant roots. The most negative values were detected in the OX-AtGPXL5 plants reaching –251±5.81 mV in the salt treated shoots (Fig. 26E, F).

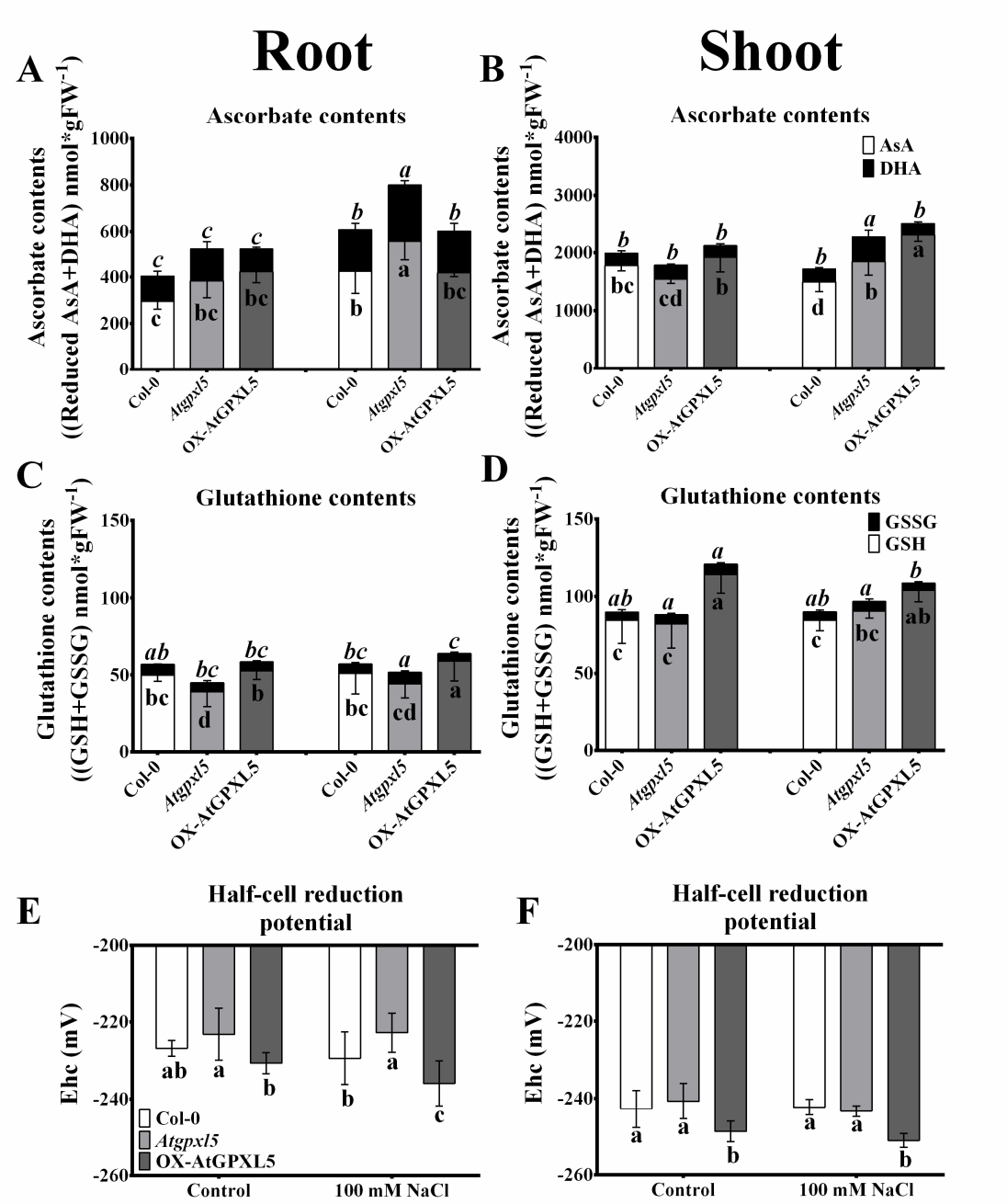

Fig. 26. Effect of salt stress on the ascorbate (A, B), glutathione (C, D) contents and glutathione half cell reduction potential (E_{hc}) (E, F) in roots and shoots, respectively, of 6-week-old *Arabidopsis thaliana* wild type (Col-0), *Atgpxl5* mutant and OX-AtGPXL5 plants. In the case of AsA and GSH contents the dark segment of the bars represents the oxidized dehydroascorbate (DHA) and oxidized glutathione (GSSG), respectively.

6. Discussion

6.1 Role of plant GPXLs in stress tolerance

Each organism on Earth is exposed to the influence of various environmental conditions and of other living organisms. These factors can trigger stress and make the organism more vulnerable. Salinity is one of the most brutal environmental factors affecting plant growth and productivity. To adapt to saline environment, plants have evolved elaborate mechanisms to preserve growth and yield. These mechanisms incorporate many physiological and genetic processes, which can function very efficiently in halophytes. Comparative studies between salinity stress adaptation in *Arabidopsis thaliana* and its salt-tolerant relatives have provided insights into the physiological and genetic bases of halophytism (Orsini et al., 2010; Rigó et al., 2016). *Thellungiella salsuginea* (also named as *T. halophile* or salt cress) proved to be more tolerant than Arabidopsis not only to high salinity but also to oxidative stress (Taji et al., 2004).

