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Selection, stress response and characterization of *Arabidopsis thaliana* GABA mutants

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Zásady pro vypracování

Cílem práce je vyselektovat mutantní rostliny Arabidopsis thaliana s inzercí v celkem devíti genech metabolismu GABA (glutamát dekarboxyláza – GAD1-5; GABA transamináza – GABA-T; sukcinát semialdehyd dehydrogenáza – SSADH; NADPH-dependentní glyoxylát-reduktáza – GLYR1 a GLYR2). Tato in vitro selekce transgenních rostlin bude následovaná genotypizací pomocí PCR. Poté budou rostliny vystaveny působení stresů (zasolení, sucho, oxidační stres, chlad) a hormonů a bude provedena jejich fenotypizace (nadzemní část rostliny na fenotypizační lince, charakterizace kořenů pomocí in vitro kultur).

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Fait A., Fromm H., Walter D. (2008): Highway or byway: the metabolic role of the GABA shunt in plants. Trends in Plant Science 13: 14-19.

Jalil S. U., Ahmad, I., Ansari, M. I. (2017): Functional loss of GABA transaminase (GABA-T) expressed early leaf senescence under various stress conditions in Arabidopsis thaliana. Current Plant Biology 9-10: 11-22.

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SUMMARY

Gamma-aminobutyric acid (GABA), an ubiquitous non-protein amino acid, is mainly known for its role as an inhibitory neurotransmitter in animals; however, it also accumulates rapidly in plants in response to biotic and abiotic stress. GABA is synthetized from *L*-glutamate by the enzyme glutamate decarboxylase (GAD), which has five isoforms in *Arabidopsis thaliana*. The GABA metabolism occurs via a pathway called GABA shunt, which consists of enzymes glutamate decarboxylase (GAD), GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH).

The aim of this thesis was to investigate the role that GABA metabolism plays in the growth of *A. thaliana* under control and salt stress conditions. The above-ground parts of different knockouts with T-DNA insertion within nine genes of GABA metabolism were analyzed using phenotyping approaches. Firstly, homozygous plants needed to be selected – this was achieved by growing the plants on selection plates with antibiotics, followed by genotyping by PCR. After that, homozygous plants for seven genes were successfully obtained. The growth and other phenotypic related traits of these homozygous lines were then evaluated under control and salt stress conditions. Phenotypical differences among the lines were identified. While some genes were correlated with the rosette size, others were more involved in the symmetry of the plant.

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SOUHRN

Kyselina gama-aminomáselná (GABA) je běžně se vyskytující neproteinogenní aminokyselina, která je známá zejména díky své funkci jako inhibiční neurotransmiter u zvířat, dochází ale také k její rapidní akumulaci v rostlinách v reakci na působení abiotického a biotického stresu. GABA je syntetizována z *L*-glutamátu pomocí enzymu glutamát dekarboxyláza (GAD), který se v *Arabidopsis thaliana* vyskytuje v pěti isoformách. Metabolismus GABA zahrnuje enzymy glutamát dekarboxyláza (GAD), GABA transamináza (GABA-T) a sukcinát semialdehyd dehydrogenáza (SSADH).

Cílem této práce bylo prozkoumat roli GABA metabolismus v růstu *A. thaliana* v kontrolních podmínkách a podmínkách solného stresu. Nadzemní části knockoutů s T-DNA inzercí v devíti genech GABA metabolismu byly analyzovány za využití fenotypovacích přístupů. Nejprve bylo nutné vyselektovat homozygotní rostliny – toho bylo docíleno nejdříve jejich pěstováním na selekčním médiu s antibiotiky, které následovala genotypizace pomocí PCR. Selekce homozygotních rostlin byla úspěšná v případě sedmi genů. Růst a další fenotypové vlastnosti těchto homozygotních linií byly posouzeny za kontrolních podmínek a za působení solného stresu. Byly identifikovány fenotypové rozdíly mezi liniemi. Zatímco některé geny korelovaly s velikostí listové růžice, jiné geny se podílely na symetrii rostliny.

I declare that this master's thesis titled 'Selection, stress response and characterization of *Arabidopsis thaliana* GABA mutants' was carried out by me under the supervision of Nuria De Diego Sanchez, Ph.D. using only sources listed in the Reference section.

Date: 2020

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Jana Zachovalová

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CONTENTS

ABBREVIATIONS

Δ^1-Pyr	delta-1-pyrroline
AAP	amino acid permease
ABRC	*Arabidopsis* Biological Resource Center
ALMT	aluminium-activated malate transporter
CaM	calmodulin
CAT	cationic amino acid transporter
ClF	chlorophyll fluorescence
CNS	central nervous system
Col	Columbia ecotype
DAO	diamine oxidase
DAS	days after stratification
DW	dry weight
FAO	The Food and Agriculture Organization
FST	T-DNA flanking sequence
FW	fresh weight
GABA	gamma-aminobutyric acid
GABA-T	GABA transaminase
GABP	GABA permease
GAD	glutamate decarboxylase
GAT	GABA transporter
gDNA	genomic DNA
Glu	glutamate
GHB	gamma-hydroxybutyrate
GHBDH	gamma-hydroxybutyrate dehydrogenase
GLYR	glyoxylate/succinic semialdehyde reductase
GSP	gene-specific primer
HM	homozygous
HTP	high-throughput phenotyping
HZ	heterozygous
KO	knockout
LB	left border
Ler	Landsberg ecotype
NASC	Nottingham *Arabidopsis* Stock Centre
PAR	photosynthetically active radiation
POP2	pollen-pistil 2

ProT	proline transporter
RB	right border
RGB	red-green-blue
RMS	Rotational Mass Symmetry
ROI	reactive oxygen intermediate
SOL	Slenderness of Leaves
SSA	succinic semialdehyde
SSADH	succinate semialdehyde dehydrogenase
SSAR	succinic semialdehyde reductase
T-DNA	transfer DNA
UTR	untranslated region
WC	water content
Ws	Wassilewskija ecotype
WT	wild type

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1 INTRODUCTION

Plants are sessile organisms, and as such have to endure adverse conditions that limit their growth and development. These conditions are generally defined as stress and can by divided into two categories – abiotic stress and biotic stress. Abiotic stress includes ultraviolet radiation, drought, high salinity, flooding and high temperature among others, whereas biotic stress is caused by damage by pathogens (bacterial, viral and fungal) or pests. Plants have developed many mechanisms to deal with stress, understanding of which is a goal of many studies.

One of the most important plant substances participating in plant response to stress are phytohormones such as abscisic acid, auxins, cytokinins, ethylene, jasmonates and others (Wani *et al*., 2016). During abiotic stress, polyamines undoubtedly play a significant role, while under biotic stress, phenolic compounds like phytoalexins and coumarins are important (Minocha *et al*., 2014; Kulbat, 2016). Plants subjected to stress also accumulate proline and other amino acids, including non-proteinogenic amino acids such as gamma-aminobutyric acid (GABA) (Rai, 2002).

The accumulation of GABA has been observed in response to a wide variety of environmental stresses. It has long been known about its involvement in the metabolism of carbon and nitrogen and the regulation of cytosolic pH; GABA functions as an osmoregulator and there are also speculations about its possible role as a signal molecule (Bouché and Fromm, 2004).

It is no surprise that GABA has become a subject of studies regarding plant growth and response to stress. As a means to investigate the function of genes involved in GABA metabolism, plant lines disrupted in the genes of interest are used. In this thesis, we analyzed the effect of salt stress on the growth and other phenotypic related traits of *Arabidopsis thaliana* lines with T-DNA insertions in the genes of GABA metabolism using phenotyping approaches.

2 AIMS AND OBJECTIVES

Aim:

To investigate the effect of salt stress on the above-ground part of *Arabidopsis thaliana* mutant lines with disrupted genes of GABA metabolism.

Objectives:

- Selection of plants homozygous for T-DNA insertion in nine genes of GABA metabolism (glutamate decarboxylase – *GAD 1–5*; GABA transaminase – *GABA-T*; succinic semialdehyde dehydrogenase – *SSADH*; NADPH-dependent glyoxylate/succinate semialdehyde reductase – *GLYR1* and *GLYR2*)
- Genotyping of mutants using PCR
- Non-invasive phenotyping of homozygous mutants under control and salinity stress conditions

3 LITERATURE REVIEW

3.1 Gamma-aminobutyric acid (GABA)

Gamma-aminobutyric acid (GABA for short) is a four-carbon non-proteinogenic amino acid that can be found throughout plant, animal and bacteria kingdoms (Steward *et al.*, 1949; Roberts and Frankel, 1950; Reed, 1950). It is mainly known for its importance as an inhibitory neurotransmitter in central nervous system of animals (Roberts, 1988) despite first being identified in potato (*Solanum tuberosum* L.) tubers in 1949 (Steward *et al.*, 1949). In plants, its role is less straightforward. It is well-known that GABA is synthetized in response to both biotic and abiotic stress (Kinnersley and Turano, 2000), but studies also point to its involvement in regulation of nitrogen and carbon metabolism, defence against invertebrate pests, and possibly signalization (Kinnersley and Turano, 2000; Bouché and Fromm, 2004; Fait *et al.*, 2008).

3.2 GABA metabolism

3.2.1 Biosynthesis of GABA

GABA biosynthesis occurs in cytosol by the means of irreversible decarboxylation of *L*-glutamate (Glu) (Breitkreuz and Shelp, 1995). The reaction is catalyzed by the enzyme glutamate decarboxylase (GAD, EC 4.1.1.15). The number of GADs differs amongst plants. The first plant GAD was identified in petunia (Baum *et al.*, 1993), followed by the characterization of GAD1 and GAD2 in *Arabidopsis thaliana* L. (Turano and Fang, 1998). Tomato, rice and maize have five isoforms, whereas soybean has nine (Shelp *et al.*, 2012b). In *A. thaliana* there are five GAD isoforms (Shelp *et al.*, 1999), the aforementioned GAD1 and GAD2, and three other isoforms – GAD3, GAD4 and GAD5, which have been revealed by a database search and share 75–82% amino acid sequence identity with GAD1 (Shelp *et al.*, 1999).

GAD has an acidic pH optimum and is activated by a raised cytosolic concentration of H^+ and Ca^{2+} ions (Shelp *et al.*, 1999). The increase of Ca^{2+} concentration is related to various stresses, namely cold and heat shock, salinity, mechanical damage and osmotic stress (Sanders *et al.*, 1999). Ca^{2+} ions form a complex with calmodulin (CaM) and this complex then binds to GAD, resulting in its activation. GAD proteins from many plant species are thought to bind CaM (Baum *et al*., 1993). At physiological pH, the activity of GAD is virtually dependent upon the presence of the Ca^{2+}/CaM complex (Snedden *et al*., 1996).

Baum *et al*. (1996) were the first to report GAD possessing a C-terminal CaM-binding domain. This domain is necessary for normal plant development and maintenance of GABA and Glu levels (Baum *et al.*, 1996). It is present in GAD1, GAD2 and GAD4 proteins, whereas the activation of GAD3 and GAD5 is CaM-independent, as proposed by *in silico* analysis (Shelp and

Zarei, 2017). CaM-independent GAD3 has been reported in apple fruit (*Malus* x *domestica* Borkh) (Trobacher *et al*., 2013). However, Akama and Takaiwa (2007) also described rice (*Oryza sativa* L.) GAD2 protein whose C-terminal domain does not bind CaM and serves as an autoinhibitory domain.

It was hypothesized that GABA could be also produced by catabolizing polyamine putrescine (Shelp *et al*., 2012a). Further research showed that in lupine seedlings (*Lupinus luteus* L. 'Juno'), upon salt stress, GABA synthesis occurred via putrescine degradation possibly by diamine oxidase (DAO) (Legocka *et al.*, 2017). Furthermore, an alternative non-enzymatic reaction of GABA synthesis from the amino acid proline during oxidative stress was suggested. Proline could contribute to the non-enzymatic formation of delta-1-pyrroline (Δ^1-Pyr), which is then converted to GABA by pyrroline dehydrogenase (Signorelli *et al.*, 2015). Proline, same as GABA, accumulates during stress and has a protective role in cells; this pathway could be an explanation for such accumulation (Szabados and Savouré, 2010; Signorelli *et al.*, 2015).

3.2.2 GABA shunt

The metabolism of GABA occurs via pathway known as the GABA shunt. It is called GABA shunt because it bypasses two steps of the citric acid cycle (Bouché and Fromm, 2004). It is a three-enzyme pathway that consists of the aforementioned GAD enzyme, GABA transaminase (GABA-T or POP2 from 'pollen-pistil', EC 2.6.1.19) and succinate semialdehyde dehydrogenase (SSADH, EC 1.2.1.16) (Fig. 1).

As mentioned before, GABA is synthetized by GAD in cytosol and then transported into mitochondria by GABA permease (AtGABP) (Michaeli *et al.*, 2011). In mitochondria, GABA is converted to succinic semialdehyde (SSA) by two GABA transaminases (GABA-T); each of them uses a different compound as amino acid acceptor. GABA-TK utilizes α-ketoglutarate and leads to the production of Glu, GABA-TP produces alanine from pyruvate (Bouché and Fromm, 2004). GABA-TP also has irreversible glyoxylate-dependent GABA-T activity – the transamination generates glycine from glyoxylate, possibly connecting GABA metabolism and photorespiration (Clark *et al.*, 2009). Thus far, only the gene for GABA-TP has been isolated (Van Cauwenberghe *et al.*, 2002), despite the fact that the activity of both transaminases has been detected in crude plant extracts (Van Cauwenberghe and Shelp, 1999). Mammals only exhibit GABA-TK activity (Bouché and Fromm, 2004).

The activity of GABA-T is essential for some biological processes in plants. For example, in *Arabidopsis*, the knockout of GABA-T (also known as *pop2* mutant, abbreviated from pollen-pistil-interaction2) is unable to degrade GABA, leading to its accumulation. In these mutants, the growth of pollen tubes in pistils is affected (Wilhelmi and Preuss, 1996). In WT plants, there is a GABA gradient along the path pollen tubes travel in the pistil, whereas in *pop2* mutants, which are unable to synthetize GABA-TP, this gradient is disturbed, and they accumulate GABA in

flowers (Bouché and Fromm, 2004). GABA has a biphasic effect; low concentrations stimulate growth of pollen tubes *in vitro*, however, higher concentrations have an inhibitory effect both *in vitro* and *in vivo* (Palanivelu *et al.*, 2003; Renault *et al.*, 2011). Exogenous GABA in *pop2* mutants causes defects in hypocotyl and primary roots elongation (Renault *et al.*, 2011).

SSA produced by GABA-T is a reactive carbonyl which presumably causes oxidative stress and an increase of reactive oxygen intermediates (ROI) (Ludewig *et al.*, 2008). There are two known pathways of its elimination – oxidation to succinate by SSADH (Breitkreuz and Shelp, 1995) and reduction to gamma-hydroxybutyrate (GHB) by NADPH-dependent glyoxylate/succinic semialdehyde reductase (GLYR1 and GLYR2, EC 1.1.1.79), which was initially designated as SSA reductase (SSAR), GHB dehydrogenase (GHBDH) or GR1 and GR2, respectively (Breitkreuz *et al.*, 2003; Shelp *et al.*, 2012b). There is evidence for the latter reaction occurring under high light and hypoxic conditions and also in response to cold, salinity and drought (Allan *et al.*, 2003; Fait *et al.*, 2005; Allan *et al.*, 2008). GLYR1 and GLYR2 predominantly catalyse the conversion of glyoxylate to glycolate and have considerably higher affinity for glyoxylate than SSA (250-fold and 350-fold higher, respectively) (Hoover *et al.*, 2007; Simpson, 2008). The removal of SSA by SSADH is thought to be more important out of the two reactions, as *ssadh* mutants exposed to stress accumulate GHB and H_2O_2 and exhibit distinctive phenotype characterized by dwarfism, necrotic lesions and bleached leaves and have fewer flowers than wild type plants (Bouché *et al.*, 2003; Fait *et al.*, 2005; Ludewig *et al.*, 2008). The activity of SSADH is negatively regulated by ATP and NADH (Bouché and Fromm, 2004).

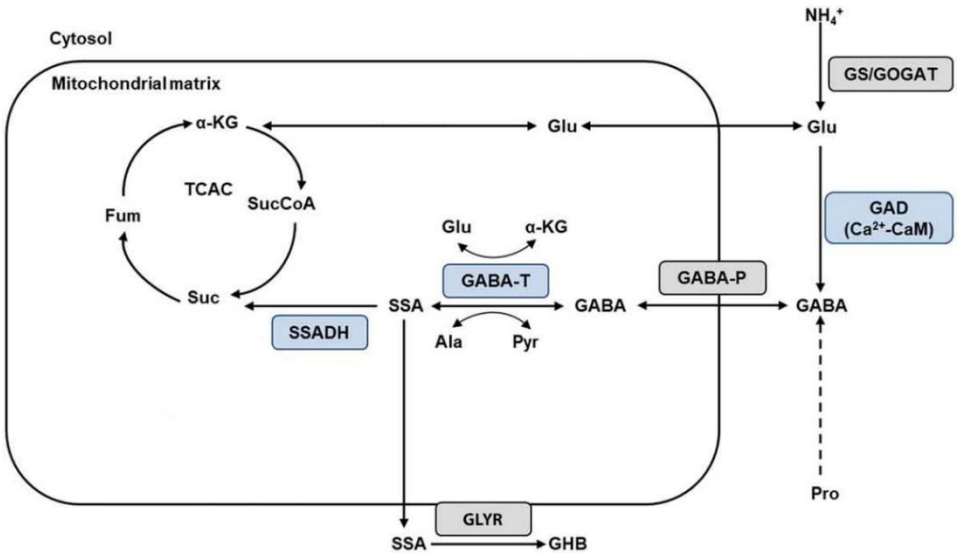

Figure 1. GABA metabolism in plants. Abbreviations: Ala, alanine; α-KG, *α*-ketoglutarate; Fum, fumarate; GABA-P, GABA permease; GABA-T, GABA transaminase; GAD, glutamate decarboxylase; GHB, gamma-hydroxybutyrate; Glu, glutamate; GLYR, glyoxylate/succinic semialdehyde reductase; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamine synthetase; Pro, proline; Pyr, pyruvate; SSA, succinic semialdehyde; Suc, succinate; SucCoA, succinyl-coenzyme A; SSADH, succinate semialdehyde dehydrogenase; TCA, tricarboxylic acid cycle. Modified from Ramos-Ruiz *et al.* (2019).

3.2.3 Organ distribution of GABA related enzymes

The expression of *GAD* isoforms of *A. thaliana* varies in different plant organs (Fig. 2). *GAD1* is expressed only in roots (Bouché and Fromm, 2004), while the transcript of *GAD2* was found in all plant organs, although the expression in siliques was nearly undetected (Turano and Fang, 1998; Zik *et al.*, 1998). The expression of remaining *GADs* is low with the exception of *GAD4* and *GAD5* in flowers (Miyashita and Good, 2008).

The two GLYR isoforms have a different localization in the cell. GLYR2 of apple, *A. thaliana* and rice are localized to the plastid stroma and mitochondria (Simpson *et al.*, 2008; Brikis *et al.*, 2017), whereas GLYR1 is localized exclusively to the cytosol (Ching *et al*., 2012). In *A. thaliana*, both isoforms are expressed in the entire plant at all stages of development, the expression is weak only in roots (Hoover *et al.*, 2007). The transcript of *GLYR2* was detected in *Arabidopsis* rosette leaves in higher abundance than that of *GLYR1* (Zarei *et al.*, 2017).

SSADH and GABA-T have both been detected in mitochondria of *Arabidopsis* and soybean (*Glycine max* L.) (Breitkreuz and Shelp, 1995; Busch and Fromm, 1999; Shelp *et al.*, 1999). *GABA-T* is expressed in all plant organs, especially in flowers, siliques and shoots (Miyashita and Good, 2008).

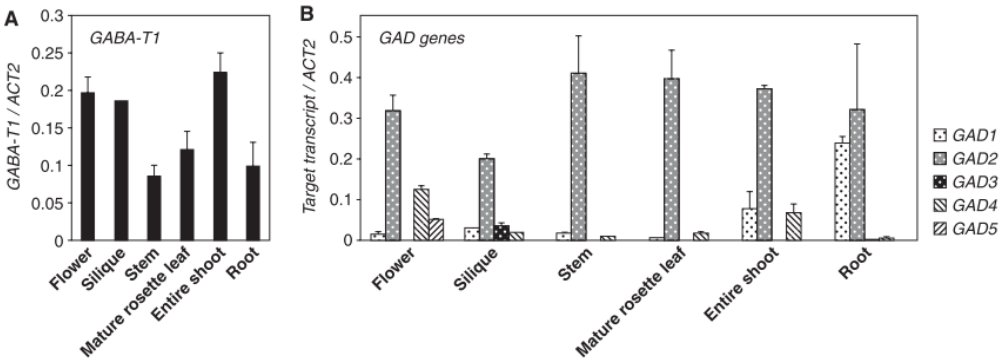

Figure 2. Organ-specific expression of *GABA-T* and five *GAD* genes in *A. thaliana*. The transcript abundance was measured using quantitative real-time RT-PCR and normalized against ACT2 (Actin2). **(A)** GABA transaminase expression profile. **(B)** Expression profiles of five glutamate decarboxylases. Miyashita and Good (2008).

