Overexpression of Pyruvate, Orthophosphate Dikinase Facilitates Phosphate Uptake and Allows Better Growth of Tobacco in Alkaline Soil

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Atheel

Abstract

Phosphorus (P) is an essential nutrient for plant growth and reproduction. It is required for many molecules such as DNA, RNA, phospholipids and ATP. Lack of P causes problems in agricultural production. P concentration is low in soil compared with other nutrients. In alkaline soil, P forms unavailable compounds for plant uptake. To alleviate this, millions of tons of P are applied every year. Up to 80% of the applied P fertilizer is lost because it becomes unavailable for plant uptake. Therefore, the development of more efficient plant varieties to use P represents the best alternative to reduce use of P fertilizers, and achieve more sustainable agriculture.

Pyruvate, Orthophosphate dikinase (PPDK) is an enzyme that catalyzes the interconversion of ATP, Pi, and pyruvate with AMP, PPi, and phosphoenolpyruvate (PEP). Some studies reported enhance activity of PPDK in plants under abiotic stress, like absence of P. Others reported that overexpression of PPDK increased exudation level of organic acid, and enhanced the tolerance of plants to Al-stress. Organic acids play an important role in mobilizing the insoluble P and make it available for plant uptake.

Transgenic tobacco plants expressing plastidic (PPDK) or cytosolic (ΔPPDK) *M. crystallinum* PPDK, under the control of B33 and CaMV-35S promoters were studied. To study the PPDK overexpression plants efficient use of sparingly soluble P (HAp), the plants were grown in alkaline MS medium pH 8.0 with absence (Pi-), presence (Pi+) of P or in HAp, and in alkaline soil with different amounts of $CaCO_3$ (0.0 g kg^{-1}, 256 g kg^{-1} and 512 g kg^{-1}). 1mM of KH_2PO_4 was added as soluble P source and the sparingly soluble hydroxyapatite (HAp) containing tacitly 1mM of P was added as insoluble P source in growth medium.

Several physiological parameters were measured. Both the transgenic and wild-type plants grown in absence or presence of P, behaved similar in each of them (absence / presence of P). Interestingly, we found remarkable differences in most of the collected data from HAp treatment concerning the transgenics compared with the wild-type, where the growth of the transgenic plants in HAp was better compared with the wild-type. The transgenics increased exudation of organic acids, accumulated more biomass, and their content of P was significantly higher than that of the wild-type. The transgenic seedlings grown in solid MS medium containing HAp, significantly produced less root:shoot ratio

compared with the wild-type. Acid phosphatase activity of root surface of both transgenic and wild-type seedlings was similar in all of P treatments of all growth periods. Transgenic seedlings acidified the growth medium in the presence of HAp better than that of wild-type. Furthermore, production of secondary root of transgenic seedlings in HAp was more in number and length than that of wild-type. Surprisingly, the transgenic seedlings content of P in HAp and Pi+ was similar, and significantly higher than that of wild-type.

Growth of transgenic plants in alkaline soil was further studied. They strongly reduced the pH of the soil, grew significantly better and fruited successfully. In contrast, wild-type plants grown in the same soil, showed only a little acidification and the growth was strongly restricted. Moreover, they failed to flower. Whole seedlings and shoot samples of both transgenic and wild-type showed an increase in their content of citric and malic acid in Pi- compared with those in HAp and Pi+. Interestingly, transgenic and wild-type root samples showed decrease in citric and malic acid in Pi- and HAp compared with those in Pi+. In general, the level of amino acid in Pi- was high in all samples, compared with that in HAp and Pi+. The whole seedlings, root and shoot samples of both transgenic and wild-type, showed differences in the content of sugars among P treatments.

PPDK enzyme plays an anaplerotic role in the replenishment of intermediates of the citric acid cycle. The overexpression of PPDK leads to enhance the metabolism of organic acids. The better growth of transgenics that overexpress PPDK is related to their ability of high amount of organic acid exudation to the soil, which enhances the availability of P for plant uptake.

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Abbreviations

ADP:	adenosine diphosphate
Al^{3+} :	aluminium
Al-P:	aluminium-phosphate complex
AMP:	adenosine monophosphate
AP:	alkaline phosphatase
APase:	acid phosphatase
APS:	ammonium persulfate
ATP:	adenosine triphosphate
B33:	root specific promoter
BCIP:	5-bromo-4-chloro-3-indolyl-phosphate
BCP:	bromocresol purple
BPB:	bromophenol blue
BSA:	bovine serum albumin
BSC:	bundle-sheath cell
CA:	carbonic anhydrase
Ca:	calcium
CAM:	crassulacean acid metabolism
CaMV-35S:	cauliflower mosaic virus -35S promoter
Ca-P:	calcium-phosphate complex
C-atoms:	carbon atoms
cDNA:	complementary DNA
CL:	citrate lyase
CO_2:	carbon dioxide
CS:	citrate synthase
DMF:	N, N-dimethylformamide
DNA:	deoxyribonucleic acid
DTT:	1, 4-dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
et al.:	and other
EtOH:	ethanol
Fe^{3+}:	ferric
Fe-P:	ferric-phosphate complex
Fig.:	figure
GC-MS:	gas chromatography mass spectrometry
Glc-6-P:	glucose-6-phosphate
GOT:	glutamate-oxaloacetate transaminase
GPDH:	glycerol-3-phosphate dehydrogenase
H^+:	hydrogen ion
H_2O:	water
H_2PO^{4-}:	dihydrogen phosphate ion
HAp:	hydroxyapatite treatment
HCl:	hydrochloric acid
HCO_3^-:	bicarbonate
HEPES:	N-2-Hydroxyethalpiperazun-N'-2-ethanesulfonic acid
His:	histidine
HPO_4^{2-}:	monohydrogen phosphate ion

KOH:	potassium hydroxide
L-LDH:	L-lactate dehydrogenase
L-MDH:	L-malate dehydrogenase
LR:	lateral root
MC:	mesophyll cell
MC-CAM:	malic enzyme-CAM
MDH:	malate dehydrogenase
ME:	malic enzyme
MeOH:	methanol
Mg^{2+}:	magnesium
Mg-P:	magnesium phosphate complex
MS medium:	Murashige and Skoog medium
MSTFA:	*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide
N:	nitrogen
n:	number of Determination
NAD^{+}:	nicotinamide adenine dinucleotide
NAD-ME:	NAD-malic enzyme
NADP+	β-nicotinamide adenine dinucleotide phosphate
NADP-MDH:	NADP-malate dehydrogenase
NADP-ME:	NADP- malate enzyme
NaOAc:	sodium acetate
NaOCl:	sodium hypochlorite
NaOH:	sodium hydroxide
NBT:	4-Nitroblautetrazodiumchlorid
OAA:	oxaloacetate
P:	phosphorus
PAGE:	polyacrylamide gel electrophoresis
PEP-CK-CAM:	phosphoenolpyruvate carboxykinase- CAM
PEP:	phosphoenolpyruvate
PEPC:	phosphoenolpyruvate carboxylase
PEP-CK:	phosphoenolpyruvate carboxykinase
PGK:	phosphoglycerate kinase
PGM /e:	phosphoglucomutase
pH:	negative logarithm of the hydronium ion
Pi:	phosphate
Pi-:	phosphate starved growth medium
Pi+:	phosphate sufficient growth medium
PMSF:	Phenylmethanesulfonylfluoride
***P*-NPP:**	*P*-nitrophenylphosphate
PPDK:	Pyruvate, Orthophosphate dikinase
PPi:	pyrophosphate
PSI:	Pi-starvation inducible genes
PSR:	Pi-starvation response
PVP-40:	polyvinylpyrrolidone
Rep	replicate
RNA:	ribonucleic acid
RP:	regulatory protein
RPM:	revolutions per minute
RT:	room temperature
RuBisCO:	ribulose-1,5-bisphosphate carboxylase oxygenase

RuBP:	ribulose-1,5-bisphosphate
SD:	standard deviation
SDS:	sodium dodecyl sulfate
TBS:	Tris-buffered saline
TBST:	Tris-buffered saline + Tween 20
TCA cycle:	tricarboxylic acid cycle
TCA:	trichloroacetic
T-DNA:	transfer DNA
TEMED:	N, N, N ', N'-tetramethylethylenediamine
TP:	triose phosphate
TRIS:	Tris (hydroxymethyl) aminomethane
Tween 20:	polyoxyethylene sorbitan Monolaurate
WT:	wild-type
ΔPPDK:	cytosolic PPDK
1,3-bis-PGA :	1,3-bisphosphoglycerate
3-PGA:	3-phosphoglycerate

Units

bp:	base pair
°C:	degrees Celsius
cm:	centimeter
cm^2:	square centimeter
D:	Dalton
g:	gram
h:	hour
IU:	International Units (enzyme unit)
KDa:	kilo Dalton
M:	molar
min:	minute
mL:	milliliter
mM:	mill molar
ng:	nano-gram
nm:	nano-molar
µE M^{-2} s^{-1}	micro-Einstein
µg:	micro-grams
µl:	microliter
µM:	micro-molar
s:	second
UV:	ultraviolet
Var:	variety
V:	volt
v / v:	volume per volume
w / v:	weight per volume
% :	percent

1. Introduction

1.1. Pyruvate, Orthophosphate Dikinase

Pyruvate, orthophosphate dikinase (PPDK) is an enzyme that catalyzes the reversible phosphorylation reaction of pyruvate, adenosine triphosphate (ATP), and phosphate (Pi) yielding, phosphoenolpyruvate (PEP), adenosine monophosphate (AMP) and pyrophosphate (PPi), in the presence of Mg^{2+} ion (Evans and Wood, 1968; Carroll *et al.*, 1990; Wei *et al.*, 2001). The reaction takes place according to the following steps: 1-The γ and β-phosphate move from ATP to a conserved histidine residue in the active region of PPDK enzyme. 2-The removal of γ-phosphate to orthophosphate and 3- Transferring the remainder of β-phosphate to pyruvate (Xu *et al.*, 1995; Herzberg *et al.*, 1996; Wei *et al.*, 2001) as shown in (Fig.1.1).

1) PPDK-His + ATP $\rightleftharpoons$ PPDK- His ~ $\underset{\beta}{P}$-$\underset{\gamma}{P}$· AMP (reaction step 1)

2) PPDK-His ~ $\underset{\beta}{P}$-$\underset{\gamma}{P}$· AMP + Pi $\rightleftharpoons$ PPDK-His ~ $\underset{\beta}{P}$ + AMP ·$\underset{\gamma}{P}$-Pi (reaction step 2)

3) PPDK-His ~ $\underset{\beta}{P}$ + pyruvate $\rightleftharpoons$ PPDK-His + $\underset{\beta}{P}$-EP (reaction step 3)

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4) Pyruvate + ATP + Pi $\xleftarrow{Mg^{2+}}$ **PEP + AMP + PPi**

Figure 1.1: Chemical steps of pyruvate, orthophosphate dikinase (PPDK) catalyze the phosphorylation of pyruvate to phosphoenolpyruvate (Evans and Wood 1968). 1- The enzyme combines with ATP to form an enzyme-diphosphate compound and AMP, with the, β and γ phosphates from ATP bound to the enzyme (reaction step 1). **2-** The β-phosphate of the enzyme-diphosphate then combines with orthophosphate to give pyrophosphate and enzyme-phosphate (reaction step 2). **3-** The β -phosphate is then transferred from the enzyme-phosphate to pyruvate to produce free enzyme and Phosphoenolpyruvate (reaction step 3). And **4-** Summary of the three steps reaction catalyzed by pyruvate, orthophosphate dikinase, **PPDK**: pyruvate, orthophosphate dikinase, **ATP**: adenosine triphosphate, **AMP**: adenosine monophosphate, **PEP**: phosphoenolpyruvate, **Pi**: phosphate, **His**: histidine, **PPi:** pyrophosphate.

1.2. Existence of PPDK in Living Organisms

Pyruvate, orthophosphate dikinase **(**PPDK) enzyme is found in various living organisms like bacteria (Evans and Wood, 1968; Reeves *et al.*, 1968; Petzel *et al.*, 1989;

Watt and Evans, 1999), protozoa (Bruderer *et al.*, 1996; Slamovits and Keeling, 2006) and plants (Hatch and Slack, 1968; Agarie *et al.*, 1997; Moons *et al.*, 1998), but it is not found in animal cells (Reeves, 1968; Maldonado and Fairlamb, 2001).

1.2.1. PPDK Role in C_4 Plant

Pyruvate, orthophosphate dikinase (PPDK) is essential (Parsley and Hibberd, 2006) and most recognized as a chloroplastic enzyme in C_4 photosynthesis (Matsuoka *et al.*, 1993; Chastain *et al.*, 2002). C_4 plants include some of the most powerful commercial crops in the world like, sugarcane, crabgrass (Sage, 2004) sorghum and maize (Kirchhoff *et al.*, 2013).

In the sixties of the last century Kortschak *et al.* (1965) observed that the primary product of photosynthesis in sugarcane is not the C_3 unit (3-phosphoglycerate), but a unit of four C atoms. This four C-atom is confirmed and identified as oxaloacetate (OAA), what is so called, the metabolic pathway of C_4 photosynthesis (Hatch and Slack, 1966). These plants can be characterized anatomically, by Krantz-anatomy which is spatially separated to mesophyll cells (MC) and bundle-sheath cells (BSC) (Hausler *et al.*, 2002). Biochemically, the C_4 plants are classified into three subtypes: NAD-malic enzyme (NAD-ME), NADP-malate enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PEP-CK) (Edwards *et al.*, 2004).

The first step in C_4 cycle is fixation of the atmospheric carbon dioxide (CO_2) into C_4 acid oxaloacetate (OAA), by carboxylation of phosphoenolpyruvate (PEP) through PEP carboxylase (PEPC) in the mesophyll cells. Then the produced OAA is transferred to the chloroplast of mesophyll cells, and reduced to malate by NADP-malate dehydrogenase (NADP-MDH). Thereafter, malate is exported to the chloroplast of bundle-sheath cells, where it is decarboxylated by NADP-ME, producing CO_2 and the reducing power (Weissmann and Brutnell, 2012). The produced CO_2 is re-fixed by ribulose-1,5 bisphosphate carboxylase oxygenase (RuBisCO) in the Calvin-Benson cycle. Pyruvate is returned to chloroplasts of the mesophyll cells, where it is phosphorylated by PPDK to regenerate the primary carbon dioxide acceptor or PEP (Fig. 1.2), (Hausler *et al.*, 2002; Leegood, 2002; Miyao, 2003; Burnell and Chastain, 2006; Omoto *et al.*, 2012)

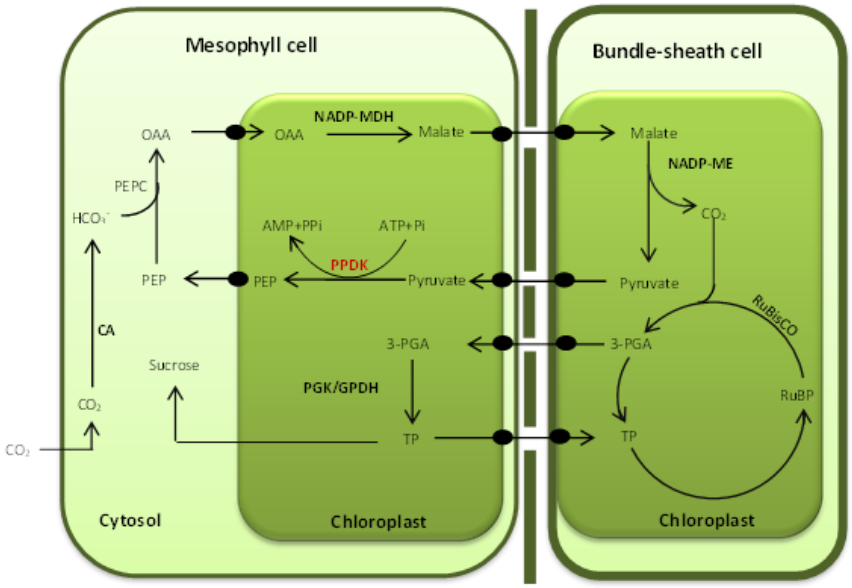


Figure 1.2: Schematic diagram illustrates the role of the enzyme PPDK in C_4 plants. CO_2: carbon dioxide, **CA:** carbonic anhydrase, **HCO_3^-:** bicarbonate, **RuBisCO:** ribulose-1,5 bisphosphate carboxylase oxygenase, **PEPC:** phosphoenolpyruvate carboxylase, **OAA:** oxaloacetate, **NADP-ME:** NADP-malate enzyme, **NADP-MDH:** NADP-malate dehydrogenase, **ATP:** adenosine triphosphate, **AMP:** adenosine monophosphate, **Pi:** inorganic phosphate, **3-PGA:** glycerate-3-phosphate, **TP**: triose phosphate, **PPi:** pyrophosphate, **PEP:** phosphoenolpyruvate, **RuBP:** ribulose-1,5 bisphosphate, **PGK:** phosphoglycerate kinase, **GPDH:** glycerol- 3-phosphate dehydrogenase, **PPDK:** pyruvate, orthophosphate dikinase (Leegood, 2013).

PPDK catalyzes the regeneration of PEP in the pathway of C_4 metabolism, (Hatch, 1987; Carroll *et al.*, 1990; Parsley and Hibberd, 2006; Chastain *et al.*, 2011). The enzyme activity of PPDK is rapidly regulated by a reversible protein phosphorylation, which is mediated by a bi-functional regulatory protein, or PPDK regulatory protein (RP) that has both kinase and phosphotransferase activities. Phosphorylation of PPDK leads to an inactivation of the enzyme activity, and dephosphorylation of PPDK results in the activation of the enzyme activity (Astley *et al.*, 2011), in response to the intensity of light (Fukayama *et al.*, 2001), while it is activated in light, it is deactivated in dark (Burnell and Hatch, 1986; Nakamoto and Edwards, 1986).

1.2.2. PPDK Role in CAM Plant

Crassulacean acid metabolism (CAM) is a specialized mode of photosynthesis. It improves the assimilation of the atmospheric CO_2 under / or in response to unfavorable conditions of growth, like water-limited, terrestrial and epiphytic habitats, and in CO_2-limited aquatic environments (Silvera *et al.*, 2010). CAM is taxonomically widespread among vascular plants. It is present in many succulent species that occupy the semi-dry lands, tropical forests and water ecosystems (Lüttge, 2010). This pathway of water-conserving metabolism (Winter *et al.*, 2005) that has the capacity of CAM metabolism, is present in nearly 6% of the vascular plant species from at least 35 families (Crayn *et al.*, 2004; Holtum *et al.*, 2007; Silvera *et al.*, 2010).

CAM is a mechanism of CO_2 acquisition, transient, storage and concentrating, based on the synthesis of organic acid. In this type of photosynthesis, the plant fixes CO_2 during the night and uses it during the day in the light (Lüttge, 2001). The fixed CO_2 from the dark period is converted to the organic acid (malic acid) using the enzyme phosphoenolpyruvate carboxylase (PEPC), which has affinity toward the inorganic carbon substrate, bicarbonate (HCO_3^-). The produced malate is accumulated in vacuoles during the dark period (Lüttge, 2001). In this type of pathway, the fixed CO_2 during the night is used during the daylight, when the light-dependent C_3-cycle runs once again (Cushman and Bohnert, 1999).