Various types of normal metabolic activities (e.g. photosynthesis and respiration) and different types of adverse environmental factors (such as high concentration of salt, drought) stimulate the overproduction of ROS, including hydrogen peroxide and superoxide radical anions (Zhai et al., 2013). Uncontrolled generation of ROS can cause damages to membrane lipids, proteins, nucleic acids and initiate the plant cell death. ROS homeostasis regulated mainly by the enzymatic (among them APX, CAT, SOD, TPOX, GPOX, GPXL) and non-enzymatic (like AsA and GSH) antioxidant defense mechanisms to withstand the oxidative damages and to ensure the right answer having role even in signaling.

Previous studies had proved that glutathione peroxidases (GPXs), the non-heme thiol peroxidase enzymes characterized in various organisms as one of the most important enzymes, are involved in ROS scavenging (Arthur, 2000; Noctor et al., 2012; Yang et al., 2016; Bela et al., 2017). GPXs catalyse the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols (Herbette et al., 2007; Passaia et al., 2014). The plant enzymes were suggested to call glutathione peroxidase-like (GPXL) enzymes (Attacha et al. 2017). It was reported that exogenously applied hydrogen peroxide can elevate the *GPXL* mRNA level in the *Panax ginseng* and *Oryza sativa* plants (Li et al., 2000; Passaia et al., 2013; Kim et al., 2014; Bela et al., 2017). GPXLs also participate in the removal of organic hydroperoxides and lipid peroxides (Bela et al., 2017). *Arabidopsis thaliana* harbors eight isoenzymes. Their role in salt- and osmotic stress tolerance was investigated earlier by using T-DNA insertional mutants (Bela et al. 2018). According to the previous results all of the AtGPXLs have some role in abiotic

stress responses, but AtGPXL5 was suggested to have most important function in salt stress tolerance (Gao et al. 2014, Bela et al. 2018). Here the *AtGPXL5* cDNA was overexpressed under the control of CaMV35S promoter. We have investigated the role of AtGPXL5 in development and responses to salt stress using T-DNA insertional mutant and *AtGPXL5*-overexpressing lines.

6.2 AtGPXL5 isoenzyme has a key role in the regulation of the ROS homeostasis and in the maintenance of the cell's vitality in the *Arabidopsis thaliana* seedlings

To examine whether a deficiency of AtGPXL5 might affect the of ROS scavenging activity and the vitality of plant cells, we investigated the effect of salt stress on the sensitivity of 12-day-old *Atgpxl5* seedlings. In our study, the untreated roots of the *Atgpxl5* mutant showed a higher $O_2^{\bullet-}$ level in comparison to the wild type roots. In *Atgpxl5* shoots the total ROS level was enhanced and led to decreased vitality compared to wild type shoot. Salt stress further increased the higher level of $O_2^{\bullet-}$ in the *Atgpxl5* roots and became significantly higher compared to the treated Col-0 roots. The H_2O_2 content in the shoots and roots of 6-week-old mutant plants was higher in the insertional mutant than in the other genotypes, and its level increased in the highest extent due to salt treatment. These results indicate that AtGPXL5 has an important role in the ROS processing system and maintaining the cell's vitality in the root and shoot (Figs. 13-15, 23).

There are other peroxidases, such as catalase, ascorbate peroxidase, GSTs with glutathione peroxidase activities, that may have a similar function to those of GPXLs in the maintenance of ROS level. In our experiments, among the ROS-processing enzymes the APX and POX activities were enhanced in mutant shoots, while the CAT and GR activities was decreased compared to the non-treated 6-week-old Col-0 plants grown hydroponically. In the OX-AtGPXL5 plants the GPOX, TPOX and the other ROS detoxifying enzymes generally exhibited similar activity to the Col-0 wild type, but not the APX (in root) and GR (in shoot) (Figs. 22, 24). This can be explained by the similar H_2O_2 levels in the OX-AtGPXL5 and wild type plants. Furthermore, the lower activity of APX and GR correlated with the elevated AsA and GSH levels of OX-AtGPXL5 plants compared to the wild type.

It was reported that Arabidopsis *gpxl1* mutant lines had higher H_2O_2 level under both low-light and high-light stresses (Chang et al., 2009). *Atgpxl3* knockdown mutant and *Osgpxl3* knockdown mutant plants showed higher level of H_2O_2 compared to the wild type (Miao et al., 2006; Passaia et al., 2013). Moreover, AtGPXL3 was suggested to be involved not only in H_2O_2

scavenging but also in ABA signaling (Miao et al., 2006). Overexpression of the *Rhodiola crenulata GPXL5* gene in *Salvia miltiorrhiza* increased tolerance to oxidative stress, induced by H_2O_2 and drought (Zhang et al., 2019). The relative expression levels of several *Salvia miltiorrhiza* genes were altered including transcription factor genes and genes connected to ROS processing and ABA signaling (such as CAT, APX, GR, monodehydroascorbate reductase and ABI2 coding genes) (Zhang et al., 2019). Constitutive expression of *RcGPXL5* caused a reduced production of ROS and MDA, higher levels of reduced and total glutathione and increased GR, APX and GPOX enzyme activities compared to the wild type.