3.2.4 GABA transport

The transport of GABA in plants has been extensively studied. The first characterised transporters are amino acid permease 3 (AAP3) and proline transporter 2 (AtProT2) localized in the plasma membrane, however, their affinity for GABA is significantly lower than their affinity for amino acid proline and derivatives such as glycine betaine, meaning that their ability to transport GABA *in planta* is uncertain (Breitkreuz *et al*., 1999; Grallath, 2005).

To this day, there are only two high-affinity GABA transporters described, the first being AtGAT1 localized in the plasma membrane. AtGAT1 is also capable of transporting ω-amino

fatty acids but does not recognise proline and glycine betaine. Out of all tested organs, *AtGAT1* expression was elevated in flowers; the expression also increased in response to raised concentration of GABA accompanying senescence and wounding (Meyer *et al.*, 2006).

Michaeli *et al.* (2011) described the second specific GABA transporter in *A. thaliana*, aforementioned mitochondrial GABA permease (AtGABP), which is the first GABA transporter with its transporter function shown *in planta*. The evidence that the uptake of GABA into mitochondria is not entirely eliminated in *gabp* mutants with disrupted gene for AtGABP suggests that there are probably other mitochondrial GABA transporters with overlapping function.

The transport of GABA from vacuole to cytosol in exchange for aspartate or Glu in tomato (*Solanum lycopersicum* L.) is facilitated by cationic amino acid transporter (SlCAT9) (Snowden *et al.*, 2015). Which member of the CAT family localized in tonoplast is responsible for transport between vacuole and cytosol in *A. thaliana* is still unknown, however AtCAT9 shares 68% amino acid sequence identity with SlCAT9 and appears to be the prime candidate (Shelp and Zarei, 2017).

3.3 GABA in plant development

The change of GABA concentration can also have an effect on normal development of plants and humans alike. In humans, SSADH and GABA-T deficiency both result in rare diseases manifested by psychomotor retardation, hypotonia, ataxia and seizures (Chambliss *et al.*, 1998; Medina-Kauwe *et al.*, 1999). Transgenic tobacco (*Nicotiana tabacum* L.) plants expressing truncated GAD display constitutive GAD activity resulting in high GABA levels leading to shorter stem cortex parenchyma cells and other developmental defects such as lack of pollen; the plants were also shorter and more branched when compared to plants with full-length GAD (Baum *et al.*, 1996). GABA gradient is also necessary for pollen tube guidance and its absence has a negative effect on fertility (Palanivelu *et al*., 2003).

GABA accumulates in cherry tomato fruits before the breaker stage, constituting up to 50% of free amino acid content, however, its role in tomato fruit development is still largely unclear (Rolin *et al.*, 2000; Takayama and Ezura, 2015).

GABA has been also shown to accumulate in *A. thaliana* leaves during senescence. As an example, the gene encoding GABA-TP is upregulated in senescent rice leaves (Ansari *et al*., 2005). Taken together, these findings suggest a role for GABA shunt in anaplerotic reactions (Ansari *et al.*, 2005; Diaz, 2005).

3.4 GABA and signalling

The role of GABA in animals has been known for 60 years, so its function as an inhibitory neurotransmitter is well established (Elliott and Jasper, 1959). Although there is no direct proof yet, the speculations about GABA as a signal molecule in plants have been going on for more than two decades (Kinnersley and Turano, 2000) and the evidence has been mounting ever since (Kinnersley and Lin, 2000; Bouché *et al.*, 2003; Palanivelu *et al.*, 2003).

In general, signalling molecules bind to their receptors and are able to modify metabolism and expression of genes (Kinnersley and Turano, 2000). In plants, GABA was shown to regulate the expression of genes such as *arginine-decarboxylase* (Turano *et al.*, 1997), *14-3-3* (Lancien and Roberts, 2006) and also the genes encoding secreted and cell wall-related proteins (Renault *et al.*, 2011). GABA-mediated interactions between plants and bacteria were also reported (Shelp *et al*., 2006). In growing pollen tubes, exogenous GABA was shown to modulate Ca^{2+} levels through activating Ca^{2+}-permeable channels, suggesting a signalling function of GABA rather than a metabolic one (Yu *et al.*, 2014). Some GABA receptor agonists and antagonists in the CNS elicit a response in plants (Kinnersley and Lin, 2000). Additionally, GABA binding sites have been detected on pollen and somatic protoplasts using quantum dot probes, hinting at the existence of GABA receptors (Yu *et al.*, 2006).

Recently, a GABA-binding motif has been discovered in aluminium-activated malate transporters (ALMTs) (Ramesh *et al.*, 2015). The aluminium trivalent cation (Al^{3+}) in soil is toxic to roots and is chelated by carboxylates such as citrate, oxalate or malate released from the roots, forming harmless complexes and thus accounting for aluminium tolerance in plants (Ryan *et al.*, 1995; Ma *et al.*, 2001; Ryan *et al.*, 2011). TaALMT1 in wheat roots mediates malate efflux (Ryan *et al.*, 2011). GABA in apoplast causes inhibition of this efflux, yet there is no known transporter capable of exporting GABA from cytoplasm to apoplast (Shelp and Zarei, 2017), although high concentration of external GABA, especially in roots, prove that such transporter must exist (Chung *et al.*, 1992; Crawford *et al.*, 1994; Warren, 2015). Ramesh *et al*. (2018) suggest that TaALMT1 could facilitate this transport. The discovery of such GABA regulation of ALMT activity and subsequent modulation of plant growth make ALMT a main candidate for a plant GABA receptor, implying that GABA might not be just a metabolite (Ramesh *et al.*, 2015).

3.5 GABA and stress

The rapid accumulation of GABA in response to a range of biotic and abiotic stresses has been observed since the 1950s. Increased GABA levels were reported upon hypoxia, drought, salinity, cold, heat, UV radiation, as well as fungal and bacterial infection and even mechanical stimulation (Ramputh and Bown, 1996; Kinnersley and Turano, 2000; Fait *et al.*, 2005; Chevrot *et al*., 2006; Renault *et al.*, 2010; Mustroph *et al*., 2014; Faës *et al.*, 2015). The application of exogenous GABA on many different plant species during various stresses alleviated the stress damage (Song *et al*., 2010; Krishnan *et al*., 2013; Salvatierra *et al*., 2016; Mahmud *et al*., 2017; Yong *et al*., 2017; Li *et al*., 2018). GABA has been proposed to act as osmolyte and could thereby help alleviate osmotic stress by maintaining turgor pressure and protecting cell membranes from cell dehydration (Shelp *et al.*, 1999; Krishnan *et al*., 2013). Understanding how GABA affects plant response to stress could help engineer stress resistant or stress tolerant crops.

3.5.1 Biotic stress

The majority of articles about GABA and stress in plants is focused on abiotic stress; however, the number of studies concerning intertwinement of GABA metabolism and plant immune response to various pests and pathogens is steadily increasing (Bown *et al*., 2006; Scholz *et al.*, 2015; Tarkowski *et al*., 2020). It has long been known that GABA concentration is raised in response to mechanical stimulation and damage of soybean leaves and also of insect larvae crawling (Ramputh and Bown, 1996; Bown *et al*., 2002). Similarly, in *A. thaliana* the wounding by both *Spodoptera littoralis* and mechanical caterpillar resulted in an increase of GABA level in the wounded leaf and, surprisingly, also in some of the adjacent leaves. Altered growth of larvae was also observed (Scholz *et al.*, 2015). In addition, the survival and growth of *Choristoneura rosaceana* cv Harris larvae was reduced; the development of the larvae was delayed when they were feeding on a diet with high concentration of GABA (Ramputh and Bown, 1996). It is suggested that GABA might deter feeding of herbivores and contributes to the general and rapid first line of plant defence (Bown *et al.*, 2006; Scholz *et al*., 2015). However, GABA accumulation is neither dependent on the increase of cytosolic Ca^{2+} concentration caused by wounding by insects nor on the transport of GABA from wounded to adjacent systemic leaves, so there is still a long way to uncover and understand the GABA-induced mechanisms of resistance against herbivory (Scholz *et al.*, 2017).

3.5.2 Abiotic stress

3.5.2.1 Salt

Salinity stress is a major problem affecting the germination, growth and yield of crop plants worldwide (Parihar *et al.*, 2015). The Food and Agriculture Organization (FAO) estimated that of the 230 million ha of irrigated land, nearly 20 % is affected by salinity, and according to another estimation, 10 million ha of agricultural land is destroyed every year by salinization (Pimentel *et al.*, 2004). Additionally, the world population is expected to reach 9.3 billion by 2050, and to satisfy growing demand for food, the production will need to increase by 59–98 % (Valin *et al.*, 2014). Breeding crops for salinity tolerance is one of possible approaches to overcome this challenge.

The breeding of salt-tolerant cultivars using conventional breeding has been slow due of the fact that salt stress tolerance is a complex trait controlled by multiple genes (Flowers, 2004). Advances in plant phenotyping enabled simultaneous studies of traits such as plant growth and photosynthetic traits. The method can be used to determine genes associated with salt tolerance (Awlia *et al*., 2016; Ismail and Horie, 2017). *A. thaliana*, as well as many important crops including rice and durum wheat (*Triticum turgidum* ssp. *durum*), is sensitive to NaCl, therefore it serves as a convenient model for studying salinity tolerance (Munns and Tester, 2008).

Renault *et al.* (2010) documented GABA accumulation in *A. thaliana* in response to salt stress. Similar results were also reported for other plant species such as tomato (*Lycopersicon esculentum* Mill) and alfalfa (*Medicago sativa* L.) (Fougère *et al*., 1991; Bolarín *et al*., 1995). Almost all genes of GABA metabolism were upregulated. Out of the five genes whose transcripts were detected, *GABA-T* was the most responsive to NaCl, followed by *GAD4* and *GAD2*. *Pop2-1* mutants accumulating GABA were sensitive to ionic stress, suggesting that GABA itself doesn't have a protective role against salt stress. However, recent study disagreed with this suggestion, as it showed *pop2-5* mutants to be salt-tolerant and *gad1/2* double mutants, which are unable to synthetize GABA, to be salt-sensitive. This finding would then mean that high GABA concentration, in fact, enhances salt stress tolerance (Su *et al.*, 2019). However, further studies must be done to clarify these controversial results.

In *pop2-1* mutants, the growth of primary roots was inhibited; moreover, the development of roots and hypocotyl was also adversely affected during salt stress (Renault *et al*., 2010; Renault *et al*., 2013). Additionally, the roots were depleted in sugars and accumulated amino acids, hinting at a role of *GABA-T* in the central carbon/nitrogen metabolism (Renault *et al*., 2010).

The overview of gene expression in various plants subjected to salinity stress is in Table 1.

Table 1. Expression of genes involved in GABA metabolism during salt stress

Species	Tissue	Gene expression	Reference
Arabidopsis	roots and shoots	*GAD3/4*↑	Shelp *et al.*, 2012b
Arabidopsis	shoots	*GAD4*↑	Zarei *et al.*, 2017b
Arabidopsis	plantlets	*GAD1*↓; *GAD2*↑; *GAD3* nd; *GAD4*↑; *GAD5* nd; *POP2*↑; *SSADH*↑	Renault *et al.*, 2010
Tomato	fruit	*SlGAD2*↓; *SlGAD3*↓	Yin *et al.*, 2010
Maize	leaves, roots	*ZmGAD1*↑	Zhuang *et al.*, 2010

nd, not detected, ↑, upregulation; ↓, downregulation

3.5.2.2 Drought

Drought is a major stress factor limiting the development and growth of plants. Plants are sessile organisms; thus, they have evolved certain mechanisms to survive adverse conditions. When subjected to drought stress, plants react by closing the stomata, thereby reducing water loss through transpiration (Harb *et al.*, 2010). GABA-depleted *gad1/2* double mutant showed oversensitivity to drought as a result of defect in stomata closure (Mekonnen *et al.*, 2016). Exogenous application of GABA in white clover (*Trifolium repens*) resulted in increased endogenous GABA concentration, which in turn led to decreased GABA synthesis by suppressing GAD activity and increased Glu concentration as a result of higher GABA-T activity (Yong *et al.*, 2017). Most importantly, exogenous GABA has been shown to mitigate drought damage in many species including white clover, perennial ryegrass (*Lolium perenne*) and creeping bentgrass (*Agrostis stolonifera*) and could thereby improve drought tolerance (Krishnan *et al.*, 2013; Yong *et al*., 2017; Li *et al*., 2018).

3.5.2.3 Hypoxia

Hypoxia, i.e., oxygen deficiency, is a condition when mitochondrial respiration is impaired (Drew, 1997). It is a result of submergence, poor soil drainage or soil compaction (Fukao and Bailey-Serres, 2004). Excess water (waterlogging or soil flooding) has adverse effect on crop yield (Drew, 1992; Sasidharan *et al.*, 2017).

Under hypoxic conditions, *Arabidopsis* roots accumulate alanine and GABA (Mustroph *et al.*, 2014). *Gaba-t1* and *gad1* mutants were shown to accumulate lower amount of alanine in roots when compared to wild type plants, implying that GABA metabolism partially contributes to hypoxia-induced alanine accumulation in roots. Only the expression of *GAD4* increased in response to oxygen deficiency (Miyashita and Good, 2008).

In the roots of hypoxia-sensitive genotype of *Prunus*, *GAD2* and *GAD4* transcript levels were increased; furthermore, there was also increased transcription of *GAD4* in the hypoxia-tolerant genotype. In both genotypes, the effect was even more apparent after the application of

exogenous GABA. In hypoxia-sensitive genotype, there was higher chlorophyll content and improved leaf gas exchange compared to untreated plants (Salvatierra *et al*., 2016).

3.5.2.4 Heavy metals

Some heavy metals (for example Zn, Cu, Co) are referred to as micronutrients and are essential for plant processes. However, same as As, Pb, Hg and Cd, they can be toxic when their concentration reaches values greater than optimal (Tiwari and Lata, 2018). Accumulation of heavy metals in soil has a negative effect on crop productivity. Plants have evolved many mechanisms to alleviate damage caused by heavy metal stress (Shahid *et al.*, 2015)

Several studies focused on the effect of GABA on the accumulation and toxicity of heavy metals in plants. In cadmium-treated tomato plants, the GABA level in roots, xylem and phloem sap was decreased (Chaffei *et al.*, 2004). Long term GABA treatment and its subsequent accumulation in rice seedlings caused a decrease in expression of Lsi1 and Lsi2 transporters which transport As^{3+} into the plant. The plants accumulated less arsenic, revealing that GABA provides tolerance against arsenic stress (Kumar *et al.*, 2017). When exogenous GABA was applied to mustard (*Brassica juncea* L.) seedlings under chromium stress, the plants showed increased relative water content (RWC), chlorophyll content and improved growth overall (Mahmud *et al.*, 2017). Similar results were observed in barley (*Hordeum vulgare* L.) under aluminium stress, where exogenous GABA improved root growth and induced activity of antioxidant enzymes, thereby alleviated damage caused by Al^{3+} and H^{+} toxicity (Song *et al.*, 2010).

3.6 *Arabidopsis* as a model organism

Arabidopsis thaliana (L.) Heynh. is an annual flowering plant native to Europe, Asia and northern Africa and has been also naturalized worldwide (for example in North America and Australia). It is a member of the mustard family (*Brassicaceae*) which includes plants like rapeseed, cauliflower or cabbage, but unlike these, *Arabidopsis* doesn't have any use in agriculture and is considered a weed. Its characteristic traits such as small genome, short generation time, large seed production and low demands on space rendered it a popular model organism for scientific research, particularly for molecular, genetic, physiological and biochemical studies.

3.6.1 Morfology

Arabidopsis is a small plant reaching just up to 25 cm. There have been over 750 natural accessions or ecotypes collected from all around the world that can vary in their morphology, development and physiology. Three ecotypes of *A. thaliana* are most widely used – Columbia (Col), Wassilewskija (Ws) and Landsberg (Ler). They show differences in seed size, number of leaves in a rosette, number of trichomes, morphology of flowers and length of siliques, among others (Passardi *et al*., 2007).

The entire life cycle of *A. thaliana* is completed in six weeks (Fig. 3). It reproduces by self-pollination (selfing), however, for genetic experiments, pollen can be easily applied to stigma to produce crosses. Its leaves form a rosette ranging from 2–10 cm in diameter and are covered with trichomes. The flowers are 2 mm in length. The plants produce several thousand 0.5 mm seeds in hundreds of siliques. Their roots have a simple structure and are often studied in *in vitro* cultures (Meinke *et al.*, 1998).

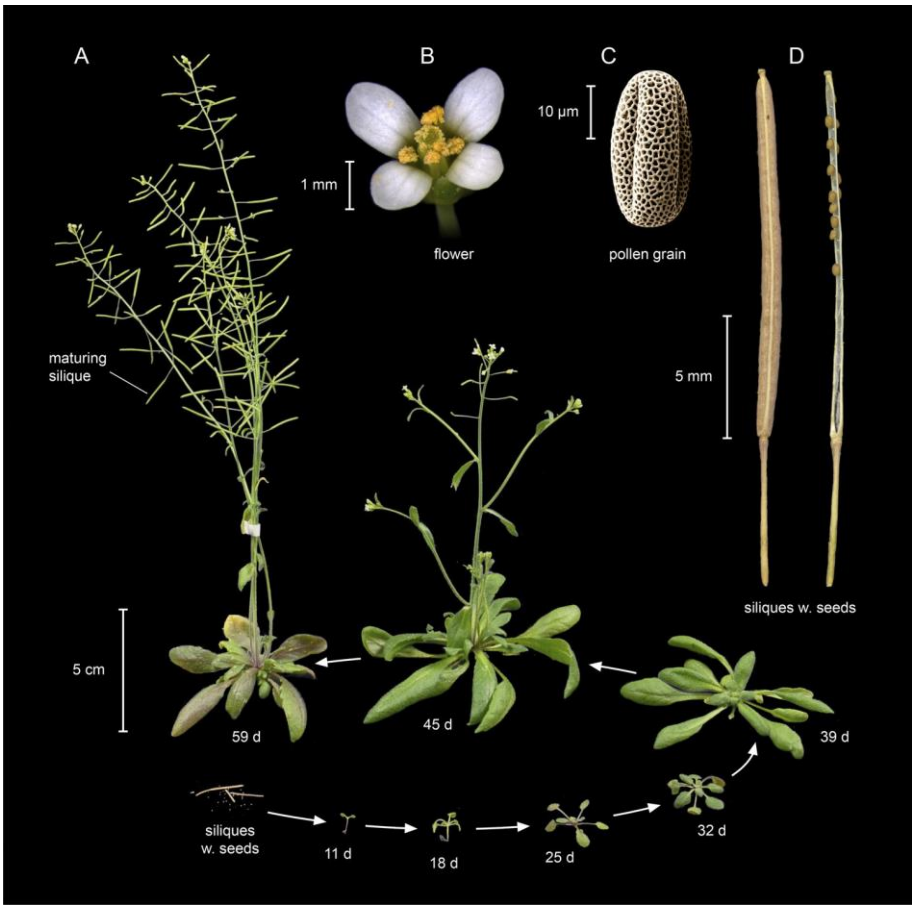

Figure 3. Life cycle of *A. thaliana*. **(A)** *A. thaliana* of ecotype Columbia (Col) during different stages of development. **(B)** A flower, **(C)** pollen grain, **(D)** mature siliques (left: closed seed pods, right: open seed pods with several unshattered seeds). Images: B and C – Maria Bernal and Peter Huijser; other photographs – Ines Kubigsteltig and Klaus Hagemann

3.6.2 Genome

A. thaliana is a diploid plant and its genome is organized into five chromosomes. For each of the chromosome, there are genetic and physical maps available. The sequencing of its genome was finished in 2000 and it revealed that *A. thaliana* has a relatively small genome with approximately 135 Mb; around 27 000 protein-coding genes have been annotated (Swarbreck *et al.*, 2008). Across chromosomes there are 24 large duplications making up 58 % of genome; that is explained by the fact that *Arabidopsis* has undergone whole-genome duplication followed by diploidization (The Arabidopsis Genome Initiative, 2000; Bowers *et al*., 2003).

3.6.3 Transformation

Plant transformation is a way to insert foreign genetic material into the plant genome. One of the methods used to prepare transgenic plants utilizes soil bacterium *Agrobacterium tumefaciens* which is a pathogen causing crown gall disease. It is able to transfer its transfer DNA (T-DNA) from Ti (tumour-inducing) plasmid into the nuclear DNA of a host plant (Chilton *et al.*, 1977). This method can be used to introduce new genes to the plant genome or to disrupt existing ones, making it an excellent tool for genetic engineering (O'Malley *et al*., 2015).

