Laboratory of Plant Physiology

MSc Thesis Plant Physiology (PPH-80424)

Phenotyping and genotyping of Arabidopsis GABA related mutants in respect to seed quality

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Abstract

Seed quality is strongly affected by the environmental conditions during maturation stages, germination is a pivotal representation of seed quality. Seed germination is the most vulnerable stage to a number of adverse environmental conditions such as salt and heat stress. Interests in plant γ-aminobutyric acid (GABA) have increased due to its accumulation in response to a wide range of environmental stimuli. However, it is still not clear whether genetic manipulation of GABA levels can also influence or improve the seed germination performance. We tested the gene expression and germination performance of GABA related mutants. The results show that the Arabidopsis *GAD1* and *GAD2* are not expressed in the seeds. The good germination performance of *gad2-1* and *gad1/2* mutants might be influenced by the phenotype of mother plants during seed maturation. The *GLYR2-1* gene is not knocked out in *glyr2-1* mutant used in this study. The *POP2-5* and *SSADH-2* genes are indeed knocked out in *pop2-5* and *ssadh-2* related mutants. The GABA-enriched *pop2-5* knocked out mutants perform better in germination, when seed dormancy is removed by stratification.

Contents

1. Introduction

Seed quality, referring to the capability of seeds to germinate under various environmental conditions, is composed of different attributes, including genetic and physical purity, germination, dormancy, vigor and uniformity (Dickson 1980; Hilhorst and Toorop 1997; Hilhorst 2007; Hilhorst and Koornneef 2007; Hilhorst et al. 2010). Seed quality is one of the most important factors determining the success of crops, since it strongly determines the success of germination, seedling establishment, later growth and development of every agricultural crop (Finch-Savage 1995).

Seed quality is immensely affected by the environmental conditions during maturation stages. Seed maturation is an important phase of seed development during which embryo growth terminates, food reserves accumulate, and desiccation and seed dormancy are acquired. Besides temperature and light, nutrient availability to the maternal plant during seed maturation could potentially affect seed production and ultimate seed quality (Roach and Wulff 1987; Donohue 2009).

Nitrate is an important nitrogen source of plants, but also a signal molecule that regulates seed dormancy. Alboresi et al. (2005) revealed that the depth of seed dormancy of Arabidopsis is inversely correlated with seed nitrate content. Higher nitrate concentrations (50mM) favoring nitrate accumulation in mother plants and in seeds led to a lower dormancy of seeds than seeds produced under standard nitrate concentrations (10mM). He et al. (2014) also showed that nitrate deficiency (0mM) during seed maturation after dispersal decreased germination rate, and decreased germination in mannitol. Nitrate probably affects seed dormancy by enhancing gibberellins (GAs) synthesis and signaling, and/or by reducing abscisic acid (ABA) levels in Arabidopsis seeds (Hilhorst and Karssen 1988; Ali-Rachedi et al. 2004; Matakiadis et al. 2009). ABA and GAs are two major hormones in determining dormancy/germination of seeds: ABA is a key hormone promoting dormancy, whereas GAs promote germination (Koornneef et al. 2002; Finkelstein et al. 2002; Yamaguchi and Kamiya 2002).

Phosphorus, in the form of inorganic phosphate, is another important macronutrient for growth and development of plants (Kornberg et al. 1999; Yang and Finnegan 2010; Dick et al. 2011). Phytate is the main storage form of phosphate in dry seeds (Zhao et al. 2008). He et al. (2014) reported that the level of phytate increased in high phosphate maturation environment, and increasing phosphate content accordingly increased seed germination in stress conditions.

Germination is a pivotal representation of seed quality. In many crops, seed germination and early seedling growth are the most sensitive stages to a number of adverse environmental

conditions such as water deficit, high salinity, and extreme temperature (Jacab et al.2005; Demir et al. 2006; Zhao et al. 2007; Jisha et al. 2013). Drought or salinity causes a variety of biochemical, physiological and metabolic changes, which are responsible for both inhibition or delayed seed germination (Almansouri et al. 2001; Xiong and Zhu 2002). Salinity may influence seed germination either by generating a lower osmotic potential external to the seed preventing water uptake, or through the toxic effects of Na^+ and Cl^- ions on the germinating seed (Khajeh-Hosseini et al. 2003). Heat stress differentially affects the stability of various proteins, membranes, and alters the efficiency of enzymatic reactions in the cell for which the major physiological processes obstacle and creates metabolic imbalance (Hasanuzzaman et al. 2013). Inhibition of seed germination often occurs in high temperature through induction of ABA (Essemine et al. 2010).

Although seed germination is vulnerable to abiotic stress, plants gain resistance to abiotic stress by reprogramming metabolism and gene expression (AL-Quraan et al. 2013). The activation of various defense mechanisms results in a qualitative and/or quantitative change in plant metabolite production, activation of hormone signaling pathways, as well as reactive oxygen species (ROS) signaling pathways (Fujita et al. 2006). Among stress responsive metabolites, γ-aminobutyric acid (GABA) is of special interest due to its accumulation in response to a wide range of environmental stimuli in Arabidopsis and many other species (Shelp et al. 1999; Kinnerlsey and Turano 2000; Allan et al. 2008). GABA is a four-carbon, ubiquitous, non-proteinogenic amino acid, produced in the GABA shunt, a short pathway bypassing two steps of the tricarboxylic acid (TCA) cycle (Fait et al. 2008; Michaeli and Fromm 2015; Bown and Shelp 2016)(Fig. 1). Environmental stresses increase GABA accumulation through H^+ or Ca^{2+}/calmodulin activation of glutamate decarboxylase (GAD), which catalyses the decarboxylation of glutamate to GABA (Shelp et al. 2012). Catabolism of GABA occurs by the action of GABA transaminase (GABA-T) to produce succinic semi-aldehyde (SSA) (Clark et al. 2009; Shelp et al. 2012). Subsequently, SSA is converted by SSA dehydrogenase (SSADH) to succinate as a component of the TCA cycle (Bouché et al. 2003a; Shelp et al. 2012). Alternatively, SSA can be converted to γ-hydroxybutyric acid (GHBA) by NADPH-dependent glyoxylate/SSA reductases (GLYR) (Allan et al. 2011).

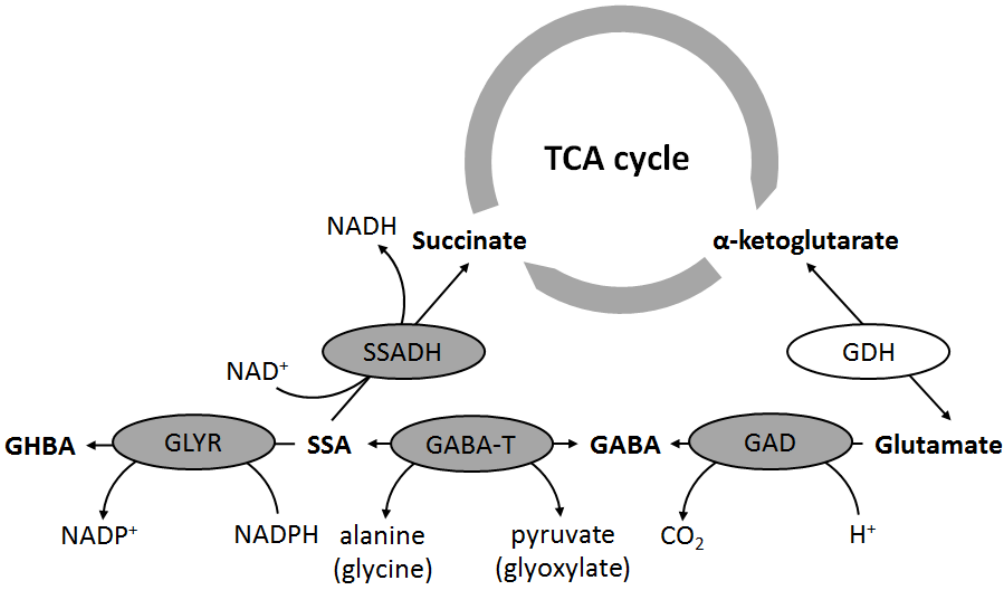

Fig. 1. Schematic representation of the GABA shunt. A component of the TCA cycle, α-ketoglutarate, serves as a precursor of glutamate through glutamate dehydrogenase (GDH) (Bown and Shelp 1997). GABA is mainly produced by the irreversible reaction of the GAD that consumes a proton and releases CO_2 (Baum et al. 1993; Fait et al. 2008). GABA-T converts GABA to SSA, using both pyruvate and glyoxylate (Clark et al. 2009). Subsequently, SSA is converted either to succinate or GHBA (Image source: Renault et al. 2010; Michaeli and Fromm 2015; Brikis et al. 2017, adapted).

Recent findings implicate the direct involvement of GABA in the signaling pathway and its potential role in plant defence against abiotic stress, since the disruption of certain GABA shunt elements may alter GABA levels and impair GABA-associated physiological processes (Bown and Shelp 2016; Ramesh et al. 2016). In Arabidopsis genome, two paralogs, *GAD1* and *GAD2*, are responsible for more than 90% of GABA production, and knockout of these two copies dramatically reduced the GABA content (Mekonnen et al. 2016). The *gad1/2* mutant plants are wilted earlier than the wild type following a prolonged defect in stomata closure under drought conditions (Mekonnen et al. 2016; Mekonnen 2017). Arabidopsis genome contains only one GABA-T encoding gene, subsequently termed *POP2* (*Pollen-Pistil Incompatibility 2*) (Palanivelu et al. 2003). The loss-of-function *pop2* mutant is shown to be oversensitive to ionic stress in spite of more than tenfold GABA levels in its tissue, suggesting a specific role in salt tolerance (Renault et al. 2010; Ramesh et al. 2016). However, the GABA-enriched *gad1/2*×*pop2* triple mutant behave like a wild type following drought stress, indicating the suppression of the *gad1/2* phenotype (Scholz et al. 2015; Mekonnen et al. 2016). The disruption of single *SSADH* gene in Arabidopsis causes necrosis, constant higher GABA and H_2O_2 over accumulation, and leads to hypersensitivity to light and heat stress (Bouché et al. 2003b; Fait et al. 2005; Miyashita and Good 2008). Interestingly,

crossing *ssadh* with *pop2* generates *pop2*×*ssadh* line that has higher GABA levels, rescues the severe phenotype of *ssadh* mutants (Ludewig et al. 2008). Plant GLYRs are considered to detoxify harmful reactive aldehydes into their corresponding less toxic alcohols, thereby preserving plant health during various abiotic stresses (Allan et al. 2009). In Arabidopsis, *GLYR* gene expression could increase under stress conditions, resulting in the diversion of SSA from succinate production to GHBA (Brikis et al. 2017). Allan et al. (2011) reported that the submergence-induced accumulation of GHBA is decreased in both Arabidopsis *glyr1* and *glyr2* single knocked out mutant.

The evidence that GABA shunt plays a central role in plant stress tolerance suggests that GABA may also be a promising metabolite for improving the stress resilience regarding seed quality and germination. Seed maturation is associated with significant decreases in sugar, organic acid, and amino acid levels (Fait et al. 2006). The glutamate to GABA conversion during seed development has a profound effect on the carbon (C) and nitrogen (N) balance, seed storage reserve accumulation, and germination performance (Fait et al. 2011). For example, GABA could possibly be used to mitigate the reduction in N under nitrate deficiency maternal environment, and consequently contribute to the C/N balance (Fait et al. 2008; He et al. 2016). Moreover, exogenously applied GABA can substantially improve seed acclimation to salinity, as evidence by increased germination rate in citrus seeds (Ziogas et al. 2017). Nevertheless, it is still not clear whether genetic manipulation of GABA levels can be used to enhance seed quality, the ability to cope with maternal nutrient starvation or abiotic stress during germination.