In our experiments, the elevated peroxide levels of *Atgpxl5* mutant compared to wild type indicates that the AtGPXL5 protein has a role in fine-tuning the ROS homeostasis. Remarkably, these results suggested that GPXLs might have a significant function in maintaining the cell's vitality and the ROS homeostasis through ROS scavenging in the plants under normal and abiotic stress conditions.

6.3 The expression level of *AtGPXL5* gene can influence the main antioxidant mechanisms in the short-term salt stress condition

M'rah et al. (2007) reported that the level of lipid hydroperoxides in the *Thellungiella* leaves under salt stress was remarkably lower than in Arabidopsis. It was hypothesized that differences in salt tolerance mechanisms between salt-sensitive glycophytes, such as *A. thaliana*, and salt-tolerant halophytes, such as *T. salsuginea,* are resulted from changes in the regulation of the same basic set of genes involved in salt tolerance (Zhu, 2001). Comparing the protein and gene expression patterns of glutathione peroxidases in *Arabidopsis* and *Thellungiella*, Gao and his co-workers found that more *GPXL* genes were induced under salt and osmotic stress conditions in *Thellungiella*. They concluded that the salt stress inducible TsGPXL5 can be implicated in the enhanced stress tolerance of *Thellungiella* (Gao et al., 2014). We have confirmed the importance of AtGPXL5 in salt tolerance by salt hypersensitivity of the *Atgpxl5* knockdown mutant compared to the Col-0 wild type plants, which well-correlated with the enhanced H_2O_2 content of the mutant (Fig. 23A, B), and by the enhanced salt stress tolerance of OX-ATGPXL5 plants.

Gao et al. (2014) reported that the amounts of *TsGXPL* transcripts and/or proteins were elevated at least at one time-point in both leaves and roots of *Thellungiella* during the 24 h salt treatment. By analysing the data of the AtGenExpress (Kilian et al., 2007) database, these authors found that 6-24 h salt stress caused up-regulation of several *AtGPXL* genes (*AtGPXL1*, *-2*, *-4*, *-6* and *-7* in roots and *AtGPXL2*, *-6* and *-8* in shoots) (Gao et al., 2014). In our earlier experiments, salt

stress elevated the expression of the same *AtGPXL*s in the shoot of Col-0 as above, although partially different genes were up-regulated in the root (Gao et al., 2014; Bela et al., 2018). Analysis of the *AtGPXL* expression pattern of the Col-0 and *Atgpxl* mutants under control conditions and after applying salt and osmotic stress revealed that in the *Atgpxl5* mutant's root the expression of *AtGPXL2* and *-3* decreased along with that of *AtGPXL5* even under control conditions. After applying 100 mM NaCl to the plants, the expression of *AtGPXL1*, *-3*, and *-8* were upregulated in the root and that of the *AtGPXL2* and *-6* in the shoot. Interestingly, strong down-regulation was found in the transcript amount of the chloroplastic *AtGPXL1* (beside that of the *AtGPXL5* gene) in *Atgpxl5* shoots (Bela et al., 2018). Chang et al. (2009) reported that depletion of *AtGPXL1* and *AtGPXL7* transcripts led to decreased tolerance against photooxidative stress but increased the resistance of the Arabidopsis plants against virulent *Pseudomonas syringae* (Chang et al., 2009). A knockout mutation of *AtGPXL8* led to increased sensitivity to salt and osmotic stress as well as to paraquat when compared to wild type, leading to suppressed root growth and higher level of oxidized proteins (Gaber et al., 2012).

In the present experiments, investigation of the main antioxidant enzyme activities in 6-week-old plants revealed that although in the *Atgpxl5* mutant some peroxidases worked in an elevated level compared to the Col-0 (APX in the treated shoots), other enzymes had lower activity (CAT, GR and SOD in the NaCl treated shoot) (Fig. 24).

6.4 AtGPXL5 affect the redox homeostasis by the enhancement of reduced glutathione and thus the reduction potential becomes more negative than in the wild type

According to recent conception, the ROS-processing antioxidants not only keep ROS levels low but also allow the cells to sense and signal ROS availability and redox perturbations (Noctor et al., 2018). Glutathione is classically considered as one of the main low molecular weight antioxidants playing a role in ROS detoxification in cooperation with AsA in the ascorbate-glutathione cycle and as an electron donor of diverse antioxidant enzymes (Foyer and Noctor, 2005, 2011; Noctor et al., 2011, 2012). In the last decade, the involvement of the GSH/GSSG and AsA/DHA redox couples in the redox homeostasis and signaling came into the forefront (Noctor, 2006; Foyer and Noctor, 2013; Chen et al., 2017; Gullner et al., 2017; Hasanuzzaman et al., 2017; Locato et al., 2017). Enhanced ROS production suggests to temporarily shifts the redox potential to more oxidizing values (Foyer and Noctor, 2016). Many reports indicate that the [GSH]:[GSSG] ratio and, eminently, the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$), are effective markers of the overall redox homeostasis (Szalai et al., 2009; Schafer, and Buettner, 2001; Meyer and Hell, 2005; Kranner et al., 2006; Meyer et al.,