The CAM metabolism is classified into two parts based on their decarboxylation process type, the phosphoenolpyruvate carboxykinase (PEP-CK) type, and the malic enzyme (ME) type (Peckmann *et al.*, 2012). Among the (PEP-CK)-CAM, the plants produce OAA from malate by pyruvate carboxykinase decarboxylation (Hong *et al.*, 2004). In malic enzyme-dependent plants ME-CAM (Fig. 1.3), malic acid is decarboxylated by NADP-ME and NAD-ME, and generates pyruvate in the chloroplast. Pyruvate is phosphorylated to phosphoenolpyruvate (PEP) by pyruvate, orthophosphate dikinase (PPDK), and then is conserved in gluconeogenesis after transferring to the cytosol (Kondo *et al.*, 2000a). Then the glucose-6-phosphate (Glc-6-P) is transported to the chloroplast, and stored in the form of starch. The starch in the chloroplast is dismantled in the dark period to triose phosphate (TP). The TP is transported to the cytosol and is converted by glycolysis to PEP which returns to the starting point. This means that

the starting point of PPDK reaction is gluconeogenesis and not the regeneration of the CO_2 acceptor (Cushman, 1992; Kondo *et al.*, 2000b).

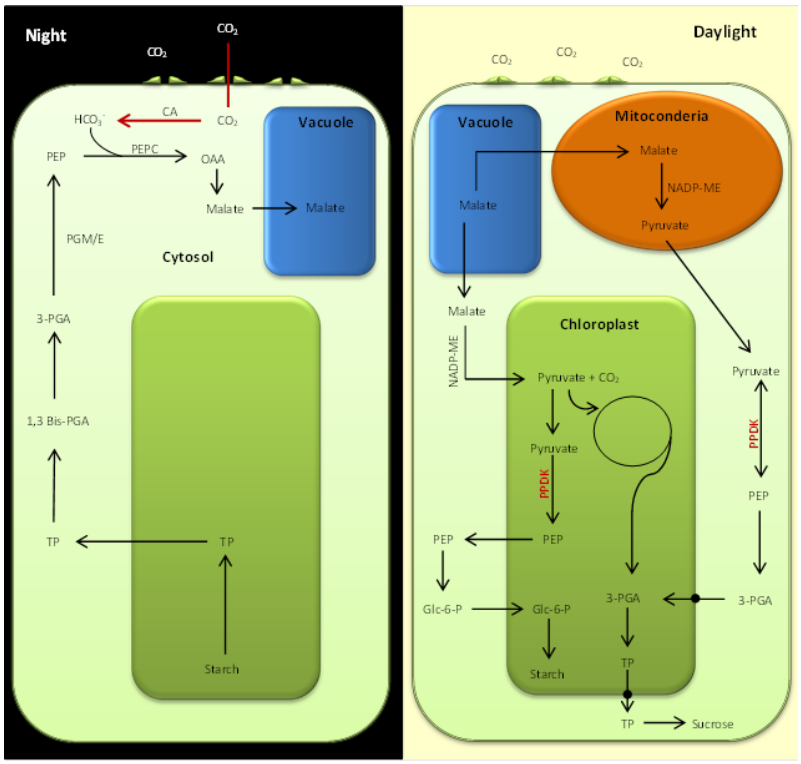


Figure 1.3: Function of PPDK in plants with CAM metabolism. 1,3-Bis-PGA :1,3-bisphosphoglycerate, **Glc-6-P:** glucose-6-phosphate, **NADP-ME:** NADP-malate enzyme, **OAA:** oxaloacetate, **PEPC:** phosphoenolpyruvate carboxylase, **PEP:** phosphoenolpyruvate, **3-PGA:** 3-phosphoglycerate, **PGM / E:** phosphoglucomutase, **PPDK:** pyruvate, orthophosphate dikinase, **TP:** triose phosphate, **CO_2**: carbon dioxide, **CA:** carbonic anhydrase, **HCO_3^-** : bicarbonate, **PEPC:** phosphoenolpyruvate carboxylase (Taiz and Zelger, 2010).

1.2.3. PPDK Role in C_3 Plant

The vast majority of terrestrial plants, which include many of agronomic substantial crops such as rice, wheat, soybean, and potato are all classified as C_3 metabolism plants, that assimilate atmospheric CO_2 directly through the C_3 pathway of photosynthesis (Miyao, 2003). However, the pathway of C_3 photosynthesis suffers from low affinity of RuBisCO to atmospheric CO_2 and is limited by the photorespiration, that costs more energy (Furbank and Taylor, 1995).

PPDK is ubiquitous in the cells and tissues of C_3 plants in very low amounts, and unconfirmed role (Chastain *et al.*, 2008). In these plants, it is proposed that the enzyme PPDK is in conjunction with the enzyme PEPC. The activity of PEPC increases with the increase of PPDK amount, and decreases with the decrease of PPDK in the cell (Wang *et al.*, 2012). PEPC plays a central role in the anaplerotic supply of the carbon skeleton for amino acid biosynthesis in leaves of C_3 plants. The produced amino acids could contribute with the activity of PPDK, in PEPC activity to refix CO_2. (Melzer and O'Leary M, 1987; Rademacher *et al.*, 2002). Consequently, PPDK enzyme enables the PEPC enzyme to re-fix CO_2 into metabolic intermediates of the cell (Jansen *et al.*, 1992). As is shown in (Fig. 1.4) the reaction of alanine and OAA forms aspartate and pyruvate, the formation of aspartate is catalyzed by aspartate transaminase (AspAT), while, pyruvate formation is catalyzed by alanine transaminase (AlaAT). The yield of pyruvate from this reaction can be phosphorylated in the chloroplast by PPDK to produce PEP. Thus, the substrate for PEPC is provided (Latzko and Kelly, 1983). PPDK could also play a consistent role with NADP-ME in the gluconeogenesis of C_3, generating PEP from pyruvate during stomata closure of guard cells of (*Vicia faba* L). The increase in the activity of PPDK in the guard cells of stomata increases the supply of PEP. PEP is a substrate for PEPC for the synthesis of organic acids (malate), malate promotes the opening of the stomata (Schnabl, 1981).

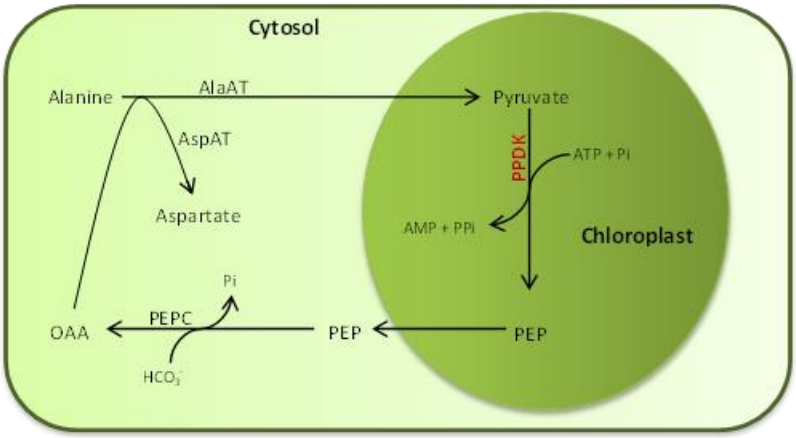


Figure 1.4: Schematic diagram illustrates the role of PPDK in C_3 plants. **PPDK:** pyruvate, orthophosphate dikinase, **AMP:** adenosine monophosphate, **PPi:** pyrophosphate, **ATP:** adenosine triphosphate, **Pi:** inorganic phosphate, **PEP:** phosphoenolpyruvate, **HCO_3^-:** bicarbonate, **PEPC:** phosphoenolpyruvate carboxylase, **OAA:** oxaloacetate, **AspAT**: aspartate transaminase, **AlaAT:** alanine transaminase, **(**Latzko and Kelly, 1983).

1.2.4. PPDK-overexpression in C_3 Plants

PPDK role in C_3 plants is still unclear and not fully understood (Burnell and Chastain, 2006). Many studies have been conducted in the last decades to understand the PPDK role, through producing various transgenic C_3 plants able to overexpress the C_4 PPDK. For example, Arabidopsis (Ishimaru *et al.*, 1997; Parsley and Hibberd, 2006; Wang *et al.*, 2012*)*, rice (Imaizumi *et al.*, 1997; Moons *et al.*, 1998; Taniguchi *et al.*, 2008; Zhang *et al.*, 2010), potato (Ishimaru *et al.*, 1998), overexpress the maize chloroplastic PPDK. The photosynthetic activity of C_3 plant that overexpresses the C_4 PPDK did not alter, though the PPDK activity of transgenic plants showed an increase compared with wild-type. For example some of the transgenic lines of rice increased the PPDK 40-fold (Fukayama *et al.*, 2001), and up to 5.4-fold in potato (Ishimaru *et al.*, 1998).

According to (Taylor *et al.*, 2010) the cytosolic PPDK is associated in N remobilization in Arabidopsis. Overexpression of PPDK during senescence, can significantly accelerate remobilization of nitrogen from leaves. Thus, data indicate the important role for the cytosolic PPDK in the leaves of C_3 plants. This role is responsible for the production of transport amino acids during leaf senescence. It increases the size of seed and the content of nitrogen that are desirable in Arabidopsis. The efficient remobilization of nitrogen within the plant, reduces the requirement for more applications of fertilizer. PPDK and the pathway in which it operates, can be used for commercial crop improvement.

1.3. Phosphorus Importance for Plant Nutrition

Phosphorus (P) is an essential nutrient element (next to nitrogen) limiting of plant growth and agricultural crop yield (Holford, 1997; Vance *et al.*, 2003; Shenoy and Kalagudi, 2005; Niu *et al.*, 2012). P is considered as one of the basic components of many key molecules, like nucleic acids, phospholipids, energy transporters, that form the structure and function of the cell. P involves in the pathways of energy metabolism and of signal transduction chains, as well as it regulates the activities of enzymes (Pao *et al.*, 1998; Plaxton, 2004; Kuo and Chiou, 2011; Péret *et al.*, 2011). P is taken up from the solution of soil by the root of plants as orthophosphate, basically $H_2PO_4^-$ and less as HPO_4^{2-} form (Schilling *et al.*, 1998; Frossard *et al.*, 2000; Syers *et al.*, 2008). P is

available for uptake of the plant in a small range of neutral pH of soil (Fig. 1.5). In alkaline and calcareous soils, the combination of P with calcium (Ca^{2+}) and magnesium (Mg^{2+}) forms a sparingly soluble complex of calcium-phosphate (Ca-P) and magnesium-phosphate (Mg-P). In acid soils, P combines with (Al^{3+}) and (Fe^{3+}) forming the low soluble complex of aluminum-phosphate (Al-P) and ferric-phosphate (Fe-P) (Toro, 2007). Therefore, the soil content of P could be high, but not available for plant uptake (Holford, 1997).

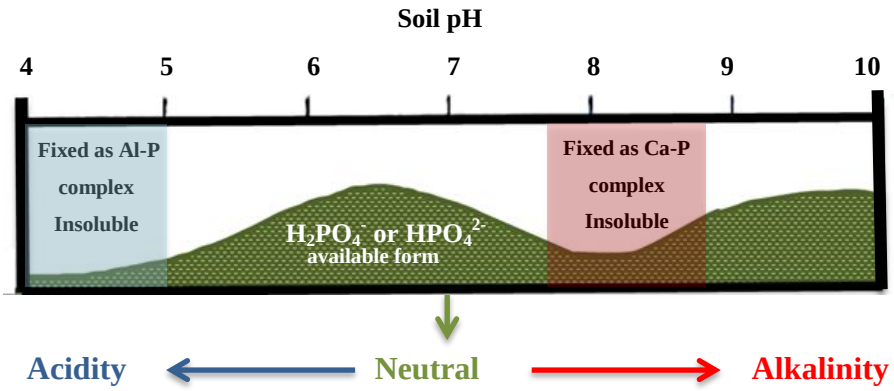


Figure 1.5: Effect of the pH of soil on the availability of P nutrient for plant uptake. The relationship between the pH of soil and the availability of P for plant uptake, for example: in alkaline soil, most of phosphate is fixed with (Ca) to form the insoluble complex of calcium-phosphate (Ca-P), and in acid soil, phosphate is fixed with (Al) to make the insoluble complex of aluminium-phosphate (Al-P), (Troeh and Thompson, 2005).

1.3.1. Phosphate Deficiency Symptoms in Plant

Pi-deficiency symptoms in plants include stunting, and dark green coloring of the leaves. In some of the plants, the dark green color of leaves changes to greyish-green or bluish-green metallic with the increase of Pi-deficiency. In others, the dark green color of the leaves changes to brown with conspicuous venation. Purple color of leaf is commonly observed in corn, and other grasses due to the accumulation of anthocyanin under Pi-deficiency. Old leaves become necrotic due to the Pi translocation from old leaves to the newly developing tissues under the conditions of severe Pi-deficiency. In the stage of reproduction; Pi translocates to fruit and seeds. Thus, if Pi-deficiency happens late in the

season of growing, it will affect the development of seed and the maturity of fruit. (Gaxiola *et al.*, 2011).

1.3.2. Plant Response to Phosphate Deficiency

A complex array of mechanisms is developed by plants, to acquire and utilize phosphate in low-phosphate soil. These mechanisms include: morphological, physiological and biochemical changes, collectively known as Pi-starvation response (PSR), (Theodorou and Plaxton, 1993; Niu *et al.*, 2012). This response is a consequence of the coordinated expression of hundreds of Pi-starvation inducible genes (PSI). These mechanisms involve conserving and using of the internal phosphate efficiently, or allowing greater access of the soil content of phosphate. Thereby, these modifications increase the availability of phosphate of the rhizosphere and enhance uptake and use of phosphate (Raghothama, 1999; Lambers *et al.*, 2006; Richardson *et al.*, 2009; Plaxton and Tran, 2011). The success of the adaptation mechanisms is displayed in numerous plant species, for example White lupin (Cheng *et al.*, 2011).

1.3.2.1. Morphological Adaptation of Roots under Pi-deficiency

The morphological response of root to the availability of phosphate in soil is a key adaptive behaviour. It allows plants to cope with environmental conditions (Lynch, 1995; Al-Ghazi *et al.*, 2003). Therefore, the architecture of the root system determines the ability of plants to acquire the nutrients. The architecture modification could be a change in terms of number of lateral root, length of lateral root, density of root hair and length of primary root (López-Bucio *et al.*, 2002; Péret *et al.*, 2011). Modification of the root growth is a well-demonstrated response in Pi-starvation (Lynch, 1995). The increase of root:shoot ratio under Pi-starvation is a distinguishing feature of the plant response to Pi-starvation. It enhances the total surface of root area for soil exploration and acquisition of phosphate (Martín *et al.*, 2000; Nielsen *et al.*, 2001; Agren and Franklin, 2003; Wissuwa, 2003). This increase of root:shoot ratio leads to an increase of the relative biomass allocated to root (Nielsen *et al.*, 2001; Hermans *et al.*, 2006). The more

proliferated root system allows plants a better uptake and utilization of phosphate from the soil (Robinson, 1994; Hodge, 2004).

A soil of low phosphate changes the distribution of growth between the primary and lateral roots (Hodge, 2009), that is well-documented in the model plant *Arabidopsis thaliana*. While, the primary root (PR) growth of Arabidopsis is reduced, the growth of the lateral root (LR) is induced (Bates and Lynch, 1996; Williamson *et al.*, 2001; Pérez-Torres *et al.*, 2008; Niu *et al.*, 2012). Furthermore, a high increase of the length and density of root hairs are commonly observed in Arabidopsis during Pi-starvation (Bates and Lynch, 1996, 2000; Ma *et al.*, 2001). Some of the plants respond to phosphate deficiency by forming proteoid or cluster roots. This type of root modification is characterized in White lupin (Johnson *et al.*, 1996; Abdolzadeh *et al.*, 2010; Cheng *et al.*, 2011). Cluster roots are specialized in synthesis and secretion of organic acids to the rhizosphere (Johnson *et al.*, 1994; Watt and Evans, 1999; Uhde-Stone *et al.*, 2003).

Another adaptation of root to Pi-deficiency is by exploring the rhizosphere and minimization of metabolic cost. Plants reduce the metabolic responsibility, from the relatively high biomass root parts, to that metabolically more efficient in P acquisition, such as root hairs. The production and maintenance of these parts need less biomass, compared with the basal roots. Moreover, the efficiency of P acquisition is increased relatively with the enlarged value of the absorptive surface area (Lynch, 2007; Den Herder *et al.*, 2010). Some of the plants such maize and bean, use another mechanism to adapt to Pi-deficiency. They reduce the root growth need of P through formation of aerenchyma in the root. They replace the cells of cortical tissue with air space through breaking the cortical tissue. Therefore, the liberation of P of the broken cells could be used for supplying root growth demand of phosphate (Fan *et al.*, 2007).

1.3.2.2. Secretion of Organic Acids under Pi-deficiency

Secretion of organic acids from the root of the plant to the rhizosphere, such as the citrate, malate and oxalate, is induced under Pi-deficiency and /or the accumulation of Al^{3+} (Aono *et al.*, 2001; Wang *et al.*, 2007; Carvalhais *et al.*, 2011; Lin *et al.*, 2011). These organic acids are involved in many physiological activities, including; enhancing of

solubiliziation and liberation of P from Al-P complex of acidic soil, or from Ca-P complex of alkaline soil (Ryan *et al.*, 2001; Yang *et al.*, 2013). Also they displace the toxic Al^{3+} cations of the soil, which leads in Al-detoxification or reducing the toxicity of aluminum to the root of plant under the conditions of Pi-deficiency (Miyasaka *et al.*, 1991; Delhaize and Ryan, 1995; Pellet *et al.*, 1996; Chen *et al.*, 2009; Trejo-Téllez *et al.*, 2010; Arunakumara *et al.*, 2013).

In general dicots; particularly legumes are more efficient than monocots in producing, and exuding organic acids under the conditions of Pi-deficiency (Raghothama, 1999). The mechanism of organic acids in mobilization of phosphate is through dissolution of phosphate minerals, or by desorption of adsorbed phosphate (Geelhoed *et al.*, 1999; Dakora and Phillips, 2002). A role of citrate has been observed in Ca chelating from hydroxyapatite (HAp) particles of alkaline soil, citrate dissolves Ca-P efficiently by weakening the nanoparticle stability of HAp, through controlling the availability of free Ca; thereby, the nucleation rate (Martins *et al.*, 2008). Thus, the release of organic acid from roots can improve the plant acquisition of P, with the increase of the solubility of some of the stable pools of soil P, which is important to increase the availability of P in calcareous and alkaline soils (Gang *et al.*, 2012). In common bean, it is indicated that the increase of exudation of organic acid, in genotypes of high Pi-solubilizing activity, increased the acquisition of P, compared with genotypes of less activity. (Shen *et al.*, 2002). Also, using of Pi-efficient soybean genotypes which secrete more malic acid, improved the nutrition of P and the tolerance of plant to Al toxicity (Liao *et al.*, 2006).

1.3.2.3 Acid Phosphatase Activity under Pi-deficiency

Acid phosphatase is widely distributed in plants, and varies in different species of plants. This enzyme has intracellular and extracellular activity (Ehsanpour and Amini, 2003). Acid phosphatase activity is stimulated under Pi-deficiency, as one of the biochemical adaptation of strategies of the plant (Del Pozo *et al.*, 1999). Plants that are grown under low-P of soil, produce and secrete acid phosphatase into the rhizosphere. This acid phosphatase releases the Pi group of organophosphates which are present in the rhizosphere to increase the availability of Pi to the plants (Baldwin *et al.*, 2001; Hurley *et al.*, 2010; Li *et al.*, 2011; Wang and Liu, 2012). The intracellular acid phosphatase is

ubiquitous within the plant tissues. Most of the plant intracellular acid phosphatase is assumed to be localized in the vacuoles (Nishimura and Beevers, 1978), and they may help remobilize and recycle of Pi of the expendable intracellular Pi-monoesters. This is accompanied by a marked reduction of cytoplasmic phosphate metabolites during the long-term of Pi-deficiency (Duff *et al.*, 1994; Tran *et al.*, 2010).