T-DNA region is bordered by two imperfect 25 bp direct repeats, commonly referred to as right and left borders (RB, LB) (Gelvin, 2003). The T-DNA region normally contains genes for auxin and cytokinin synthesis, which cause uncontrollable growth and formation of characteristic tumours, after they are expressed in the host cells (Akiyoshi *et al.*, 1984; Schröder *et al.*, 1984). However, these genes can be removed and replaced with genes of interest along with a selectable marker (Wang *et al.*, 1984). Plant selection markers are necessary for the selection of successfully transformed plants; genes conferring resistance to antibiotic (kanamycin, hygromycin, gentamycin etc.) or herbicide (Basta) are used (Lee and Gelvin, 2008).

Unfortunately, the insertion of T-DNA into the plant genome is nearly random, so hundreds of thousands of independent T-DNA insertion events are needed for saturation of the genome (Krysan *et al*, 1999). In addition, multiple insertions in different parts of the chromosome in a single plant are common, the average being 1.5 inserts per line (Alonso *et al.*, 2003).

When T-DNA insertion disrupts a gene, the result is called gene knockout (KO); this technique can be used to study a loss-of-function phenotype (Krysan *et al*, 1999). For *A. thaliana*, there are many collections (SALK, SAIL, GABI-KAT, FLAG and others) containing more than 300 thousand T-DNA insertion mutants with inserts within most of the genes (O'Malley and Ecker, 2010). The lines can be ordered from *Arabidopsis* Biological Resource Center (ABRC) and The Nottingham *Arabidopsis* Stock Centre (NASC).

3.7 Plant phenotyping

With the continued rapid population growth, it is necessary to achieve food security in the decades to come. For that, the yield and quality of agronomically important crops need to increase; however, the production is affected by many unfavourable conditions such as changes in weather patterns or salinity and drought stress, which make it even more difficult to meet the food demand (Tester and Langridge, 2010). Plant phenotyping represents a method to identify traits responsible for enhanced crop performance under stress and as such can aid in the breeding of stress-tolerant crops (Ghanem *et al*., 2015).

The term 'phenotyping' is interpreted in many different ways. Fiorani and Schurr (2013) define phenotyping as 'the set of methodologies and protocols used to measure plant growth, architecture, and composition with a certain accuracy and precision at different scales of organization, from organs to canopies'. Plant phenotyping approaches can evaluate a large number of traits often related to plant growth. One of parameters describing the growth of plants is biomass formation. Above-ground biomass is defined as a mass of plant shoots at a certain point in their lifespan (Roberts *et al.*, 1993). Traditionally, it can be determined simply by weighing fresh (FW) and dry (DW) plants. However, this sampling involves harvesting the plants and destroying them in the process, presenting a disadvantage. In general, conventional phenotyping techniques are often destructive and manual measurement of parameters is time-consuming and tedious, slowing the progress in plant breeding (Berger *et al*., 2012).

Recently, technological advancement enabled the development of high-throughput phenotyping (HTP) platforms. HTP offers a large-scale, non-invasive and non-destructive analysis of morphometric parameters describing plant morphological, physiological and pathological traits (Zhang and Zhang, 2018). HTP platforms can use a simple red-green-blue (RGB) digital cameras to acquire images, but they can be also equipped with other imaging sensors; for example chlorophyll fluorescence (ClF) imaging that is able to measure photosynthetic activity, or thermal imaging used for monitoring transpiration (Humplík *et al.*, 2015b). There are many advantages to HTP – the platforms enable monitoring of individual plants during their lifecycle that can be automated to various extents, and a range of sensors makes it possible to study traits beyond the visible light spectrum, which would be impossible to do using conventional methods (Berger *et al*., 2012). HTP are becoming more popular, as the increase of phenotyping capacity allows to screen a large number of plants, even more than a thousand (Flood *et al.*, 2016).

On the whole, plant phenotyping is a very useful tool for studying stress response in plants. HTP has been used to study the effects of cold and drought stress, among others (Bresson *et al*., 2014; Humplík *et al.*, 2015a). However, there is a need for optimized experimental protocols to better compare achieved results. Awlia *et al.* (2016) developed a protocol to investigate early and

late plant response to salt stress using RGB and ClF imaging in PlantScreen Compact System (PSI, Czech Republic) to evaluate the growth, photosynthetic activity and greenness of *A. thaliana* plants. Additionally, an assay to evaluate *in vitro A. thaliana* rosette growth under salt stress conditions in multi-well plates using OloPhen platform has been also described (De Diego *et al*., 2017).

A recent study has demonstrated that the effect of compounds, which were identified as growth regulators in *A. thaliana*, was translatable to important crops such as lettuce, tomato or maize (Rodriguez-Furlán *et al.*, 2016). This finding, along with the small size of the plant, makes the genetic model ideal for studying plant phenotype under different growth conditions to clarify the mechanisms related to stress tolerance.

4 MATERIALS AND METHODS

4.1 Plant material and growth conditions

The wild type (WT) and mutant seeds of *Arabidopsis thaliana* L. (accession Col-0) bearing T-DNA insertion in genes of GABA metabolism (*GAD1*, *GAD2*, *GAD3*, *GAD4*, *GAD5*, *GABA-T*, *SSADH*, *GLYR1* and *GLYR2*) were obtained from The Nottingham *Arabidopsis* Stock Centre (NASC). The SALK, SAIL and GABI-Kat lines are listed in Table 3 (Sessions *et al*., 2002; Alonso *et al.*, 2003; Kleinboelting *et al*., 2012).

Prior to the genotyping, homozygous transgenic lines were selected. First, the seeds were surface-sterilized with 200 µl of sterilization solution and vortexed for 10 minutes. After that, the solution was removed, and the seeds were washed three times with 200 µl of the sterilization solution. The seeds were sown on round Petri dishes (90 x 15 mm) containing 0.5x Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with agarose and an antibiotic corresponding to the T-DNA collection (Table 2).

Table 2. Antibiotics used for the selection of T-DNA insertion mutant collections

Antibiotic	Working concentration [µg/ml]	Collection
Kanamycin	25	SALK
Sulfadiazine	5	GABI-Kat
Basta	12	SAIL

The Petri dishes were maintained at 4 °C in the dark for four days to synchronize germination. After that, the Petri dishes were transferred to a growth chamber and placed in a horizontal position. The seedlings were grown under controlled conditions (20 °C, 16/8 h light/dark cycle, 60% relative humidity and a photon irradiance of 120 µmol photons of PAR m^{-2} s^{-1}) for approximately 14 days. When the first true leaves formed, the seedlings were transferred into separate square pots filled with substrate (Potgrond H, Klasmann-Deilmann) and grown under identical conditions.

For the non-invasive phenotyping, 6 mg of seeds from WT and obtained homozygous lines (see Table 3) were placed into individual 1.5 ml microtubes. The seeds were surface sterilized as described above. Then they were sown on a filter paper to avoid the growth of roots into the media, on square Petri dishes (12 x 12 cm) containing 0.5x Murashige-Skoog (MS) medium supplemented with 0.6% Phytagel. The Petri dishes were maintained at 4 °C for four days for cold stratification. Thereafter, they were placed vertically in a growth chamber and grown under controlled conditions (22 °C, 16/8 h light/dark cycle, a photon irradiance of 120 µmol photons of PAR m^{-2} s^{-1}).

Table 3. Genotyped mutant lines with T-DNA insertion in genes of GABA metabolism

Gene	Locus	NASC ID	Line	Insertion location	End result after genotyping
GAD1	AT5G17330	N860068	SALK_047648	2nd intron	HM
		N666827	SALK_096492C	7th intron	HM
GAD2	AT1G65960	N662815	SALK_067677C	1st intron	HM
		N445461	GK-474E05	4th exon	HZ
GAD3	AT2G02000	N533307	SALK_033307	3rd intron	HM
		N2106898	SALK_033307C	3rd intron	HZ
		N506072	SALK_006072	5th intron	HZ
		N664931	SALK_138534C	6th exon	HZ
GAD4	AT2G02010	N664262	SALK_146398C	2nd exon	HM
		N668479	SALK_072198C	promoter	HM
GAD5	AT3G17760	N692387	SALK_203883C	4th exon	HM
GABA-T	AT3G22200	N415022	GK-157D10	8th intron	HZ
		N878670	SAIL_1230_C03	4th intron	HM
SSADH	AT1G79440	N503223	SALK_003223	8th intron	HZ
		N657867	SALK_003223C	8th intron	HZ
		N879160	SAIL_1278_B12	1st intron	HZ
GLYR1	AT3G25530	N557410	SALK_057410	2nd intron	HZ
		N691939	SALK_203580C	3rd exon	HZ
		N840352	SAIL_894_G08	6th exon	HZ
GLYR2	AT1G17650	N665949	SALK_047412C	300-UTR5’	HM
		N2103841	GK-933D03	7th exon	HM

HM, homozygous; HZ, heterozygous; UTR, untranslated region

4.2 Chemicals and solutions

Chemicals

- Acetic acid (Lach-Ner, cat. no. 10047-A80)
- Agarose (Sigma-Aldrich. cat. no. A9539-500G)
- Chloroform:Isoamyl alcohol 24:1 (CIA) (Sigma-Aldrich, cat. no. C0549)
- Deoxynucleotide (dNTP) set (Invitrogen, cat. no. 10297018)
- Ethanol, 96% (Lach-Ner, cat. no. 20025-A96)
- Ethidium bromide (neoLab, cat. no. 1254ML015)
- Ethylenediaminetetraacetic acid (EDTA) (Penta, cat. no. 18010-30500)
- GeneRuler 100 bp Plus DNA Ladder (ThermoFisher Scientific, cat. no. SM0321)
- GeneRuler 1 kb Plus DNA Ladder (ThermoFisher Scientific, cat. no. SM1331)
- Glufosinate-ammonium (Basta) (Sigma, cat. no 45520-100MG)
- GoTaq® G2 Flexi DNA Polymerase (Promega, cat. no. M7801)
- 5X Green GoTaq® Reaction Buffer (Promega, cat. no. M7911)
- Kanamycine sulphate monohydrate (Duchefa, cat. no. K0126.0005)
- MES (4-Morpholineethanesulfonic acid) hydrate (Sigma-Aldrich, cat. no. M8250)
- $MgCl_2$ (Promega, cat. no. A3511)
- Murashige & Skoog medium including vitamins (MS) (Duchefa, cat. no. M0222.0050)
- NaCl (Lach-Ner, cat. no 30093-AP0-G1000-1)
- Phytagel (Sigma-Aldrich, cat. no. P8169)
- Sodium dodecyl sulfate (SDS) (Lach-Ner, cat. no. 40089-AP0)
- Tris base (Duchefa, cat. no. T1501.1000)
- Triton® X 100 (Sigma, cat. no. X100-1L)
- Sulfadiazine (Sigma, cat. no. S8626-25G)

Solutions

- Agarose gel: 1% (w/v) agarose in 1x TAE buffer
- Basta: dissolve 12 mg of glufosinate-ammonium in 1 ml of distilled water, filter-sterilize
- 70% ethanol
- gDNA extraction buffer: mix 100 ml of 1M Tris-HCl, 20 ml of 0.5M EDTA, 31.25 ml of 4M NaCl and 25 ml of 10% SDS, adjust the volume of the solution to 500 ml, sterilize by autoclaving
- Kanamycin: dissolve 25 mg of kanamycin in 1 ml of distilled water, filter-sterilize

- 0.5x MS medium: dissolve 2.2 g of MS medium including vitamins, 0.5 g of MES, 0.6% of gelling agent Phytagel, adjust pH to 5.7 using KOH, adjust the volume of the solution to 500 ml, sterilize by autoclaving
- 150 mM NaCl: dissolve 96.426 g of NaCl in 11 l of distilled water
- Sterilization solution: 70% ethanol, 0,01% Triton X 100
- Sulfadiazine: dissolve 5 mg of sulfadiazine in 1 ml of distilled water, filter-sterilize
- 50x TAE (Tris-acetate-EDTA): dissolve 242 g of Tris base in 700 ml of distilled water, add 57.1 ml of acetic acid and 100 ml of 0.5M EDTA, adjust pH to 8.5, adjust the volume of the solution to 1 l
- TE (Tris-EDTA) buffer (pH 8): add 1 ml of 1M Tris-HCl and 0.2 ml of 0.5M EDTA, adjust the volume of the solution to 100 ml, sterilize by autoclaving
- 1M Tris-HCl (pH 8): dissolve 60,55 g of Tris in 400 ml of distilled water, adjust pH with HCl, adjust the volume of the solution to 500 ml, sterilize by autoclaving

4.3 List of equipment and software

Equipment

- Analytical balances XA 110/2X (Radwag)
- Autoclave STERIVAP HP IL (BMT Medical technology)
- Balances Scout SKX (OHAUS)
- Centrifuge 5417R (Eppendorf)
- Documentation System Gel Doc EZ Imager (Bio-Rad)
- Dry Heat Sterilizer/Oven FN500 (Nüve)
- Electrophoresis System ENDURO Gel XL (Labnet)
- Fitotron EGR Economy Growth Rooms (Weisstechnik)
- Magnetic stirrer MM4 (Lavat)
- Mini-Centrifuge/Vortex FVL-2400N (Biosan)
- Mixer mill MM 400 (Retsch)
- NanoDrop One (Labtech)
- pH meter Orion Star A111 (Thermo Scientific)
- PlantScreen Modular System (PSI)
- Thermal Cycler Veriti 96-Well (Applied Biosystems™); PrimeG (Techne)
- Vertical Laminar Airflow Cabinet MERCI® M - 1200 (MERCI)

Software

- ImageLab™ Software
- SnapGene Viewer

4.4 Methods

4.4.1 Genotyping

For every line, a pair of gene-specific primers (GSP) needed to be designed to amplify the putative T-DNA insertion site. The first PCR reaction, which was performed only with GSP primers, detected a WT copy of a gene (without a T-DNA insertion). A band was amplified for WT and heterozygous plants, whereas for homozygous plants the amplification could not proceed because the fragment with the T-DNA insert was too big to amplify. The second PCR reaction used a combination of a left border primer and a GSP in a correct orientation, i.e. on the opposite strand from the left border primer (LB). In this selective reaction, the sequence flanking the T-DNA insertion site (FST) was amplified in heterozygous and homozygous plants; PCR for WT plants produced no band.

The principle of genotyping is depicted in Fig. 4.

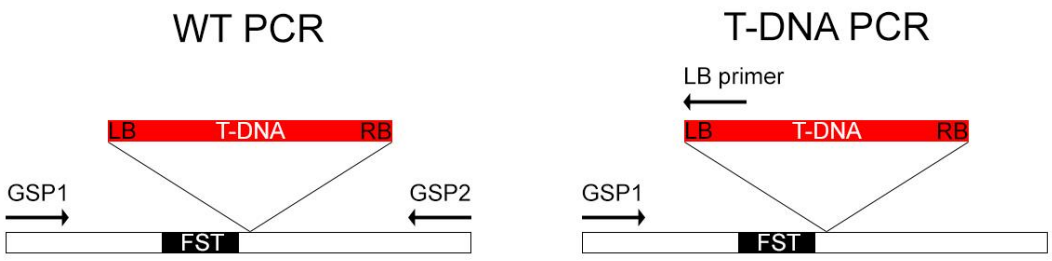

Figure 4. PCR reactions for genotyping of T-DNA insertion lines. "WT PCR" amplifies the part of genome present in WT and heterozygous lines, but not in homozygous lines. "T-DNA PCR" detects the presence of a T-DNA insert. FST, flanking sequence tag; GSP, gene-specific primer; LB, left border of the T-DNA; RB, right border of the T-DNA.

The genotype of plants was determined by the presence or absence of a band for the GSP product. In Fig. 5 there is an illustrative example. In the first well of the gel there is only a product of 900 bp, which means that the plant is WT. The presence of a single 500 bp band is characteristic for homozygous plants. If there are both products in the well, it signifies that the plant is heterozygous. Ideally, the products of WT PCR and T-DNA PCR should have a different size; in that case all three primers (both GSP and LB) can be put into one reaction.

Figure 5. PCR products of WT PCR and T-DNA PCR. WT, wild type plant; HZ, heterozygote; HM, homozygote.

4.4.1.1 Genomic DNA isolation

Approximately 0.5 cm long leaf was taken from a seedling and placed into 1.5 ml tube (SafeLock, Eppendorf) with a tungsten carbide bead (3 mm, QIAGEN, cat. no. 69997). 300 µl of gDNA isolation buffer and 80 µl of CIA (Chloroform:Isoamyl alcohol 24:1) were added. The plant tissue was then homogenized by mixer mill (1 minute, frequency of 25 Hz). The homogenate was centrifuged for five minutes at maximum angular speed of 14 000 rpm. Upper phase containing genomic DNA was transferred to a new microtube. 700 µl of 96% ethanol was added and the microtube was immediately mixed by inversion. The solution was incubated for five minutes at room temperature and then centrifuged at 14 000 rpm for 15 minutes. The supernatant was decanted, and the remaining solution was completely removed. DNA was rinsed by 70 µl of 70% ethanol and the microtube was centrifuged for three minutes at 14 000 rpm. All liquid was removed. As a final step, 50 µl of TE was added to dissolve DNA.

4.4.1.2 Polymerase chain reaction (PCR)

For every insertion line, a pair of gene-specific primers was designed. They were either designed in SALK T-DNA Primer Design Tool with default settings or in SnapGene Viewer following general guidelines for primer design. The primers were obtained from Sigma-Aldrich, dissolved in distilled water in an amount specified by the manufacturer, and diluted to 10 µmol·dm^{-3}.

PCR reaction mix was prepared according to Table 4.

Table 4. PCR reaction mix

Chemical	Final concentration
Green buffer	1x
$MgCl_2$	2.5 mmol·dm^{-3}
dNTPs	0.15 mmol·dm^{-3}
GoTaq polymerase	0.025 U
Left primer	0.8 μmol·dm^{-3}
Right primer	0.8 μmol·dm^{-3}
T-DNA primer	0.8 μmol·dm^{-3}
gDNA	30 ng
PCR water	Added to 10 μl
Final volume	10 μl

Because the size of products differed for WT and T-DNA PCR, only one PCR reaction with gene-specific primer pair and T-DNA primer was performed. T-DNA primers for mutant collections are listed in Table 5, GSP amplifying WT allele are listed in Table 6. PCR conditions are listed in Table 7. Two of the conditions were modified for individual lines – annealing temperature and extension time (Table 8). Annealing temperature of the PCR reaction depended on the melting temperature of primer pairs, which should, in general, be within 5 °C of each other. Extension time for *Taq* polymerase is generally 1 minute per 1000 base pairs, so the time was adjusted when shorter or longer product was expected.