2007). Earlier we have found strong positive correlations between the GSH content of the Col-0 roots and the expression of *AtGPXL1, -2, -3, -5, - 6* and *-8* genes under different stress conditions which have been changed in the *Atgpxl* mutants. Correlations were found between the GSH level and TPOX activity in the wild type shoots and roots too, which were altered especially in the roots of *Atgpxl* mutants (Bela et al., 2018). The enhanced germination and seedling growth rates or higher chlorophyll contents of the OX-AtGPXL5 seedlings under salt stress in the present experiments suggest that constitutive overexpression of *AtGPXL5* increased the salt stress tolerance of plants. Our results revealed that the key point in the AtGPXL5's effect might be the modification of the redox status. Overexpression of *AtGPXL5* increased the amount of total GSH, thus the redox potential became more negative than that in the wild type even in the untreated shoots, and the differences increased and were significant both in the shoots and roots under salt stress (Fig. 26C-F).

Herbette et al. (2005) reported that overexpressing a mammalian selenium-independent *GPX* in tomato plants maintained a significantly higher photosynthesis rate and fructose-1,6-bisphosphatase activity under chilling stress. In the sustained viability, the role of modified levels of AsA and GSH was suggested (Herbette et al. 2005). Investigating the role of the mitochondrial OsGPXL3, Paiva et al. (2019) found that this isoenzyme is involved in redox equilibrium both in mitochondria and chloroplast. The rice OsGPXL3 has a role in the salt stress tolerance, but also in germination and growth of plants (Paiva et al., 2019). Passaia and Margis-Pinheiro (2015) emphasized that the rather low peroxidase activity of plant glutathione peroxidases compared to the selenocysteine-containing animal GPXs, the presence of the thiol catalytic centre in them, their capacity to interact with regulatory proteins make GPXLs strong and promising candidate to be efficient redox sensors. Our results indicate that AtGPXL5 can be such protein having a role in salt stress response.

6.5 AtGPXL5 has a role in the seed germination, growth and development of Arabidopsis seedlings under control and in the presence of 100 mM NaCl

Shifts in the cellular glutathione redox status may reversibly modify redox-sensitive thiol groups of proteins either through glutathionylation or formation of cysteine cross bridges. A relatively small global shift in the glutathione E_{hc} is associated with a very large change in gene expression and plant development (Aller et al., 2013; Schnaubelt et al., 2015). It is likely that the enzymatic and non-enzymatic elements of the complex ROS-processing system and the signaling pathways controling growth and development overlap (Noctor et al., 2018). Either AsA, GSH or several GSH-related enzymes can be among the components linking ROS- and

redox signaling to growth (Schnaubelt et al., 2015; Bela et al., 2017; Bielach et al., 2017; Gietler and Nykiel, 2017; Horváth et al., 2019). According to our results, AtGPXL5 has a function both in development and oxidative stress response. *In silico* promoter analysis of the *AtGPXL5* gene revealed the presence of several abiotic (ARE, HSE, MBS) and biotic (Box-W1) stress-related *cis*-acting elements in its 5′ up-regulatory region (Bela et al., 2015). Furthermore, two *cis*-regulatory sequences of this promoter were connected to seed development (AAGAA-motif and Skn-1_motif, (Bruce et al., 1991; Washida et al., 1999). According to data found in the Genevestigator database, *AtGPXL5* is expressed in all developmental stages of Arabidopsis plants, both in shoots and roots (Bela et al., 2015).

The roles of Arabidopsis GPXLs were investigated using *Atgpxl1*, *Atgpxl2*, *Atgpxl3*, *Atgpxl4*, *Atgpxl6*, *Atgpxl7*, and *Atgpxl8* T-DNA insertion mutants by Passaia et al. (2014). The shoot phenotypes of the mutants were largely similar to wild type plants, with small differences observed in the *Atgpxl2*, *Atgpxl3*, *Atgpxl7*, and *Atgpxl8* mutants, which displayed minor differences in the number of rosette leaves and lateral roots of the 4-week-old plants (Passaia et al., 2014). These authors confirmed the connections among the AtGPXLs and plant hormones such as auxin, ABA, strigolactone hormones, thereby demonstrating the importance of AtGPXLs in the hormone-mediated regulation of lateral root development. The relationship between the changes in redox status and the auxin transport was proved by Jiang et al (2016). Using the redox sensitive green fluorescent protein (roGFP1) they demonstrated that treatment of the Arabidopsis seedlings with 50, 100 and 150 mM NaCl caused initially a more oxidized redox status in the roots, but in the presence of relative low salt concentration (< 100 mM) after 1-3 days it could be re-establised. Coincident with the salt-associated changes in the redox profiles of roots were changes in the distribution of auxin transporters (AUX1, PIN1/2), which became more diffuse in their localization (Jiang et al. 2016). It was suggested that the adaptation to salinity in Arabidopsis might be mediated partly by an auxin/redox interaction (Iglesias et al., 2010; Jiang et al., 2016).