1.4. Objectives

The role of PPDK in C_3 plants is still unclear up now. In non-photosynthetic parts of the plants, it plays an important role in the maintenance of pH and replenishment of citric acid cycle intermediates, thereby, contributing to the biosynthesis of amino acids. PPDK was found in plants under various types of abiotic stress, such as drought, high salinity, absence of phosphate and iron, or presence of heavy metals in soil (Doubnerova and Ryslava, 2011). Trejo-Téllez *et al.* (2010) found that overexpression of PPDK in tobacco, leads to the exudation of large amounts of organic acids (citrate and malate), and increases the plant tolerance to Al-stress. Role of organic acids in mobilization of insoluble P is well documented in many studies. Our aim is to find out whether overexpression of PPDK could help plants to utilize insoluble phosphate (HAp) for growth in alkaline conditions by exudation of organic acid in the root space. Expression of PPDK of plants that express either plastidic (PPDK) or cytosolic (ΔPPDK) was examined. To achieve our aim, we grew the transgenic and wild-type seedlings in MS alkaline pH 8.0 medium supplied, with different supplements of P and in alkaline soil. Several physiological parameters are measured.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Materials

2.1.1.1. *Mesembryanthemum crystallinum* L.

Mesembryanthemum crystallinum L. (ice plant), possesses a pathway of C_3 metabolism when unstressed, but shifts to CAM (an ecophysiological modification of photosynthetic carbon acquisition) (Lüttge, 2004) under water, salt stress or with the increase in age (Ostrem *et al.*, 1987; Cushman *et al.*, 1989; Fißlthaler *et al.*, 1995; Tallman *et al.*, 1997; Cushman *et al.*, 2000). The plant belongs to the family of Aizoaceae, order of Caryophyllales. Its native place is South Africa, and is spread worldwide in arid and hot climates as in Western Australia, along the coasts of the United States and in Mexico (Bohnert *et al.*, 1988; Adams *et al.*, 1998). This plant produces the enzyme PPDK under salt stress or with the increase in age (Fißlthaler *et al.*, 1995), (Fig. 2.1).

Figure 2.1: *Mesembryanthemum crystallinum* L. plant in flowering stage (Ibtissem *et al.*, 2010).

2.1.1.2. *Nicotiana tabacum* L.

Transgenic tobacco lines were constructed by transformation with *Agrobacterium tumefaciens* by Sheriff (1994) and Stenzel (1997). The transgenic tobacco expresses the cDNA of pyruvate, orthophosphate dikinase (PPDK) from *Mesembryanthemum*

crystallinum into a binary expression vector (Bevan, 1984; Lee and Gelvin, 2008). Sheriff (1994) overexpressed the gene for PPDK under the control of 35S promoter of cauliflower mosaic virus (CaMV). The CaMV-35S promoter is well characterized and is the most used for driving transgene expression in monocotyledonous and dicotyledonous plants. (Benfey *et al.*, 1990; Benfey. and Chua., 1990; Sunilkumar *et al.*, 2002; Anuar *et al.*, 2011). In another construct to express the PPDK in the cytosol, the region of the sequence that codes the chloroplast recognition was deleted. In order to achieve specificity in roots, PPDK gene expression was carried out under the control of the root-specific promoter B33 (Stenzel, 1997). B33 is a promoter of the class I patatin gene of potato (Rocha-Sosa *et al.*, 1989; Naumkina *et al.*, 2007). Figure 2.2 shows the schematic representation of the constructs used for the transformation. Figure 2.3: Schematic drawing shows expression of *Mesembryanthemum crystallinum* PPDK in tobacco leaves and roots.

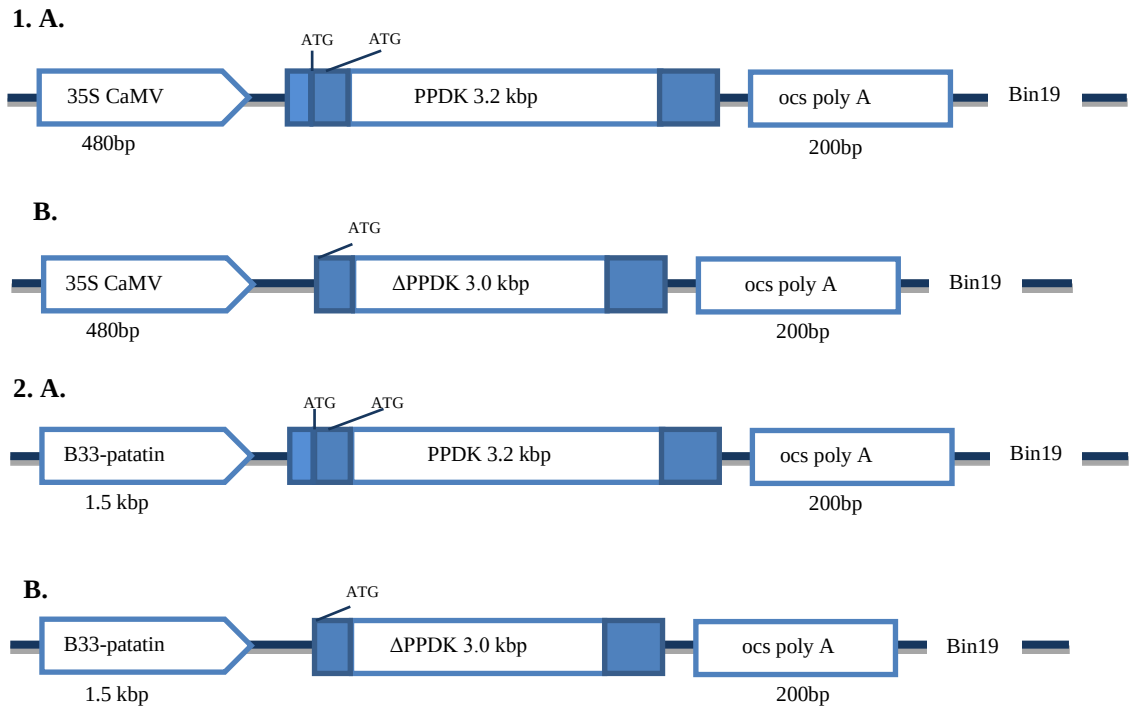


Figure 2.2: Schematic drawing represents the T-DNA construct of the binary vectors containing the *Mesembryanthemum crystallinum* PPDK- insertion.

1. PPDK expression controlled via 35S promoter.
2. PPDK expression controlled via B33 promoter.

A. Partial T-DNA scheme of putative plastidic PPDK construct, harboring complete PPDK cDNA sequence from *M. crystallinum* (3173 bp).

B. Partial T-DNA scheme of putative cytosolic ΔPPDK construct with a partially deleted pre-sequence of PPDK cDNA from *M. crystallinum* (2990 bp). Δ: represents deletion of (183 bp) at the 5'-end.

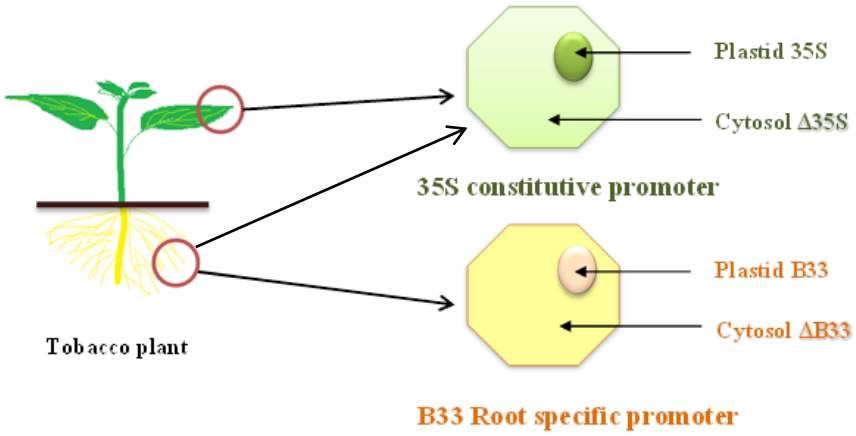


Figure 2.3: Schematic drawing shows expression of *Mesembryanthemum crystallinum* PPDK in tobacco leaves and roots using non-specific 35S, Sheriff, (1994) **and roots-specific B33 promoters,** Stenzel, (1997).

2.1.2. Chemicals and Solutions

All chemicals used in this study were analytically pure and from trusted companies such as Sigma-Aldrich and Roth.

2.1.3. Proteins

Bovine serum albumin (BSA) …….…….. Roth, Karlsruhe
Casein…….…….. Roth, Karlsruhe
Skimmed milk…….…… AppliChem, Darmstadt

2.1.4. Laboratory Materials

Aluminum foil ……..... Roth, Germany
Eppendorf tubes (1.5, 2,10,15,50 ml) ……..…… Eppendorf, Germany
Hybridization transfer membranes (Hybond ™-N)…… Amersham Biosciences, Freiburg
Nitrocellulose membrane (Hybon ™-c extra) ……… Amersham Biosciences, Freiburg
Parafilm ……..……...……..…… Germany
Petri dishes (tissue culture dishes)…….…….... Greiner Labortechnik, Food

UV-Cuvette micro ……..……...…..... Roth, Germany

2.1.5. Apparatus

Autoclave (Systec DX-150) ……..... Germany

(Arbus) ……. Germany

Dissecting microscope ……...…….……..…….. Germany

Digital camera ……...… Canon EOS 550, Japan

Deep freezer (Ultra Low) ……..Sanyo, Japan

Electrophoresis Power Supply PHERO rod. 300 ……..……. Biotec-Fischer, Germany

GC-MS Unit …….…..…Thermo Scientific

Growth chamber …….……...…….…. FU-Berlin, Germany

Greenhouse …….…….. FU-Berlin, Germany

Ice-maker …….…….…..... Ziegra, Germany

Illuminator supplier (Schott KL 1500) ……..……..…….…….…… Germany

Lab-shaker (Kuhner) …….…. Germany

(GFL 3015) ……..……. Germany

(MS) ……..…….…….. Germany

Laminar air flow hood ……...…….……. Gelaire, Germany

Lyophilizer ……. Germany

Magnetic stirrer (Heidolph)…….…….……..……. Germany

Magnetic (Electronic speed) ……..……..…… IKA Labortechnik, Germany

MotoDirect ……...…….…….…… H+P Labortechnik, Germany

MR 3001 …….……...…. Heidolph, Germany

Oven (Memmert) ……..……..……..……..…… Germany

pH meter ……...……....….. Schott, Berlin, Germany

pH meter ……..……... Knick, Berlin, Germany

pH meter (Flat surface electrode)…….……..….. Schott, Germany

Protein gel electrophoresis equipment …..... workshop at the Free University of Berlin

Refrigerated (2K15 cooled) centrifuge ……..…….....…. Sigma, Germany

Refrigerated super speed centrifuge …….…….……. Germany

Refrigerated economic super automatic …….…..... Bosch, Germany

Radiometer/Photometer Model IL 1400A …….……. Germany

Retsch mill (Type MM301) ……..... Haan, Germany

Refrigerated centrifuges (Biofuge 28 R)…….. Heraeus Sepatech. Berlin, Germany
(Tabletop centrifuge 5415 C) ……..…… Eppendorf, Germany
Scanner (Epson V100 photo) ……..……..……..…China
Speed vacuum (Univapo 150 H)……..……..……..……Germany
Scales (GT 480) OHAUS …….……U. S. A.
(H110) Sartorius …….……..…… Germany
(KERN ALJ160-4NM) …… Germany
Spectrophotometer (Novaspec ® II)…….Pharmacia LKB Biochrom, England
(Ultrospec II 4050)……..Pharmacia LKB Biochrom, England
Thermostat (BlockThermostat BT 100) ……..……. Kleinfeld Labortechnik, Germany
(Thermostat Plus) …… Eppendorf, Germany
Vortex (K) ……..……. IKA Labortechnik, Germany
(Heidolph)…….……..……..…… Elektro KG, Germany
(Reamix 2789)...……..……..….. Germany
Water baths (3042) ……..……... Köttermann, Germany
(Minitherm S) ……..……...……..…. Dinkelberg Labortechnik, Germany
(10S) GFL .....……..…… Germany
Western-blot-chamber …….……. Roth, Germany

2.1.6. Enzymes and Kit-systems

2.1.6.1. Enzymes

Acid phosphatase enzyme …….……. Sigma, Germany

2.1.6.2. Kit-systems.

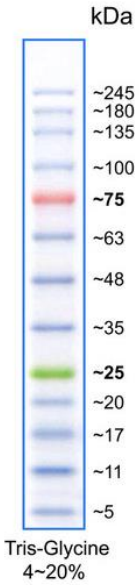
L-Citric acid UV test ……..…..… Boehringer Mannheim / R-Biopharm
L-Malic acid UV test ……..……..…… Boehringer Mannheim / R-Biopharm

2.1.7. Antibody

Anti-PPDK ……..…….…….. Schmitt *et al.*, 1989
Anti-mouse IgG alkaline phosphatase (Anti-GAR-AP) …….. Bio Rad

2.1.8. Protein Marker

Blue-Star Pre-stained Protein Marker ….....….. NIPPON GENETICS EUROPE GmbH



2.1.9. Soil and lime

1. Soil of tobacco planting …… Floragard, Germany

2. Calcium carbonate (Lime) **.....**……Vereinigte Kreidewerke Dammann, Germany

2.2. Methods

2.2.1. Preparing Stock Solutions of MS Medium

Stock solutions of MS medium (Murashige and Skoog) have been prepared manually in the laboratory, by collecting the chemical elements of the medium according to "Murashige and Skoog formula 1962" (Murashige and Skoog, 1962). The prepared stock solutions were free of phosphate salts. The stock solutions were stored in the refrigerator after preparation.

A. Macroelements Stock Solution Components

NH_4NO_3 ……..	1650 mg/l
KNO_3 ……...…	1900 mg/l
$CaCl_2.2H_2O$ …….....	440 mg/l
$MgSO_4.7H_2O$ ……....….	370 mg/l
KH_2PO_4 ……...….....	--.-- mg

B. Microelements Stock Solution Components

KI ……...	0.83 mg/l
H_3BO_3 ……..…	6.2 mg/l
$MnSO_4.4H_2O$……...…	22.3 mg/l
$ZnSO_4.7H_2O$……...….	8.6 mg/l
$Na_2MoO_4.2H_2O$ …….....	0.20 mg/l
$CuSO_4.5H_2O$ ……..	0.25 mg/l
$CoCl_2.6H_2O$…….....	0.025 mg/l

C. Iron Stock Solution Components

$FeSO_4.7H_2O$…….	27.8 mg/l
Na_2 EDTA. $2H_2O$ …….....	37.3 mg/l

2.2.2. Seed Germination

Tobacco seeds *Nicotiana tabacum* L., var Samsun NN (SNN) were germinated to measure the viability of the seeds under alkaline environment. The seeds were obtained from our laboratory (Institute of Biology, FU-Berlin, Berlin, Germany). A total of 9 lines, wild-type (WT) and 8 PPDK overexpression were tested. The tested transgenic lines are 35S, Δ35S, B33 group (B33-1, B33-2, B33-3) and ΔB33 group (ΔB33-1, ΔB33-2, ΔB33-3). The seeds were surface sterilized in 3.3% sodium hypochlorite (NaOCl) solution for 15 min, and then washed 3 times for 5 min in bi-distilled sterilized water. Then the seeds were placed on a sterile filter paper for drying. After that, they were planted (100 seed / petri-dish) on the surface of MS solid (0.7 % agar) medium pH 8.0, within 9 cm diameter petri-dishes (three replicates/line) under sterilized conditions, and the petri-dishes were sealed by parafilm. The seeds were allowed to germinate in controlled growth conditions, in growth chamber in 16/8h light/dark duration at 24/19 ºC, with light intensity of 150 µE m^{-2} s^{-1}. The germination rate was observed daily under a dissecting microscope.

2.2.3. Preparing Plant Material for Protein Extraction

To prepare the plant material for protein extraction, the WT and the transgenic lines were cultivated on cotton gauze in 15 cm diameter petri-dishes. The petri-dishes were watered every other day with sterilized liquid MS medium pH 8.0, supplemented with 1mM KH_2PO_4. The medium was previously sterilized by autoclave at 121 ºC, for 20 min. The pH was adjusted to pH 8.0 after autoclaving at room temperature using a sterilized pH meter electrode. The petri-dishes were incubated for three weeks under controlled growth conditions, in growth chamber in 16/8h light /dark duration at 24 /19 ºC, with light intensity of 150 µE m^{-2} s^{-1}.

For the same purpose, a soil-sand (2/3) mixture was used for *Mesembryanthemum crystallinum* plant growth in a greenhouse. The plants were watered with tap water for 6 weeks after germination then they were saline stressed by daily watering with 0.5 M NaCl for two weeks.

2.2.3.1. Protein Extraction

Tobaccos WT, transgenic lines roots and leaves tissue of *M. crystallinum* were harvested, weighed (1 g fresh weight) and homogenized immediately to fine powder, with the help of liquid nitrogen in a pre-cooled mortar. All the other steps were performed in ice. The homogenized material was well mixed with 2 ml of extraction buffer, then transferred to a 2 ml pre-cooled eppendorf tube, centrifuged in an eppendorf pre-cooled centrifuge at 4 ºC at 14000 rpm for 10 min. The supernatant was transferred to a 2 ml eppendorf tube and mixed with 1/1 volume of phenol and 1% β-mercaptoethanol, incubated in ice bath for 30 min with continuous shaking. After 30 min of incubation the tubes were centrifuged for 5 min at 4 ºC and 14000 rpm to separate the phases. Protein was then precipitated from 250 µl of the lower phenol phase by adding 15µl of 7.5M ammonium acetate, 1.25 ml of ethanol-glycerin mixture (95/5, v/v) and 10 µl of 2 mM of 1, 4-dithiothreitol (DTT). The precipitation of protein was carried out overnight at -20 ºC. The precipitated protein samples were centrifuged for 15 min at 4 ºC and 14000 rpm. Thereafter, the protein sediment was washed 3-5 times in ethanol-glycerin mixture. The washing step was conducted by adding 1 ml of (95/5, v/v) ice cooled ethanol-glycerin to the protein sample, then centrifuged for 5 min at 4 ºC and 14000 rpm (Höfner *et al.*, 1989). The extracted protein was re-suspended in sample buffer for SDS-PAGE.

Protein Extraction Buffer Components

HEPES-Tris ……	250 mM
DTT *……..	2 mM
ε -amino-n-capronic acid ……	5 mM
Benzamidine	1mM
Leupeptin	20 µM
PMSF *……	1mM
PVP- 40.....	1mM
Sodium dithionite ……	1mM

* These substances were added from stock solution to the extraction buffer immediately at the protein extraction time.

DTT was freshly prepared in water at extraction time.

PMSF stock solution 0.1 M in isopropanol (stored at -20 ºC).

Sample Buffer Components

Tris / HCl pH 6.8…….	0.125 M
Bromophenolblue ……...	0.025% (w/v)
SDS …….....	5%
Urea …….....	4 M
*DTT ……..	2 mM

*Added freshly to the sample buffer solution

2.2.3.2. Determination of Protein Quantity

Total protein concentration was determined using dot-blot method following the modified protocol described by (Zlatanova *et al.*, 1994). Protein samples were mixed with 100 µl of sample buffer solution and 5 µl of 2 mM DTT. The mixture was boiled at 100 ºC for 5 min. 5 µl of each sample was taken and applied by dot-plotting on a nitrocellulose membrane and left to dry at room temperature (RT). After that, the membrane was stained with amido-black stain for 5 min with continuous careful shaking, and then washed three times for 5 min with 50% methanol / water (v/v). Identical membrane surfaces of the samples and blank areas (free of protein as a control sample) were cut out, and the bound dye was eluted with 1 ml of 25 mM sodium hydroxide (NaOH) in 50% methanol (v/v). The absorbance was measured by spectrophotometer at 640 nm. The standard curve was acquired using the bovine serum albumin (BSA) standard of 5-100 µg /l, which was treated the same way.