The main objective of this research is phenotyping and genotyping of Arabidopsis GABA related mutants in respect to seed quality. In this way, we aim to understand the role of GABA shunt elements on seed quality, under different maternal nutrient availability and germination conditions.

2. Materials and Methods

2.1 Plant Materials

The *Arabidopsis thaliana* Columbia (Col-0, Col) was used as wild type (WT, CS60). Seven GABA shunt related mutants with the Col genetic background were used in this study: *gad1-1*, *gad2-1* (Mekonnen et al. 2016), *pop2-5* (Renault et al. 2010), *glyr2-1* (Allan et al. 2012), the *gad1/2* double mutant (Mekonnen et al. 2017), the *pop2-5*×*ssadh-2* double mutant (Ludewig et al. 2008), and the *gad1/2*×*pop2-5* triple mutant (Mekonnen et al. 2016). These mutants were obtained by T-DNA insertion in the promoter region of *GAD1* (At5g17330), *GAD2* (At1g65960), *POP2* (At3g22200), *SSADH* (At1g79440), and *GLYR2* (At1g17650).

2.2 Growth Conditions

Seeds were sown in petri dishes on two sheets of water-soaked filter paper followed by a 4-d cold treatment at 4°C, and transferred to a climate room at 22°C with continuous light for 3 d before planting. Germinated seeds were grown on 4 × 4 cm Rockwool blocks in a growth chamber at 20°C/18°C (day/night) under a 16-h photoperiod provided by white fluorescent tubes (150 µmol $m^{-2}s^{-1}$), and 70% relative humidity. Plants were grown in a standard nutrient solution and watered three times per week. Upon the start of flowering, five nutrient solution treatments were applied: standard nutrient solution as control (Hyponex nutrient solution; N:P:K=7:6:19; http://www.hyponex.co.jp), low nitrate (0 mM), high nitrate (20 mM), low phosphate (0.0125 mM), and high phosphate (3 mM); for each treatment there were two biological replicates containing four plants per genotype per replicate (Supplemental Table 1).

2.3 RNA Extraction and cDNA Synthesis

RNA was isolated from seeds (eight lines) with the hot-borate extraction method modified from Wan and Wilkins (1994). For each sample, 5 mg of seeds (or three leaf blades/ten seedlings) were added to 2 ml tubes containing one iron ball. The material was frozen in liquid nitrogen and ground at 2000 rpm for 1 min with a dismembrator. After that, 800 µmol XT-buffer was added to the samples. XT-buffer was freshly prepared by adding 60 mg ml^{-1} polyvinylpyrrolidone and 2 mg ml^{-1} dithiothreitol to 80°C pH 9.0 XT-buffer (0.2 M Na borate decahydrate; 30 mM ethylene glycol tetraacetic acid; 1% sodium dodecyl sulfate; 1% Na deoxycholate; Sigma-Aldrich). After vortexing the samples, 4 µl freshely prepared Proteinase

K solution (3 mg μl^{-1}; Sigma-Aldrich) was added. After incubating the samples at 42°C water bath for 15 min, 64 µl 2 M KCl was added. After incubating the samples for 30 min on ice, the tubes were centrifuged at 12,000 g for 20 min at 4°C (Heraeus Fresco 17 Centrifuge; Bio-Rad). After that, the supernatant was transferred to a new tube and added with 259 µl ice-cold LiCl. The samples were subsequently incubated on ice in the coldroom overnight.

On the next day, the tubes were centrifuged at 12,000 g for 20 min at 4°C and the supernatant was discarded. The pellets were dissolved in 750 µl ice-cold 2 M LiCl and subsequently centrifuged at 12,000 g for 10 min at 4°C. Again the supernatant was removed, and the pellets were dissolved in 80 µl diethylpyrocarbonate (DEPC; Sigma-Aldrich) treated Milli-Q water, and supplemented with 10 µl DNase buffer and 10 µl DNase (Promega). After incubating the samples for 30 min at 37°C, the samples were added with 100 µl phenol chloroform (1:1; Sigma-Aldrich) and vortexed. A 200 µl RNA/phenol mixture was transferred to the phaselock. After spinning the phaselock in a table centrifuge (Eppendorf 5415C Micro Centrifuge Gray; Thermo-Scientific) at maximum speed for 5 min, 90 µl of upper phase was transferred to a new eppendorf containing 9 µl NaAC and 225 µl ice-cold absolute ethanol. After precipitating for 2 h at -20°C, the samples were centrifuged at maximum speed for 20 min at 4°C. The supernatant was removed and 250 µl cold 70% ethanol was added, followed by centrifuging at maximum speed for 5 min at 4°C. After drying, the pellets were dissolved in 20 µl DEPC Milli-Q water. RNA samples were measured on the nanodrop-1000 (Thermo-Scientific) to determine the concentration and quality. RNA samples were then added to a gel-electrophoresis to ensure that the RNA was intact and free of DNA (Fig. 2).

For copy-DNA (cDNA) synthesis, 750 ng RNA was transferred to PCR eppendorf strips and DEPC water was added to an end volume of 15 µl. After that, 4 µl cDNA buffer and 1 µl reverse transcriptase (iScript Selecet cDNA Synthesis Kit; Bio-Rad) were added. The samples were subsequently incubated according to a program of: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C in the S1000 Thermal Cycler (Bio-Rad). After the reaction, 180 µl Milli-Q water was added to dilute the samples (Fig. 2).

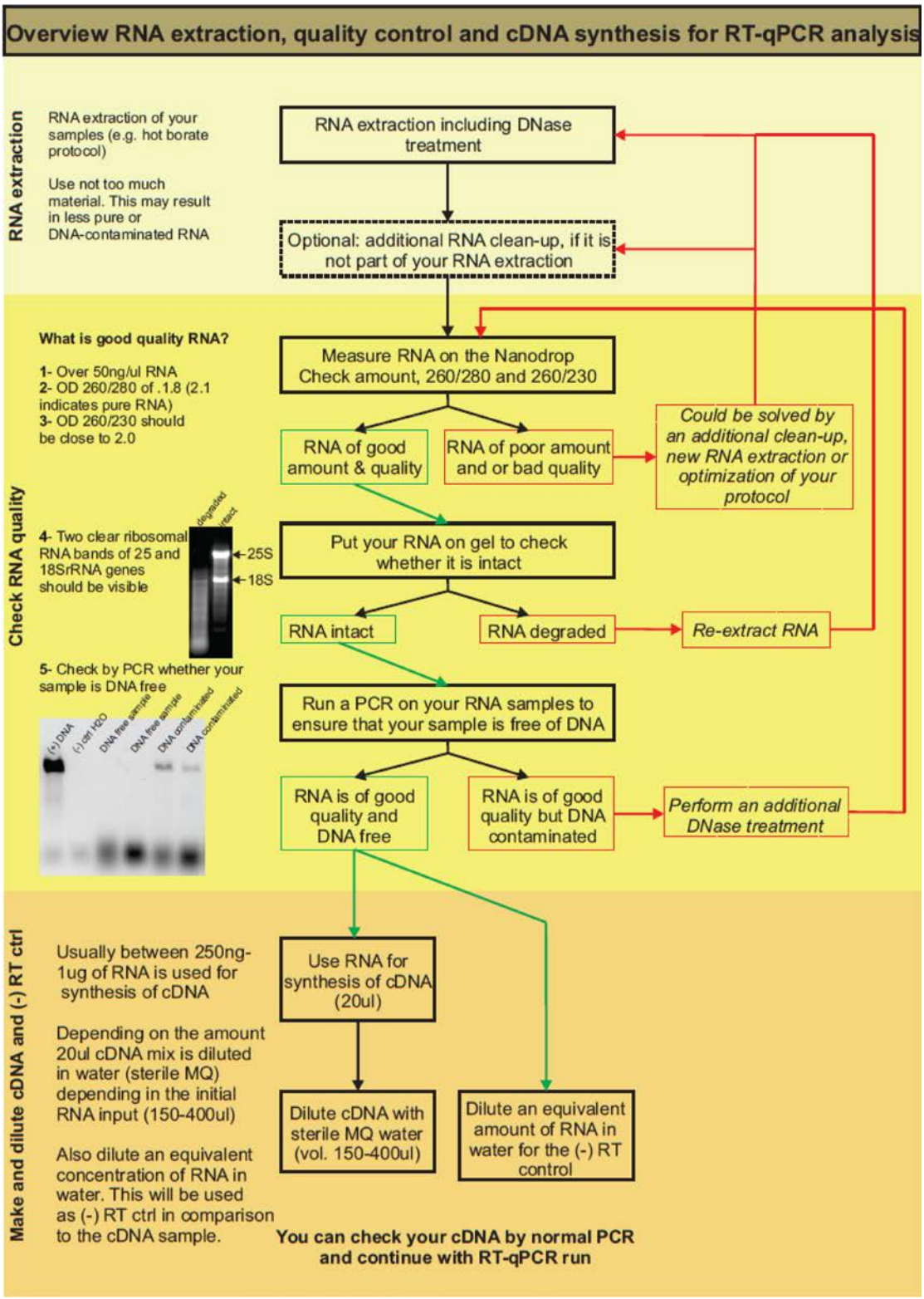

Fig. 2. Overview of RNA isolation of RT-qPCR. The quality of the samples was ensured by this step-by-step procedure including RNA extraction, quality control experiments, and cDNA synthesis (Image source: unpublished file by Dekkers 2014).

2.4 Primer Design and Primer Efficiency

In order to design the primers for mutant analysis, coding DNA sequence and genomic sequence information of five Arabidopsis GABA shunt related genes (*GAD1* (At5g17330), *GAD2* (At1g65960), *POP2* (At3g22200), *SSADH* (At1g79440), and *GLYR2* (At1g17650)) was retrieved from The Arabidopsis Information Resource (http://arabidopsis.org). Primer design was executed by alignment analysis and subsequent primer analysis in CLC-Bio (Version 7.6.2; CLC-Bio, Katrinebjerg, Denmark). The primers were designed to span an exon-exon junction, which aims to reduce the risk of false positives from amplification of any contaminating genomic DNA. Another strategy we used is to design primers near the 3' end, because mRNAs start to get degraded at the 3' end. Parameters that were used for primer design were: melting temperature 58-62°C and 18-22 base pairs long. Primers were ordered at Integrated DNA technologies (IDT; Leuven, Belgium) and dissolved in Milli-Q water to an end concentration of 100 µM (Primers information, see Supplemental Table 2). Primer mixes were prepared by mixing 10 µM of the forward and reverse primers each, where 80 µl Milli-Q water was added.

The functionality of the primers was checked by FIREPol PCR. For each reaction, the primer mix was added to a prepared Master mix (Supplemental Table 3A). The PCR programs were loaded in S1000 Thermal Cycler (Bio-Rad) (Supplemental Table 3B). 5 µl of PCR products were added with 1 µl loading buffer, and then loaded on a 1% agarose gel. The electrophoresis gel was run for 30 min at 80 Volt. All five primers were functional as shown in Supplemental Fig. 1.