GSH is specifically required to activate and maintain the cell division cycle in the root's apical cells (Cheng et al., 1995; Vernoux et al., 2000; Frendo et al., 2005; Schnaubelt et al., 2015). Severe GSH depletion specifically inhibited root meristem development, and low root GSH levels decreased lateral root densities (Schnaubelt et al., 2015). A low level of GSH was reported to cause more oxidized redox potentials and to arrest the cell cycle in roots but not in shoots (Schnaubelt et al., 2015). It was shown that low GSH level modulates developmental responses through alteration of hormonal homeostasis of plants (Considine and Foyer, 2014;

Passaia et al., 2014; Schnaubelt et al., 2015; Jiang et al., 2016). Passaia et al. (2014) suggested that GPXLs may be required to mediate GSH and reduced TRX functions in roots that impact on lateral root production or growth. Plant GPXLs are considered to function as redox sensors (Milla et al., 2003; Miao et al., 2006; Herbette et al., 2007; Passaia et al., 2014, Passaia and Margis-Pinheiro, 2015). According to their relatively high expression levels, the AtGPX2, -3, -5, -6 and -8 gene products may play a role in the growth and differentiation of roots.

Passaia et al. (2013) reported that the mitochondrial OsGPXL3 was essential for normal *Oryza sativa* shoot development and seed production and according to the results of Lima-Melo et al. (2016) even for photosynthesis and root development. Mutation in *OsGPXL5* caused lower germination rate, reduced growth, and less filled grains compared to wild-type plants (Wang et al. 2017). In Arabidopsis, the relatively high transcription level of *AtGPXL1*, *AtGPXL2*, *AtGPXL3*, and *AtGPXL6* in shoot apical meristems, seedlings, and rosette leaves suggests the physiological importance of the encoded isoenzymes in shoot development (Bela et al., 2015). Interestingly, 4-week-old Arabidopsis knockout mutants of *GPXL7* under short-day conditions have greater rosette and under long-day photoperiod have more leaves than wild-type plants, indicating the role of GPXL7 in shoot development (Passaia et al. 2014). The relevance of AtGPXL7 in hormone-mediated root development, especially in lateral root development, was also demonstrated by using 1-naphtaleneacetic acid and synthetic strigolactone treatments (Passaia et al. 2014).

It was reported that some *GPXL* genes are activated (*AtGPXL2*, *-3* and *-8*), while others (*AtGPX1*, *-4*, *-6* and *-7*) are repressed during the seed germination (Bela et al., 2017). According to our results, overexpression of *AtGPXL5* resulted in maintained seed germination rate in the presence of 100 mM NaCl. There was no difference among the germination of the investigated lines under control conditions, indicating that the coded protein has a specific role in germination under salt stress. However, the decreased root length of the *Atgpxl5* mutant and the reduced development of the shoot of seedlings indicate that this protein has a function even in normal development. The decreased growth of the primary root in the *Atgpxl5* mutant caused higher lateral root density value than that in the Col-0. Interestingly, increased lateral root density was found also in the OX-AtGPXL5 seedlings after applying 100 mM NaCl for 10 days compared to the wild type, but in these plants the number of lateral roots remained higher in the presence of salt than in the other genotypes (Fig. 18E). Likewise, the un-treated 15-day-old OX-AtGPXL5 seedlings had similar rosette area, shoot biomass, convex area, chlorophyll and anthocyanin contents than the wild type (Fig. 17-20), but after applying salt stress these

parameters suggested less-impaired growth of the *AtGPXL5*-overexpressing lines. The differences found in the phenotype and salt stress response of the *Atgpxl5* mutant and *AtGPXL5* overexpressing plants suppose a complex interaction of AtGPXL5 with plant hormones.

7. Summary

Plant's glutathione peroxidase-like (GPXL) enzymes are thiol-based peroxidases catalysing the reduction of H_2O_2 or hydroperoxides to water or alcohols using reduced glutathione (GSH) or thioredoxin as an electron donor (Navrot et al., 2006). *Arabidopsis thaliana* possesses eight isoenzymes located in different plant's organelles and having different roles in redox-dependent processes. Among them, AtGPXL5 is a poorly known plasma membrane-associated enzyme, although its role in salt stress tolerance was suggested (Gao et al. 2014). We have constitutively overexpressed the *AtGPXL5* cDNA and investigated the role of AtGPXL5 in response to NaCl treatment and in development. Experiments were performed by using *AtGPXL5*-overexpressing lines (OX-AtGPXL5) and *Atgpxl5* mutant plants. Based on our results, we have made the following observations:

1) 12-day-old *Arabidopsis thaliana Atgpxl5* insertional mutants had higher level of superoxide radical anion and total ROS in untreated roots and shoots, respectively compared with Col-0. The higher level of ROS decreased the cells' vitality in the shoot of *Atgpxl5* seedlings even under control condition. After applying 7-day treatment with the concentration of 100 mM NaCl, the $O_2^{\bullet-}$ level in the root was elevated further and reached higher level than in the wild type. These indicate that AtGPXL5 might play an important role in the ROS homeostasis and maintaining the cell's vitality.