Amido black …...……..……	0.1% (w/v)
Methanol (MeOH) ….……...	30% (v/v)
Acetic acid ……..……	10% (v/v)

2.2.3.3. SDS Polyacrylamide Gel Electrophoresis

Protein separation was performed according to the method of (Laemmli, 1970). In this method, the protein separation is according to size under denaturation conditions in a vertical SDS acrylamide electrophoresis gel. The gel used in protein separation was with dimensions 10 cm width and 7 cm in height and 1 mm thickness. The lower part or the separating gel consisted of 12.5 % polyacrylamide, the upper part or stacking gel consisted of 3.96 % polyacrylamide. 10 µl of each sample and 3 µl of pre-stained protein marker were put into 12.5 polyacrylamide gel, and subjected to electrophoresis for two hours at 30 V followed by three hours at 150 V. After that, the gel was stained for 60 min in a coomassie staining solution in a 10 cm^2 petri-dish with continuous careful shaking. The protein band was visualized by destaining in 50 % mixture of methanol/water for 2 to 3 h.

Stacking (upper gel) Components

Acrylamide ……...	3.96%
Tris / HCl pH 6.8 …….	0.20 M
SDS ……..	0.1% (w/v)
APS ……..	0.05% (w/v)
Temed ……..	3µl

Separating (lower gel) Components

Acrylamide ……...	12.5%
Tris / HCl pH 8.8 …….	0.38 M
SDS ……..	0.1% (w/v)
APS ……..	0.05% (w/v)
Temed …….……...	3µl

SDS-PAGE Running Buffer Components

Tris (pH not fixed) ……...	0.025 M
Glycine (w/v) ……...	0.192 M
SDS (w/v) ……..……..	0.1%

SDS-PAGE Coomassie Staining Solution

Coomassie R-250 …….	1.25g
MeOH ……...…….....	225 mL
H_2O ……...	225 mL
Glacial acetic acid ……	50 mL

2.2.3.4. Protein Transfer to Nitrocellulose Membrane (Western Blot)

Protein analysis was performed after the blot method of Towbin *et al.* (1979). The protein was transferred from the gel to a nitrocellulose membrane. The protein transfer was carried out overnight at 100 V and 4 ºC.

Western Blot Buffer Components

Tris/HCl pH 8.3 ……..…….	0.025 M
Glycine (w/v) ……..…..….	0.192 M
MeOH (v/v) ……...……..	20%

2.2.3.5. Ponceau S Staining of Protein on Nitrocellulose Membrane

Nitrocellulose membrane was transferred into a square Petri dish 10 cm^2, and incubated for 5 min with 20 ml of ponceau S stain. Then the strained membrane was washed under running tap water with gently shaking by hand until the background became colorless and the protein bands were visible.

Ponceau S Staining Solution Components

Ponceau S (w/v) …….……	0.2%
TCA (v/v) …….…..……	3%

2.2.3.6. Immunological Detection by Specific Antibodies

Membrane was incubated in blocking solution with continuous shaking for about one hour at RT. The blocking step was followed by three times 5 min washing with TBST (TBS + 0.05% Tween 20) at RT. After that, the membrane was incubated overnight in 20 ml freshly prepared primary antibodies, Anti-PPDK, Schmitt *et al.* (1989), 1/3000 at 4 ºC with gently shaking. Then it was washed three times for 5 min by TBST at RT with shaking. Followed by incubation in secondary antibodies, Anti-GAR, (1/1000) for 30 min at 4 ºC, and washed one time for 5 min in TBST at RT.

TBS Buffer

Tris / HCl pH 7 ……..……...	25 mM
NaCl	150 mM

Blocking Solution

Skimmed milk powder (w/v)……..….	5%
Tween 20 (v/v) ……..….....	0.1%
in TBS	

Antibodies Incubation Buffer

Casein (w/v) ……..…	0.2%
Tween 20 (v/v) ……..…	0.1%
Sodium azide (w/v) ……..…	0.01%
in TBS	

Antibodies Dilution Rate

Primary Anti-Bodies

Anti-PPDK (Everl 13) ……..	1/3000

Secondary Anti-Bodies

Anti-GAR-AP …….....……	1/1000

2.2.3.7. Colorimetric Detection by Alkaline Phosphatase

According to the colorimetric detection method for protein, based on BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (4-Nitroblautetrazodiumchlorid), the alkaline phosphatase (AP) catalyzes the phosphate group hydrolysis of BCIP, converts it into appropriate indoxyl derivatives, oxidizes by NBT, and dimerizes to insoluble deep blue color (Sambrook J. and D. W. Russell. 2001) . The washed membrane was incubated in 20 ml of BCIP-NBT solution for 30 s to 2 min with gently shaking in dark at RT until the band color appeared. The reaction was stopped by washing in tap water.

Alkaline Phosphatase Buffer Components

Tris / HCl pH 9.5 …….….....	100 mM
Magnesium chloride ……..……..…….	5 mM
Sodium chloride …….……..	10 mM

Membrane Reaction Solution Components

AP buffer …….……	10 ml
BCIP stock …….…...…..	33 µl
NBT stock …….…...…....	66 µl

Composition of Membrane Reaction Stock Solution

BCIP Stock: 50 mg BCIP in1 ml 100 % DMF

NBT Stock: 50 mg NBT in 1 ml 70 % DMF

2.2.4. Cultivation of Seedlings in Alkaline Liquid MS

In order to determine the growth of the seedlings in insoluble P source, transgenic and WT seedlings were grown in alkaline liquid MS medium pH 8.0. The medium was supplied with three different Pi treatments, Pi-starved (Pi-): growth medium free of phosphate, hydroxyapatite (HAp): growth medium supplemented with hydroxyapatite containing tacitly 1mM of insoluble phosphate, and Pi-sufficient (Pi+): growth medium supplemented with 1mM KH_2PO_4.

2.2.4.1. Preparation of MS Liquid Medium

Liquid MS medium was prepared by mixing the medium stock solutions that were previously prepared (see 2.2.1) in a liter volume size flask. The medium was supplemented with Pi-, HAp or Pi+. pH of the medium was adjusted to pH 8.0 with 1 N NaOH using pipette, and then sterilized by autoclave for 20 min. The medium was cooled to RT, and the pH was re-adjusted to pH 8.0. Then it was distributed (20ml /flask) into 100 ml volume sterilized erlenmeyer flasks.

2.2.4.2. Seed Cultivation and Growth Conditions

Twenty mg of transgenic and WT seeds were weighed and surface sterilized as described previously in paragraph (2.2.2). Then seeds were transferred to 20 ml of liquid MS medium in 100 ml flasks, some flasks were left without seeds as a control. Cultivation process was conducted under antiseptic conditions on laminar airflow hood. All flasks were transferred to the growth chamber. Growth was performed under continuous shaking at 150 round /min and 150 µE m^{-2} s^{-1} of light intensity in 16 / 8 h light / dark duration and 24 /19 ºC.

2.2.4.3. pH Determination of Growth Medium

In order to determine the ability of transgenic and WT seedlings to change the pH of the growth medium, the growth medium was collected and pH was measured (using a pH meter). The experiment was performed by growing transgenic and WT seedlings in alkaline liquid MS medium pH 8.0 in Pi-, HAp and Pi+ treatments. 20 mg seed from each line was germinated in 20 ml of MS medium in 100 ml erlenmeyer flasks (medium pH was adjusted to pH 8.0 before autoclaving, and re-fixed after autoclave). The growth of the seedlings was stopped at day 10 of growth. The growth medium was then collected and the pH of the medium was determined. pH of control (negative) medium was also determined at the same time (Narang *et al.*, 2000).

2.2.4.4. Fresh and Dry Biomass Determination

Seedlings were harvested after 10 days of growth; this was done by pouring flask contents into a plastic strainer, and washed three times with bi-distilled water. After that, they were counted on wet filter paper. The counted seedlings on the wet paper were centrifuged for 3 min at 5000 rpm and 15 ºC (Broyer and Hoagland, 1940) to remove any water particles and keep them moist. Fresh biomass was determined by weighing. Dry biomass was determined by weighing samples after they were dried for 72 h at 65 ºC.

2.2.4.5. Determination of Total Phosphorus in Whole Seedlings

Total phosphorus of the transgenic and WT seedlings was extracted according to the method described by (Wang *et al.*, 2009a) with some modifications. 20 mg of seed from each line was germinated in 20 ml of MS medium in 100 ml erlenmeyer flask (medium pH was adjusted to pH 8.0 before autoclaving, and re-fixed after autoclave). The growth medium was supplied with Pi-, HAp and Pi+ treatments.

Seedlings were harvested at day 10 of growth, washed thoroughly in bi-distilled water then dried in oven for 72 h at 65 ºC. 20 mg of dried and powdered samples were pre-digested with 20 µl of 5 M H_2SO_4 for (1 h) at 100 ^{o}C in 5 ml volume glass tubes. 2 ml of bi-distilled water was added and the digestion continued at 100 ºC for extra (2 h). The color of the solution was removed by adding 50 mg active charcoal in three steps. After each step the samples were vortexed and centrifuged for 10 min at 14000 rpm at RT. After that, the colorless supernatant was transferred to a 2 ml eppendorf tube. (All glass wares used in phosphorus determination were acid washed).

The total phosphorus was determined by using the method of (Bencini *et al.*, 1983). This method is based on a one-step spectrophotometric assay that needs a single stable reagent solution. The reagent solution consists of ammonium molybdate and zinc acetate at pH 5.0, which produces a stable complex with orthophosphate. To prepare samples for phosphorus determination, 200 µl of sample was added to 600 µl of the phosphate reagent mixture in 2ml eppendorf tubes, mixed with care and left in RT for at least 30 s. The absorbance of the sample solution was determined at 350 nm using a spectrophotometer.

Reagent Mixture

Ammonium molybdate ……..…..... 15 mM

Zinc acetate ……..……..… 100 mM

(in deionized distilled water).

2.2.4.6. Determination of Organic acid in Growth Medium

It is known that organic acid exudation is enhanced in the roots of Pi-stressed plants (Aono *et al.*, 2001). In order to determine the amount of exuded organic acid in growth medium of the transgenic roots in hydroxyapatite treatment, we used enzyme kits L-citric acid and L-malic acid. The transgenic and WT seedlings were grown in 20 ml alkaline MS liquid medium pH 8.0 in Pi-, HAp and Pi+ treatments. Growth was performed by incubating the culture in a growth chamber with continuous shaking at 16/8 h light/dark duration at 150 µE m^{-2} s^{-1}. The experiment was stopped at day 10 of growth. The growth medium was collected and lyophilized. Lyophilized samples were re-dissolved in 2ml ultrapure bi-distilled water, and then analyzed for malic and citric acids using the enzyme kits, following procedures provided by the company.

2.2.4.6.1. Citric Acid Determination

Production of oxaloacetate and acetate occurs during the conversion of citric acid (citrate) in reaction catalysed by the enzyme citrate lyase (CL). In the presence of enzymes L-malate dehydrogenase (L-MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product (pyruvate) are reduced to L-malate and L-lactate respectively, by reducing nicotinamide-adenine dinucleotide (NADH). The amount of NADH oxidized in reactions of oxaloacetate and pyruvate is stoichiometric to the amount of citrate. NADH is determined at 340 nm.

$$\text{Citric acid} \xrightarrow{\text{CL}} \text{oxaloacetate} + \text{acetate}$$

$$\text{Oxaloacetate} + \text{NADH}+\text{H}^+ \xrightarrow{\text{L-MDH}} \text{L-malate} + \text{NAD}^+$$

$$\text{Pyruvate} + \text{NADH}+\text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+$$

2.2.4.6.2. Malic Acid Determination

Detection of L-malic acid requires two enzyme reactions. The first reaction is catalyzed by L-malate dehydrogenase (L-MDH), L-malic acid is oxidized to oxaloacetate by nicotinamide-adenine dinucleotide (NAD+). The equilibrium of this reaction lies on the side of L-malate. Removal of oxaloacetate from reaction system causes displacement of equilibrium in favor of oxaloacetate. The second reaction is catalyzed by the enzyme glutamate-oxaloacetate transaminase (GOT), oxaloacetate is converted to L-aspartate in presence of L-glutamate. The amount of nicotinamide adenine dinucleotide (NADH) formed is stoichiometric to the amount of L-malate. The increase in nicotinamide adenine dinucleotide (NADH) is measured by means of its light absorbance at 340 nm.

$$\text{L-Malate} + \text{NAD}^+ \xleftrightarrow{\text{L-MDH}} \text{oxaloacetate} + \text{NADH}+\text{H}^+$$

$$\text{Oxaloacetate} + \text{L-glutamate} \xleftrightarrow{\text{GOT}} \text{L-aspartate} + \text{2-oxoglutarate}$$

2.2.5. Cultivation of Seedlings in Alkaline Solid MS

In order to determine the growth of the transgenic seedlings and their response to insoluble P source in alkaline conditions, we measured the length of the primary and secondary root, the biomass of the seedling shoot and root, root:shoot ratio, ability of seedlings to lower the pH of the rhizosphere, and the root surface acid phosphatase activity.

2.2.5.1. Preparation of Growth Medium

Growth medium was prepared by collecting and mixing appropriate volumes from previously prepared stock solutions (see 2.2.1). The medium was supplemented with

different P treatments, Pi-, HAp or Pi+. pH was adjusted to pH 8.0 with 1N NaOH. The medium was solidified by adding (1%) low melting point agar and boiled to complete dissolving of agar and then sterilized. After that, pH was re-adjusted to pH 8.0 and poured into petri-dish after sterilization.

2.2.5.2. Seed Cultivation

Seeds of WT and the transgenic lines were sterilized, washed and dried on sterilized filter paper. 7 to 8 seeds were transferred onto MS agar surface in 9 cm petri-dish. Then the petri-dishes were sealed with parafilm. After that, they were incubated diagonally at 70 º in growth chamber with 16/8 h light /dark duration at 24 /19 ºC, with light intensity of 150 µE m^{-2} s^{-1}.

2.2.5.3. Determination of the Length of Root and Shoot

The length of seedlings root and shoot were determined after 12, 24 and 36 days of growth in alkaline MS medium at pH 8.0, using a ruler.

2.2.5.4. Determination of Fresh and Dry Biomass of Root and Shoot

In order to determine fresh and dry biomass of root and shoot, seedlings were harvested and washed with bi-distilled water, then the water was removed using filter-paper. After that, the roots were separated from the shoots. Fresh biomass of the collected shoot and root was determined by weighing. Then, the shoots and roots were collected in small paper sacks, and dried in oven for 72 h at 65 ºC. The dry biomass was determined by weighing.

2.2.5.5. Determination of Shoot Content of Total Phosphorus

Total phosphorus of the shoot was extracted according to method of Wang *et al*. (2009b) and measured according to Bencini *et al.* (1983), as mentioned in (see 2.3.5).

2.2.5.6. Phosphatase Activity Assay

Acid phosphatase activity (E.C.3.11.3.2) orthophosphoric-monoester phosphohydrolase was measured using the modified protocol as mentioned by (Dodd *et al.*, 1987).

Assay Reaction Solution

P-nitrophenylphosphate (*P*-NPP) ……	50 mM
Sodium acetate buffer (pH 5.2)…….....	0.1 M

Seedlings at 12, 24 and 36 days of growth were harvested; roots were separated from shoots. The excised fresh roots were washed with bi-distilled water, and the water was removed by spreading the roots on filter paper. 20 mg of each of fresh root samples was weighed, using the end of primary root (2-3 cm) length and incubated with 1ml of the assay solution, 200 µl of *P*-NPP and 800 µl of Na-acetate (NaOAc) at pH 5.2 for 30 min. at 37 ºC with continuous shaking at 50 rpm. The reaction was stopped by adding 1 ml of 0.5 M NaOH. The control sample was assayed by adding 200 µl of *P*-NPP to NaOAc buffer solution immediately before adding NaOH. The released nitrophenol amount during incubation was determined spectrophotometrically at 405 nm. The recorded activity represented primary root surface acid phosphatase activity, with small amount of internal root phosphatase exuded from the cut edge. Standard curve was prepared in NaOAc buffer solution containing 0, 10, 20, 30, 40 and 50 mM *P*-nitrophenylphosphate using 10 µl of 1mg / ml acid phosphatase enzyme extracted from sweet potatoes (Sigma).

2.2.6. Staining Root Surface Acid Phosphatase Activity

Acid phosphatase activity was visualized on the surface of root by incubating the roots with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) using the method of (Bozzo *et al.*, 2006). The BCIP is a colorless soluble substrate, but acid phosphatase hydrolysis converts it to an insoluble blue color product (Goldstein *et al.*, 1988). Eradicated roots from 15 days old WT and transgenic seedlings grown in Pi-, HAp or Pi+ were washed in distilled water, and placed on 15 % agar in a petri-dish. Then they were covered by a layer of 0.1 mg / ml of BCIP in 50 mM NaOAc, pH 5.3; 10 mM $MgCl_2$ mixed with 0.6 % low

melting point agar. After that, the petri-dishes were sealed, incubated in the dark at 24 ºC, watched and scanned after 4, 8, 12, 24 and 48 h of incubation.

2.2.7. Following Acidification of the Roots

Root ability to change the pH of growth medium was tested using pH color indicator bromocresol purple (BCP). This color indicator is non-toxic for plants, and its color changes from violet at pH 6.8 to yellow at pH 5.2. After three weeks of germination on alkaline MS solid medium pH 8.0, in Pi-, HAp or Pi+, WT and transgenic seedlings were removed, washed with sterilized bi-distilled water, then placed on alkaline MS medium at pH 8.0, containing 100 mg / liter BCP in a petri-dish. The petri-dishes were sealed with parafilm; the colored medium parts of the petri-dishes were covered to protect roots and medium from light. Then they were incubated in a growth chamber at 24 /19 ºC and 16/8h light /dark duration, with light intensity of 150 µE m^{-2} s^{-1}, they were scanned every 12 h. pH values for the medium were measured and recorded after 72 h of seedlings incubation by using a pH meter with flat surface electrode (Marschner and Römheld, 1983; Gollany and Schumacher, 1993).

2.2.8. Cultivation of Plants in Alkaline Soil under Greenhouse Conditions

2.2.8.1. Soil Preparation

To test the ability of plants to grow and yield in low-P soil, various amounts of $CaCO_3$ were added to the soil, thus obtaining alkaline soil. The prepared ratios were 16, 32, 64, 128, 256 and up to 512 g $CaCO_3$ / kg of soil, and soil without $CaCO_3$ as control soil treatment. The soil was sterilized for 24 h before use. After that, the pH of the soil was determined, two combinations were chosen (256 and 512 g kg^{-1} $CaCO_3$ / soil) with pH of (7.2 and 8.3) respectively, in addition to control soil treatment (0.0 g kg^{-1} $CaCO_3$) with pH (5.8).

2.2.8.2. Cultivation of Seeds

Seeds of WT and transgenic seedlings were spread at the surface of well watered soil in 2.5 volume size liters pots, 12 pots / line for each treatment. They were allowed to grow under greenhouse conditions with around 24± ºC and 16/8 h light/dark cycle. After 5 to 7 days of germination, one of the best growing seedlings was kept for each pot and the rest were removed. The plants were watered daily with tap water. To ensure balanced exposure of plants to light, pots positions were changed every two days.