Table 5. T-DNA specific primers for T-DNA collections

Collection	primer ID	Sequence (5'–3')
SALK	LBa1	GGTTCACGTAGTGGGCCATCGCCCTG
SAIL	LB2m	TATTATATCTTCCCAAATTACCAATACA
GABI-Kat	o8760	GGGCTACACTGAATTGGTAGCTC

Table 6. Primers for all acquired lines and the expected size of products

Gene	NASC ID	Line	Sequence of left primer	Sequence of right primer	WT Product size [bp]	Mutant product size [bp]
GAD1	N860068	SALK_047648	ACAAAAACGGTGCAATGAATC	TTATTTCCGCAAATACCATCC	1171	740
	N666827	SALK_096492C	TTATCTTGGTGCTGACCAACC	CTCTGCTTGTCGATATCGCTC	1149	684
GAD2	N662815	SALK_067677C	GGTCGGTGTTTTTGACAAAAC	CCTCTCAAATCTAATAATTGAAATGC	1136	446
	N445461	GK-474E05	TCGTTTGTTACGTTTATGACTCTGG	CACACACCATTCATCTTCTTCC	527	680
GAD3	N533307	SALK_033307	CCTGACAAAGCGGTTGAAATGG	GGAAGATAAGTTCATCAGGCAAATCG	664	446
	N506072	SALK_006072	GCTCAGTACTACCAGTTGATTCG	CGTGTAGAACCTTCTCGAAATCGGCTACC	554	365
	N664931	SALK_138534C	TGCTTTTGTAGAACCACGAGAC	AGGATTAGAGAAAACGGGACG	1160	1089
	N2106898	SALK_033307C	TCGAATCCAAGACGAATCAAC	AGAACAAGCGTAAGGCCCTAG	1075	676
GAD4	N664262	SALK_146398C	CAATAGTAACATGTAATGATGTAAACCTTGG	CGTCTTCGAATTCACCGGTTAACG	817	648
	N668479	SALK_072198C	TTTCTGAAAATCATTTGGGGG	CGAGAAGCAAAAGTTGAATGG	1172	1043
GAD5	N692387	SALK_203883C	TCCTTCTCTTAGCCTCCTTGC	CTGCTATTGGGTGTGGAACTG	993	635

Gene	NASC ID	Line	Sequence of left primer	Sequence of right primer	WT Product size [bp]	Mutant product size [bp]
GABA-T	N878670	SAIL_1230_C03	ATCTGTGTGCAACACAAATGG	TAAAGGGCATGATATGCTTGC	1110	626
	N415022	GK-157D10	GGTTCTTTTAGAGATGTTCACGGC	GAAACATTTACAACTTGTCAGGGG	1285	781
SSADH	N657867	SALK_003223C	GCTTCTGGATGCACGGTGG	GCAAAGCATCCCCAATTTCAGG	529	480
	N879160	SAIL_1278_B12	CGATTGAAGTTTGGGAAGTGG	GAACTCCTAAGCTTTTCAGAAACGC	430	408
	N503223	SALK_003223	GCCCCTAACAGCACTTGC	GGACTCGTAAGCAAAGCATCCCC	488	660
GLYR1	N691939	SALK_203580C	GCTTGCAAAAGTTTGATCACC	CTGTATGGAACAGAACACTCTCC	1081	658
	N840352	SAIL_894_G08	CCTCTGGACCAATATCGAGTG	TTCGTAGAAGGTCCGGTTTC	1131	718
	N557410	SALK_057410	GCTTGCAAAAGTTTGATCACC	CTGTATGGAACAGAACACTCTCC	1081	554
GLYR2	N665949	SALK_047412C	CTTGAAATAAGCGCTTCCATG	TCGAAACTTGTGGGTTTTGTC	1095	663
	N2103841	GK-933D03	AAAAATTTCCCGGGGTTTATAGC	ATGTCTCACTTTTGTTTGCGTCTC	1160	913

Table 7. PCR conditions

Process	Temperature [°C]	Time	Number of cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 s	
Annealing	57–62	30 s	35
Extension	74	50–80 s	
Final extension	72	5 min	1

Table 8. Annealing temperature and extension time

Gene	Line	Annealing temperature [°C]	Extension time [s]
GAD1	SALK_047648	57	70
	SALK_096492C	57	70
GAD2	SALK_067677C	61	70
	GK-474E05	57	50
GAD3	SALK_033307	59	50
	SALK_006072	62	50
	SALK_138534C	57	70
	SALK_033307C	57	70
GAD4	SALK_146398C	57	60
	SALK_072198C	57	70
GAD5	SALK_203883C	57	60
GABA-T	SAIL_1230_C03	57	70
	GK-157D10	57	80
SSADH	SALK_003223C	57	50
	SAIL_1278_B12	57	50
	SALK_003223	59	50
GLYR1	SALK_203580C	57	70
	SAIL_894_G08	57	80
	SALK_057410	57	70
GLYR2	SALK_047412C	57	70
	GK-933D03	57	80

The amplification products along with a molecular weight marker were loaded into wells of a 1% agarose gel in 1x TAE buffer with 12 µl of 0.5% ethidium bromide added and were separated at 100 V for 30 minutes. Image of the gel was acquired using a documentation system Gel Doc EZ Imager (Bio-Rad) and its ImageLab™ Software.

4.4.2 Non-invasive phenotyping

Non-invasive plant phenotyping was conducted according to the protocol created by Awlia *et al*. (2016) with minor modifications. Seven days after stratification (DAS), similar-sized homozygous and control (WT) seedlings were transferred from square plates into pots containing approximately 140 g of substrate (Florcom) that were watered one day prior to the transplanting. Two seedlings were put into every pot. The pots were put into trays (5 x 4 pots per tray) and then placed into FytoScope Walk-In growth chamber (PSI, Czech Republic) and grown under controlled conditions (22 °C/20 °C, 12 h/12 h light/dark cycle with a relative humidity of 60% and an irradiance of 150 μmol m^{-2} s^{-1}). At 14 DAS, only one plant was left in every pot and the plants were placed on conveyor belts into a growth chamber with PlantScreen Modular System (PSI, Czech Republic). The plants were watered with distilled water and continued to grow under the same conditions. At 21 DAS, the plants were placed to a 150 mM NaCl solution or distilled water for 1 hour to saturate the substrate. Twenty plants per line and growth conditions were used as biological replicate and the position of each plant in the trays was randomized. The plants were imaged three to four times a day for three days.

During imaging, nine parameters were measured – area, perimeter, roundness1, roundness2, isotropy, compactness, eccentricity, Rotational Mass Symmetry (RMS) and Slenderness of Leaves (SOL). Pavicic *et al.* (2017) classified the parameters into four groups according to their characteristics – Raw, Circularity, Symmetry and Centre distance (Fig. 6). Area and perimeter are raw parameters and represent a number of pixels of the image of the whole rosette and its edges, respectively; the value can be then converted to millimetres (Fig. 6A). The rest of the parameters are dimensionless. The parameters roundness1, roundness2 and isotropy were grouped into category Circularity (Fig. 6B). Roundness1 compares rosette to a circle with the same perimeter; value 1 represents a perfect circle. Roundness2 is computed using rosette convex hull area (in the picture marked in pink) and perimeter, for WT plants the number lies usually in the range of 0.7–1. Isotropy is a parameter which uses the area of a polygon drawn on top of the rosette. Eccentricity and RMS represent symmetric parameters (Fig. 6C). Eccentricity describes how much is the rosette similar to an ellipse. RMS is a ratio between the non-overlapping area of a rosette convex hull and a circle that has the same area and is centered in the rosette centroid, and the area which overlaps in both. The category Centre distance comprises of parameters compactness and SOL (Fig. 6D). Compactness is described as a ratio between the rosette area (in the picture marked in grey) and the rosette convex hull area. SOL describes the shape of leaves, particularly their sharpness.

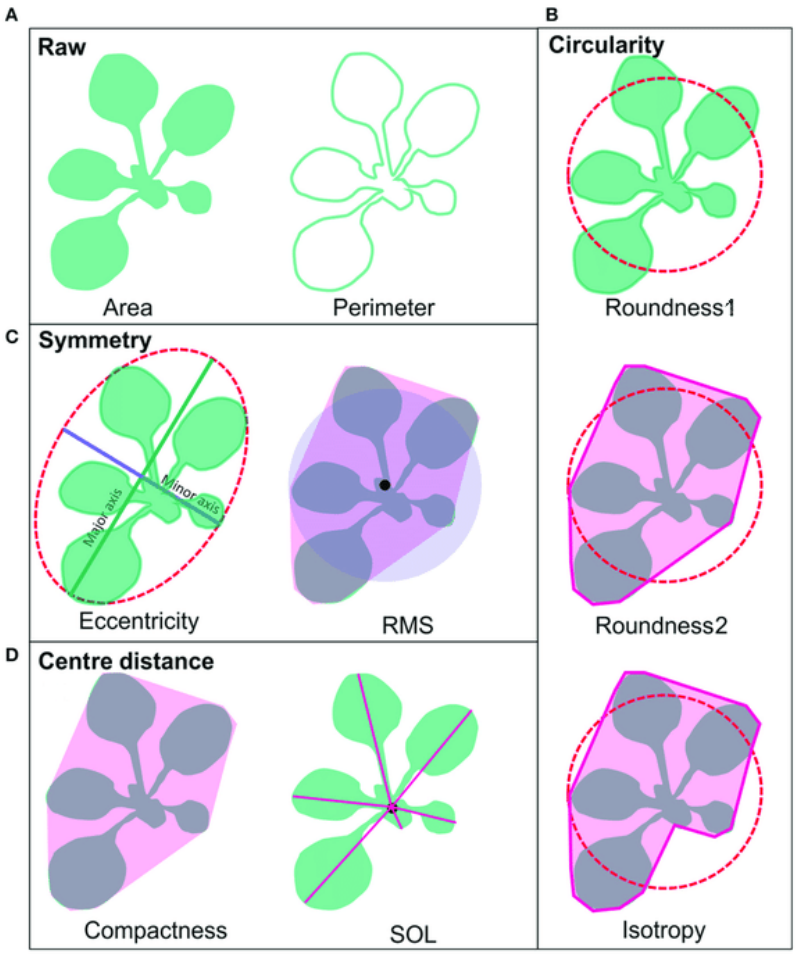

Figure 6. Rosette morphology parameters. Modified from Pavicic *et al*. (2017).

4.4.3 Invasive phenotyping

At 28 DAS and seven days of salt stress, the aerial parts of the mutant lines and WT were harvested for measurement of fresh weight (FW). Samples were then left to dry for two days at 80 °C in forced air oven to acquire dry weight (DW). From the fresh and dry weight, water content was calculated using an equation described by Turner (1986).

$$\mathrm{WC}\ [\%] = \left(\frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{FW}}\right) * 100$$

5 RESULTS

5.1 Selection of homozygous plants

To obtain homozygous plants, *Arabidopsis thaliana* L. seeds with T-DNA insertion were firstly sown on a culture medium supplemented with antibiotic (according to the line). For simplification, all selected lines have the same background *A. thaliana* accession Col-0. The plants that survived the selection were transferred to soil and their genotype was verified by PCR. One PCR reaction with both of the GSP and a specific T-DNA primer was performed for every line. From the size of the obtained products, it could be determined whether the plant was WT, heterozygous or homozygous. WT plants had a band corresponding to the expected size of a product without the T-DNA insert, while homozygous plants had a band corresponding to the size of a product obtained with LB primer and GSP primer in a correct orientation. For heterozygous plants, the presence of both products was characteristic.

For the gene *GAD1*, two insertion lines were ordered (SALK_047648 and SALK_096492C). After genotyping, both lines were found homozygous. Genotyping of the line SALK_096492C produced a 700 bp product for mutant plants with T-DNA insertion, whereas the presence of approximately 1100 bp band was characteristic for WT plants (Fig. 7). Similarly, the knockout plants from SALK_047648 line had a product with the size of 700 bp, while WT plants could be identified by a 1100 bp band (Fig. 8).

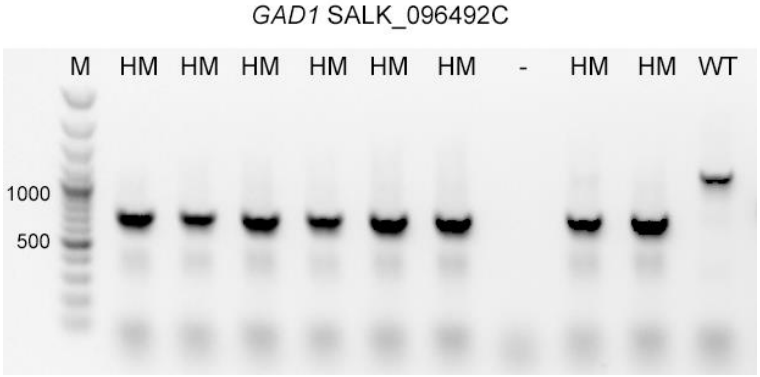

Figure 7. Genotyping of SALK_096492C mutants with T-DNA insertion in the gene *GAD1*. HM, homozygous; M, molecular weight marker; WT, wild type.

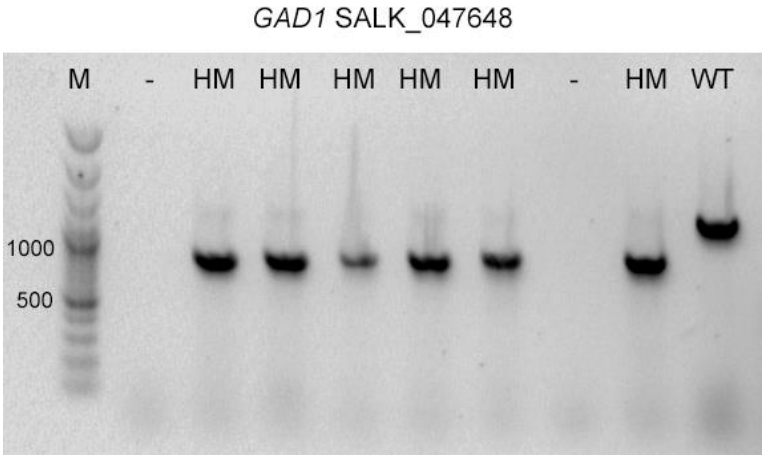

Figure 8. Genotyping of SALK_047648 mutants with T-DNA insertion in the gene *GAD1*. HM, homozygous; M, molecular weight marker; WT, wild type.

Two lines were selected for the gene *GAD2*; SALK_067677C and GK-474E05. Only the SALK_067677C line was homozygous; the mutant plants could be identified by the presence of an 800 bp product, while WT plant had a band with the size of 1100 bp (Fig. 9).

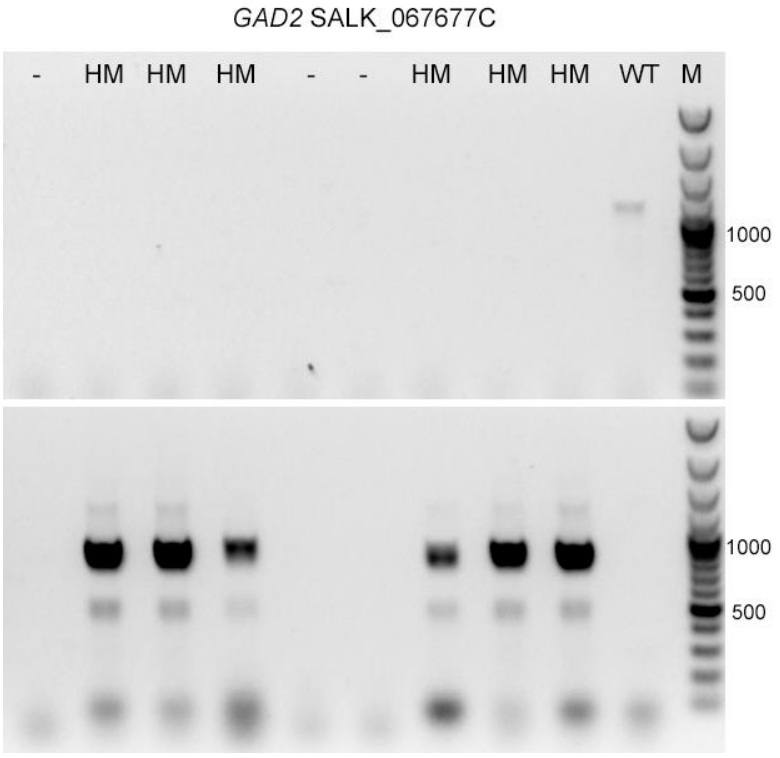

Figure 9. Genotyping of SALK_067677C mutants with T-DNA insertion in the gene *GAD2*. Upper picture is a result of WT PCR, lower picture depicts products of T-DNA PCR. HM, homozygous; M, molecular weight marker; WT, wild type.

As for *GAD3*, four lines were genotyped (SALK_033307, SALK_033307C, SALK_006072, SALK_138534C). Only SALK_033307 line was found homozygous. In this case, the homozygous line had a product with the size of 450 bp compared to the 600 bp product from WT. PCR reaction with WT primers also produced a strong non-specific product that has approximately 400 bp (Fig. 10).

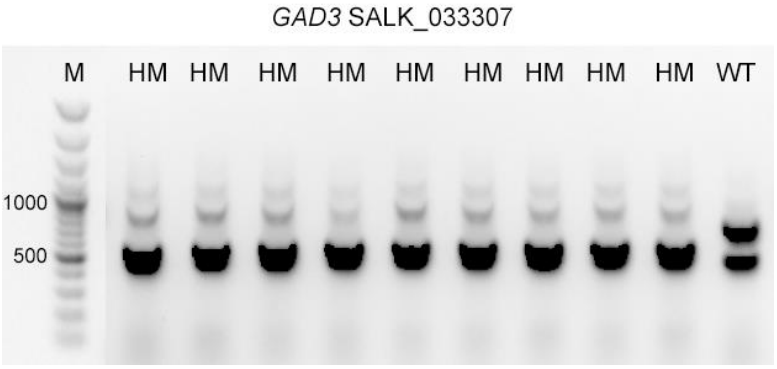

Figure 10. Genotyping of SALK_033307 mutants with T-DNA insertion in the gene *GAD3*. HM, homozygous; M, molecular weight marker; WT, wild type.

In the case of *GAD4*, both lines were homozygous (SALK_146398C and SALK_072198C). As for the line SALK_072198C, 900 bp product meant that the plant was a mutant, whereas a 1100 bp product signified a WT plant (Fig. 11). WT plants of the line SALK_146398C could be identified by the presence of a product of the size of 800 bp and mutant plants with the size of 600 bp (Fig. 12).

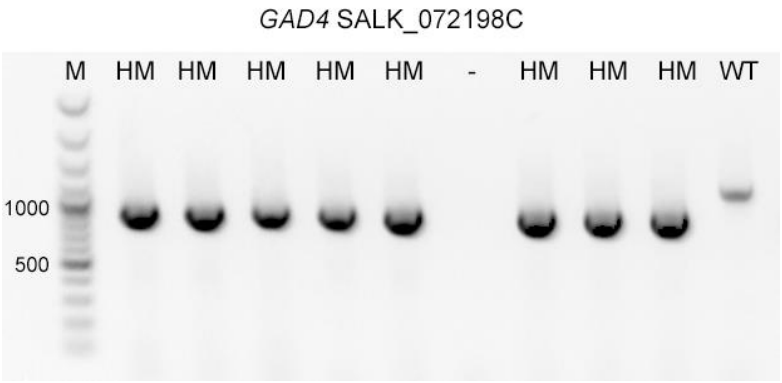

Figure 11. Genotyping of SALK_072198C mutants with T-DNA insertion in the gene *GAD4*. HM, homozygous; M, molecular weight marker; WT, wild type.

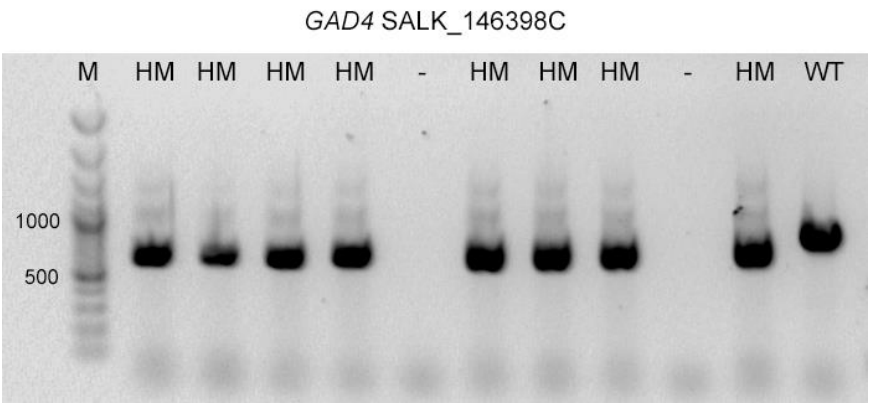

Figure 12. Genotyping of SALK_146398C mutants with T-DNA insertion in the gene *GAD4*. HM, homozygous; M, molecular weight marker; WT, wild type.

Regarding *GAD5*, the line SALK_203883C was genotyped and proved to be homozygous. WT product had 900 bp and mutant product had 650 bp (Fig. 13).

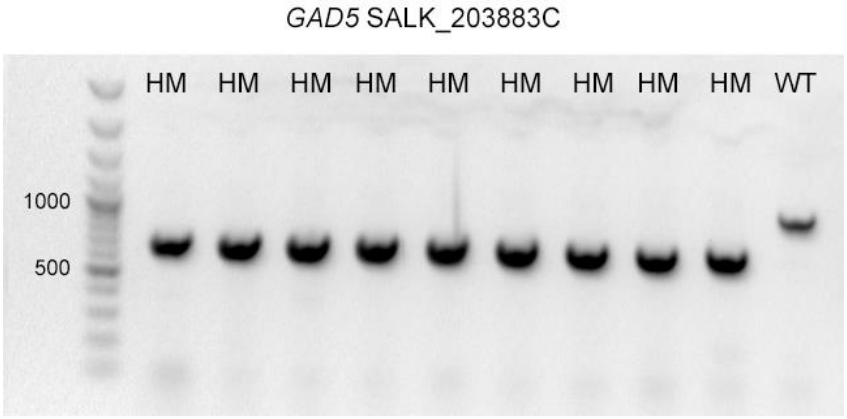

Figure 13. Genotyping of SALK_203883C mutants with T-DNA insertion in the gene *GAD5*. HM, homozygous; M, molecular weight marker; WT, wild type.

There were two lines ordered for the gene *GABA-T;* GK-157D10 and SAIL_1230_C03, the latter proved to be homozygous after genotyping, which produced a 1100 bp product for WT and 600 bp product for mutant plants (Fig. 14).

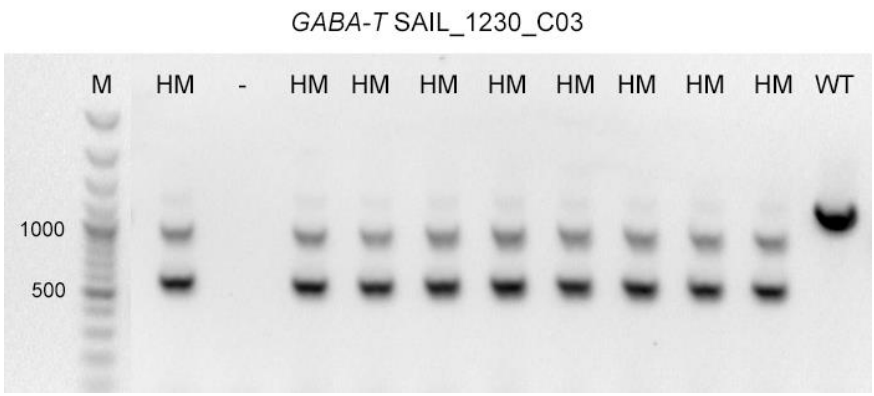

Figure 14. Genotyping of SAIL_1230_C03 mutants with T-DNA insertion in the gene *GABA-T*. HM, homozygous; M, molecular weight marker; WT, wild type.