The efficiency of the primers was measured by a qPCR reaction. The cDNA samples of all eight lines were combined (20 µl each; 160 µl in total), and diluted five times (0×, 2×, 4×, 8×, 16×, 32×). Each reaction contained 2.5 µl cDNA or diluted cDNA sample, 5 µl iQ Sybr Green supermix (Bio-Rad), 0.5 µl primer mix, and 2 µl Milli-Q water. The blank control contained 2.5 µl Milli-Q water instead of cDNA sample. Through the qPCR reaction, cDNA sample was amplified till a fluorescent signal was detected as cycle threshold. The linear correlation, between the cycle threshold value and the logarithmic scale of the executed dilutions, was fitted. The primer efficiency was calculated using the slope of the trendline, by the following formula:

$$Efficiency = \left(10^{-\frac{1}{slope}} - 1\right) \times 100\%$$

The quality of the primers was then determined. The ideal primer should have efficiency between 80-120%, and the R^2 of the trendline should above 0.98, and our primers all have high efficiency (Supplemental Table 4).

2.5 RT-qPCR

The RT-qPCR procedure was performed by mixing 2.5 µl cDNA sample with 5 µl iQ Sybr Green supermix (Bio-Rad), 0.5 µl primer mix, and 2 µl Milli-Q water. Samples were pipetted in duplicate in a 96-well plate (iQ 96-Well PCR Plates; Bio-Rad). Five Arabidopsis GABA shunt related genes and two reference genes were measured. The plate was placed in the MyiQ iCycler (Bio-Rad) and experienced the following reaction protocol: 3 min at 95°C, 39 steps of alternating between 10 s at 95°C and 30 s at 60°C (during these steps the data was collected in real-time), and then 1 min at 95°C; a melt curve analysis of 80 steps of each 10 s, during which the temperature was increased by 0.5°C per step, starting from 55°C. The relative gene expression was calculated from qPCR Data using a ΔΔCq calculation method.

2.6 Germination Assays and the GERMINATOR Package

In order to evaluate the germination characteristics for the GABA related mutants, germination assays were carried out. The germination assays were performed in plastic (15 × 21 cm) trays (DBP Plastics) containing 50 ml water or NaCl (125 mM) solution and two layers of blue filter paper (5.6’ × 8’ Blue Blotter Paper; Anchor Paper Company). Six samples of approximately 50-75 Arabidopsis seeds were dispersed on the filter paper using a mask to ensure an accurate and reproducible spacing. Clustering of seeds was prevented as much as possible. A maximum of 20 trays were piled with, on both the top and the bottom of the stack, two empty trays with 50 ml water and two layers of blue filter paper to ensure equal light distribution and prevent unequal evaporation. The whole pile was wrapped in a transparent plastic during the assay.

The seeds were treated with or without stratification after sowing. For the stratification treatment, the pile was placed at 4°C for 4 d before transferring to an incubator. The rest of the piles (without stratification) were placed in the incubator directly after sowing. In order to break the dormancy of seeds without using stratification, one extra treatment, supplementation of 10 mM KNO_3 and 10 µM GA in water or NaCl solutions, was used. The incubator (type 5042; Seed Processing Holland) provides light from three sides and was set to a temperature of 22°C, 30°C or 32°C. The combinations of stratification treatments and environmental conditions of the germination assays were shown in Table 1.

Table 1. Stratification and environmental conditions of the germination assays.

Stratification	**Environmental Conditions**
With Stratification	Water, 22°C (Control)
	Water, 30°C
	NaCl (125 mM), 22°C
Without Stratification	Water, 22°C (Control)
	Water, 30°C
	NaCl (125 mM), 22°C
Without Stratification,	Water, 22°C (Control)
KNO_3 (10 mM) and GA (10 µM)[1]	Water, 30°C
	Water, 32°C
	NaCl (125 mM), 22°C

[1] This treatment was only for the WT and the *pop2-5* related mutants.

The content of these trays, consisting of the blue filter paper with six samples of seeds, were manually photographed using a digital camera (Nikon D80 with Nikkor AF-S 60 mm f/2.8 G Micro ED; Nikon) twice a day after sowing. The blue filter paper was used to obtain optimal contrast between seed, radicle and filter paper. Completion of germination was defined as the protrusion of the radicle through the endosperm and seed coat, according to Bewley (1997). The digital photographs were automatically analysed and the germination performance was scored by using GERMINATOR package, developed by Joosen et al. (2010). The package mathematically fitted the germination-time curve using the four-parameter Hill function (4PHF) as the method described by (El-Kassaby et al. 2008). This function allowed extraction of biologically relevant parameters such as maximum percentage of germination (G_{max}), time to reach 50% of germination (t_{50}), the uniformity of germination (for example U_{7525}: time interval between 25% and 75% of viable seeds to germinate), and the integration of the area under the curve (AUC). AUC provided a value that enumerates those parameters and showed a high discriminative power between samples (Fig. 3).

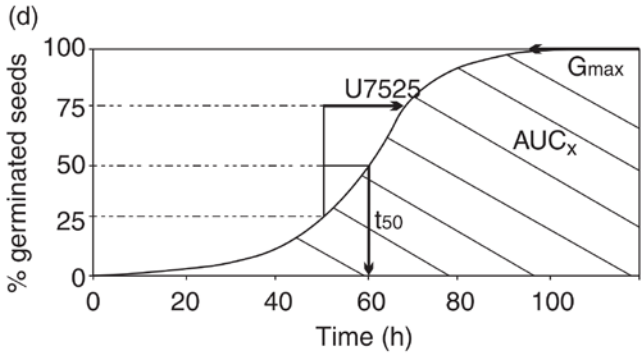

Fig. 3. Multiple germination parameters extracted from the curve fitting module. (Image source: Joosen et al. 2010)

3. Results

3.1 RNA Extraction

RNA samples (Code: 1-24) were isolated from the seeds of all eight lines in order to determine whether the target genes were indeed knocked out in certain mutants. However, Arabidopsis *GAD1* and *GAD2* were shown not expressed in the seeds according to the results of FIREPol PCR (Supplemental Fig. 1) and primer efficiency analysis (Supplemental Table 4), in line with the results of Zik et al. (1998) and the Arabidopsis eFP Browser (http://bar.utoronto.ca). Therefore, RNA samples (Code: 25-34) were isolated from the leaves of WT, *GAD1* and *GAD2* related mutants. Lastly, RNA samples (Code: 35-42) were isolated from the roots of WT and *GAD1* related mutant seedlings (seeds germinated under 4 d control conditions after 4 d cold stratification), since Arabidopsis *GAD1* was only expressed in roots.

The quality of our RNA samples was measured on the nanodrop (Table 2). The RNA concentration was high enough (around or more than 50 ng/µl) for cDNA synthesis. For pure RNA, the A260/A280 and A260/A230 ratios should be over 2.0 and 2.2, respectively. The A260 is the absorption peak of nucleic acids, whereas the A280 and A230 are the absorption peaks of proteins and carbohydrates. Our samples were very close to the optimal values, and therefore the contamination by proteins and carbohydrates can be ruled out.

Possible RNA degradation and DNA contamination were determined by adding RNA samples to a gel-electrophoresis. Non-degraded RNA should show two clear ribosomal RNA bands of 25 and 18 SrRNA genes (Fig. 2). In our RNA samples, these two bands were apparently visible, and therefore the RNA was not degraded (Fig. 4). Moreover, no upper band was shown below the origin slots, indicating that the DNase treatment worked and the RNA samples were free of DNA (Fig. 4).

Table 2A. Nanodrop concentrations of RNA samples (from seeds and leaves).

Sample		Replicate	Code	RNA (ng/µl)	A260/A280	A260/A230
Seed	CS60	1	1	96.0	2.05	2.17
	pop2-5		2	67.2	1.58	2.12
	glyr2-1		3	198.0	1.93	2.12
	gad1-1		4	211.7	2.01	2.30
	gad1/2		5	328.2	2.00	2.35
	gad1/2×pop2-5		6	314.9	2.03	2.31
	pop2-5×ssadh-2		7	266.5	2.02	2.10
	gad2-1		8	46.3	1.97	2.01
	CS60	2	9	232.1	2.03	2.25
	pop2-5		10	355.1	1.95	2.17
	glyr2-1		11	350.8	1.95	2.21
	gad1-1		12	171.2	1.99	2.28
	gad1/2		13	94.7	2.04	1.97
	gad1/2×pop2-5		14	236.9	1.95	2.27
	pop2-5×ssadh-2		15	339.5	2.03	2.31
	gad2-1		16	95.4	1.98	2.16
	CS60	3	17	220.6	2.09	2.43
	pop2-5		18	238.9	2.06	2.32
	glyr2-1		19	101.2	2.02	2.23
	gad1-1		20	117.4	2.06	2.38
	gad1/2		21	489.9	2.05	2.40
	gad1/2×pop2-5		22	134.5	2.04	2.22
	pop2-5×ssadh-2		23	151.4	2.06	2.40
	gad2-1		24	204.8	2.00	2.27
Leaf	CS60	1	25	348.3	2.02	2.39
	gad1-1		27	319.8	2.04	2.45
	gad1/2		29	313.7	2.01	2.41
	gad1/2×pop2-5		31	209.0	1.99	2.36
	gad2-1		33	384.0	2.06	2.42
	CS60	2	26	405.8	2.07	2.39
	gad1-1		28	189.3	2.08	2.51
	gad1/2		30	302.7	2.01	2.41
	gad1/2×pop2-5		32	896.3	2.07	2.36
	gad2-1		34	174.3	1.88	2.33

Table 2B. Nanodrop concentrations of RNA samples (from seedlings).

Sample		Replicate	Code	RNA (ng/µl)	A260/A280	A260/A230
Seedling	CS60	1	35	106.2	1.99	2.29
	gad1-1		36	25.6	1.63	2.10
	gad1/2		37	73.6	1.61	2.18
	gad1/2×pop2-5		38	50.6	1.72	2.10
	CS60	2	39	128.1	1.86	2.23
	gad1-1		40	40.2	1.65	2.27
	gad1/2		41	69.1	1.87	2.30
	gad1/2×pop2-5		42	132.7	2.04	2.29

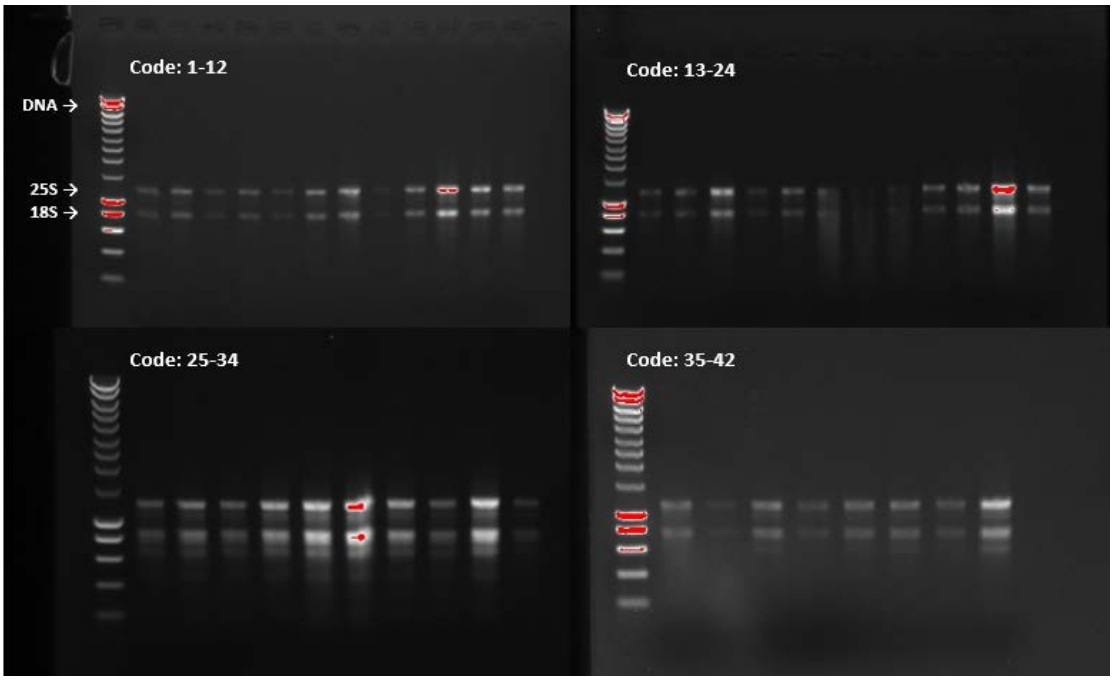

Fig. 4. Determining the quality of the RNA samples by gel-electrophoresis. The DNA band was inexistent, whereas the 25S and 18S ribosomal subunits were visible on gel. The code number of RNA samples was consistent with those in Table 2.