2) The antioxidant mechanisms of the 6-week-old plants have altered, especially in the *Atgpxl5* mutants compared to OX-AtGPXL5 plants. Several ROS processing enzymes worked in elevated level in *Atgpxl5* mutant, but OX-AtGPXL5 plant exhibited similar activity to the Col-0 wild type. The GPOX activity was elevated in the lowest extent in *Atgpxl5* plants while GPOX and TPOX enzymes in the *AtGPXL5*-overexpressing plants worked about on the level of wild type.

3) Under control conditions, significantly lower GSH was found in the *Atgpxl5* mutant roots while its amount was elevated in the OX-AtGPXL5 shoot. The applied salt stress caused accumulation of the highest amount of reduced glutathione and the less oxidized form (GSSG) in the *AtGPXL5*-overexpressing plants among the investigated lines, while the GSSG increased most in the *Atgpxl5* roots. The amount of reduced glutathione was higher and the calculated redox potential was more negative in the overexpressed line than in Col-0. The result confirms that AtGPXL5 has function in regulating the redox state, through which they can also influence the growth and development.

4) AtGPXL5 enzymes are required for healthy growth and development of the *Arabidopsis thaliana* seedlings. Deficiency of AtGPXL5 led to reduce the length of primary roots, biomass, rosette size, convex area, chlorophyll and anthocyanin contents compared to other investigated lines under normal conditions. In the presence of 100 mM NaCl, *Atgpxl5* mutant and the Col-0 wild type seeds showed delayed germination, while germination of the OX-AtGPXL5 lines was not inhibited in the presence of 100 mM NaCl. Untreated OX-AtGPXL5 lines exhibited similar phenotype as Col-0, however the overexpressing plants grew better in the presence of 100mM NaCl: they had larger rosettes, larger convex area and lower convex percentage values with higher content of chlorophyll and anthocyanin than that of the wild type and *Atgpxl5* plants. The reduced development of the shoots and decreased root length of the *Atgpxl5* mutant indicate that this protein has a function even in the normal development.

8. Összefoglalás

A tiol peroxidázok közé tartozó növényi glutation peroxidáz-szerű enzimek (GPXL-ek) redukált glutation (GSH) vagy tioredoxin elektron donort használva katalizálják a H_2O_2 vagy egyéb hidroperoxidok vízzé vagy alkohollá történő redukcióját (Navrot és mtsai., 2006). Az *Arabidopsis thaliana* 8 GPXL izoenzimet tartalmaz, amelyek különböző sejtorganellumokban lokalizáltak és eltérő redox-függő folyamatokban játszanak szerepet. Az AtGPXL5 egy kevéssé ismert plazmamembrán-kapcsolt izoenzim, bár szerepét feltételezték a sóstressz tolerancia kialakításában (Gao és mtsai. 2014). Kísérleteinkben konstitutívan túltermeltettük az *AtGPXL5* cDNS-t és a növények fejlődésében és NaCl kezelésre adott válaszában vizsgáltuk az AtGPXL5 szerepét. Vizsgálatainkat *AtGPXL5*-túltermelő (OX-AtGPXL5) és *Atgpxl5* mutáns növények felhasználásával végeztük. Eredményeink alapján az alábbi megállapításokat tettük:

1) A 12 napos *Arabidopsis thaliana Atgpxl5* inszerciós mutánsok gyökerében magasabb szuperoxid gyök anion és össz ROS szint található kontroll körülmények között is, mint a vad típusú (Col-0) gyökerekben. A magasabb ROS szint az *Atgpxl5* hajtások csökkent életképességét eredményezte. 7 napig tartó 100 mM-os NaCl kezelés tovább emelte a gyökerekben a $O_2^{\bullet -}$ mennyiségét, szintje meghaladta a vad típusban kialakultat. Ezek alapján az AtGPXL5 fontos szerepet tölthet be a reaktív oxigénformák homeosztázisában és a sejt életképességének fenntartásában.
2) A hat hetes *Atgpxl5* mutáns és OX-AtGPXL5 növények antioxidáns mechanizmusait összehasonlítva jelentős különbségek mutatkoztak. Míg az *Atgpxl5* mutánsban több antioxidatív enzim emelkedett aktivitást mutatott, az *AtGPXL5*-túltermelő növényekben a vad típushoz hasonló aktivitásokat mértünk. Míg az Atgpxl5 növények magasabb glutation peroxidáz (GPOX) aktivitással rendelkeztek, az OX-AtGPXL növényekben a GPOX és TPOX (tioredoxin peroxidáz) aktivitás is a vad típuséval azonos szinten működött.
3) Kontroll körülmények között a GSH tartalom szignifikánsan alacsonyabb volt az *Atgpxl5* mutáns gyökerekben, míg az OX-AtGPXL5 hajtásban magasabb. Az alkalmazott sóstressz után az *AtGPXL5*-túltermelő növények rendelkeztek legmagasabb GSH és legalacsonyabb GSSG szinttel, a GSSG mennyisége az *Atgpxl5* mutáns gyökerekben nőtt legnagyobb mértékben. Az OX-AtGPXL5 vonalak számított redukciós potenciál értéke negatívabb volt, mint a Col-0 vad típusé. Az eredmények megerősítették, hogy az AtGPXL5 fehérje szerepet játszik a redox állapot szabályozásában, amelyen keresztül a növekedést és fejlődést is befolyásolhatják.