For *GLYR2*, two lines, SALK_047412C, GK-933D03, were available and both were found homozygous. For SALK_047412C, WT had a product of 1200 bp, whereas for the knockouts the product had 700 bp (Fig. 15). In the case of GK-933D03, the presence of a 1100 bp product signified a plant without a T-DNA insert, while mutant plants could be identified by an 800 bp product (Fig. 16).

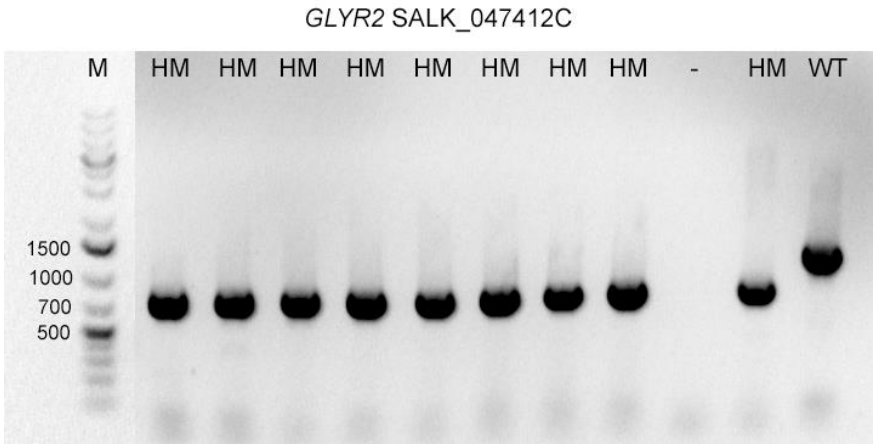

Figure 15. Genotyping of SALK_047412C mutants with T-DNA insertion in the gene *GLYR2*. HM, homozygous; M, molecular weight marker; WT, wild type.

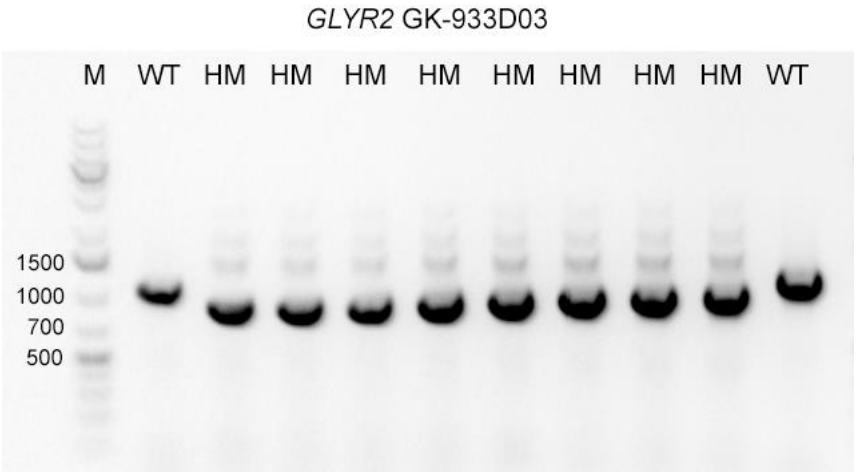

Figure 16. Genotyping of GK-933D03 mutants with T-DNA insertion in the gene *GLYR2*. HM, homozygous; M, molecular weight marker; WT, wild type.

For *SSADH* (lines SALK_003223C, SAIL_1278_B12 and SALK_003223) and *GLYR1* (lines SALK_203580C, SAIL_894_G08 and SALK_057410), the selection of homozygous plants was not successful; all genotyped plants were either WT or heterozygous.

5.2 Non-invasive phenotyping

To analyze the role of different genes related to GABA metabolism in *Arabidopsis* growth under control and salt stress conditions, from the aforementioned plants, 11 lines were chosen for non-destructive phenotyping – WT (Col-0) and ten homozygous lines: *GAD1* (SALK_047648, SALK_096492C), *GAD2* (SALK_067677C), *GAD3* (SALK_033307), *GAD4* (SALK_146398C, SALK_072198C), *GAD5* (SALK_203883C), *GABA-T* (SAIL_1230_C03) and *GLYR2* (SALK_047412C, GK-933D03).

Twenty plants per line and per growth conditions (control or 150 mM salt treatment) represented one biological replicate each; a total of 440 plants were phenotyped using RGB camera. Plants were imaged for three days; three times during the first day, then four times a day for the remaining two days. Nine parameters were measured – area, perimeter, roundness1, roundness2, isotropy, compactness, eccentricity, Rotational Mass Symmetry (RMS) and Slenderness of Leaves (SOL). The final values of every parameter were represented by a median value of data acquired from plants of a single line subjected to the same treatment.

5.2.1 Area and perimeter

The parameter area represents a count of green pixels in an image of a plant rosette, whereas perimeter can be described as a total of green pixels counted from the edges of the rosette. The rosette area and perimeter of all homozygous lines and the WT generally followed the same pattern during the day – there was a peak at 6:00 or 10:00 and then the values gradually decreased.

There were two *gad1* lines used – SALK_047648 and SALK_096492C. During control conditions, SALK_047648 rosette area was similar to that of WT, whereas the perimeter was larger. In the case of SALK_096492C line, smaller rosette and perimeter compared to WT was observed. Under salt stress treatment, the final area and perimeter of SALK_047648 line were significantly smaller than the area and perimeter of WT. SALK_096492C line in salt conditions had smaller rosette area and perimeter than WT, but both parameters reached higher values than in control conditions (Fig. 17A, 18A).

For the gene *GAD2*, only the line SALK_067677C was used. The mutants had slightly larger rosette area and perimeter than WT in control and stress conditions (Fig. 17B, 18B).

Mutant line SALK_033307 was used in the case of the gene *GAD3*. In control condition, smaller area and perimeter in comparison to WT were observed. Contrarily, salt-treated plants had larger rosette area than control plants, but the perimeter was nearly the same as the perimeter of WT plants (Fig. 17C, 18C).

There were two *gad4* mutant lines – SALK_072198C and SALK_146398C. Plants of the line SALK_072198C grown in control conditions had basically the same area and perimeter as WT, whereas salt treatment caused an increase in both. SALK_146398C line had larger rosette area and perimeter than WT in both conditions (Fig. 17D, 18D).

Gad5 line SALK_203883C showed similar increase in area and perimeter as WT in both conditions, in the case of perimeter the increase was more noticeable (Fig. 17E, 18E).

In control conditions, SAIL_1230_C03 plants with disrupted *GABA-T* gene had the same area and perimeter as WT; under salt stress treatment, the area and perimeter were smaller than in WT (Fig. 17F, 18F).

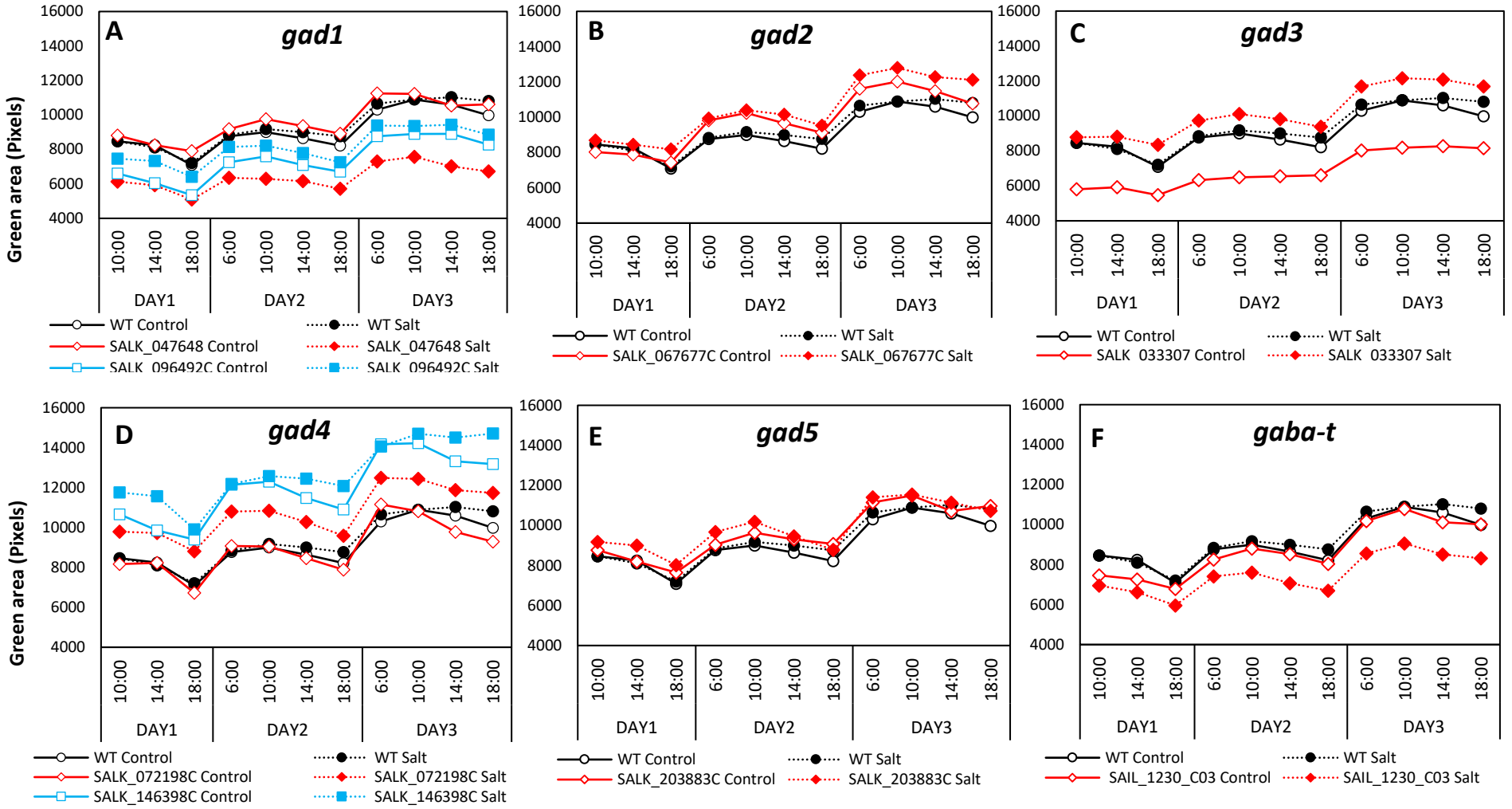

Figure 17. Rosette growth of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

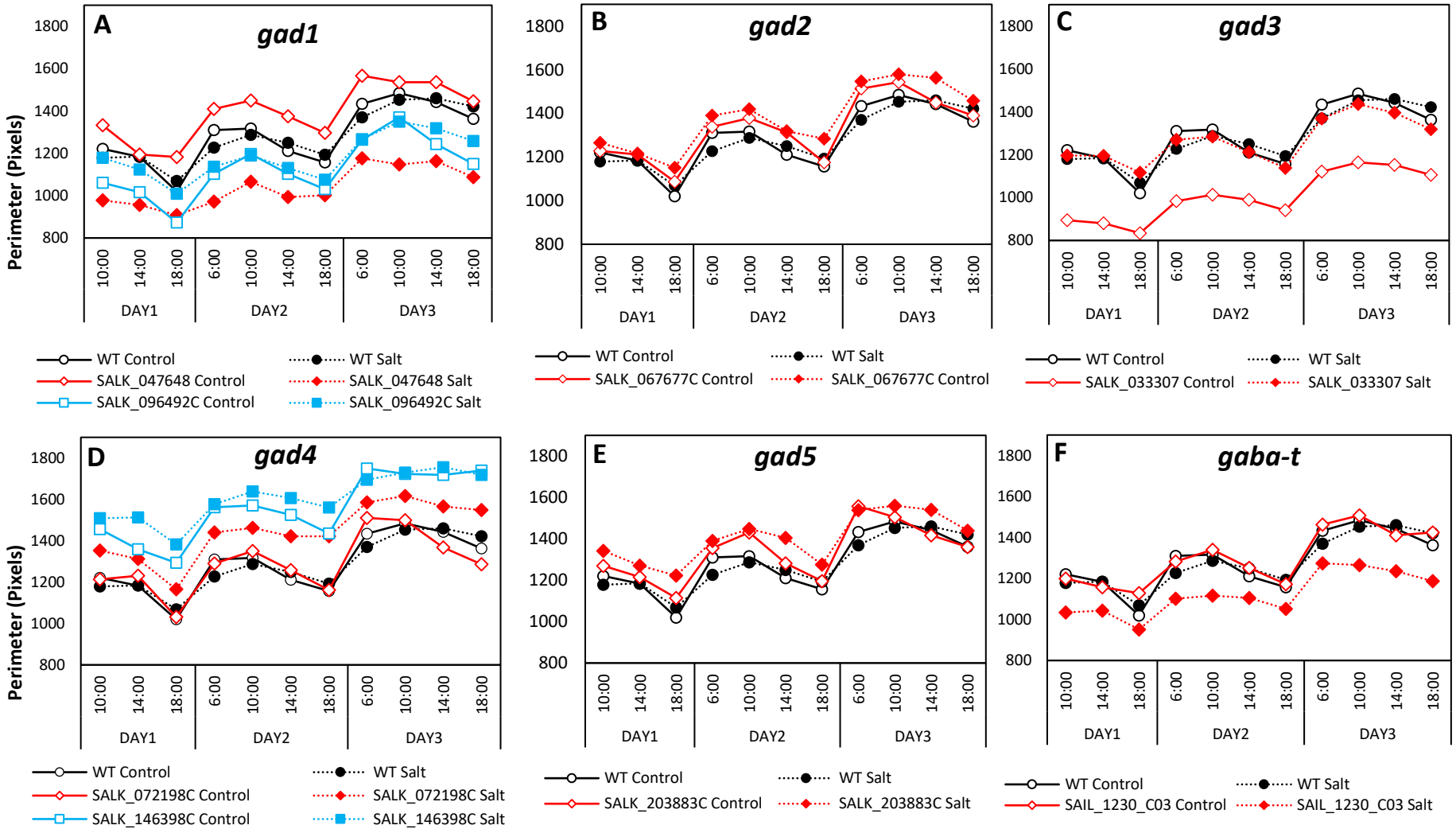

Figure 18. Perimeter of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

Two mutant *glyr2* lines were phenotyped. The line SALK_047412C in control conditions had smaller rosette area and perimeter compared to WT, in contrast, both parameters were larger than WT after salt treatment. GK-933D03 line in salt and control conditions had larger rosette and perimeter than WT (Fig. 19, 20).

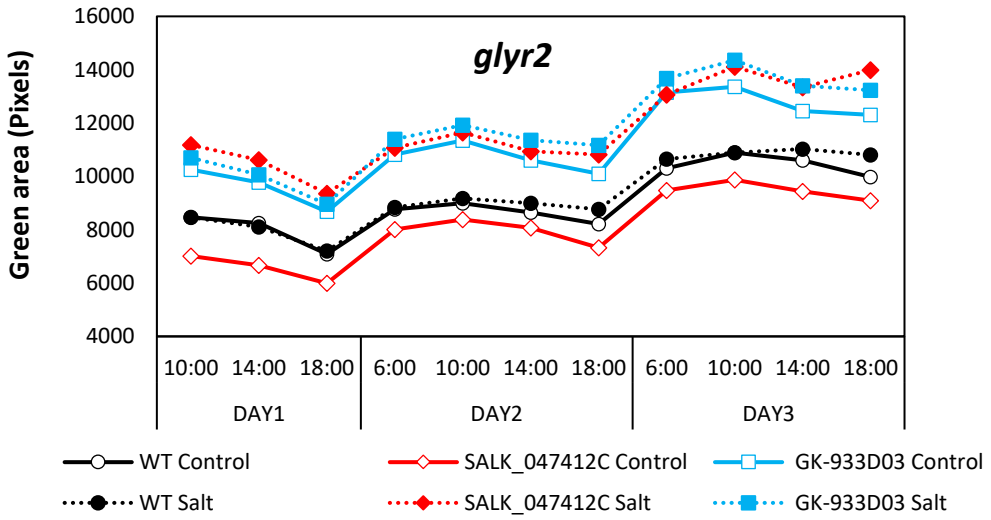

Figure 19. Rosette growth of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

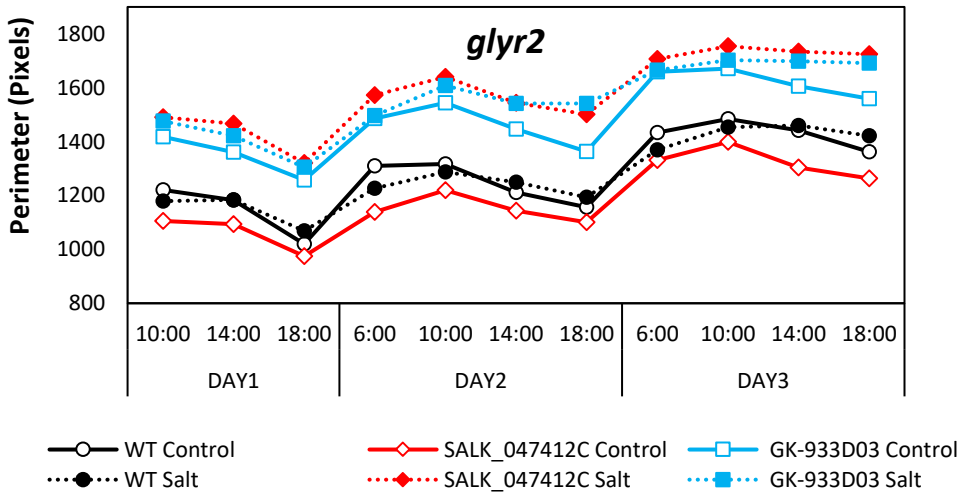

Figure 20. Perimeter of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

When we compared all the lines together, *GAD4* and *GLYR2* were found to be the genes that participated the most in the regulation of rosette area size and perimeter under control and salt stress conditions. The rosette size and perimeter of these mutants were the largest out of all lines and knockout of these genes allowed the plants to get bigger. In contrast, the *GAD1* and *GABA-T* mutants were the most sensitive to stress.

5.2.2 Circularity

The circularity of the plants includes three different traits: roundness1, roundness2 and isotropy.

Regarding the parameter roundness1, the curves usually had a peak at 18:00, overall the parameter had a slightly downward tendency.

In control conditions, *gad1* mutants of the line SALK_047648 showed decreased roundness in comparison to WT. SALK_096492C had a similar curve to WT, only with higher peaks. SALK_047648 line grown under salt stress showed increased roundness when compared to WT, but unlike WT, it didn't have clear peaks. Roundness1 pattern of salt-treated plants of the line SALK_096492C nearly corresponded to the pattern of WT (Fig. 21A).

For *gad2*, the roundness of SALK_067677C line in control conditions didn't change greatly compared to WT, but after salt treatment there was a slight decrease (Fig. 21B).

Gad3 line SALK_033307 in conditions without stress reached the highest roundness1 values out of all analyzed lines, in salt conditions the values were higher than the values of control plants as well (Fig. 21C).

In control conditions, *gad4* line SALK_072198C showed roundness1 curve similar to WT, but had sharper peaks; under salt stress conditions the curve had lower values and was flatter than the curve of WT. The curves of *gad4* SALK_146398C line in control and salt stress conditions were similar to each other; they showed lower values than WT and SALK_072198C (Fig. 21D).

Gad5 line SALK_203883C showed lower roundness1 values in control conditions compared to WT, but with high peaks; in salt stress conditions, the values were even lower (Fig. 21E).

Gaba-t mutants of the SAIL_1230_C03 line in control conditions showed lower values than WT, under salt stress the curve reached higher values but without the peaks characteristic for WT (Fig. 21F).