3.2 Gene Expression Analysis of GABA Shunt Related Mutants

We used 750 ng RNA to perform cDNA synthesis. With the diluted cDNA samples, we performed the RT-qPCR on two reference genes in order to normalize the expression data. Our target genes were *POP2* (At3g22200), *SSADH-2* (At1g79440), *GLYR2* (At1g17650), *GAD1* (At5g17330) and *GAD2* (At1g65960).

The relative *POP2* expression was very low in the seeds of Arabidopsis *pop2-5* mutant, *pop2-5*×*ssadh-2* double mutant, and *gad1/2*×*pop2-5* triple mutant (Fig. 5A). No significant difference in relative *POP2* expression was found between CS60 and the other mutants (*gad1-1*, *gad1-2*, *gad1/2*, and *glyr2-1*). For *SSADH-2*, almost no *SSADH-2* cDNA was present in the samples of *pop2-5*×*ssadh-2* seed, while the other mutants (*gad1-1*, *gad1-2*, *gad1/2*, *pop2-5*, *gad1/2*×*pop2-5*, and *glyr2-1*) produced higher amount of *SSADH-2* cDNA, equivalent to CS60 (Fig. 5B). The relative expression of *GLYR2* in *glyr2-1* seeds was lower than *gad2-1*, *gad1/2*, *gad1/2*×*pop2-5* and *pop2-5*, but no significant difference was found among *glyr2-1*, CS60, *gad1-1* and *pop2-5*×*ssadh-2* (Fig. 5C). The seeds of *gad2-1* mutant produced the highest level of *GLYR2* cDNA among all eight lines (Fig. 5C).

The relative *GAD1* expression was very low in the seedlings of Arabidopsis *gad1-1* mutant, *gad1/2* double mutant, and *gad1/2*×*pop2-5* triple mutant (Fig. 6A). The relative *GAD2* expression was very low in the leaves of Arabidopsis *gad2-1* mutant, *gad1/2* double mutant, and *gad1/2*×*pop2-5* triple mutant (Fig. 6B). In contrast, *GAD1* was abundantly expressed in WT seedlings, and *GAD2* was abundantly expressed in WT leaves, respectively (Fig. 6).

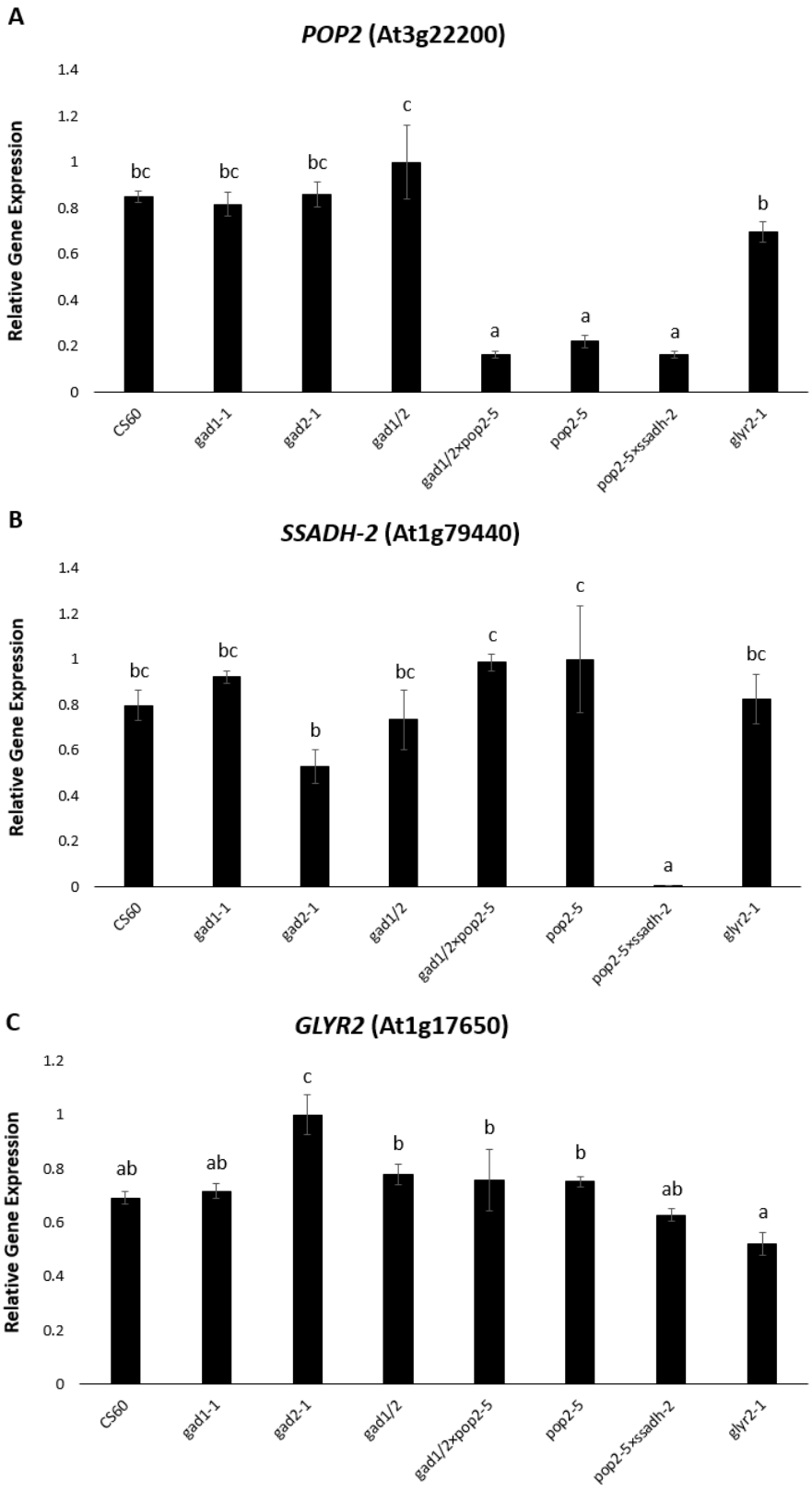

Fig. 5. Relative expression of *POP2* (A), *SSADH-2* (B) and *GLYR2* (C). The cDNA samples for RT-qPCR were synthesized from seed RNA. Error bars represent standard error. Different letters indicate significant differences according to LSD test ($P < 0.05$) calculated by one-way ANOVA.

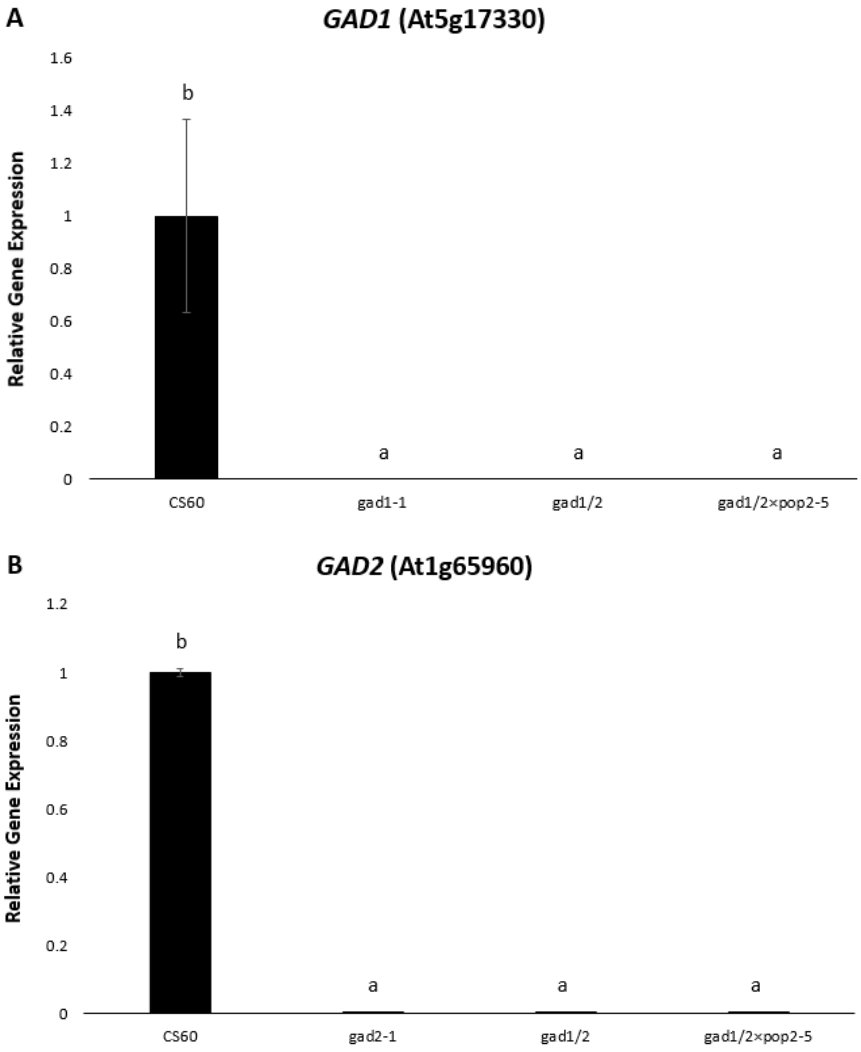

Fig. 6. Relative expression of *GAD1* (A) and *GAD2* (B). The cDNA samples for RT-qPCR were synthesized from seedling (for *GAD1*) and leaf (for *GAD2*) RNA. Error bars represent standard error. Different letters indicate significant differences according to LSD test ($P < 0.05$) calculated by one-way ANOVA.

3.3 Germination Assays

Due to the lack of time, the germination performance of Arabidopsis GABA shunt related mutants and WT was only investigated in the seeds produced under standard conditions. Almost all the seeds were germinated under standard germination conditions, no matter whether they were treated with 4 d stratification or not (Fig. 7; Fig. 9A; Fig. 10; Fig. 12A). When the seeds with stratification were germinated under salt conditions, the seeds of WT, *gad1-1*, *gad1/2* and *glyr2-1* mutants had lower G_{max} than under standard conditions (Fig. 7A). The seeds of *gad2-1* mutant, *gad1/2* mutant, and *pop2-5* knocked out mutants (*pop2-5*, *gad1/2*×*pop2-5* and *pop2-5*×*ssadh-2*) had higher maximum germination rate than the WT seeds under salt conditions, when treated with stratification (Fig. 7A).