2.2.8.3. Measurements of Shoot Height and Biomass

The height and biomass were measured after harvest of plants grown for three months. To measure height of plants, the height measure tool was put in parallel with plant stem at the point of soil surface to the top of the plant vertically. Plant green part (shoot) fresh biomass was determined after cutting off the stem from soil surface point. After that, the plant was cut into small pieces and placed in a paper bag. To measure dry biomass the shoot was dried from 96 to 120 h in oven at 75 ºC. Dry biomass was determined by weighing.

2.2.8.4. Determination of Soil pH

Soil pH of the transgenic and WT plants was determined after three months of growth in different soil / $CaCO_3$ mixtures, by removing 1-2 cm^3 from the surface layer of soil, and collecting soil from the root zone (50 grams). The collected soil samples were mixed thoroughly with 100 ml of distilled water. Then the mixture was filtered using filter paper, and pH was measured for the collected water using a glass electrode pH meter (Youssef and Chino, 1989).

2.2.8.5. Determination of Plant Leaves Content of Total Phosphorus

Total phosphorus of plant leaves was determined after three months of growth in greenhouse. This determination was accomplished by extracting plant content of

phosphorus using the method of Wang *et al.* (2009b). Plant content of phosphorus was measured according to the method of Bencini *et al.* (1983) (see 2.3.5).

2.2.8.6. Yield of Plant and Harvesting of Capsules

The floral parts of the plants were immediately covered with white paper bags at flowering stage to prevent cross fertilization among lines. The yield of seed capsules of plants was collected by cutting the flowers from the stem after ensuring that seed capsules were dry (60-75 days after flowering). Then paper bags containing capsules were transferred to the laboratory and the capsules were carefully separated from the plant floral part, counted and weighed. Ratio of seeds weight to capsules gross weight was determined by weighing the capsule content of seeds.

2.2.9. Transgenic Seedlings Metabolic Response to Insoluble P source

To determine the metabolic changes induced by growing plants under Pi-stress in alkaline conditions, GC-MS (Gas chromatography mass spectrometry) analysis was done. The method is based on the protocol of (Lisec *et al.*, 2006) with some modifications.

2.2.9.1. Collecting of Plant Material and Sampling

Transgenic and WT seedlings were harvested at light period after 2 and 3 weeks of growth in Pi-, HAp or Pi+ in alkaline MS pH 8.0 liquid, and solid medium respectively. The seedlings in solid medium were removed to a clean petri-dish containing bi-distilled sterilized water for washing. Water was removed from the washed seedlings using filter paper. Shoots were rapidly separated from roots, both shoots and roots were immediately put in 2 ml eppendorf tubes and frozen in liquid nitrogen. In liquid culture, seedlings were removed from the growth medium and rapidly washed, washing water was removed and the seedlings were frozen in eppendorf tubes. After that, all samples were stored in -84 ºC.

2.2.9.2. Extraction of Plant Material

Before extraction, all laboratory materials and tools were cooled down to prevent samples from melting. Frozen samples were crushed and homogenized using clean stainless steel metal ball mill (5 mm diameter) in mixer-mill grinder (Retsch mill) for 2 min at 25 Hz. Then 50 mg of shoots, roots or whole seedlings material were taken, put into 2 ml eppendorf tubes, labeled and stored at -84 ºC. Extraction was started by adding 360 µl methanol (pre-cooled to -20 ºC) containing internal ribitol to frozen samples. Then the samples were transferred to thermo-mixer, and incubated with continuous shaking at 1200 rpm for 15 min under 70 ºC. The pressure of hot methanol was released from the eppendorf tubes 1 min after shaking start. Then samples were cooled down to RT. After that, 200 µl of chloroform was added to each sample and the samples were shaken in thermo-mixer for 5 min at 37 ºC and 1200 rpm, 400µl of distilled deionized water was added to the samples. The samples were vortexed for around 10 s and centrifuged for 5 min at 14000 rpm, 160 µl of supernatant or upper phase (polar phase) of centrifuged samples was transferred to new 2 ml eppendorf tubes for analysis. Another 160 µl was kept as backup sample (in case of loss of sample). The samples were dried 2 h in speed vacuum concentrator at 30 ºC and then stored at -20 ºC.

2.2.9.3. Deriving of Samples

Dried stored samples were taken out of freezer, and a steel mill ball was put in each eppendorf tube to help dissolve sediment more quickly. Then (40 µl) of Methoxyaminhydrochlorid / pyridine solution (20 mg Methoxyaminhydrochlorid/1 ml of pyridine (w/v)) was added. After that, the tubes were shaken in a thermo-mixer for 90 min at 30 ºC and 1200 rpm. Sample tubes were allowed to spin down for 1 min, (10 µl) alkane mixture and (70 µl) MSTFA reagent (*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide) were added to the tubes and shaken for 30 min at 30 ºC and 1200 rpm. After that, the tubes were centrifuged for 30 s at 14000 rpm and a volume of (90 µl) of sample was collected and transferred to GC vial. Figure 2.4 illustrates experimental procedure for plant extract preparation for GC-MS analysis.

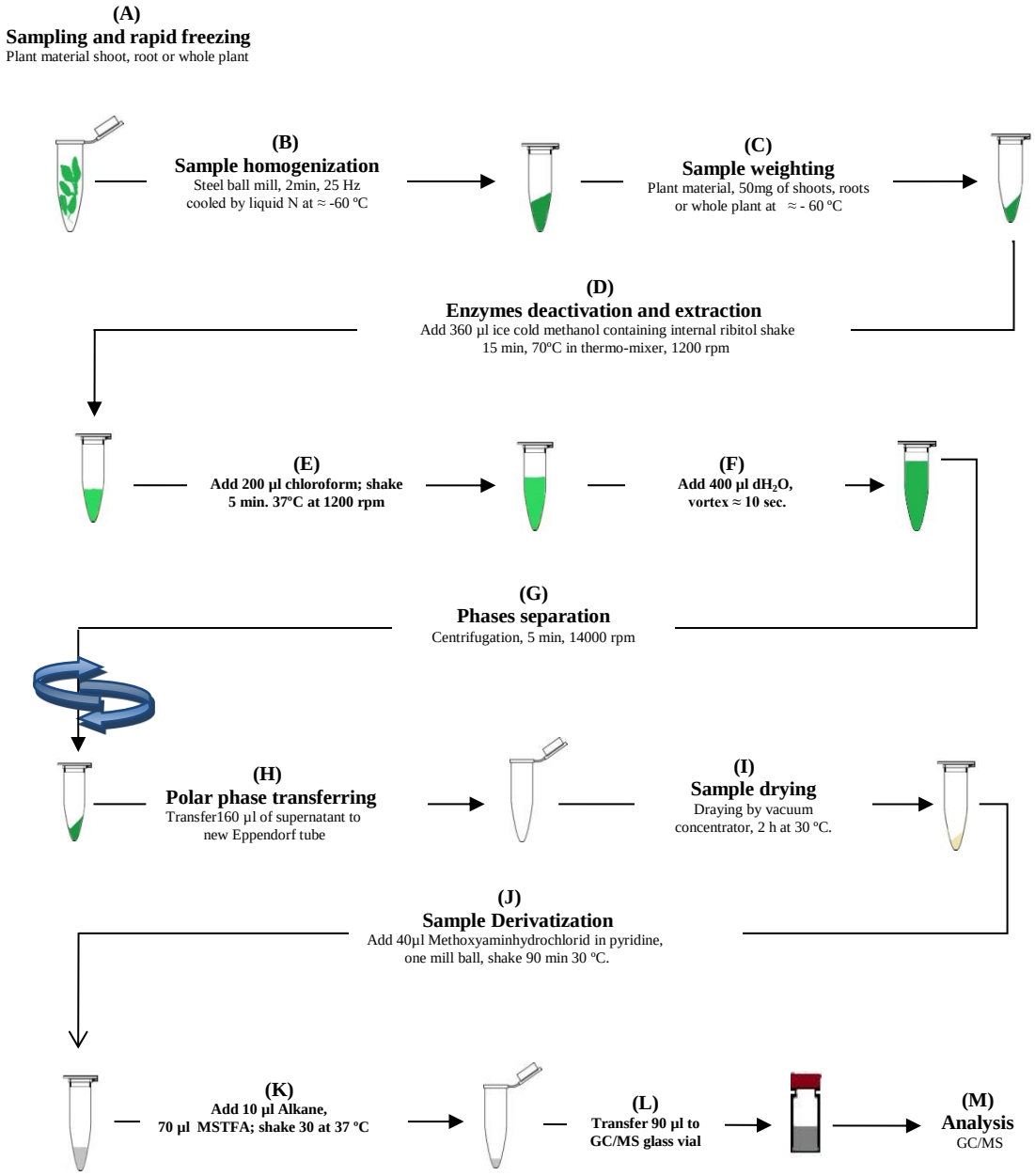


Figure 2.4: Experiment procedure for plant extract preparation of GC-MS analysis samples. A: Plant material: root, shoot or whole seedlings sampling and rapid freezing, **B:** Homogenization using steel ball mill, 2 min. 25 Hz cooled by liquid N at ≈ -60 ºC, **C:** Weighing, the homogenised plant material 50mg / sample, **D:** Enzymes deactivation and extraction: add 360 µl ice cold methanol containing internal ribitol, shake 15 min, 70 ºC in thermo-mixer, 1200 rpm, **E:** Add 200 µl chloroform; shake 5 min. 37 ºC, 1200 rpm, **F:** Add 400 µl dH_2O, vortex ≈ 10 sec, **G:** Phases separation: centrifugation 5 min, 14000 rpm, **H:** Polar phase transferring, transfer 160 µl of supernatant to new Eppendorf tube, **I:** Sample drying by vacuum concentrator, 2 h at 30 ºC, **J:** Derivatization, add 40 µl Methoxyaminhydrochlorid in pyridine, one mill ball, shake 90 min 30 ºC, **K:** Add 10µl Alkane, 70µl MSTFA, shake 30 at 37 ºC, **L:** Transfer 90 µl to GC/MS glass vial**, M:** analyze by GC-MS.

2.2.10. Statistical Analysis and Software

2.2.10.1. Statistical Analysis

The experiments were repeated at least twice independently. Number of repetitions for each variant (s) is specified in the appropriate presentation of results. Data of mean values and associated standard deviations were generated with computer program (Excel 2010) for Windows (Microsoft Corporation, USA).To compare mean value, student *t*-test was generated with graph-pad computer program at significant level of 95%. Definition of the value of significant (*** $p \leq 0, 0001$= an extreme significant; ** $p \leq$ 0,001= very significant; * $p \leq 0, 01$= significant; $p \leq 0, 01$= non-significant).

2.2.10.2. GC-MS Data Analysis Software

1. **MetAlign:** is a computer software program used for pre-processing, analysis, alignment and comparison of full-scan mass spectrometry datasets (GC-MS) data (http://www.wageningenur.nl/en/show/MetAlign-1.htm).

2. **Tagfinder:** is a computer software tool used for alignment of large GC-MS-based metabolite profiling experiments into statistically accessible data matrices (Luedemann *et al.*, 2008).

3. **AMDIS:** is a computer program that extracts spectra for individual components in a GC/MS data file (http://chemdata.nist.gov).

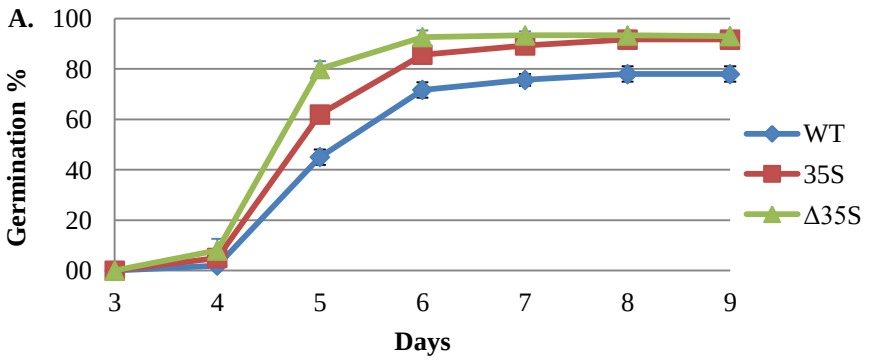
3. Results

This chapter consists of three sections: the first section summarizes the result of PPDK expression of transgenic tobacco at the protein level. The second section gives an overview of the influence of phosphate nourishment on growth. We measured the fresh and dry biomass, length of shoot, length of primary and secondary root, plant content of P, exudation of organic acid, activity of acid phosphatase, and plant yield of capsules. The last section deals with the outcome of the metabolic profiling.

3.1. Seed Germination

A germination test is often conducted on seed before planting. Monitoring the time taken to germinate seed, gives an indication of their viability. *In*-vitro, germination of seed is usually carried out on a wet filter paper (Shoemaker and Carlson, 1990; Manz *et al.*, 2005) or on MS solid medium pH 5.7 (Schernthaner *et al.*, 2003). In order to test the viability of seed, and time taken to germinate in alkaline conditions, the same age sterilized transgenic and wild-type seeds were germinated in sterilized MS solid medium pH 8.0 in petri-dishes (see 2.2.2).

As is shown in (Fig. 3.1) germination of the transgenic and wild-type seeds started at the same time at day 4. Germination rate of the transgenic seedlings was 93% and 78% for wild-type at the end of germination time.



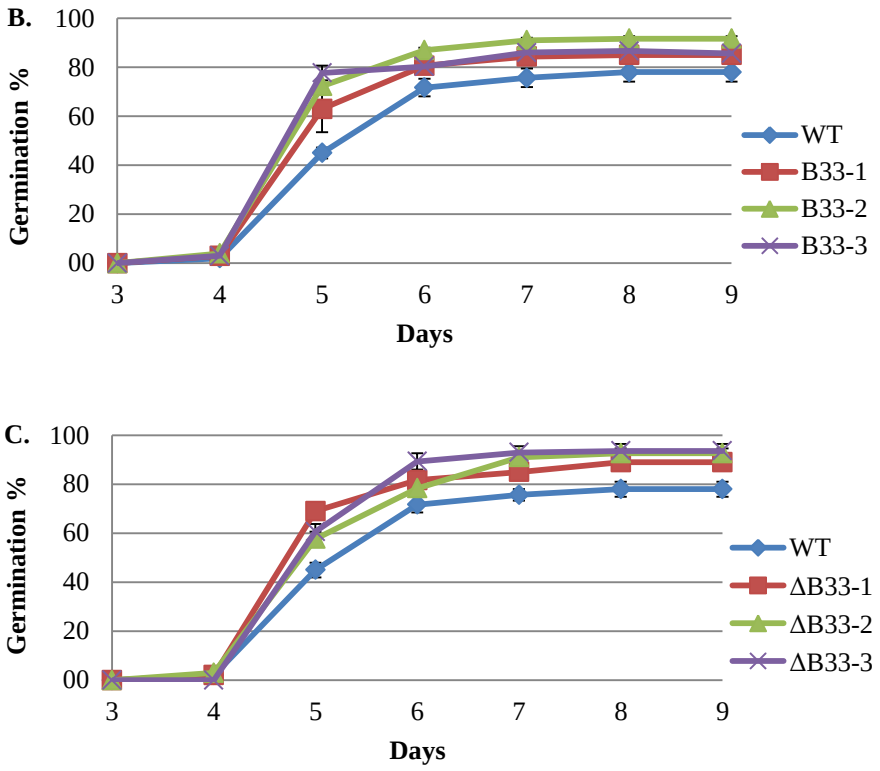


Figure 3.1: Germination rate of the seed. Seeds were germinated on alkaline solid MS medium pH 8.0. **A.** 35S and Δ35S transgenic lines rate of germination compared with that of wild-type, **B.** B33 transgenic lines rate of germination compared with that of wild-type, **C.** ΔB33 transgenic lines rate of germination compared with that of wild-type. The data represent the mean and standard error of 3 rep, 100 seeds each.

3.2. Testing the Overproduction of PPDK in the Transgenic Tobacco

The transgenic lines that are used in this study were constructed by A. Sheriff, (1994) and R. Stenzel (1997). To prove the overproduction of PPDK protein in the transgenic lines compared with that of the wild-type, SDS-PAGE and western blot were used. To achieve that, the protein of 14-day old transgenic root was extracted after the method of (Höfner *et al.*, 1989), (see 2.2.3.1). The extracted protein of the leaves of *Mesembryanthemum crystallinum* plant was used as a positive control. The plant was grown in a greenhouse for 6 weeks. The 6-week old plant continued growing for another 2 weeks under saline stress before it was used for protein extraction. The plant was stressed by daily watering with 500 mM NaCl (Bohnert *et al.*, 1988). The concentration of total protein was determined by amido-black method as mentioned previously in section (see 2.2.3.2). 10µl of the protein of the same concentration of the transgenics, wild-type and

the positive control of *M.crystallinum* was loaded on SDS-PAGE (see 2.2.3.3). The specific antibodies (anti-*PPDK*) that react with the PPDK protein (Schmitt *et al.*, 1989) was used to detect the PPDK protein on the western-blot membrane (see 2.2.3.6 and 2.2.3.7).

As is shown in (Fig. 3.2A and B) the strength of PPDK protein bands of the transgenic lines on the SDS-PAGE and western-blot was considerably higher, compared with the faint band of the wild-type. In contrast, the strength of PPDK bands of the transgenic lines and the PPDK positive control of *M. crystallinum* were similar, which is consistent with the result of (Trejo-Téllez *et al.*, 2010). The result of western blot test suggests that our transgenic lines still carry the trait of PPDK overexpression, and are still capable of overproducing of PPDK.

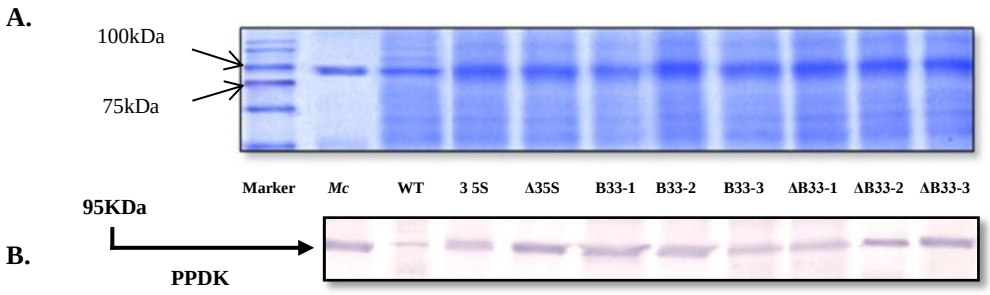


Figure 3.2: A 14-day roots of the transgenic tobacco overproduce the *Mesembryanthemum crystallinum* PPDK protein. A. SDS-PAGE, **B.** Western blot results of the roots PPDK protein for (35S, B33-1, B33-2, B33-3) lines, and ΔPPDK (Δ 35S, Δ B33-1, Δ B33-2, Δ B33-3) lines. *M. crystallinum (Mc)* leaf protein was used as positive PPDK control and wild-type roots (WT) protein was used as negative PPDK control. The PPDK protein molecular mass is shown in right in (kDa). The *M. crystallinum* plant used in this analysis was stressed at age of 6 weeks with 0.5M NaCl for 2 weeks to induce the PPDK overproduction.