4) Az AtGPXL5 protein szükséges az *Arabidopsis thaliana* csíranövények normál növekedéséhez és fejlődéséhez. Hiányában csökkent az elsődleges gyökerek hossza, biomasszája, a rozetta mérete, a hajtások klorofill- és antocián tartalma kontroll körülmények között is. 100 mM NaCl jelenlétében az *Atgpxl5* mutáns és a vad típusú növények magjainak csírázása gátlódott, azonban az *AtGPXL5* túltermelőké nem. Az OX-AtGPXL5 növények normal fiziológiás körülmények között a vad típushoz hasonló fenotípust mutattak, azonban 100 mM NaCl jelenlétében jobban növekedtek: nagyobb rozetta átmérővel rendelkeztek, a levelek nagyobb konvex területet foglaltak el, magasabb klorofill és antocián tartalommal rendelkeztek, mint a Col-0 és *Atgpxl5* mutáns növények. Az *Atgpxl5*-nél megfigyelt csökkent hajtás- és gyökérnövekedés arra enged következtetni, hogy az AtGPXL5 fehérje szerepet játszik a növények normal növekedésében.

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11. Publication list

Riyazuddin, R., Bela, K., Horváth, E., Rigó, G., Gallé, Á., Szabados, L., Fehér, A., Csiszár, J., 2019. Overexpression of the Arabidopsis glutathione peroxidase-like 5 gene (AtGPXL5) resulted in altered plant development and redox status. Environmental and Experimental Botany, 167, 103849. https://doi.org/10.1016/j.envexpbot.2019.103849. **IF: 3.712 (2019)**

Csiszár, J., Brunner, S., Horváth, E., Bela, K., Ködmön, P., **Riyazuddin, R.,** Gallé, Á., Hurton, Á., Papdi, C., Szabados, L., Tari, I., 2018. Exogenously applied salicylic acid maintains redox homeostasis in salt-stressed Arabidopsis gr1 mutants expressing cytosolic roGFP1. Plant Growth Regul. 86, 181. https://doi.org/10.1007/s10725-018-0420-6. **IF: 2.473 (2018)**

Publications in a peer-reviewed journal

Riyazuddin, R., Bela, K., Horváth, E., Rigó, G., Gallé, Á., Szabados, L., Fehér, A., Csiszár, J., 2019. Overexpression of the Arabidopsis glutathione peroxidase-like 5 gene (AtGPXL5) resulted in altered plant development and redox status. Environmental and Experimental Botany, 167, 103849. https://doi.org/10.1016/j.envexpbot.2019.103849. **IF: 3.712**

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Bela, K., **Riyazuddin, R.,** Horváth, E., Hurton, Á., Gallé, Á., Takács, Z., Zsigmond, L., Szabados, L., Tari, I., Csiszár, J., 2018. Comprehensive analysis of antioxidant mechanisms in Arabidopsis glutathione peroxidase-like mutants under salt- and osmotic stress reveals organ-specific significance of the AtGPXL's activities. Environ. Exp. Bot. 150, 127-140. https://doi.org/10.1016/j.envexpbot.2018.02.016**.** **IF: 3.712**

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Book chapter

Bela, K., Bangash, S.A.K., Riyazuddin, Csiszár, J., 2017. Plant glutathione peroxidases: antioxidant enzymes in plant stress responses and tolerance. In: Hossain, M.A., Mostofa, M.G., Diaz-Vivancos, P., Burritt, D.J., Fujita, M., Tran, L.S.P (Eds.), Glutathione in Plant Growth, Development, and Stress Tolerance. Springer, Cham, pp. 113–126. https://doi.org/10.1007/978-3-319-66682-2_5.

Poster

Riyazuddin, R., Bela, K., Horváth, E., Rigó, G., Szabados, L., Gallé, Á., Fehér, A., Csiszár, J- Overexpression of Arabidopsis glutathione peroxidase-like 5 (*AtGPXL5*) gene resulted in enhanced plant development and protection against salt stress by improving the redox potential in Free radical conference, XIII Congress of the Hungarian Free Radical Research Society, 29-30 August 2019, Szeged, Hungary.

Bela, K., **Riyazuddin, R.,** Horváth, E., Gallé, Á., Hurton, Á, Bangash, S.A.K., Ayaydin, F., Csiszár J- Effect of salinity on the redox potential of Arabidopsis glutathione peroxidase-like mutant roots in Free radical conference, XIII Congress of the Hungarian Free Radical Research Society, 29-30 August 2019, Szeged, Hungary.

Horváth, E., Bela, K., Kulman, K., Gallé, Á., **Riyazuddin, R.,** Csiszár, J-Salicylic Acid-Induced Redox Status Changes in Arabidopsis Atgstf8 mutant root in Free radical conference, XIII Congress of the Hungarian Free Radical Research Society, 29-30 August 2019, Szeged, Hungary.