When the seeds were not treated with stratification, the maximum germination rates were lower in all eight lines under salt conditions, than under standard conditions (Fig. 7B). No significant difference was found in G_{max} among WT, *gad* knocked out mutants and *glyr2-1* mutant (Fig. 7B). The *pop2-5* knocked out mutants performed worse than WT in G_{max} (Fig. 7B). Only 6% of the *pop2-5*×*ssadh-2* seeds were germinated, followed by *pop2-5* mutant (26%) and *gad1/2*×*pop2-5* triple mutant (63%) (Fig. 7B).

The seeds of all eight lines required more time to reach 50% of maximum germination rate under salt conditions, than under standard conditions, no matter whether they were treated with stratification or not (Fig. 8). When the seeds with stratification were germinated under salt conditions, the seeds of *pop2-5* knocked out mutant required less time to reach 50% of maximum germination rate, compared to the WT (Fig. 8A). The seeds of *gad1/2*×*pop2-5* triple mutant and *pop2-5* mutant had the least t_{50} maxG among all eight lines under salt conditions, with 35.4 h and 33.5 h, respectively (Fig. 8A).

When the seeds were not treated with stratification, no significant difference was found in t_{50} maxG between GABA shunt related mutants and WT, under salt conditions (Fig. 8B). However, the seeds of *gad1-1* mutant had less t_{50} maxG than *gad2-1* mutant, *gad1/2* double mutant, and *pop2-5* knocked out mutants under salt conditions (Fig. 8B).

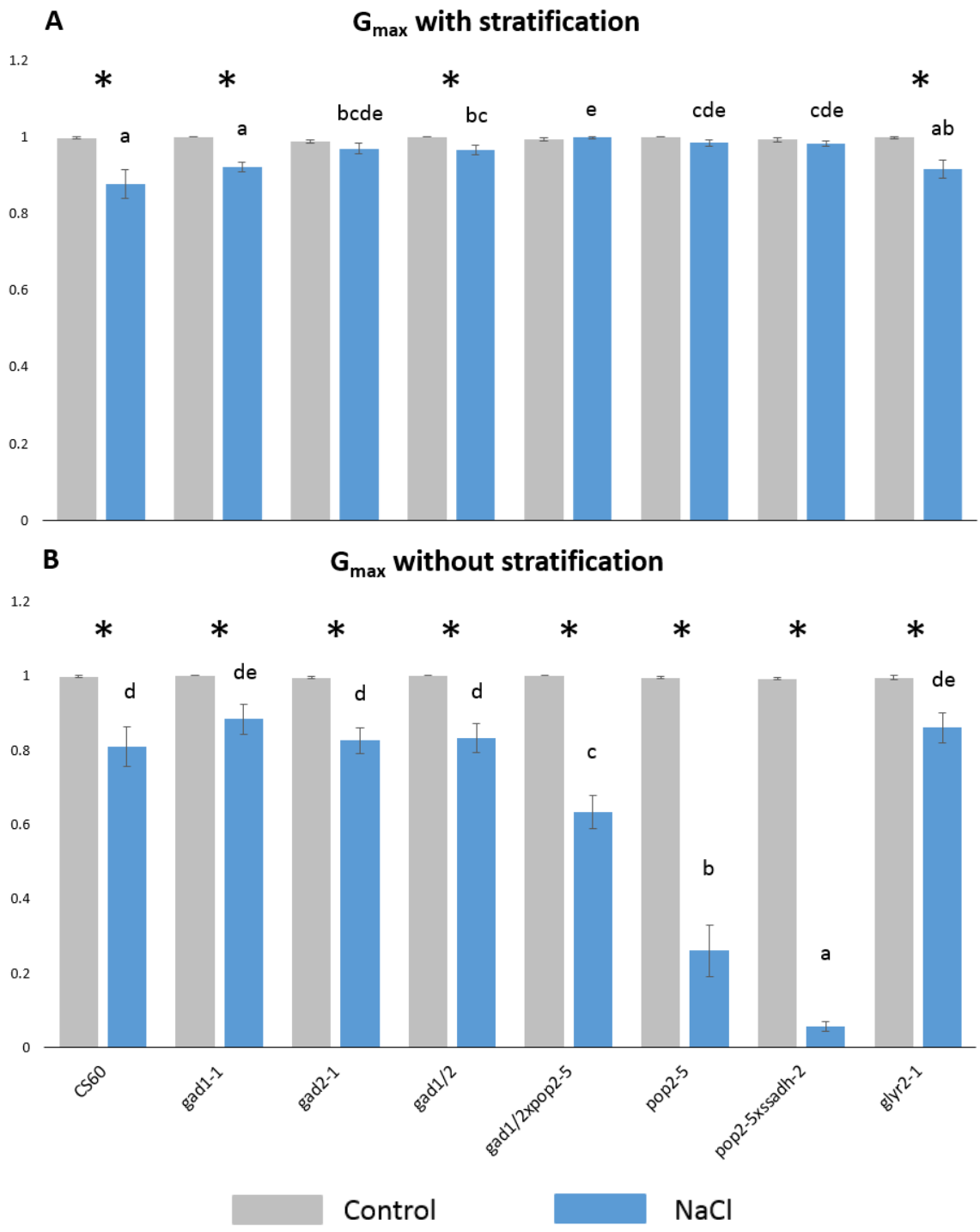

Fig. 7. The G_{max} of Arabidopsis GABA shunt related mutant and WT seeds, treated with (A) or without (B) stratification. The seeds were germinated under control (water, 22°C) and salt (125 Mm NaCl, 22°C) conditions. Error bars represent standard error. Asterisks indicate significant differences between control and salt treatment ($P < 0.05$). Different letters indicate significant differences among eight lines under salt treatment, according to LSD test ($P < 0.05$).

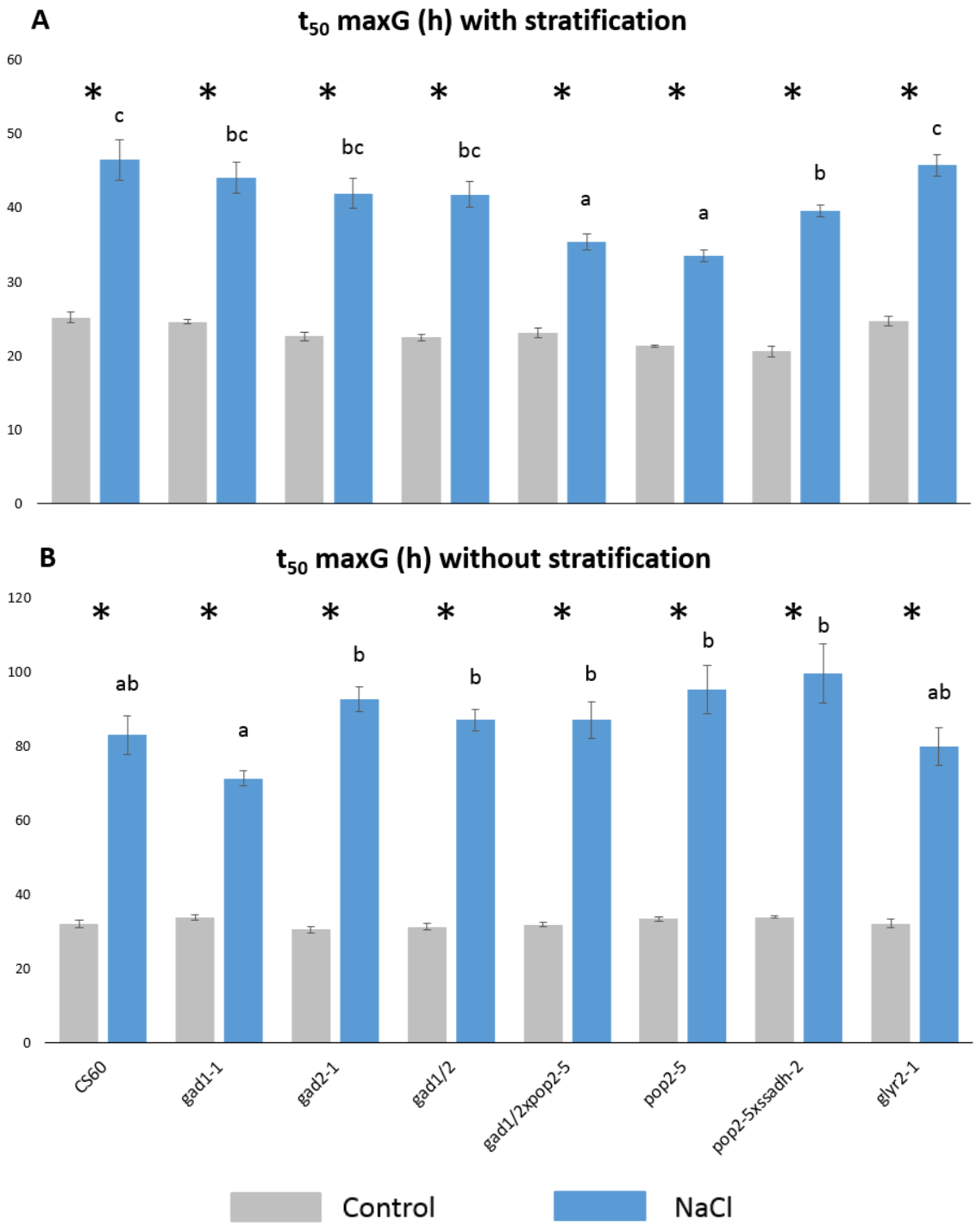

Fig. 8. The t_{50} maxG (h) of Arabidopsis GABA shunt related mutant and WT seeds, treated with (A) or without (B) stratification. The seeds were germinated under control (standard: water, 22°C) and salt (125 Mm NaCl, 22°C) conditions. Error bars represent standard error. Asterisks indicate significant differences between control and salt treatment ($P < 0.05$).

Different letters indicate significant differences among eight lines under salt treatment, according to LSD test ($P < 0.05$).

When the seeds of *pop2-5* knocked out mutants and WT without stratification were germinated under salt conditions supplemented with GA and KNO_3, theirs G_{max} increased compared to without GA and KNO_3 (Fig. 7B; Fig. 9A). Regarding G_{max}, only *pop2-5* and *pop2-5*×*ssadh2* mutants performed worse under salt conditions, than under standard conditions, when GA and KNO_3 were added (Fig. 9A). These two mutants also showed lower maximum germination rate than WT under salt conditions with GA and KNO_3 (Fig. 9A).

The non-stratified seeds of WT and *pop2-5* knocked out mutants required more time to reach 50% of maximum germination rate under salt conditions, when GA and KNO_3 were added (Fig. 9B). Under salt conditions with GA and KNO_3, the t_{50} maxG of non-stratified seeds increased according to the order of WT, *gad1/2*×*pop2-5*, *pop2-5* and *pop2-5*×*ssadh-2* (Fig. 9B).

The seeds of all eight lines were germinated under 30°C heat stress conditions when treated with 4 d stratification (Fig. 10A). The non-stratified seeds had lower G_{max} under 30°C heat stress conditions, than under standard conditions, in all lines except for *glyr2-1* mutant (Fig. 10B). The *glyr2-1* mutant showed the highest G_{max} among all eight lines under 30°C heat stress conditions without stratification (Fig. 10B). The *pop2-5* knocked out mutants had lower G_{max} than the other lines under 30°C heat stress conditions, without stratification (Fig. 10B). Among these mutants, the *pop2-5*×*ssadh-2* double mutant had the lowest G_{max}, followed by *pop2-5* mutant and then *gad1/2*×*pop2-5* triple mutant (Fig. 10B).