3.3. Transgenic Plants Efficient use of Sparingly Soluble HAp as a Source of P in Alkaline Liquid MS Medium

In order to determine the influence of different P-nutrition on the growth of seedlings, we grew the transgenic lines, which express either a plastidic pyruvate, orthophosphate dikinase (PPDK) or a putative cytosolic (ΔPPDK) and the wild-type in

alkaline liquid MS medium pH 8.0. Three different sources of P-nutrition were used, Pi-starved (Pi-) treatment: (growth medium free of P source), hydroxyapatite (HAp) treatment: (hydroxyapatite containing tacitly 1mM of phosphate, used as insoluble P source) and Pi-sufficient (Pi+) treatment: (1mM KH_2PO_4, used as soluble P source).

3.3.1. P-stress Induces Change of pH in Growth Solution

Soil alkalinity is one of the plant growth challenges. In this soil, P becomes insoluble and inaccessible for plant uptake. This adversely affects the growth and development of the plant (Hopkins and Ellsworth, 2005; Lynch and Brown, 2008). In order to investigate the ability of the transgenic and wild-type seedlings to lower the pH, the seedlings were allowed to grow for 10 days in alkaline MS liquid medium pH 8.0 in Pi-, HAp and Pi+ treatments.

An appreciable decrease of pH of growth solution was observed, when the seedlings were grown in Pi-starved (Pi-) and hydroxyapatite (HAp) treatments. In these treatments, the transgenic seedlings significantly reduced the pH of the growth solution, compared with the wild-type. In contrast, when the seedlings were grown in Pi-sufficient (Pi+) treatment, only a slight reduction in the pH of the growth solution was observed, this reduction was comparable in both the transgenic and the wild-type (Fig. 3.3), which is consistent with the results of (Dakora and Phillips, 2002; Shahbaz *et al.*, 2006). This suggests that the ability of the transgenics to reduce the pH of the growth solution was relatively higher under Pi-stress (Pi- and HAp), compared with that of the wild-type.

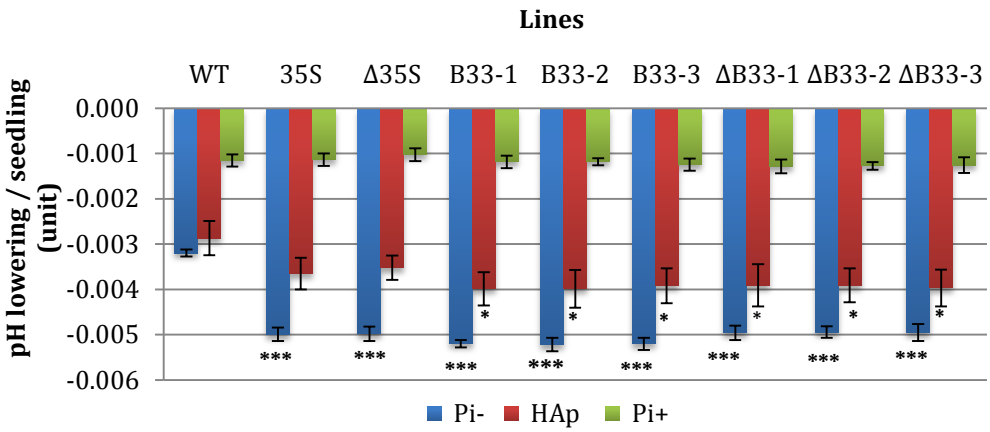


Figure 3.3: Change in the pH of the growth solution after 10 days. Transgenic and wild-type seedlings grown in alkaline MS liquid medium with a starting pH of 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments. The data are from three independent experiments. (n= 190-210 each, ±SD, t-test: *** $p \leq 0, 0001$; * $p \leq 0, 01$).

3.3.2. Determination of the Plant Biomass

Wissuwa *et al.* (2005), found a significant decrease of the biomass of plants grown in Pi-deficient medium. According to (Mollier and Pellerin, 1999) the dry biomass for maize grown for 14 days in Pi-deficient soil was 23 % compared to that in control treatment. In this study, to determine the growth of the transgenics and wild-type in different Pi-nutrition sources, the seedlings were grown in 20 ml alkaline MS liquid medium in Pi-starved (Pi-), hydroxyapatite (HAp) and Pi-sufficient (Pi+) conditions.

As is shown in (Fig. 3.4 and 3.5), the growth and accumulated biomass of the transgenic seedlings was significantly higher in HAp, compared with those of the wild-type. The biomass of the transgenic seedlings grown in HAp was comparable to those grown in Pi+. In contrast, a substantial increase in the biomass of the transgenic seedlings was observed in HAp, compared with that in Pi-. The biomass of the wild-type in HAp was comparable to that in Pi-. This suggests that, the transgenic seedlings were able to use HAp as a source of phosphate more efficiently than the wild-type which showed weak ability in using HAp.

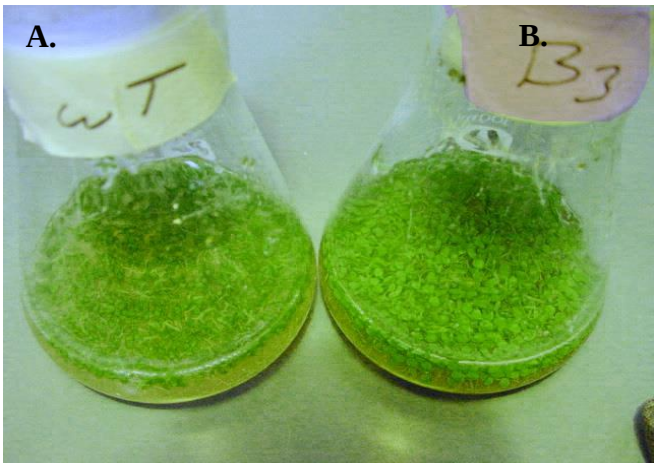


Figure 3.4: The transgenic and wild-type seedlings after 7 days of growth in alkaline MS liquid medium at pH 8.0, supplied with HAp. **A.** wild-type, and **B.** B33-3 transgenic line.

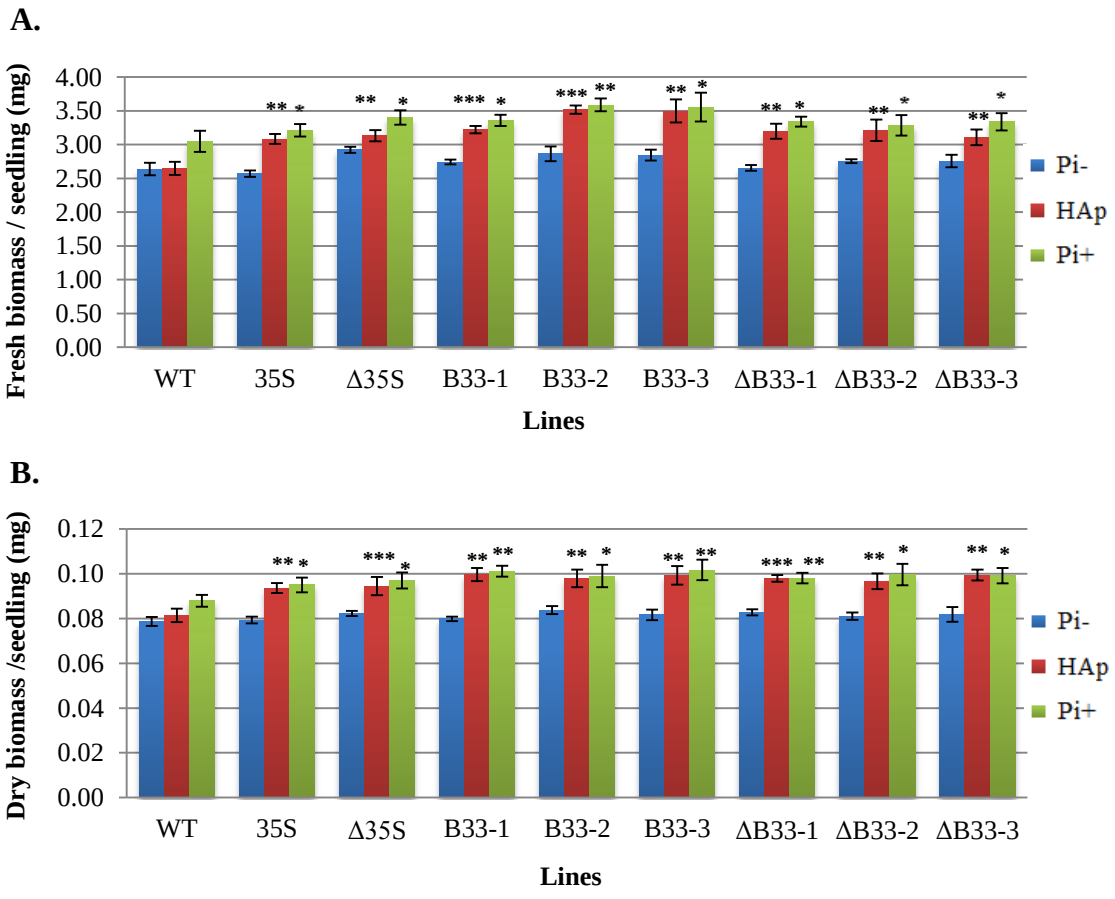


Figure 3.5: Fresh and dry biomass (mg). Transgenic and wild-type seedlings biomass after 10 days of growth in alkaline liquid MS medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments. **A.** Fresh biomass**.** **B.** Dry biomass. The data are from three independent experiments. (rep.3, n=190-210 each, t-test: *** $p \leq 0,0001$; ** $p \leq 0,001$; * $p \leq 0,01$).

3.3.3. Measurement of Whole Seedlings Content of Phosphorus

Phosphorus (P) is important for the plant growth (Hernández *et al.*, 2007). P deficiency is critical in calcareous and alkaline soils (Shenoy and Kalagudi, 2005). According to the results obtained by Zia *et al.* (1988), the P content of maize was significantly affected by different treatments of P. Therefore, determination of P content in the plant could be an indication of the plant ability to uptake P in Hydroxyapatite environment. In order to determine the efficiency of the seedlings in using of different sources of P (Pi-, HAp, or Pi+), the P content of the whole seedlings was measured as is shown in (2.3.5).

In (Fig. 3.6A: P/seedling) and (Fig. 3.6B: P/g DW), a significant increase in the transgenic whole seedlings content of P was observed in HAp, compared with that in the wild-type. In contrast, the transgenic and wild-type whole seedlings content of P in Pi- and Pi+ was similar. The P content of the transgenic whole seedlings in HAp was comparable to that in Pi+. In contrast, the P content of the whole wild-type seedlings in HAp was appreciably less than that in Pi+. As expected, the P content of the transgenic and wild-type whole seedlings in Pi- showed decrease, compared with those in HAp and Pi+ (Fig. 3.6A).

The observed increase in P content of the transgenic whole seedlings in HAp treatment could be due to their ability to utilize HAp more efficiently than the wild-type.

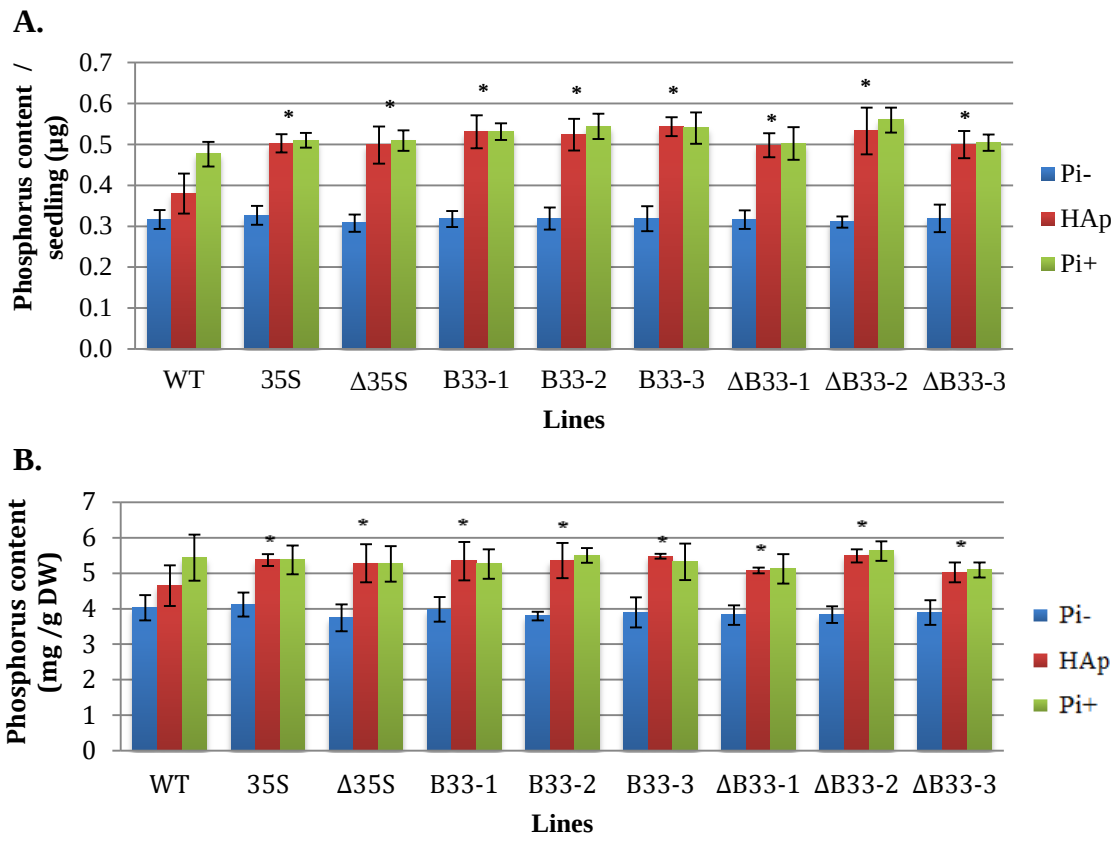


Figure 3.6: Whole seedlings content of total phosphorus. Transgenic and wild-type seedlings grown for 10 days in alkaline MS liquid pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments. **A.** phosphorus content per seedling (µg) **B.** phosphorus content (mg / g DW). The data are from three independent experiments. (rep.3, n=190-210 each, ±SD, t-test: * $p \leq 0, 01$).

3.3.4. Exudation of Organic Acids

Organic acids such as malate and citrate have been suggested to be involved in many of the processes in the rhizosphere, including uptake of nutrient and detoxification of metal. Data obtained from studies conducted on plants like, rape, radish, and white lupin in alkaline or in calcareous soils, showed an increase of organic acid exudation under phosphate deficiency (Zhang *et al.*, 1997; Guo-Hui, 2012; Siane, 2012). To determine the content of organic acids in the growth medium, we used the enzyme kits TC L-citric acid and TC L-malic acid. The transgenic and wild-type seedlings were grown in 20 ml alkaline MS liquid pH 8.0, in different sources of Pi-nutrition (Pi-, HAp, or Pi+).

The exudation of citric (Fig. 3.7) and malic acids (Fig. 3.8) of the transgenic seedlings significantly increased in HAp, compared with those of the wild-type. In contrast, they did not increase in Pi- and Pi+ compared with those of the wild-type. The exudation of citric and malic acids of the transgenic seedlings in HAp was considerably higher than in Pi- and Pi+. In contrast, the exudation of citric and malic acids of the wild-type in HAp was comparable to that in Pi- and Pi+.

The data indicate that, presence of HAp in the growth medium has stimulated the transgenic seedlings to increase the exudation of citric and malic acids significantly higher than the wild-type.

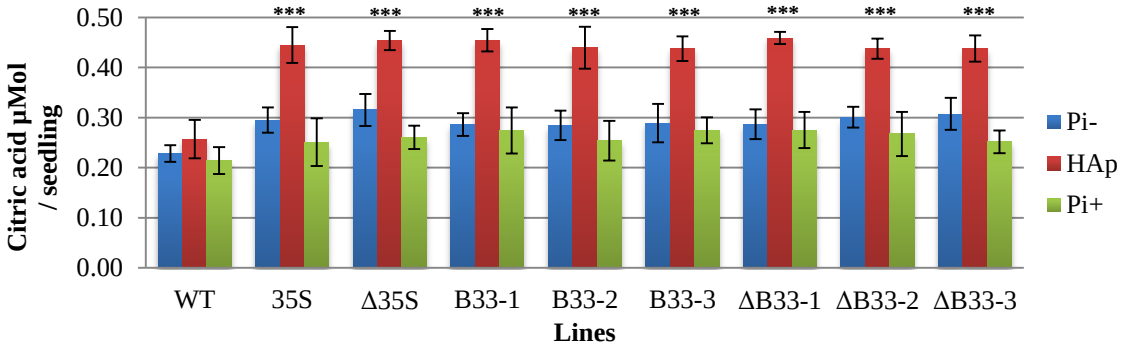


Figure 3.7: Citric acid amount (µMol/seedling). Transgenic and wild-type seedlings grown for 10 days in alkaline MS liquid pH 8.0 in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments. The data are from three independent experiments. (rep.3, n=190-210 each, ±SD, test *** $p \leq 0,0001$).

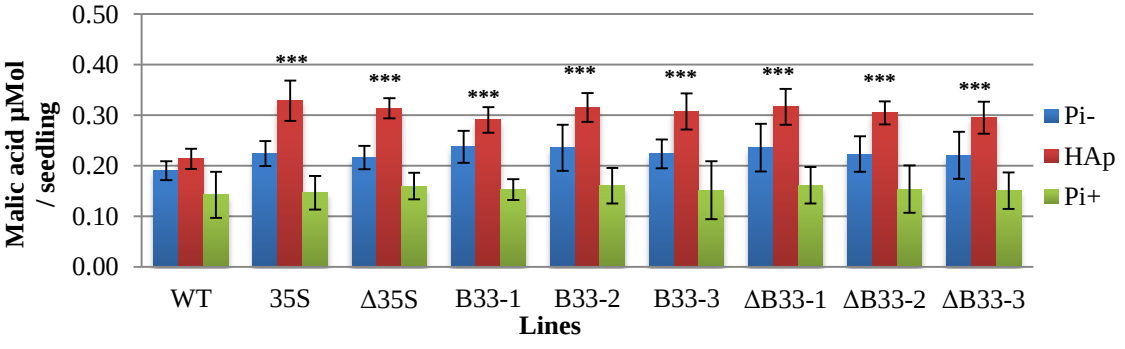


Figure 3.8: Malic acid amount (µMol/seedling). Transgenic and wild-type seedlings grown for 10 days in alkaline MS liquid pH 8.0 in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments. The data are from three independent experiments. (rep.3, n=190-210 each, ±SD, t-test: *** $p \leq 0,0001$).