Riyazuddin, R., Bela, K., Horváth, E., Rigó, G., Szabados, L., Gallé, Á., Fehér, A., Csiszár, J - Overexpression of a membrane localised Arabidopsis glutathione peroxidase (AtGPXL5) resulted in altered plant development and protection against salt stress" in Sensing and signaling in plant stress response symposium, European Molecular Biology Organization (EMBO), April 2019, New Delhi India.

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Statement

As the main author of the scientific publication, I certify that the results reported in the Ph.D. dissertation and the following publication were not used to acquire any Ph.D. degree previously and will not be used in future either.

Riyazuddin, R., Bela, K., Horváth, E., Rigó, G., Gallé, Á., Szabados, L., Fehér, A., Csiszár, J., 2019. Overexpression of the *Arabidopsis* glutathione peroxidase-like 5 gene (*AtGPXL5*) resulted in altered plant development and redox status. Environmental and Experimental Botany, 167, 103849. https://doi.org/10.1016/j.envexpbot.2019.103849.

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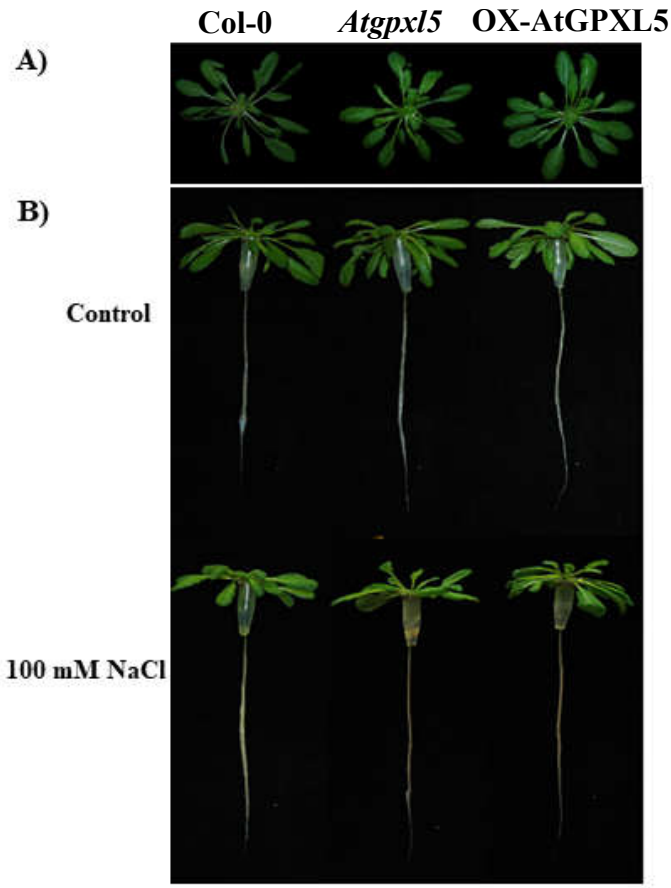
12. Supplementary data



C)

Maximum rosette diameter (cm) and length of roots of 6-week-old hydroponically grown Col-0, *Atgpxl5* and OX-AtGPXL5 plants.

	Rosette diameter (cm)	Root length (cm)
Col-0	12.17±0.20^{b}	20.55±1.05$^{n.s}$
Atgpxl5	12.14±0.22^{b}	24.30±1.38$^{n.s}$
OX-AtGPXL5-1	12.90±0.12^{a}	22.96±0.85$^{n.s}$

Figure S1 Hydroponically grown 6-week-old *Arabidopsis* Col-0 wild type, *Atgpxl5* insertional mutant and OX-AtGPXL5-1 plants treated with 100 mM NaCl for 24 h. A: Top view photos of selected un-treated 6-week-old hydroponically grown Col-0, *Atgpxl5* and OX-AtGPXL5 plants, respectively B: side view photos of selected plants after one-day-long salt treatment. C: Maximum rosette diameter (cm) and length of roots of 6-week-old un-treated plants. Data are presented by mean values ± SE, n=20. Data with different letters are significantly different at $p \leq 0.05$, determined by Duncan's test.

Supplementary Table 1. List of oligonucleotides used during identification of interested sequences and in real time quantitative polymerase chain reaction (qRT-PCR)

Vector name or gene ID	Primer	Sequence	Reference
pTCO35S new	pTCO35SNEW-F	5’ GCAGGACGATCCGTATTTTTACAAC 3’	Rigó et al. 2016
T35S/RD29rc	T35S/RD29rc-R	5’ GGACTCTAGCATGGCCGCGGG 3’	Rigó et al. 2016
At3G63080	AtGPXL5-F	5′ TCATCATCATCATCTGTGTCGGA 3’	Bela et al. 2018
	AtGPXL5-R	5′ GGACTCCGTGAATCCGCATT 3’	
At1G16300	AtGAPDH2-F	5’ GAATCAACGGTTTCGGAAGA 3’	Riyazuddin et al., 2019
	AtGAPDH2-R	5’ CTCGGTGGTGATGAAAGGAT 3’	