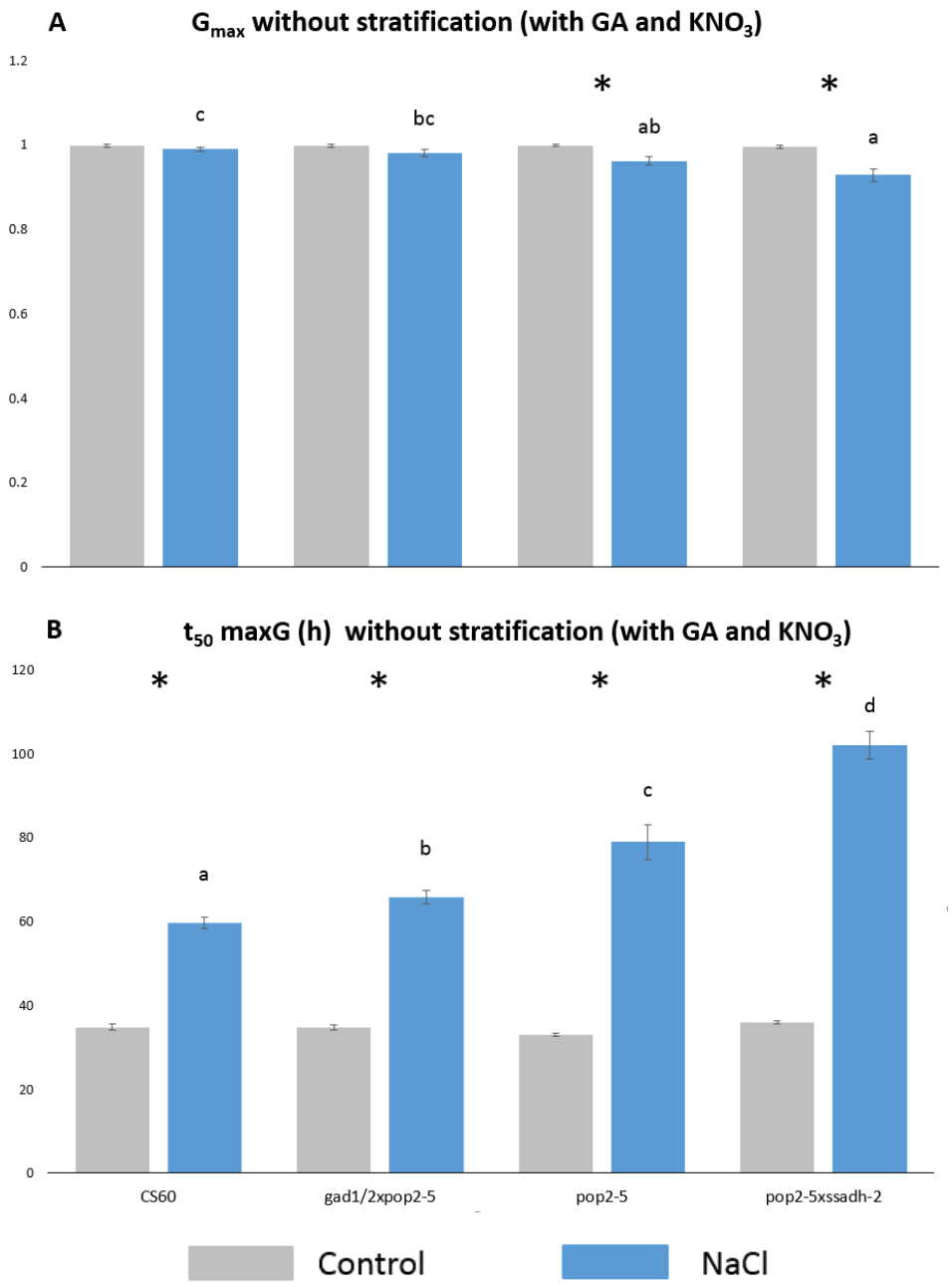

Fig. 9. The G_{max} (A) and t_{50} maxG (h) (B) of Arabidopsis *pop2-5* related mutant and WT seeds, treated with GA and KNO_3 without stratification. The seeds were germinated under control (standard: water, 22°C) and salt (125 Mm NaCl, 22°C) conditions. Error bars represent standard error. Asterisks indicate significant differences between control and salt treatment ($P < 0.05$). Different letters indicate significant differences among eight lines under salt treatment, according to LSD test ($P < 0.05$).

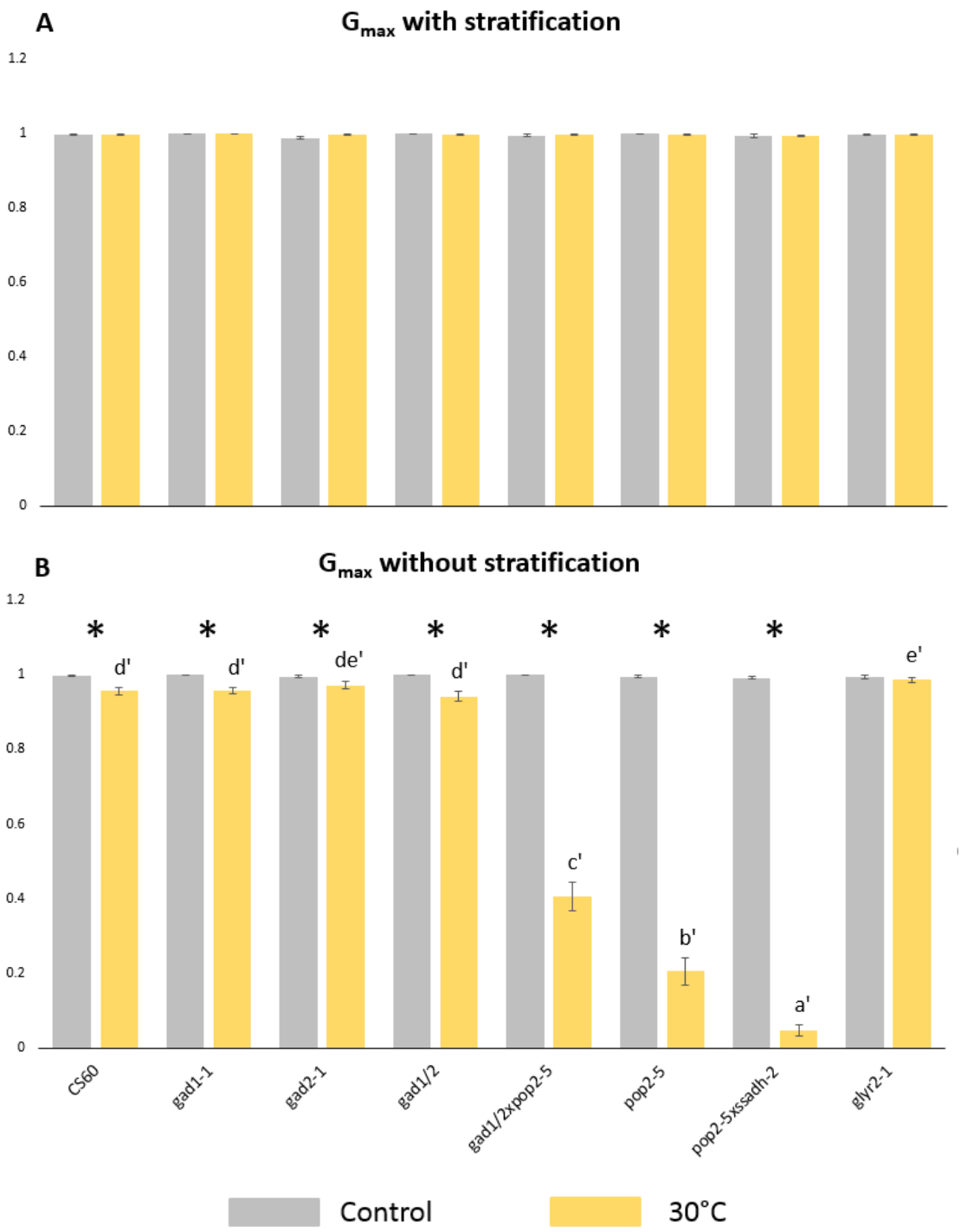

Fig. 10. The G_{max} of Arabidopsis GABA shunt related mutant and WT seeds, treated with (A) or without (B) stratification. The seeds were germinated under control (standard: 22°C) and high temperature (30°C). Error bars represent standard error. Asterisks indicate significant differences between control and 30°C (P < 0.05). Different letters indicate significant differences among eight lines under 30°C, according to LSD test (P < 0.05).

The stratified seeds of *gad2-1*, *gad1/2*, *pop2-5* and *pop2-5*×*ssadh-2* mutants required less time than the WT to reach 50% maxG under standard germination conditions (Fig. 11A). The 30°C heat stress conditions reduced t_{50} maxG in all eight lines with stratification, compared to the standard conditions (Fig. 11A). When germinated under 30°C heat stress conditions, the stratified seeds of *gad1/2*, *gad1/2*×*pop2-5*, and *pop2-5* mutants had lower t_{50} maxG than the WT (Fig. 11A).

Without stratification, only the seeds of *pop2-5* knocked out mutants had higher t_{50} maxG under 30°C heat stress conditions, when compared to standard germination conditions (Fig. 11B). When germinated under 30°C heat stress conditions, the non-stratified seeds of *gad2-1* and *gad1/2* mutants required less time to reach 50% of maximum germination rate than the WT (Fig. 11B). The longest t_{50} maxG was required by the non-stratified seeds of *pop2-5*×*ssadh-2* double mutant, followed by *pop2-5* mutant and *gad1/2*×*pop2-5* triple mutant, when germinated under 30°C heat stress conditions (Fig. 11B).

When the non-stratified seeds of *pop2-5* knocked out mutants and WT were germinated under 30°C heat stress conditions, the supplementation of GA and KNO_3 increased the G_{max} (Fig. 10B; Fig. 12A). Only the non-stratified seeds of *pop2-5*×*ssadh-2* double mutants showed lower G_{max} under 30°C heat stress conditions, than under standard conditions, when GA and KNO_3 were added (Fig. 12A). This mutant also had lower G_{max} than the WT, when they were germinated under both 30°C and 32°C heat stress conditions, without stratification, but with GA and KNO_3 (Fig. 12A). The non-stratified seeds of *pop2-5* mutant only showed lower G_{max} than WT, under 32°C heat stress conditions, when supplemented with GA and KNO_3 (Fig. 12A).

When the non-stratified seeds (with GA and KNO_3) were germinated under 30°C heat stress conditions, the t_{50} maxG was lower in WT, *gad1/2*×*pop2-5* triple mutant and *pop2-5* mutant, but higher in *pop2-5*×*ssadh-2* double mutant, than under standard conditions (Fig. 12B). The t_{50} maxG increased according to the order of WT, *gad1/2*×*pop2-5*, *pop2-5* and *pop2-5*×*ssadh-2*, except that there was no significant difference in t_{50} maxG between *gad1/2*×*pop2-5* and *pop2-5* under 32°C heat stress conditions (Fig. 12B).