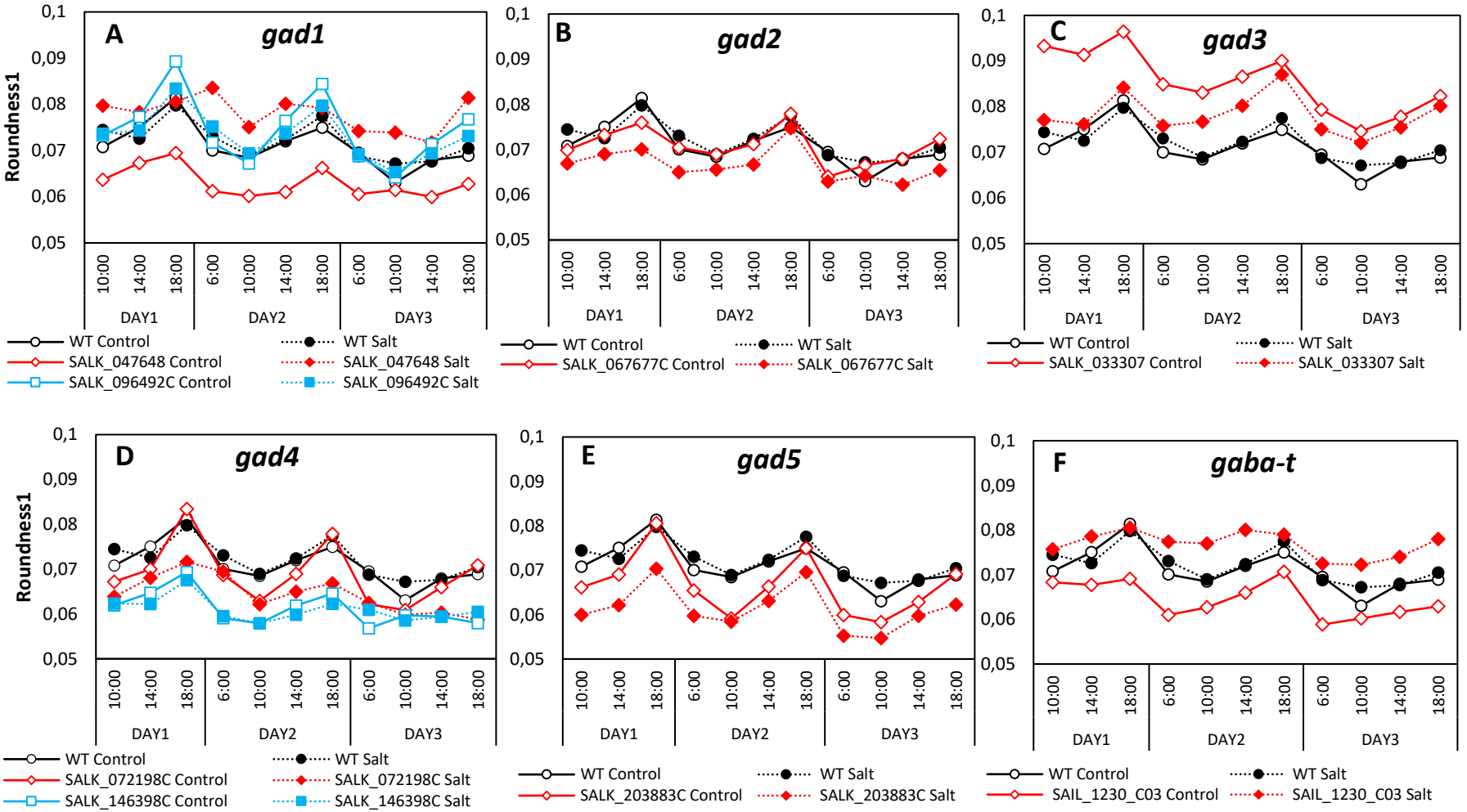

Figure 21. Parameter roundness1 of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

For *glyr2*, SALK_047412C mutants in control conditions had a similar pattern to WT, whereas in salt conditions the roundness1 values were lower. GK-933D03 in both salt and control conditions showed values lower than WT (Fig. 22).

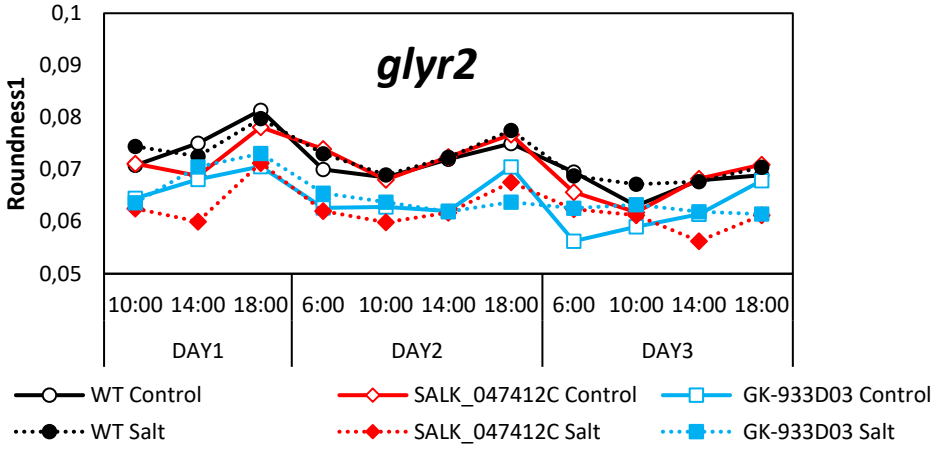

Figure 22. Parameter roundness1 of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

Another circularity parameter was roundness2. WT plants showed different pattern under salt stress and control conditions. The plants grown under control conditions had a pattern with two peaks, the first on the second day at 6:00 and the second higher peak on the third day at 10:00, whereas salt-treated plants showed lower roundness2 that increased at the beginning of the third day.

Gad1 lines SALK_047648 and SALK_096492C in both control and stress conditions had a similar pattern as WT in control conditions (Fig. 23A).

For *gad2*, the line SALK_067677C in control conditions had a similar roundness2 pattern as WT, under salt stress were the values larger than those of salt-treated WT (Fig. 23B).

The curve of *gad3* SALK_033307 line in control and salt conditions was flatter than the curve of WT, salt-stressed mutants reached higher values than WT plants under salt conditions (Fig. 23C).

The pattern of *gad4* SALK_072198C line in control and salt conditions corresponded to the pattern of WT in respective conditions. Concerning the other *gad4* mutant line SALK_146398C, in control conditions the line showed a similar pattern to the pattern of WT in the same conditions, whereas salt-stressed mutants had higher values than WT plants exposed to stress (Fig. 23D).

Gad5 SALK_203883C line in control conditions showed flatter curve than WT, but it otherwise followed the same pattern; in salt conditions the curve was similar to that of salt-treated WT (Fig. 23E).

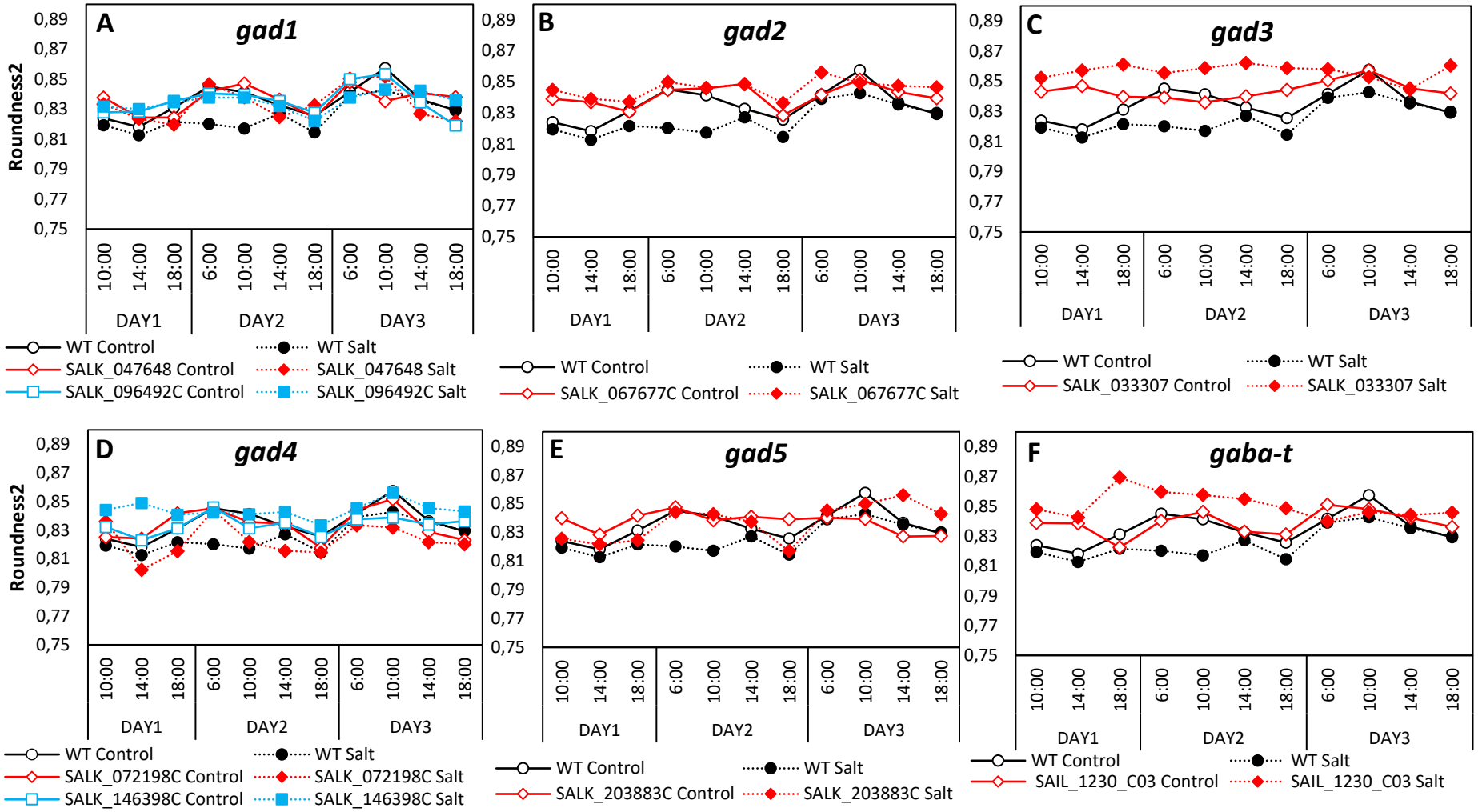

Figure 23. Parameter roundness2 of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

For the *gaba-t* SAIL_1230_C03 line, the curves of mutant and WT plants in control conditions were similar, under salt stress the mutant curve had a peak on the first day at 18:00 and then the values decreased (Fig. 23F).

Glyr2 roundness2 curve for the SALK_047412C line was similar to WT in both conditions; the same was also true for salt-treated GK-933D03 plants. In control conditions, GK-933D03 mutants showed higher values than WT (Fig. 24).

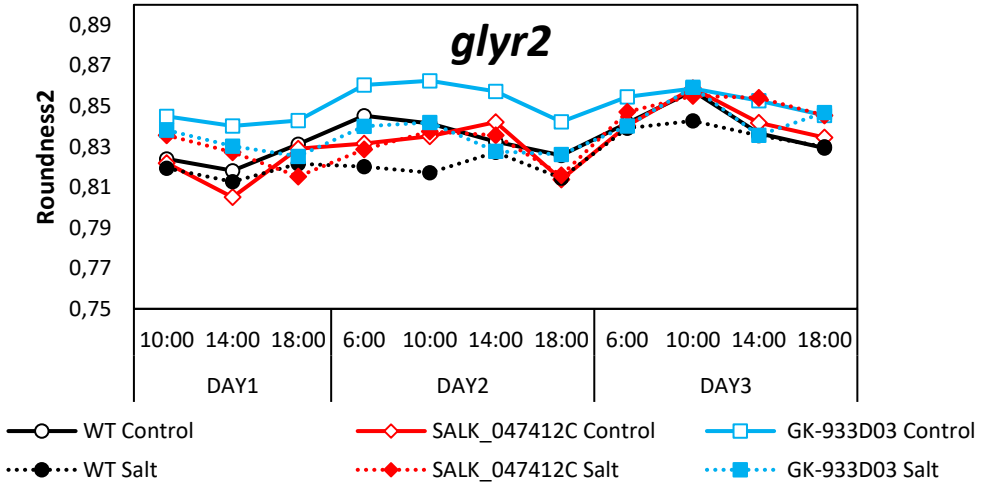

Figure 24. Parameter roundness2 of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

The parameter isotropy was difficult to describe, as the curves had no clear pattern. The isotropy values for most of the lines belonged to the range of 0.5–0.7. WT plants in control conditions had two peaks, in salt conditions the curve was flatter.

Some of the lines (SALK_047648 and SALK_096492C for *gad1*, SALK_203883C for *gad5* and GK-933D03 for *glyr2*) had a pattern somewhat similar to WT (Fig. 25A, 25E, 26).

For *gad2*, the line SALK_067677C in control conditions had a similar pattern to WT, in salt stress conditions the curve was flat and had higher values than WT plants grown under stress (Fig. 25B).

The curve of *gad3* line SALK_033307 in control conditions showed high values at the beginning of the first day, then the values decreased until the beginning of the second day. Under salt stress, the curve was flat, and its values were higher than the values of salt-stressed WT plants (Fig. 25C).

Gad4 SALK_072198C curve was flat in control conditions and had similar values in salt stress conditions. In control conditions, isotropy values for *gad4* SALK_146398C line were lower and in salt conditions higher than for WT (Fig. 25D).

In control conditions, SAIL_1230_C03 line with disrupted *GABA-T* gene had isotropy curve with a pattern similar to WT, salt-stressed mutants showed higher values than WT (Fig. 25E).

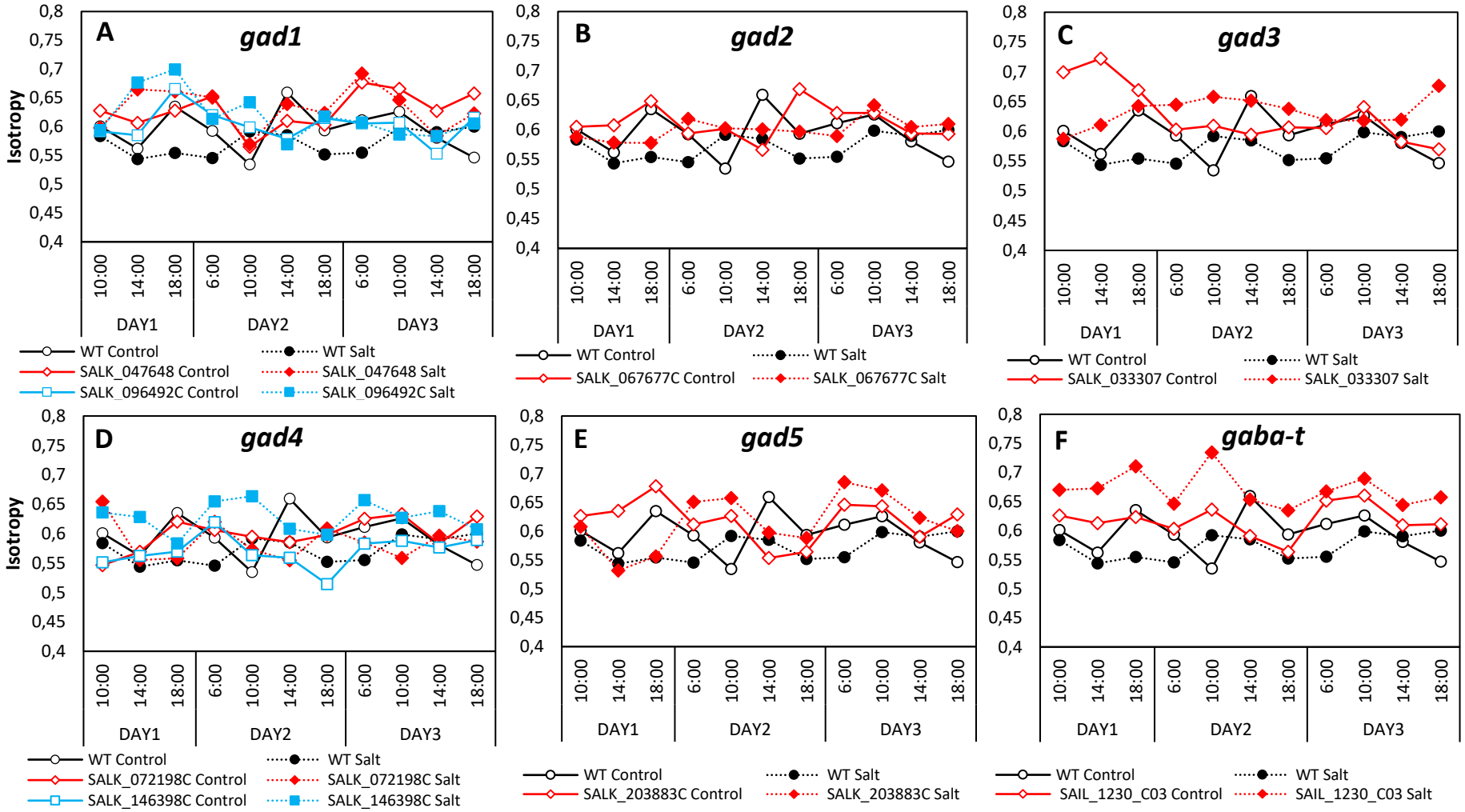

Figure 25. Parameter isotropy of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

The curve of the other *glyr2* line SALK_047412C was flat in both control and salt stress conditions (Fig. 26).

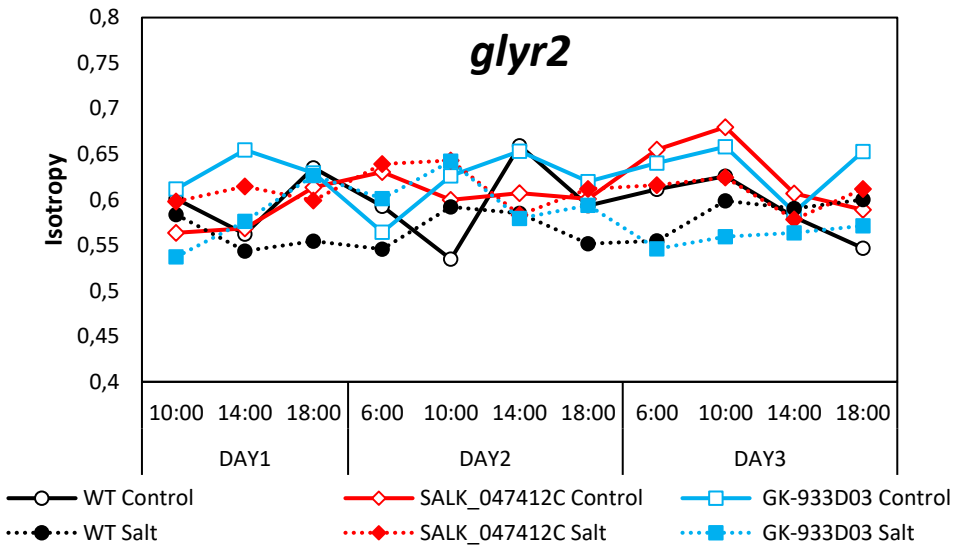

Figure 26. Parameter isotropy of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

In summary, for roundness1, *gad3* mutants the highest values, especially in control conditions; *gad4* (SALK_146398C line) and *glyr2* (GK-933D03 line) mutant plants were less round than WT. The results showed that *GAD3* gene had the most determining role in *Arabidopsis* circularity. Although the roundness2 and isotropy values of the rest of lines were changing during the day, almost no variation was observed for the knockout plants of this gene.

5.2.3 Eccentricity

Parameter eccentricity had a slightly decreasing tendency for most of the lines. For WT plants, the eccentricity curve had a peak on the second day at 18:00; under salt treatment, the curve was higher overall and, in addition, had a particularly sharp peak.

As for control conditions, *gad1* mutants of SALK_047648 line showed similar pattern as WT, whereas SALK_096492C eccentricity curve was higher that WT with its peak shifted to the left. During salt conditions, SALK_047648 line had higher eccentricity values than during control conditions, for SALK_096492C it was the opposite (Fig. 27A).

The curve of *gad2* SALK_067677C line in control conditions was similar to WT in the same conditions, there was a peak on the first day at 18:00; in salt conditions, the eccentricity values were lower than those of salt-treated WT (Fig. 27B).

Regarding *gad3* plants of the line SALK_033307, in control conditions they had an eccentricity curve similar to WT. However, as happened with the circularity parameters, under salt conditions the curve showed low values without any peaks (Fig. 27C).

The curves of the two *gad4* lines SALK_072198C and SALK_146398C were flat and had an upward tendency in control conditions; concerning salt stress conditions, the line SALK_072198C had similar values as WT in salt stress conditions and SALK_146398C had lower values than those of salt-stressed WT plants (Fig. 27D).

Gad5 SALK_203883C line grown without stress had a pattern similar to WT, under salt stress it showed lower values than salt-treated WT (Fig. 27E).

Gaba-t SAIL_1230_C03 line had a pattern similar to WT, but with a peak on the first day at 18:00; salt-treated mutants had values slightly lower than WT in stress conditions (Fig. 27F).