3.4. Transgenic Plants Efficient use of Sparingly Soluble HAp as a Source of P in Alkaline MS Solid Medium

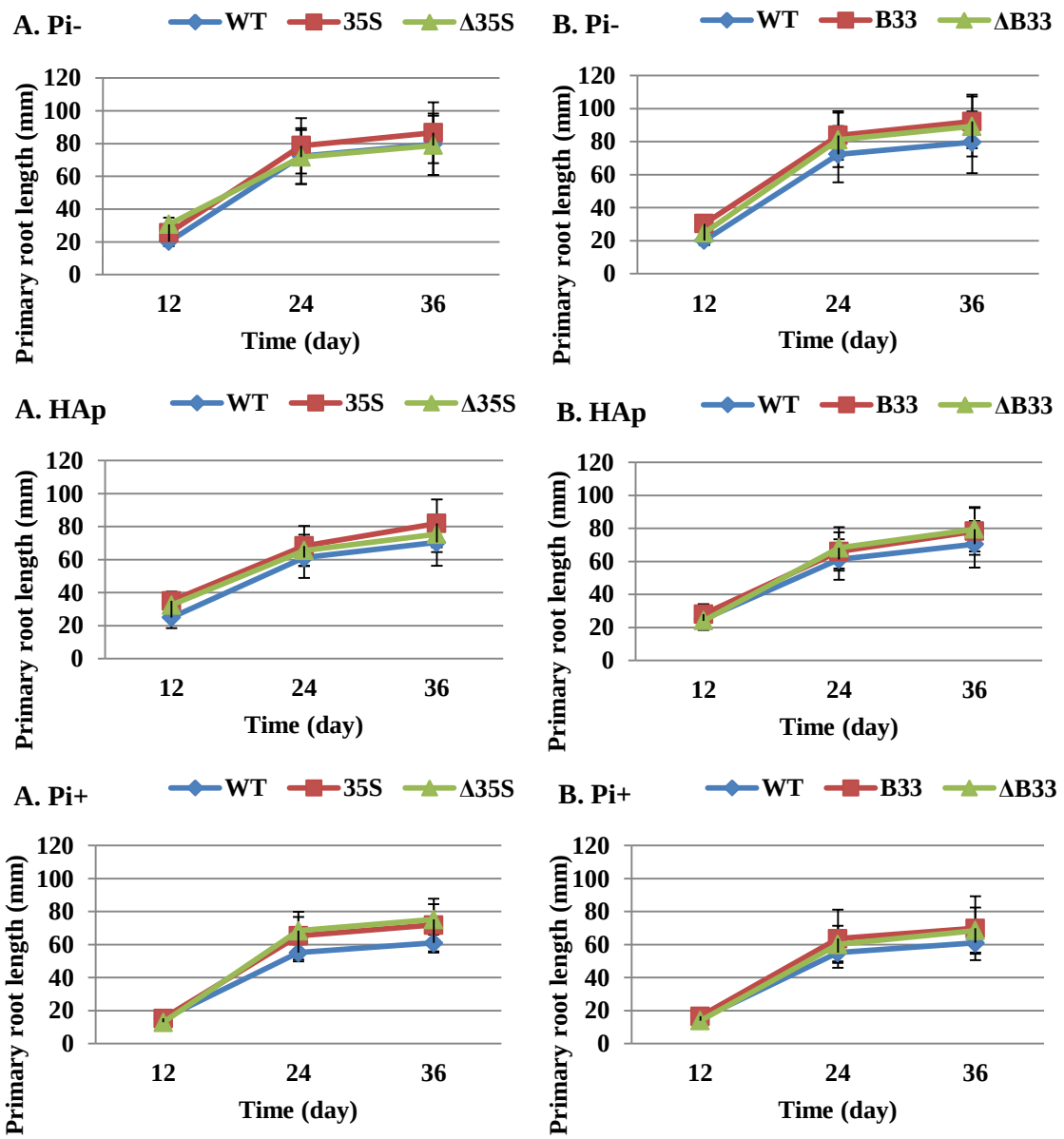
Rhizosphere is a zone where roots react with the physical, chemical and biological properties of the soil. The structural and functional characteristics of the root participate in rhizosphere operations, and influence the capability of the root to obtain nutrients (Richardson *et al.*, 2009). Adaptation of plant to the limitation of P includes architecture modification of root system (Péret *et al.*, 2011). In order to determine the ability of the transgenic seedlings to use the sparingly soluble HAp as a source of P in alkaline conditions, we measured the length of the primary and secondary root, the biomass of the shoot and root, root:shoot ratio, lowering of the pH of the growth medium and acid phosphatase activity of the root surface.

3.4.1. Influence of P-nutrition on the Length of Primary Roots

The ability of the root system architecture to respond to soil availability of phosphate, is an adaptive key allowing plants to cope with environmental conditions. The response to phosphate deprivation is reported in many studies (Al-Ghazi *et al.*, 2003; Péret *et al.*, 2011). To determine the growth of the transgenic seedlings under HAp, we measured the length of the primary roots. The experiment was performed by growing the seedlings of transgenic and wild-type on alkaline MS solid medium pH 8.0 in Pi-, HAp or Pi+ treatments.

The 12-day old seedlings grown in Pi- and HAp showed a marked increase in the length of their primary roots (Fig. 3.9A and 3.13A-B), compared with the seedlings grown in Pi+ (Fig. 3.9A and 3.13C). The length of the primary roots of the transgenic seedlings in Pi- was significantly longer than those of the wild-type. In contrast, both the transgenic and wild-type seedlings growth of the primary roots in HAp and Pi+ was comparable. The 24 and 36-day old seedlings grown in Pi- showed an increase in the length of their primary roots (Fig. 3.9B-C, 3.14A and 3.15A), compared with the seedlings grown in HAp and Pi+ (Fig. 3.9B-C, 3.14B-C and 3.15.B-C).

It is evident that, presence of HAp in the growth medium could stimulate the growth of the primary roots of the transgenic seedlings significantly higher compared to those of the wild-type.



Time (d)	Pi-				HAp				Pi+			
	35S	Δ35S	B33	ΔB33	35S	Δ35S	B33	ΔB33	35S	Δ35S	B33	ΔB33
12	***	***	***	***								
24							*	*	**	***	**	**
36					**		**	**	**	***	**	*

Figure 3.9: The length of the primary roots (mm). Transgenic and wild-type seedlings grown on alkaline MS pH 8.0 in Pi-starved (Pi-), hydroxyapatite (HAp), or Pi-sufficient (Pi+) treatments. **A.** 12-day old seedlings, **B.** 24-day old seedlings, and **C.** 36-day old seedlings. The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** $p \leq 0,0001$; ** $p \leq 0,001$; * $p \leq 0,01$).

3.4.2. Influence of P-nutrition on the Formation of Root Hairs

The availability of phosphate in the growth medium, controlled both the growth and density of the root hair of the transgenic and the wild-type. While the seedlings grown under Pi-starved and HAp treatments produced long and intensive root hair, the seedlings grown in Pi-sufficient conditions produced a small number of short root hair (Fig. 4.10).

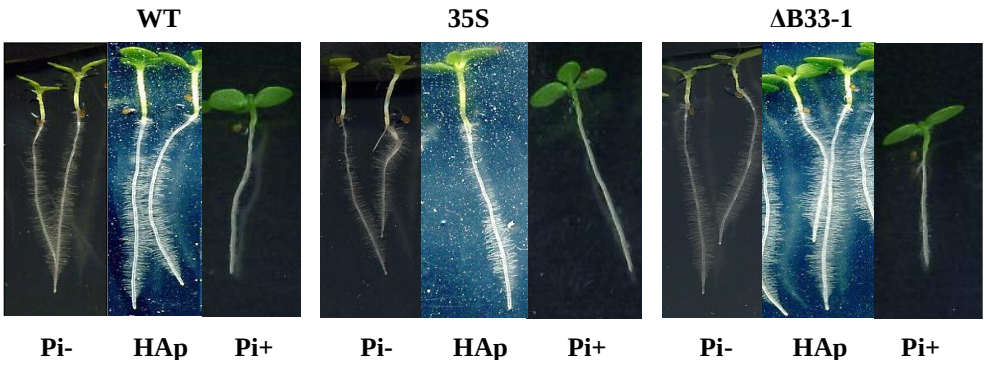


Figure 3.10: Wild-type and transgenic lines 35S and ΔB33-1 formation of root hair after 12 days of growth in alkaline MS medium, in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments.

3.4.3. Influence of P-nutrition on the Length of Secondary Roots

The morphology of plant root is dramatically affected by phosphate deficiency. The growth of the secondary root system is a clear example of this developmental plasticity. Number and length of the secondary roots are influenced by the soil content of P (Malamy and Ryan, 2001).

The 12-day old seedlings of transgenic and wild-type grown in all P treatments showed comparable length of the secondary roots (Fig. 3.11A, 3.12A and 3.13). The 24 and 36-day old seedlings of transgenic grown in HAp and Pi+ (Fig. 3.11B-C, 3.12B-C, 3.14B-C and 3.15B-C) showed significant increase in length of the secondary roots compared with those of the wild-type. In contrast, the 24 and 36-day old transgenic and wild-type seedlings grown in Pi- showed no significant difference in the length of their secondary roots (Fig. 3.11B-C, 3.12B-C, 3.14B-C and 3.15B-C). The length of the secondary roots of the transgenic seedlings in HAp was high in all growth periods

compared with those in Pi+. However, the length of the secondary roots of the wild-type in HAp decreased with the seedlings increase in age compared with those in Pi+ (Fig. 3.12).

The results show, that using the sparingly soluble HAp as a source of P in the growth medium stimulates the growth of the secondary roots with time. In contrast, Pi-starvation reduces the secondary roots growth with time. The stimulation of growth in the secondary roots of the transgenic seedlings was higher under hydroxyapatite treatment compared with that of the wild-type seedlings in all growth periods.

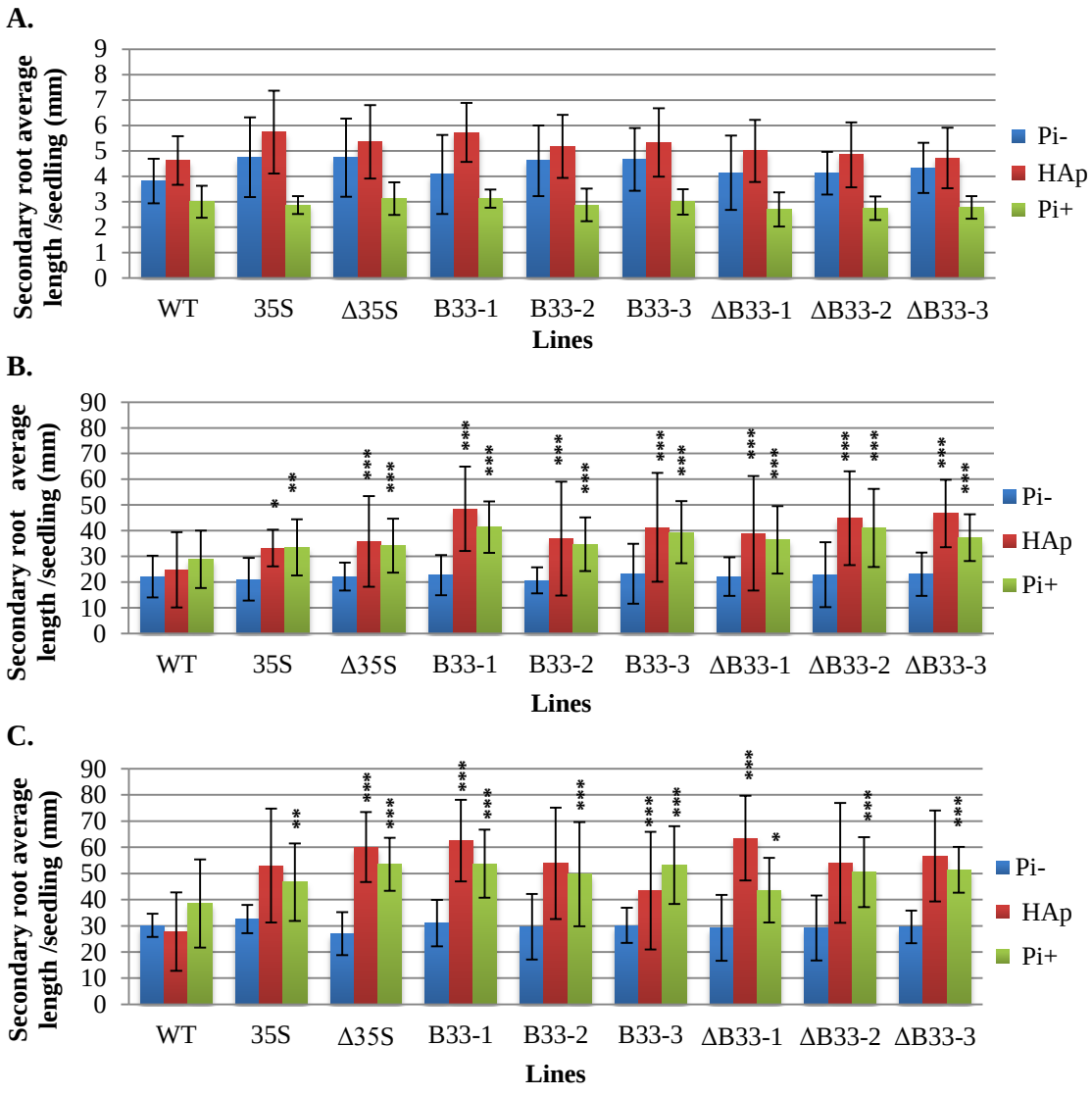


Figure 3.11: Average length of secondary roots (mm). Transgenic and wild-type seedlings grown on alkaline MS medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp), or Pi-sufficient (Pi+) treatments. **A.** 12 days old seedlings, **B.** 24 days old seedlings, and **C.** 36 days old seedlings. The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** $p \leq 0,0001$; ** $p \leq 0,001$; * $p \leq 0,01$).

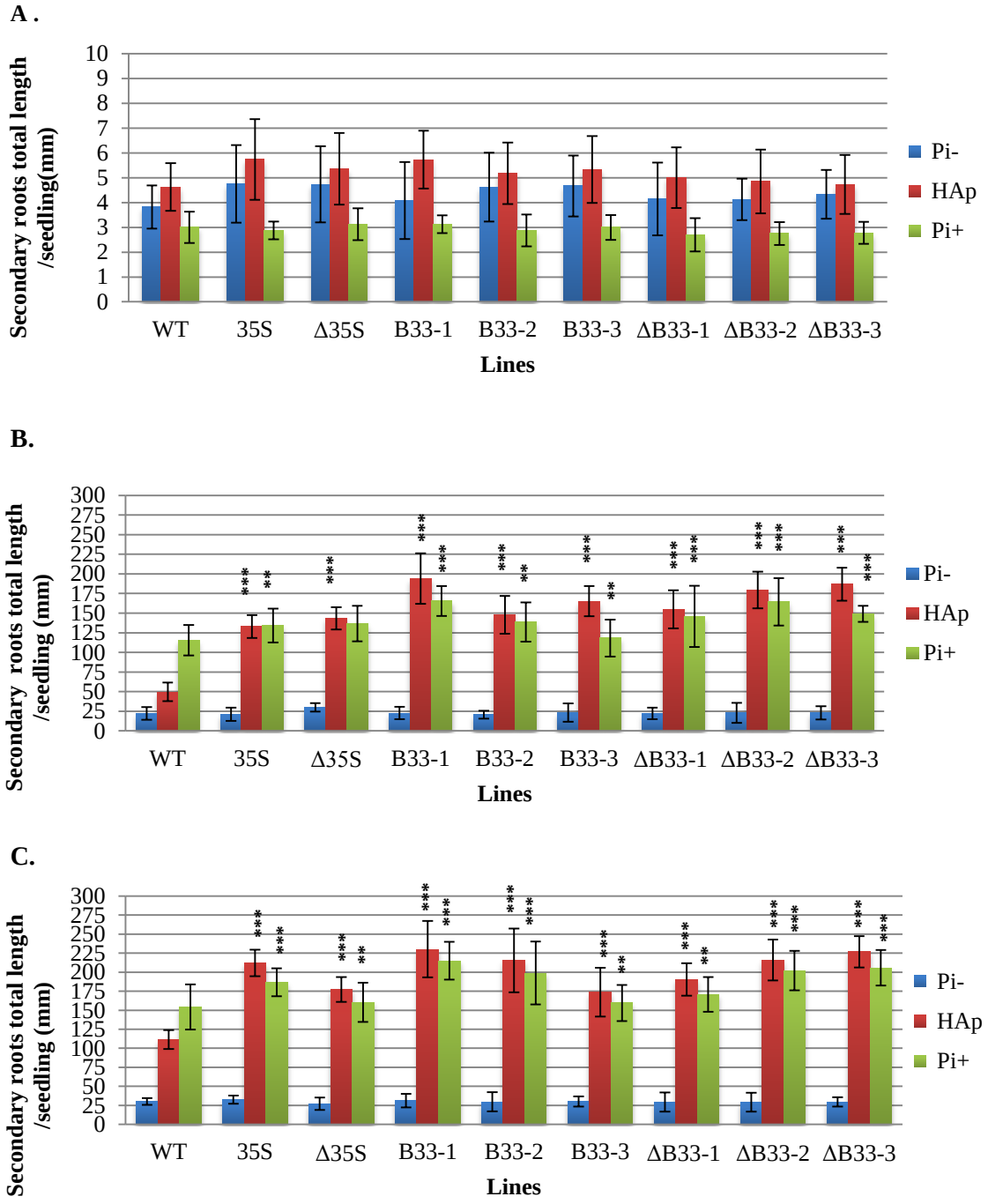


Figure 3.12: Total length of secondary roots (mm). Transgenic and wild-type seedlings grown on alkaline MS medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp), or Pi-sufficient (Pi+) treatments. **A.** 12 days age seedlings, **B.** 24 days age seedlings, and **C.** 36 days age seedlings. The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** $p \leq 0,0001$; ** $p \leq 0,001$).

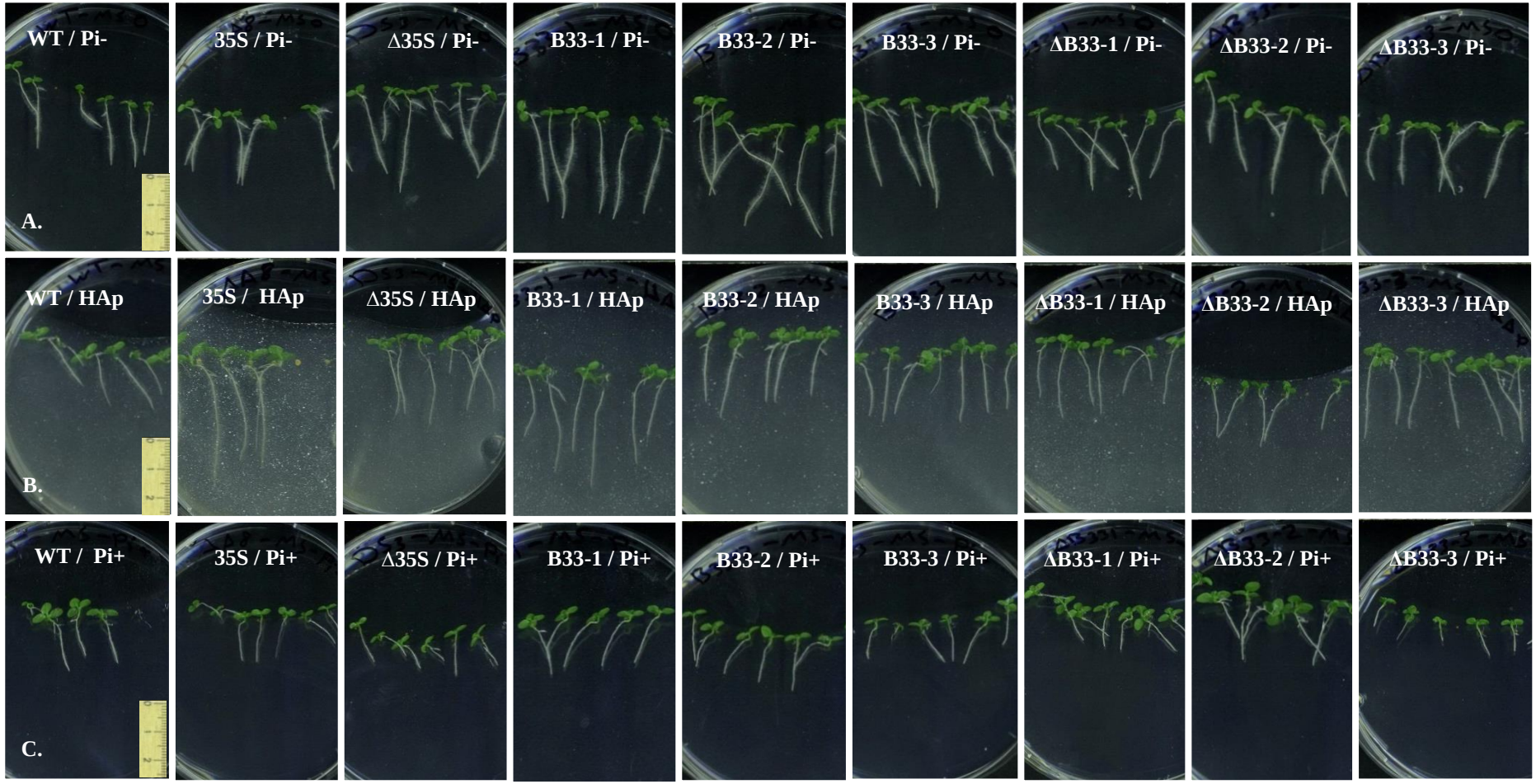


Figure 3.13: Tobacco seedlings growth (root, shoot and root hair). Transgenic and wild-type seedlings were grown for 12 days in alkaline MS solid medium at pH 8.0, in **A.** Pi-starved (Pi-), **B.** hydroxyapatite (HAp), or **C.** Pi-sufficient (Pi+) treatments.