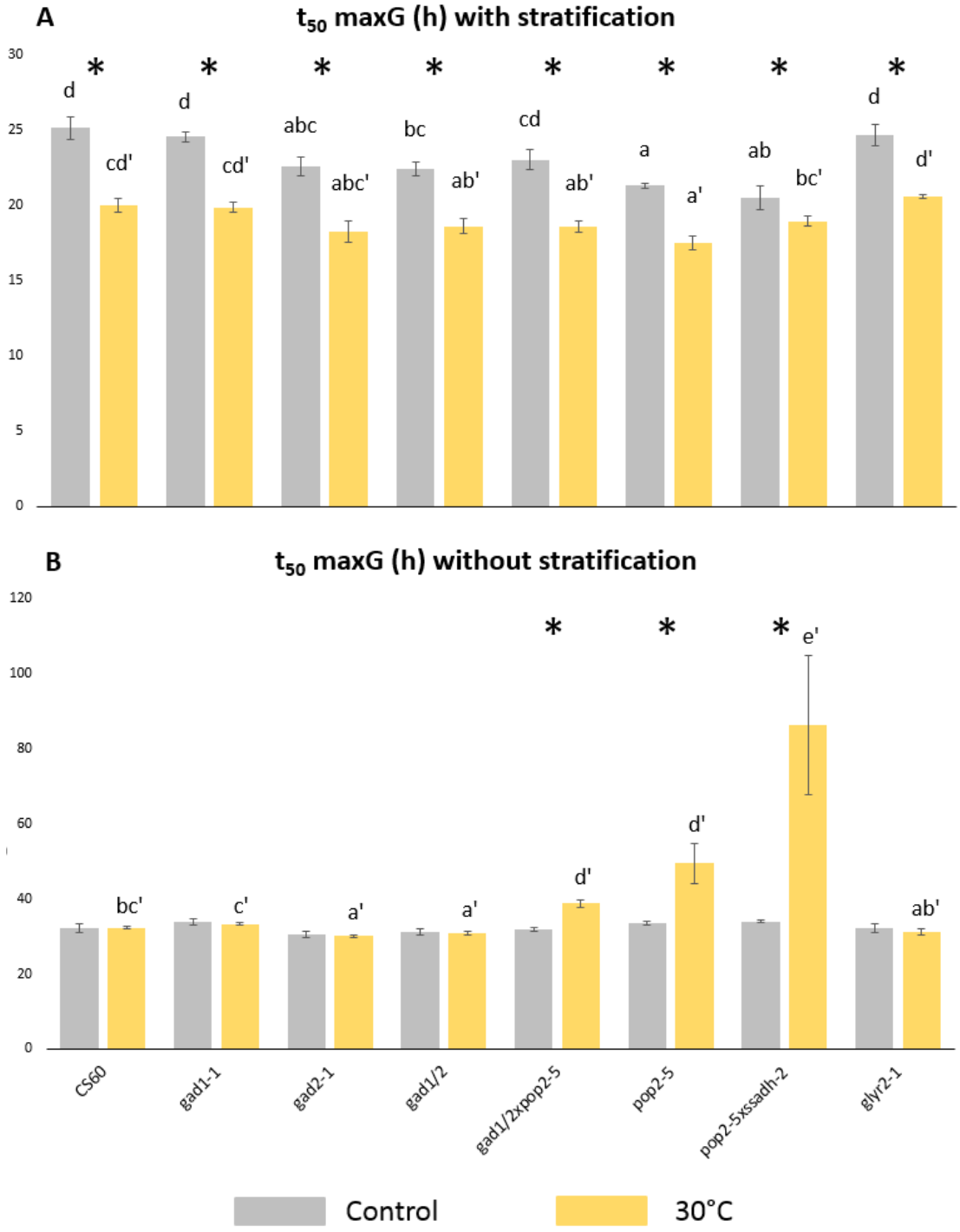

Fig. 11. The t_{50} maxG (h) of Arabidopsis GABA shunt related mutant and WT seeds, treated with (A) or without (B) stratification. The seeds were germinated under control (standard: 22°C) and high temperature (30°C). Error bars represent standard error. Asterisks indicate significant differences between control and 30°C ($P < 0.05$). Different letters indicate significant differences in control and in 30°C , respectively, according to LSD test ($P < 0.05$).

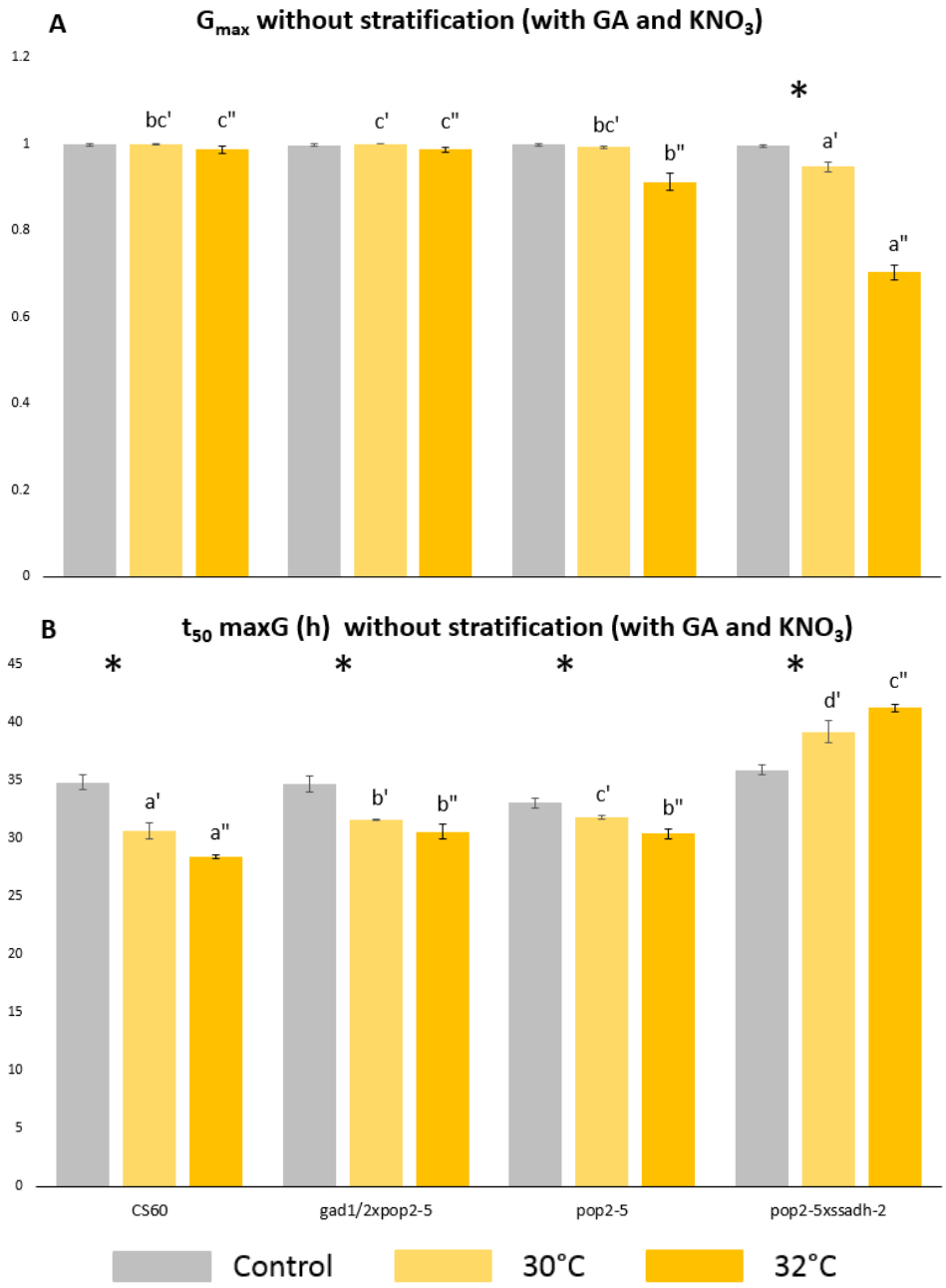

Fig. 12. The G_{max} (A) and t_{50} maxG (h) (B) of Arabidopsis *pop2-5* related mutant and WT seeds, treated with GA and KNO_3 without stratification. The seeds were germinated under control (standard: 22°C) and high temperature (30°C, 32°C). Error bars represent standard error. Asterisks indicate significant differences between control and 30°C ($P < 0.05$). Different letters indicate significant differences in 30°C and in 32°C , respectively, according to LSD test ($P < 0.05$).

4. Discussion

Interest in plant GABA has increased following the observations of GABA accumulation under different abiotic stresses (Michaeli and Fromm 2015). GABA is metabolized via a short pathway known as the GABA shunt (Fait et al. 2008; Michaeli and Fromm 2015; Bown and Shelp 2016). The role of GABA shunt elements (GAD, GABA-T, SSADH and GLYR) has been abundantly investigated in respect to plant physiology and development under stress conditions (Ludewig et al. 2008; Renault et al. 2010; Allan et al. 2011; Scholz et al. 2015; Mekonnen et al. 2016). These researches examined the phenotype of GABA related mutants, concluding that the GABA shunt plays a central role in plant stress tolerance (Bown and Shelp 2016). However, it is still not clear whether genetic manipulation of GABA levels can also influence or improve the seed germination performance. The aim of this study was to fill this knowledge gap. Therefore, the gene expression and germination performance of GABA related mutants were tested.

The accumulation of GABA under stress conditions was reported as the results from H^+ and Ca^{2+}/calmodulin activation of GAD, which catalyzes the synthesis of GABA from glutamate in the cytosol (Bown and Shelp 2016; Mekonnen et al. 2016). It was expected that the knockout of *GAD1* and *GAD2* genes would reduce the stress tolerance and seed germination performance, since these two copies are responsible for more than 90% of the GABA production (Mekonnen et al. 2016). However, these two genes were found not expressed in Arabidopsis seeds at all (Supplemental Fig. 1; Supplemental Table 4). According to the Arabidopsis eFP Browser, *GAD1* (At5g17330) is only expressed in roots, whereas *GAD2* (At1g65960) transcripts are detectable in almost all organs except for seeds. The *GAD1* and *GAD2* genes were indeed knocked out in corresponding lines (*gad1-1*, *gad2-1*, *gad1/2*, *gad1/2*×*pop*2-5), since the relative *GAD1* and *GAD2* expression were found extremely low in the seedlings or leaves of these lines (Fig. 6).

Although *GAD1* and *GAD2* are not expressed in Arabidopsis seeds, the knockout of these two genes (*gad2-1* and *gad1/2*) had higher G_{max} than WT under salt stress (Fig. 7A). The seeds of *gad2-1* and *gad1/2* knocked out mutants also germinated faster than WT under standard or heat stress conditions (Fig. 11). The good performance of *gad2-1* and *gad1/2* mutants was not expected, because the shoot GABA content of these two mutants was reported lower than *gad1* single mutant, and lower than WT (Bouché et al. 2004; Mekonnen et al. 2016). Moreover, the *gad1/2* mutants also showed early wilting, stomata malfunction and reduced shoot growth (Mekonnen et al. 2016). Our results suggest that the drought oversensitive phenotype of the *gad2-1* and *gad1/2* mutants may somehow contribute to seed quality during seed production and maturation. For example, the symptoms of withering and increased transpiration may be caused by stomata malfunction, resulting in

lowered ABA content in ripe seeds and leaves (Karssen et al. 1983). Dormancy of low-ABA seeds might have strongly reduced, and therefore these seeds performed better than WT.