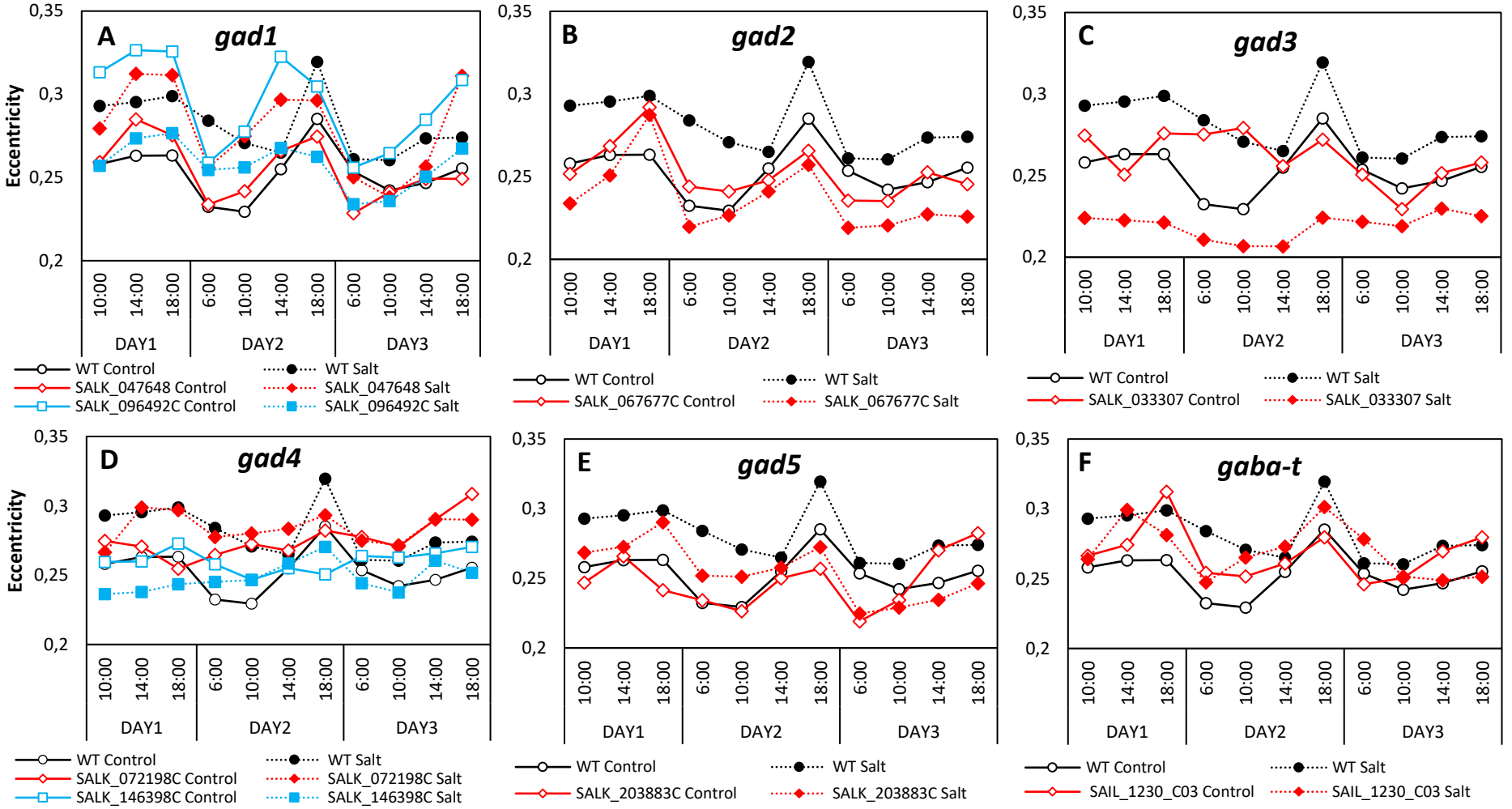

Figure 27. Parameter eccentricity of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type

Glyr2 line SALK_047412C showed higher values and a peak even higher than the peak of salt-stressed WT, under salt stress the eccentricity values were similar to those of salt-treated WT. *Glyr2* line GK-933D03 in control conditions had almost the same values as WT, in salt conditions the values were lower than salt-stressed WT (Fig. 28).

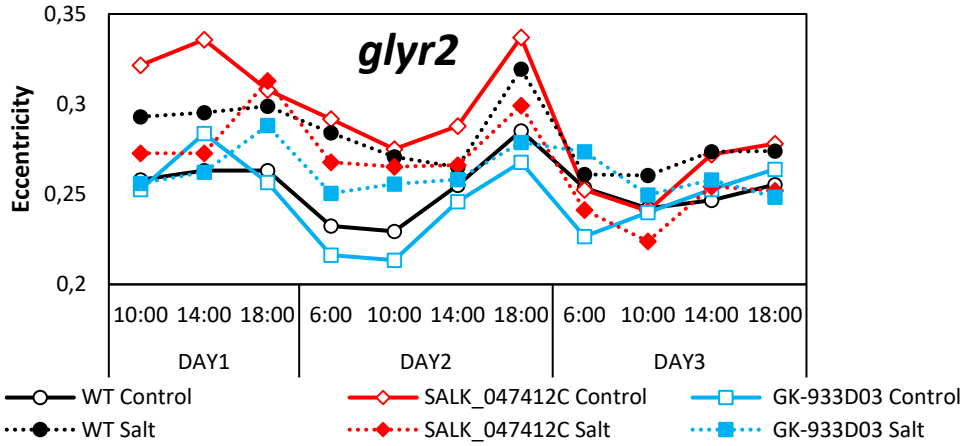

Figure 28. Parameter eccentricity of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type

To sum up, *gad3* mutants in salt stress conditions were the least eccentric, whereas the most eccentric were *gad1* (SALK_096492C) and *glyr2* (SALK_047412C) mutants.

5.2.4 Rotational Mass Symmetry (RMS)

The curves of RMS generally followed a downward trend. In control conditions, the curve for WT plants had a peak on the second day at 18:00; in contrast, under salt stress the curve was flat, and the values of the parameter didn't change greatly.

The values of *gad1* mutant line SALK_047648 in control conditions were higher in comparison to WT; the curve was flatter, yet there was still a peak, albeit it was shifted to the left. SALK_096492C showed higher RMS values than WT and SALK_047648, otherwise the pattern was similar to WT. Under salt stress, SALK_047648 curve first had a similar pattern to the salt-treated WT. The curve of SALK_096492C line under salt stress showed lower values than in control conditions, however, the curve had a similar shape (Fig. 29A).

The RMS curve of the line SALK_067677C mutant in gene *GAD2* was flat in control conditions, and had values corresponding to the highest peak of the curve of WT plants; in salt conditions, the values were lower than the values of WT (Fig. 29B).

In control conditions, *gad3* SALK_033307 line had a flatter curve than WT, mutants grown under salt treatment showed low RMS values which gradually increased (Fig. 29C).

The two *gad4* lines, SALK_072198C and SALK_146398C, were flatter in control and salt conditions in comparison to WT; all curves except for the curve of salt-stressed SALK_146398C had an increasing tendency (Fig. 29D).

For *gad5*, the curve of SALK_203883C in control conditions showed a large dip at the end of the first day, in salt stress conditions the parameter had a pattern similar to WT in control conditions (Fig. 29E).

The curve of *gaba-t* SAIL_1230_C03 line followed the pattern of WT, however, it did not have the same peak. The values of mutants under salt stress were larger than those of salt-treated WT plants (Fig. 29F).

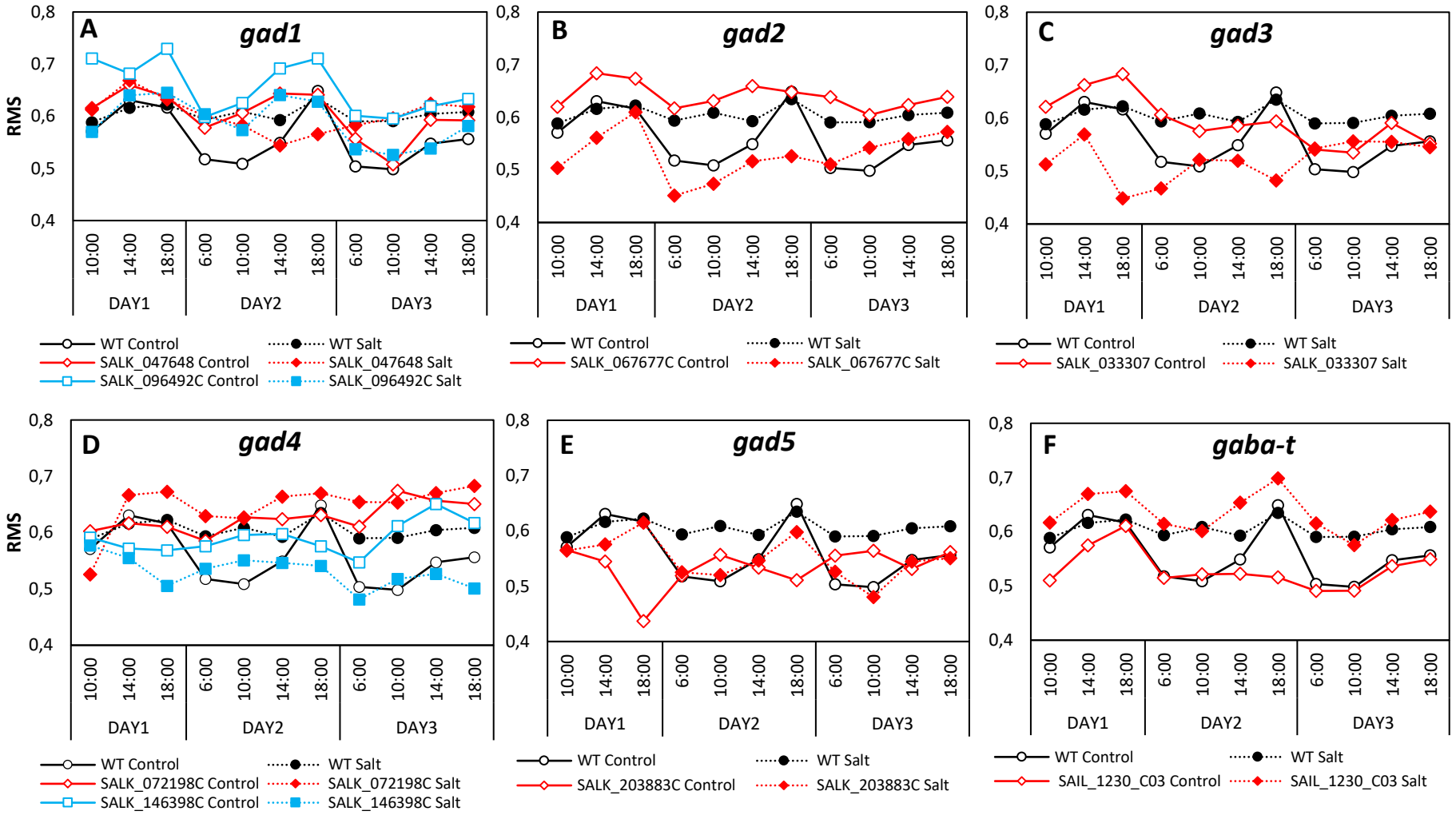

Figure 29. Parameter Rotational Mass Symmetry of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

In control conditions, the *glyr2* line SALK_047412C reached higher values than WT; in salt conditions, the values were first low and then increased. The curve of *glyr2* GK-933D03 line was flatter than that of WT (Fig. 30).

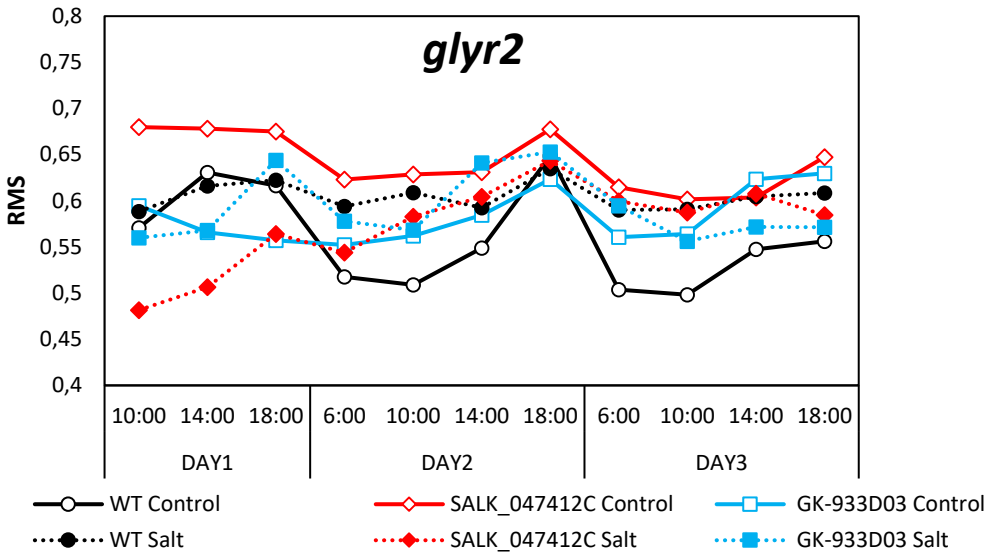

Figure 30. Parameter Rotational Mass Symmetry of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

In short, all mutants except *gad4* showed variability in this parameter under control and salt stress conditions. *Gad1* (SALK_096492C line) mutants had the highest values for RMS.

5.2.5 Compactness

The parameter compactness had a pattern with peaks every day at 6:00. WT plants grown in salt stress conditions showed slightly lower values than WT plants without stress.

In control conditions, compactness values of *gad1* mutants of the SALK_047648 line were lower than the values of WT, SALK_096492C and also SALK_047648 during salt stress. The curve of SALK_096492C line grown without salt was lower than the curve of WT; its peak was shifted to the left. In stress conditions, the compactness values were similar to salt-treated WT plants (Fig. 31A).

For *gad2*, the compactness pattern of the line SALK_067677C in control and stress conditions was nearly identical to WT (Fig. 31B).

In control and salt conditions, as occurred with the circularity, the *gad3* line SALK_033307 showed higher values than WT; in control conditions the compactness curve had a different pattern with only one peak on the second day at 18:00 (Fig. 31C).

The compactness curves for *gad4* SALK_072198C line in control and salt conditions were lower compared to WT, in the salt conditions more so. The *gad4* mutants of the line SALK_146398C showed lower values in control conditions and slightly higher values in salt conditions compared to WT (Fig. 31D).

For *gad5*, the compactness curve of the SALK_203883C line had no peaks in control conditions and the values almost didn't change over the course of the experiment; salt-treated plants had similar values as WT (Fig. 31E).

Gaba-t line SAIL_1230_C03 in control conditions showed lower values than WT, plants under salt stress had similar values to salt-treated WT plants (Fig. 31F).

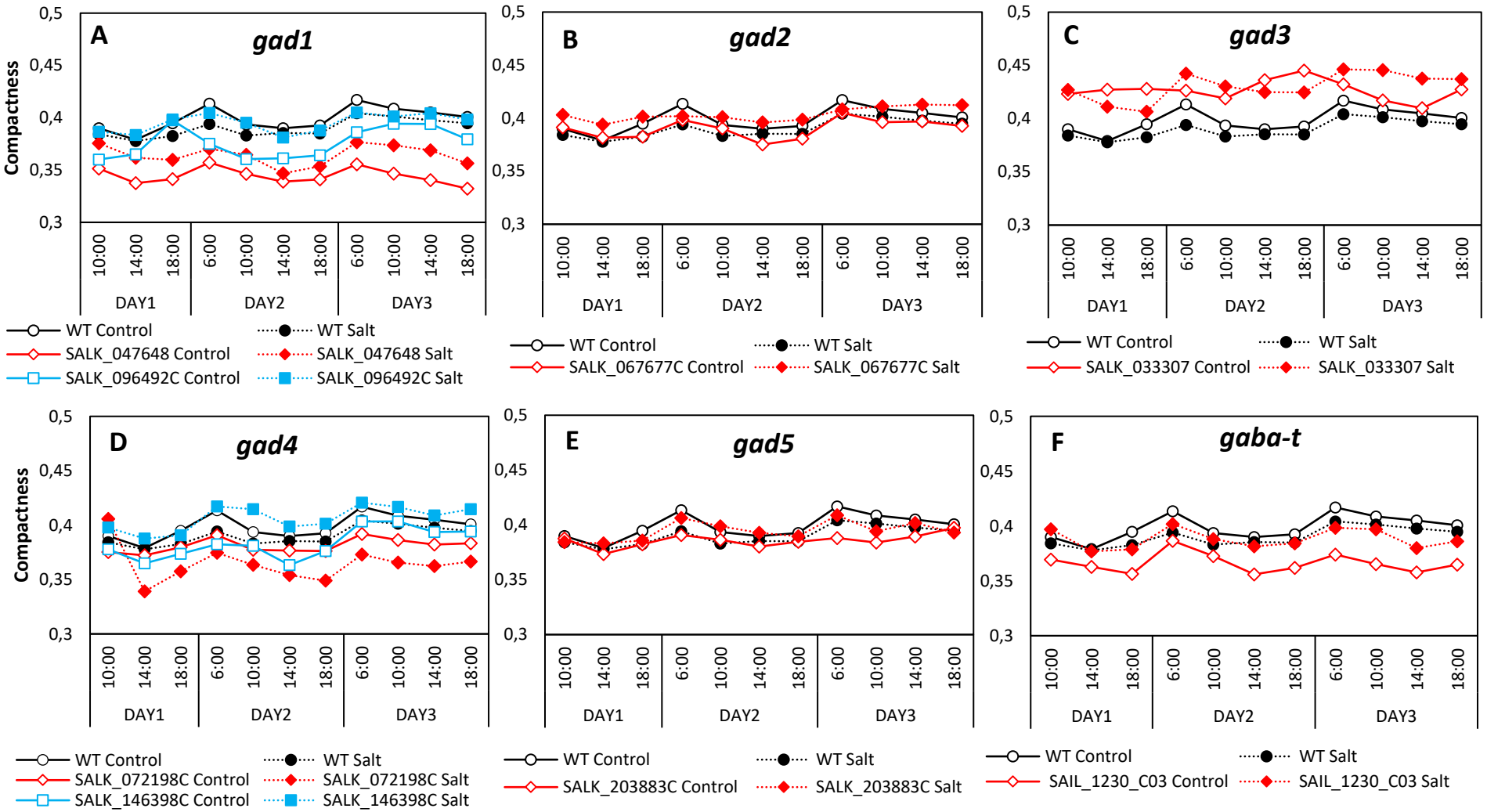

Figure 31. Parameter compactness of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

The *glyr2* SALK_047412C line showed slightly lower values in the case of salt-stressed and non-stressed plants when compared to WT, while for the line GK-933D03, lower values in control conditions and higher values in salt stress conditions in comparison to WT were recorded (Fig. 32).

In conclusion, *GAD1*, *GAD3* and *GABA-T* controlled the compactness (parameter of centre distance) of the plants in the opposite way. In the knockout lines of *gad1* and *gaba-t*, this parameter was reduced, whereas in the *gad3* mutants it increased.

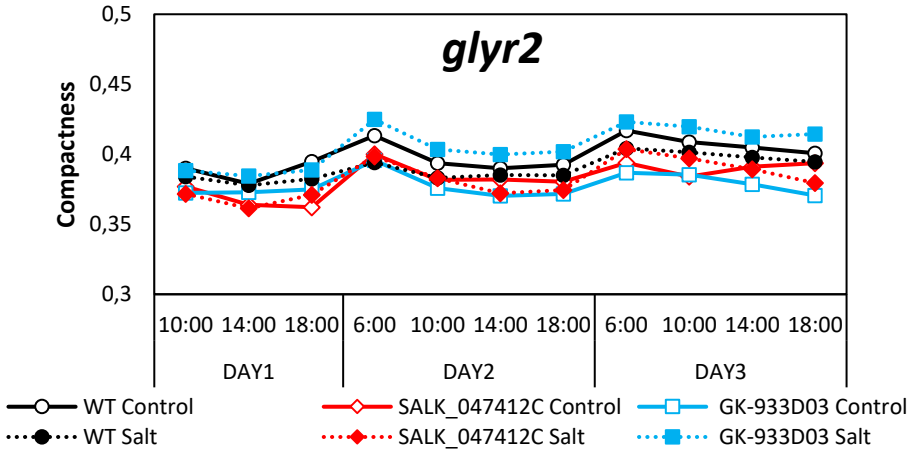

Figure 32. Parameter compactness of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

5.2.6 Slenderness of Leaves (SOL)

The parameter SOL followed an increasing tendency, there was a peak every day at 10:00. In salt and control conditions, SOL had a similar pattern for WT.

In control conditions, the curve of mutant line *gad1* SALK_047648 had higher values than WT; in the case of SALK_096492C, the curve was similar to WT. Salt-treated plants of the line SALK_047648 had slightly lower SOL values than WT, for SALK_096492C line the values were similar to those of WT (Fig. 33A).

For *gad2* mutant line SALK_067677C, the curve in control conditions resembled the curve of WT, but in salt conditions the values increased (Fig. 33B).

SALK_033307 line mutant in *GAD3* gene displayed lower SOL values in control conditions, plants grown under salt stress had a curve similar to WT (Fig. 33C).

Gad4 mutant lines SALK_072198C and SALK_146398C showed larger SOL values than WT in control and experimental conditions; the same applied to *gad5* line SALK_203883C (Fig. 33D, 33E).