Unlike *GAD*, the Arabidopsis *POP2* gene do express in seeds, and *pop2-5* mutant showed very low relative *POP2* expression (Fig. 5A). The product of *POP2* expression, GABA-T, catalyzes the first step of GABA catabolism, converting GABA to SSA (Clark et al. 2009; Shelp et al. 2012). We hypothesized that *pop2-5* mutant would show better germination behavior and salt tolerance due to high GABA levels in its tissue (Ramesh et al. 2016). Although *GAD* is not expressed in Arabidopsis seeds, GABA synthesis can still be realized by polyamine (putrescine and spermidine) degradation or non-enzymatic reactions (via proline) under stress conditions (Fait et al. 2008; Shelp et al. 2012; Signorelli et al. 2015). As expected, the *pop2-5* knocked out mutants displayed higher G_{max} and higher speed of germination than WT, under salt conditions, when stratification was applied (Fig. 7A; Fig. 8A). The non-stratified *gad1/2×pop2-5* and *pop2-5* seeds also showed similar G_{max}, but higher speed of germination than WT, under heat stress (both 30°C and 32°C), when treated with GA and KNO_3 (Fig. 12).

Interestingly, when the seeds were treated neither with stratification nor GA and KNO_3, the G_{max} of *pop2-5* knocked out mutants sharply reduced under salt and 30°C heat stress conditions, which did not occur in WT seeds (Fig. 7B; Fig. 10B). Applying GA and KNO_3 can mitigate this reduction in non-stratified *pop2-5* seeds (Fig. 9; Fig. 12). However, the *pop2-5* knocked out mutants still showed lower germination speed than WT under salt stress, when non-stratified seeds were treated with GA and KNO_3 (Fig. 9B). The aim of using stratification or GA/KNO_3 treatment is to release seed dormancy (Bewley et al. 2013). Our results indicate that the GABA-enriched seeds performed better than WT under salt or heat germination conditions, only when the dormancy of imbibed seeds had been fully removed by stratification or GA/KNO3 treatment. By contrary, if the seed dormancy was not broken by certain treatments, the GABA-enriched seeds performed even worse than WT under stress conditions. This finding gives an inspiration that exogenous GABA can be supplemented to the seed priming treatment. Seed priming is the induction of a particular physiological state in plants by the treatment of natural and synthetic compounds to the seeds before germination (Jisha et al. 2013). Further research is required to investigate the optimal combination of exogenous GABA concentrations and priming strategies, in order to maximize seed germination performance under stress conditions.

The GABA catabolic enzyme SSADH is also highly expressed in response to stress (Michaeli et al. 2011). The Arabidopsis *ssadh* mutants are significantly dwarfed, a phenotype that can be rescued by a second mutation in *POP2* (Ludewig et al. 2008). The *pop2×ssadh* double mutant has higher GABA levels in tissue, and can rescue hypersensitivity of *ssadh* single mutant to light and heat stress (Ludewig et al. 2008). The *SSADH* gene expression was very low in

pop2-5×*ssadh-2* double mutant used in this study (Fig. 5B). This double mutant performed worse than *pop2-5* single mutant in G_{max} and t_{50} maxG, under salt or heat stress without stratification (Fig. 7B; Fig. 9B; Fig. 10B; Fig. 11B; Fig. 12). In particular, the *pop2-5*×*ssadh-2* double mutant showed extremely low G_{max} and low speed of germination under heat stress without stratification, when compared to the other seven lines (Fig. 10B; Fig. 11B; Fig. 12). These results indicate that the second mutation in *SSADH* will make *pop2-5* mutant worse in germination and hypersensitive to heat stress. This severe phenotype might be cause by SSA and/or GHBA accumulation, resulting in reactive oxygen intermediates production and cell death in response to heat stress (Ludewig et al. 2008).

The Arabidopsis plants exposed to heat and salinity stress could have elevated expression of *GLYR*, resulting in the diversion of SSA from succinate production to GHBA (Allan et al. 2008). This pathway can detoxify the harmful reactive aldehydes SSA into their less toxic alcohols, thereby mitigating severe phenotype caused by abiotic stresses (Allan et al. 2009; Brikis et al. 2017). No significant difference was found in relative *GLYR2* expression between *glyr2-1* mutant and WT, indicating that the *GLYR2* gene was not knocked out in *glyr2-1* mutant used in this study (Fig. 5C). The *glyr2-1* seeds also showed similar germination patterns as the WT, under standard or stress conditions, except that *glyr2-1* seeds had higher G_{max} than WT under 30°C heat stress without stratification (Fig. 10B).

The germination performance can also be influenced by nutrient availability to the maternal plant during seed maturation (Roach and Wulff 1987; Donohue 2009). Unfortunately, the seeds produced under different maternal nutrient availability were not ready yet for the germination assay at the end of this study. We hypothesize that the phosphorus levels will not have any interaction with GABA levels, whereas GABA-enriched mutant may mitigate the reduction in N under nitrate deficiency maternal environment (Fait et al. 2008). Moreover, other seed quality parameters should also to be taken into consideration. For example, the seed size is positively related to initial seedling size, and larger seeds have more food reserves which is favorable to germination (Khan et al. 2012). Both maternal nutrient availability and genetic (GABA related genes knocked out) may strongly influence the seed size and thereby the germination performance.

5. Conclusions and Future Work

The Arabidopsis *GAD1* and *GAD2* are not expressed in the seeds. The good germination performance of *gad2-1* and *gad1/2* mutants might be influenced by the phenotype of mother plants during seed maturation. Further research is needed to confirm this hypothesis, by testing for instance the ABA levels in the seeds of *GAD* mutants.

The *GLYR2-1* gene is not knocked out in *glyr2-1* mutant used in this study. The effects of GLYR deficiency on seed germination should be further tested in another T-DNA insertion line. The *POP2-5* and *SSADH-2* genes are indeed knocked out in *pop2-5* and *ssadh-2* related mutants used in this study. The GABA-enriched *pop2-5* knocked out mutants perform better in germination, when seed dormancy is removed by stratification. The application of these results, for instance seed priming, should be further investigated.

The germination performance of GABA related mutants produced under different maternal nutrient availability need to be tested. The seed size and GABA concentration in seeds can be analyzed to confirm the effects of GABA on seed quality.

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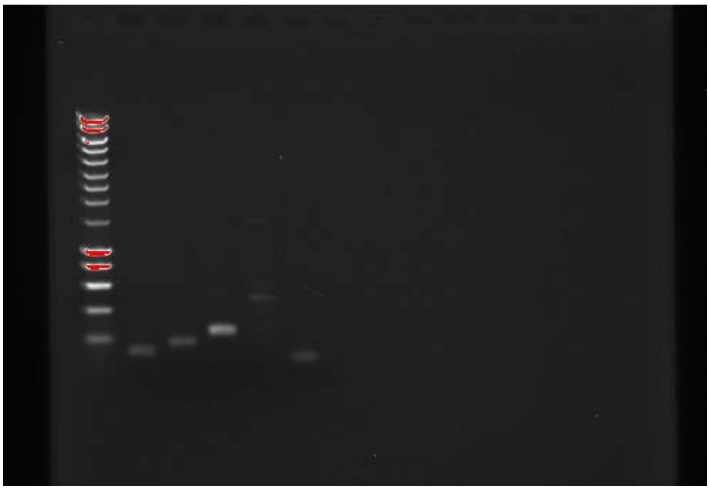
Appendixes

Supplemental Table 1. Element concentrations and EC levels of four nutrient solutions.

Element (mM)	Treatment			
	LN (0 mM N)	HN (20 mM N)	LP (0.0125 mM P)	HP (3 mM P)
N	0	20	5.8	5.7
K	4.2	7	4.1125	4.1
Ca	2	5	2.05	2
Mg	1.3	2.5	1.3	1.2
Na	13.2	0	8.5	13
Cl	17.2	2	10.2	14.7
S	1.85	1.9	3.35	2.95
P	1.1	1.1	0.0125	3
EC	2.5	2.6	2.2	2.9



Supplemental Fig 1. Determining the functionality of the primers by gel-electrophoresis. The bands of five primers were visible on gel, from left to right: marker, *GAD1*, *GAD2*, *POP2*, *SSADH2*, and *GLYR2*.

Supplemental Table 2. Primers designed for target genes for qPCR.

Target	TAIR	Forward			Reverse		
Gene	Identifier	Sequence	Length	Tm (°C)	Sequence	Length	Tm (°C)
GAD1	At5g17330	CGCAGGTATGGATGGATA	19	52.2	AGCTCACGCATCACTTTC	18	52.8
GAD2	At1g65960	CGAGGGGTACAAAAATGTGA	20	52.6	CCAGCCAAAACGACGTAG	18	53.7
POP2	At3g22200	GGTGTTGGCGCATTCTTT	18	54.1	CCTTCTCTTCCGTTGCCT	18	54.6
SSADH2	At1g79440	GCAGCAGTGCAAAAGGTAGAGA	22	57.6	AACCGAATAAGGGGAGCTACA	21	55.6
GLYR2	At1g17650	CGAGGTTGTCTCACAGGGA	19	56.5	AATGGAAAAGCCGTCGGG	18	55.8

Supplemental Table 3. The master mix (A) and reaction protocol (B) for FIREPol PCR.

A

Material	Concentration	Volume (µl)
Buffer B	10×	1.5
$MgCl_2$	25 mM	1.5
Primer mix	10 µM	0.4
dNTPs	10 mM	0.4
FirePol		0.15
Milli-Q water		9.05
DNA[1]		1
End volume		14

B

Stage	Cycle	Temperature (°C)	Time
	1×	95	5 min
Melting		95	30 s
Annealing	40×	55	30 s
Elongation		72	1 min
	1×	72	10 min
	1×	4	Infinite

[1] the DNA of WT Col was used.

Supplemental Table 4. Primer efficiency analysis by dilution series.

Primerset	Replicate	Dilution Series[1]							Slope	R^2	Efficiency (%)	
		Dilution	0	2	4	8	16	32				
		Logarithmic	0	-0.30103	-0.60206	-0.90309	-1.20412	-1.50515				
GAD1	1	**Cq**	23.23	24.04	25.16	26.45	27.37	28.24	-3.4497	1.00	94.931	92.785
	2		23.10	24.03	25.12	26.49	27.42	28.32	-3.5687	1.00	90.640	
GAD2	1		22.41	23.53	24.49	25.74	27.05	27.61	-3.5886	0.99	89.959	91.560
	2		22.62	23.63	24.79	26.01	26.89	27.79	-3.4975	1.00	93.161	
POP2	1		24.50	25.33	26.51	27.52	28.86	29.37	-3.4121	0.99	96.370	97.463
	2		24.42	25.46	26.35	27.19	28.27	29.64	-3.3570	0.99	98.557	
SSADH2	1		24.78	25.82	27.02	27.91	28.65	30.29	-3.5051	0.99	92.885	94.244
	2		24.96	25.82	26.89	27.94	29.19	29.96	-3.4320	1.00	95.603	
GLYR2	1		26.56	27.21	28.22	29.41	30.50	31.58	-3.4320	0.99	95.603	100.034
	2		26.60	27.34	28.48	29.35	30.34	31.41	-3.2194	1.00	104.464	

[1] Using cDNA samples synthesized from seed RNA (for *POP2*, *SSADH2*, *GLYR2*), leaf RNA (for *GAD2*), and seedling RNA (for *GAD1*)