Line SAIL_1230_C03 disrupted in the gene *GABA-T* had higher SOL values than WT in control conditions, while under salt stress the parameter didn't change in comparison to WT (Fig. 33F).

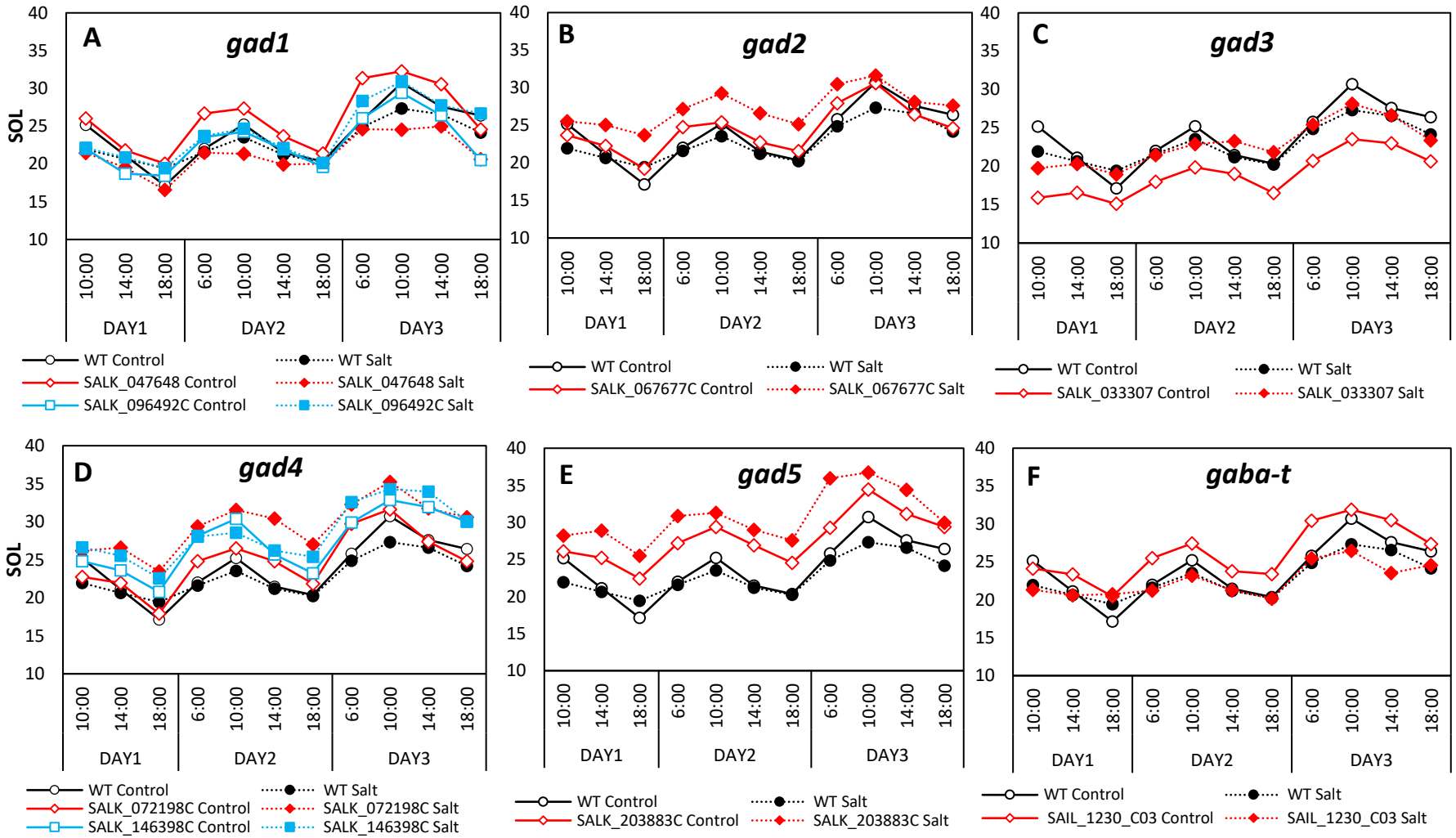

Figure 33. Parameter Slenderness of Leaves of **(A)** *gad1*, **(B)** *gad2*, **(C)** *gad3*, **(D)** *gad4*, **(E)** *gad*5 and **(F)** *gaba-t* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

Glyr2 line SALK_047412C in control conditions had nearly the same values as WT, whereas the other line GK-933D03 had higher values than WT. Salt stress led to an increase of SOL for both lines (Fig. 34).

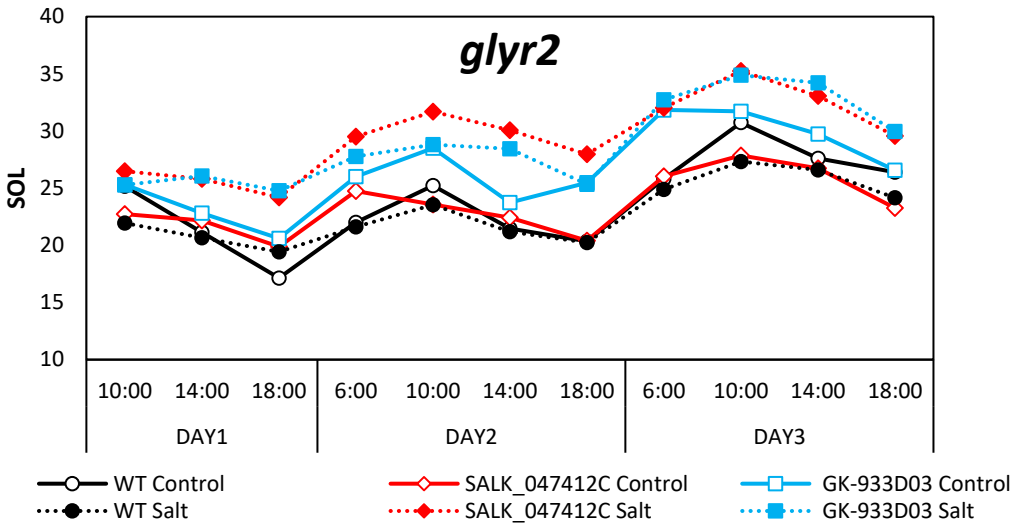

Figure 34. Parameter Slenderness of Leaves of *glyr2* mutants of *A. thaliana* under control conditions and salt treatment during imaging over the span of three days. WT, wild type.

In summary, the slenderness of leaves was increased in *gad2*, *gad4*, *gad5* and *glyr2* mutants, whereas in *gad3* mutants it decreased.

5.3 Invasive phenotyping – biomass and water content (WC)

From every mutant line and WT, the aerial parts of four plants were harvested and immediately weighed, then the plants were dried, and their weigh was measured again. The acquired fresh weight (Fig. 35) and dry weight (Fig. 36) under control conditions and salt stress were calculated as an average weight of the four plants.

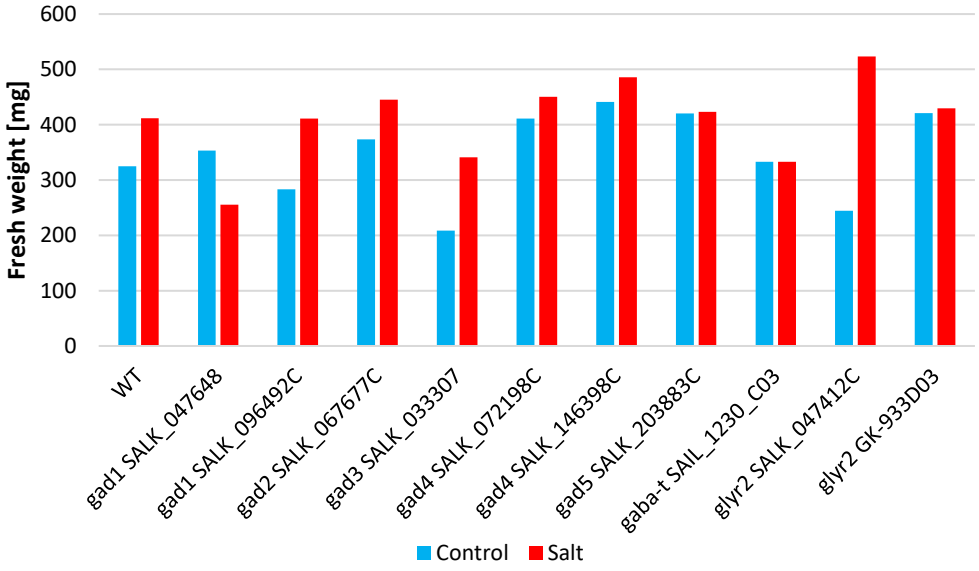

Figure 35. Fresh weight of *A. thaliana* lines with a T-DNA insertion in the genes of GABA metabolism (*GAD1–GAD5, GABA-T, GLYR2*) in control and salt stress conditions. WT, wild type.

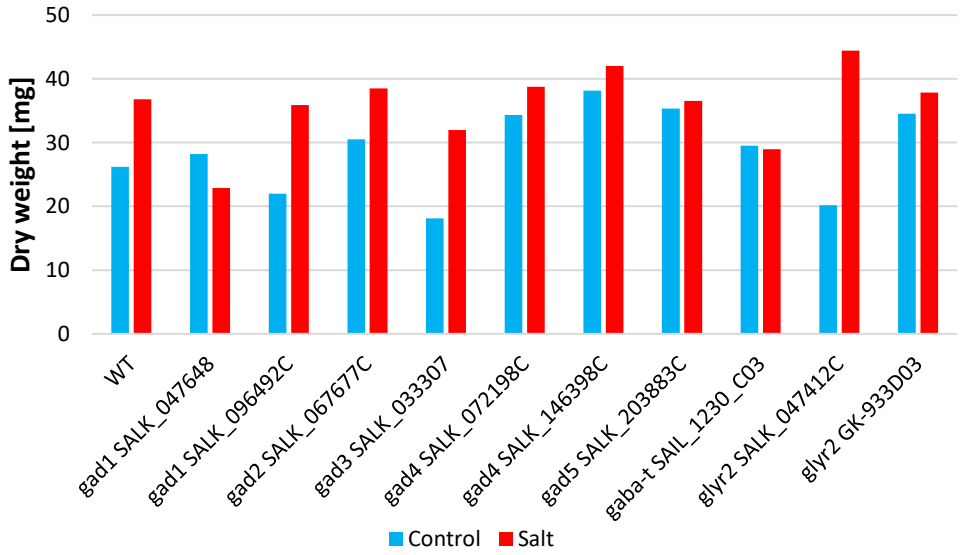

Figure 36. Dry weight of *A. thaliana* lines with a T-DNA insertion in the genes of GABA metabolism (*GAD1–GAD5, GABA-T, GLYR2*) in control and salt stress conditions. WT, wild type.

Dry weight of a biomass is considered more accurate than fresh weight because the latter can differ depending on the moisture content of biomass; another source of error can be the loss of moisture if the weight is not measured immediately after harvesting. In control conditions, the dry weight was larger for mutant lines *gad1* (SALK_047648), *gad2*, both *gad4*, *gad5*, *gaba-t* and *glyr2* (GK-933D03) when compared to WT; only for *gad1* (SALK_096492C), *gad3* and *glyr2* (SALK_047412C) was the dry weight smaller. In salt stress conditions, the dry weight was larger than that of WT for *gad2*, both *gad4* and both *glyr2* mutant lines. Surprisingly, in case of all of the lines except for *gad1* SALK_047648 line and *gaba-t* SAIL_1230_C03 line, the biomass was larger in conditions of salt stress than in control conditions. The most prominent difference between these two conditions was in the case of *glyr2* SALK_047412C line, where the dry weight of salt-treated plants more than doubled (Fig. 36).

WC was calculated using fresh and dry weight. In control conditions, WC was higher only for both *gad1* lines, the remaining lines had lower WC compared to WT. In salt stress conditions, WC reached higher values than WT in the case of *gad1* (SALK_096492C), *gad2*, both *gad4*, *gad5*, *gaba-t* and both *glyr2* lines. Most of the lines had higher WC in control conditions than in salt stress conditions, the only exception was *gaba-t* line (Fig. 37).

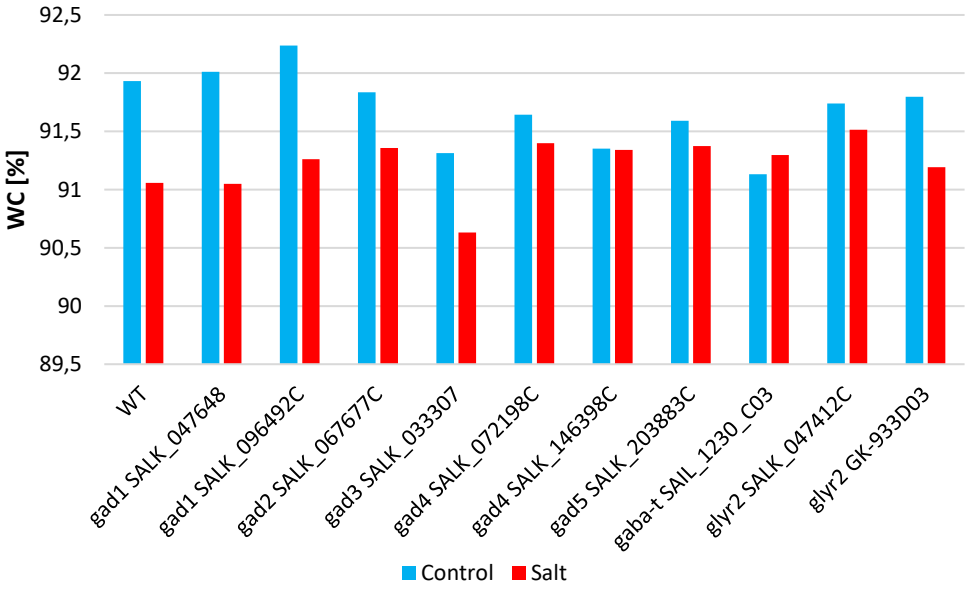

Figure 37. Water content percentage of *A. thaliana* lines with a T-DNA insertion in the genes of GABA metabolism (*GAD1–GAD5*, *GABA-T*, *GLYR2*) in control and salt stress conditions. WT, wild type.

In conclusion, *gad1* (SALK_096492C line), *glyr2* (SALK_047412C line) and *gad3* mutant plants showed the biggest difference in dry biomass during control and salt conditions; during salt stress the values were higher. *Gad3* mutants were affected the most, as the plants had the least biomass overall in both conditions. Only in *gad1* (SALK_047648 line) and *gaba-t* mutants the salt stress negatively affected biomass. However, *gad1* mutants also had the highest water content, whereas *gad3* mutants had the lowest water content.

6 DISCUSSION

The role of genes involved in the GABA metabolism has been studied in many plant species under biotic and abiotic stresses (Ramputh and Bown, 1996; Kinnersley and Turano, 2000; Fait *et al.*, 2005; Chevrot *et al*., 2006; Renault *et al.*, 2010; Mustroph *et al*., 2014; Faës *et al.*, 2015). Several of these studies focused on the effect of salt stress on GABA mutants (Renault *et al.*, 2010; Renault *et al*., 2013; Su *et al*., 2019). However, these studies are focused on a small number of lines. To the best of our knowledge, a deep and simultaneous plant phenotyping study of many of the GABA mutants hasn't been performed yet, neither under control nor salt stress conditions.

To shed light on the function of genes of the GABA metabolism during stress, mutant lines where these genes are disrupted are commonly used. Miyashita and Good (2008) used T-DNA insertion line SALK_047648 (*gad1*), Mekonnen *et al.* (2016) worked with the lines GK-474E05 (*gad2*) and GK-157D10 (*gaba-t*). Zarei *et al.* (2017b) chose the line SALK_146398C (*gad4*), Ludewig *et al.* (2008) used SAIL_1230_C03 (*gaba-t*) and Mekonnen and Ludewig (2016) worked with SALK_057410 (*glyr1*) and GK-933D03 (*glyr2*) in their study. Based on these articles, these lines were chosen for the experiment. The remaining lines were chosen with the insertion location in mind; insertion into exon was preferable. The insertion into intron still creates a knockout of the gene, but the T-DNA insert can be spliced out with the intron, which then leads to a WT transcript (Wang, 2008).

During the experiment, nine parameters were measured. As previously said, there is no similar study regarding the effect of salt stress on the growth and other phenotypic traits of GABA mutants to compare the results to, so only the results for WT could be compared. In our experiment, the rosette area of WT in control and salt conditions almost did not differ, whereas Awlia *et al.* (2016) observed smaller area in salt stress conditions than in control conditions. However, the growth parameters changed in some of the mutant lines, such as *gad1*, *gad4* and *glyr2*.

Regarding the other morphological parameters such as roundness1 and compactness, the WT values we obtained were lower compared to those showed by Pavicic *et al.* (2017), the rest of the parameters reached very similar values. However, the salt treatment did not modify these parameters; only for *gad3* line the morphological parameters varied compared to the rest of the lines. Further studies on this *gad3* mutant are needed because there is not enough information available about the role of this gene in the regulation of *Arabidopsis* rosette morphology.

In the experiment, for several genes there were two different mutant lines used – SALK_047648 and SALK_096492C for *GAD1*, SALK_146398C and SALK_072198C for *GAD4*, and SALK_047412C and GK-933D03 for *GLYR2*. It is interesting to note that in several parameters, the results of the two lines varied. That could be explained by the presence of multiple insertions in different loci, which could be causing the observed phenotype (O'Malley and Ecker,

2010). Another possibility is that the variability in phenotype depends on the position of the insertion, as suggested by Valentine *et al.* (2012) who reported great variation for traits among lines with T-DNA insertions in exons, introns and untranscribed regions (UTR) within a single locus.

Based on all results, *GAD1*, *GAD4*, *GABA-T* and *GLYR2* could be considered as genes regulating *Arabidopsis* growth, as the mutants in these genes showed either increase (in the case of *gad4* and *glyr2*) or decrease (in the case of *gad1* and *gaba-t*) in area. *Gaba-t* mutants being sensitive to stress corresponds to the findings of Renault *et al.* (2010), who reported that *GABA-T* is upregulated in WT and that the *gaba-t* mutants are oversensitive to salt stress. However, in a recent study, *gaba-t* mutants showed a salt-tolerant phenotype (Su *et al.*, 2019). The contrasting results could have been in part caused by the different *Arabidopsis* ecotype used – Renault *et al.* (2010) used plants of Ler background, while Su *et al.* (2019) worked with ecotype Col. In addition, between these two studies, there was a 2-fold difference in GABA content in the mutants, so different GABA concentrations could have had varying effect in the plants.

GAD1, GAD2 and GAD4 are calmodulin dependent and require Ca^{2+} for activation (Shelp and Zarei, 2017). Increase of Ca^{2+} concentration is the first response to salt stress, however, only *GAD2* and *GAD4* upregulation was reported under salt stress, while *GAD1* expression decreased (Knight *et al.*, 1997; Renault *et al.*, 2010; Zarei *et al.*, 2017b). In our experiment, the *gad4* knockout lines grew better than *gad1* knockouts, and no significant changes were observed for the *gad2* mutant line. There is a need for further studies on gene expression and also for research at metabolic level to be able to determine which of the GAD isoforms is most involved in plant stress tolerance.

GAD3 and GAD5 are calmodulin-independent (Shelp and Zarei, 2017). To our knowledge, there is no study focused on these two *GAD* genes. Our results did not show any relevant changes in the *GAD5* lines compared to the WT under both control and salt conditions. However, the results of *gad3* mutants were very atypical and in many cases they were different from the results of all the other genes – the *gad3* plants had the highest values for roundness1 and were the least eccentric, showed almost no variation in roundness2 and isotropy, and showed increased compactness values and decreased SOL values. That is interesting considering that *GAD3* is thought to express only in siliques, and its transcript was not detected upon salt stress treatment (Miyashita and Good, 2008; Renault *et al.*, 2010). It would be also interesting to know how the suppression of *GAD3* can affect GABA metabolism and the expression of the rest of the GAD isoforms.

In summary, this study corroborated that the genes involved in GABA metabolism are altered by salt stress, and the knockout of some of them can improve plant growth. Additionally, our results also showed that some of these genes (e.g. *GAD3*) can be involved in the phenotype (especially compactness and roundness) of the *Arabidopsis* plant. However, these data were only a preliminary experiment, but the results gave us promising data that will be validated with further experiments under long-term stress with different intensities of salt.

7 CONCLUSION

In this master's thesis, we demonstrated that non-invasive phenotyping is a good approach to study plant tolerance using *A. thaliana* mutants. From the selected homozygous plants with T-DNA insertion within the genes involved in GABA metabolism, *gad1*, *gad4*, *gaba-t* and *glyr2* lines were the ones that modified the plant growth under control and salt stress conditions. The *gad3* mutants presented different morphology of rosette form compared to WT and the rest of the mutant lines under control and salt stress conditions.

In conclusion, the acquired results show promise and the genes should be investigated in detail in the future. Another experiment with GABA mutants in control and salt conditions could be performed.

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