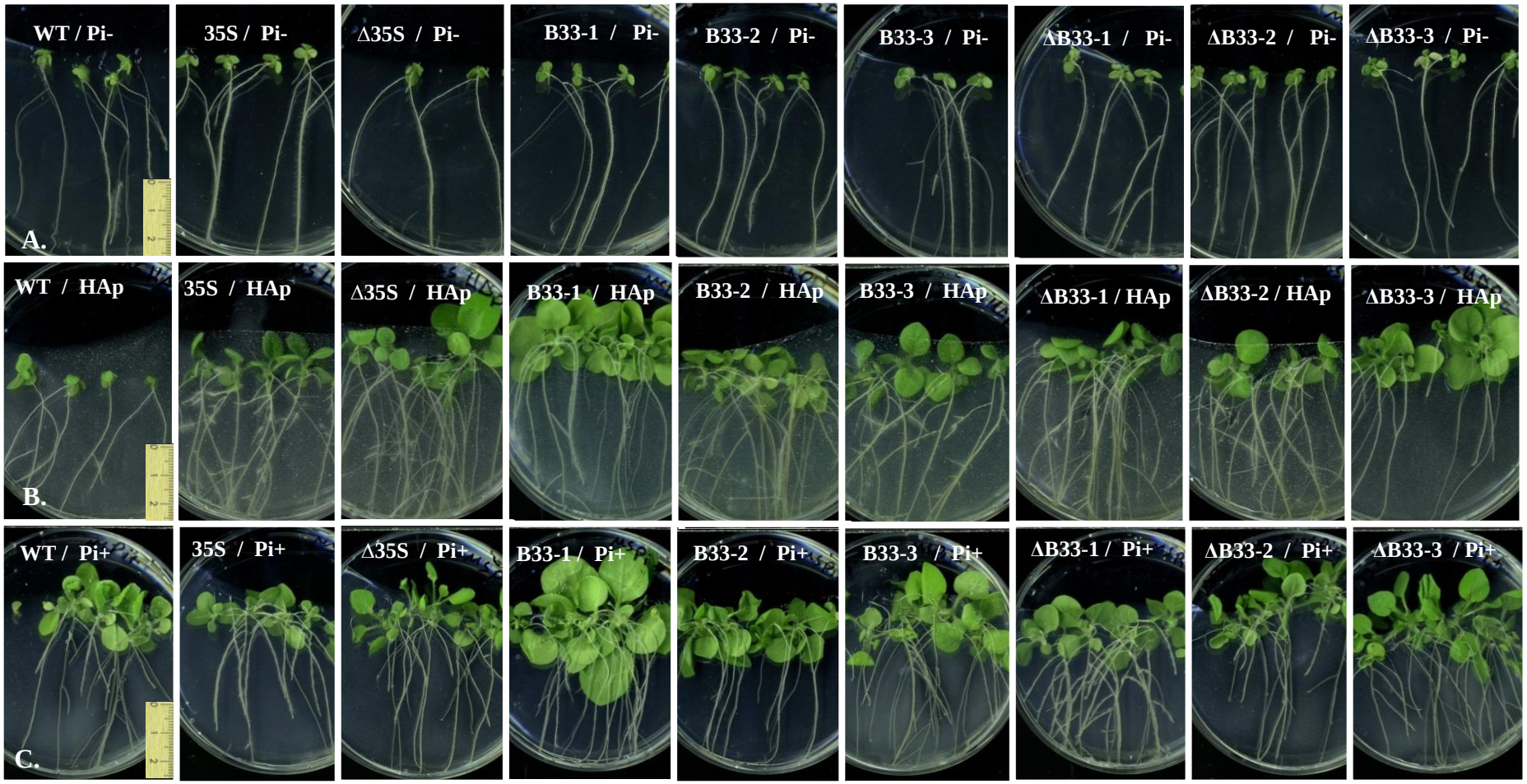


Figure 3.14: Tobacco seedlings growth (root, shoot and root hair). Transgenic and wild-type seedlings were grown for 24 days in alkaline MS solid medium at pH 8.0, in **A.** Pi-starved (Pi-), **B.** hydroxyapatite (HAp), or **C.** Pi-sufficient (Pi+) treatments.

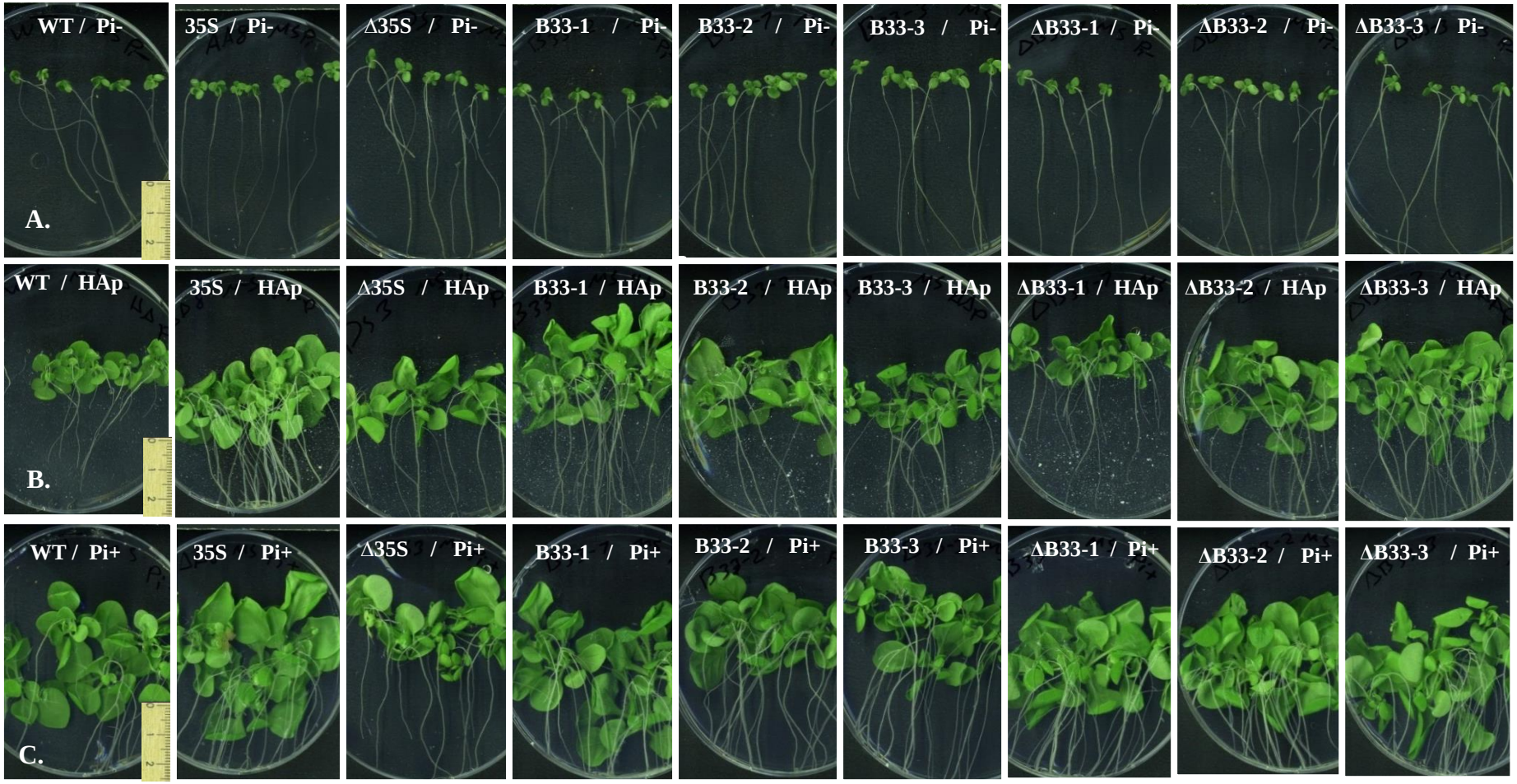


Figure 3.15: Tobacco seedlings growth (root, shoot and root hair). Transgenic and wild-type seedlings were grown for 36 days in alkaline MS solid medium at pH 8.0, in **A.** Pi-starved (Pi-), **B.** hydroxyapatite (HAp), or **C.** Pi-sufficient (Pi+) treatments.

3.4.4. Influence of P-nutrition on the Biomass of Transgenic Seedlings

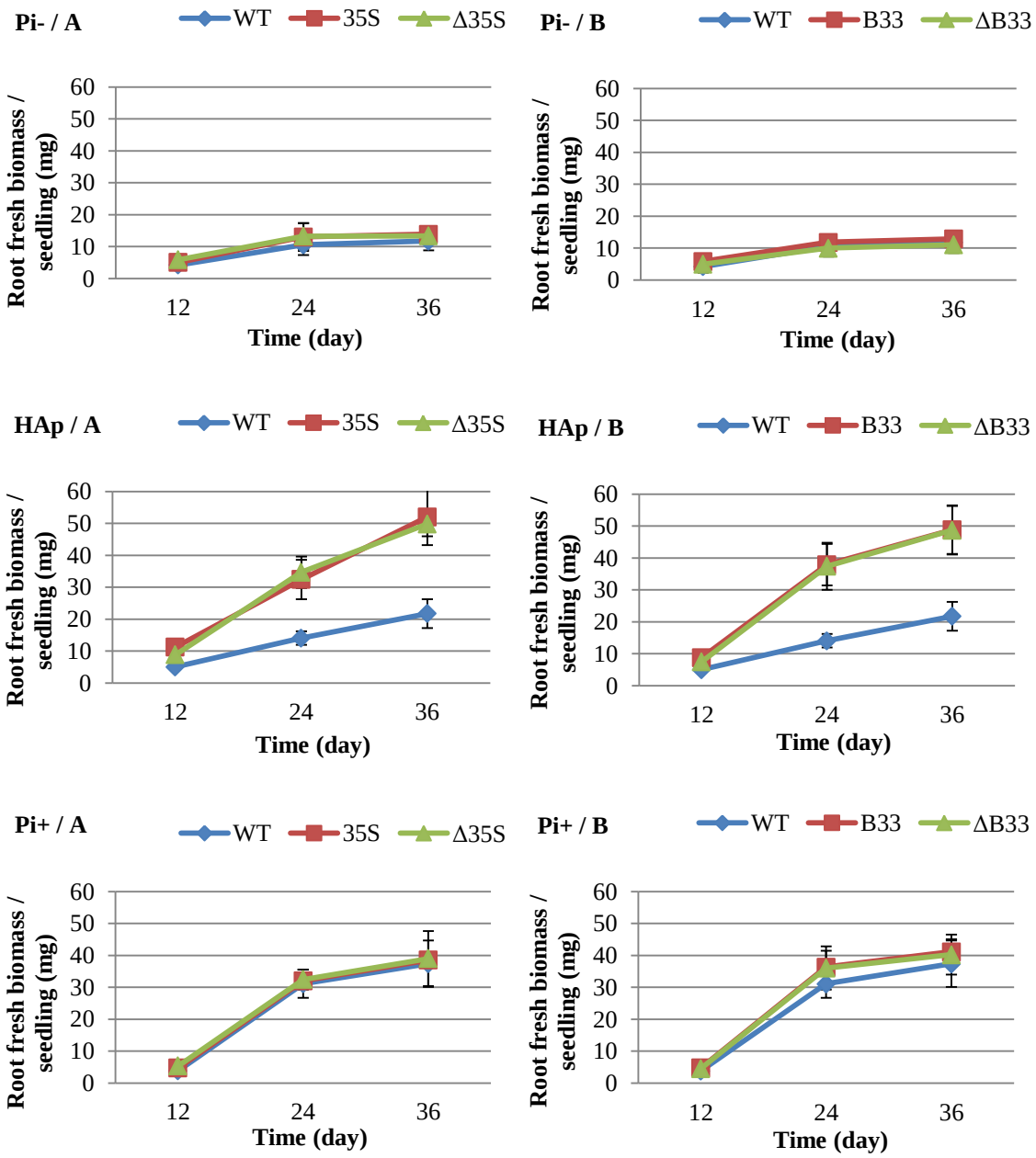
The biomass of the root and shoot in the seedlings is directly affected by phosphate deficiency. Plants grown in phosphate deficient soil produce less shoot dry biomass and more root dry biomass (Li *et al.*, 2009). To determine the effect of Pi-nutrition on tobacco root and shoot biomass, fresh and dry biomass of the roots and shoots were measured after 12, 24 and 36 days of seedlings growth on alkaline MS medium pH 8.0 in Pi-, HAp or Pi+ treatments.

3.4.4.1. Root Fresh and Dry Biomass

The root fresh and dry biomass of the transgenic seedlings increased significantly compared with that of the wild-type, when the seedlings were grown in HAp (Fig. 3.16 and 3-17 HAp / A-B) for all growth periods. No significant difference in fresh and dry biomass of the transgenic roots was observed, compared with that of the wild-type, when the seedlings were grown in Pi- (Fig. 3.16 and 3.17 Pi- / A-B) and Pi+ (Fig. 3.16 and 3.17 Pi+ / A-B).

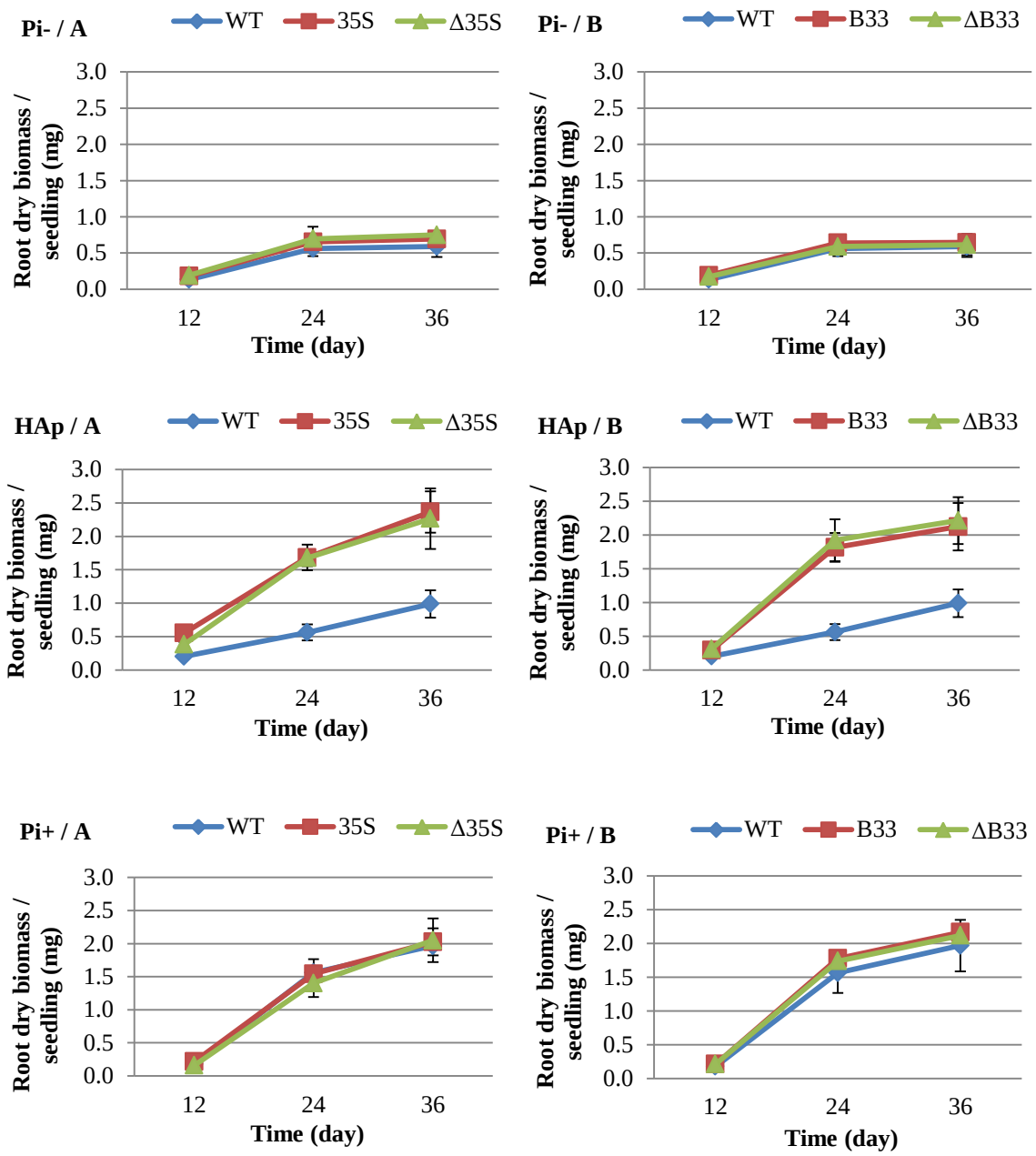
As is shown in (Fig. 3.16 and 3.17 HAp / A-B), the fresh and dry biomass of the transgenic roots in HAp was higher than in Pi- and Pi+ (Fig. 3.16 and 3.17 Pi-, Pi+ /A-B) in all growth periods. The fresh and dry biomass of the transgenic roots in Pi- was lower than in HAp and Pi+ in all growth periods. In contrast, the fresh and dry biomass of the wild-type roots was considerably higher in Pi+ than that in HAp.

The increase of the root biomass of the transgenic seedlings in HAp, indicates the ability of the plants to use HAp as a source of phosphate more efficiently for growth, compared with the wild-type, which showed an inadequate ability to use HAp.



Time (d)	Pi- 35S	Δ35S	B33	ΔB33	HAp 35S	Δ35S	B33	ΔB33	Pi+ 35S	Δ35S	B33	ΔB33
12					*	*						
24					***	***	***	***				
36					***	***	***	***				

Figure 3.16: Root fresh biomass (mg). Transgenic and wild-type seedlings grown for 12, 24 and 36 days on alkaline MS solid medium at pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp), or Pi-sufficient (Pi+) treatments, **A:** WT with 35S and Δ35S lines, **B:** WT with B33 group (B33-1, B33-2, B33-3) and ΔB33 group (ΔB33-1, ΔB33-2, ΔB33-3). The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** $p \leq 0,0001$; * $p \leq 0,01$).



Time (d)	Pi- 35S	Δ35S	B33	ΔB33	HAp 35S	Δ35S	B33	ΔB33	Pi+ 35S	Δ35S	B33	ΔB33
12					**	*						
24					***	***	***	***				
36					***	***	***	***				

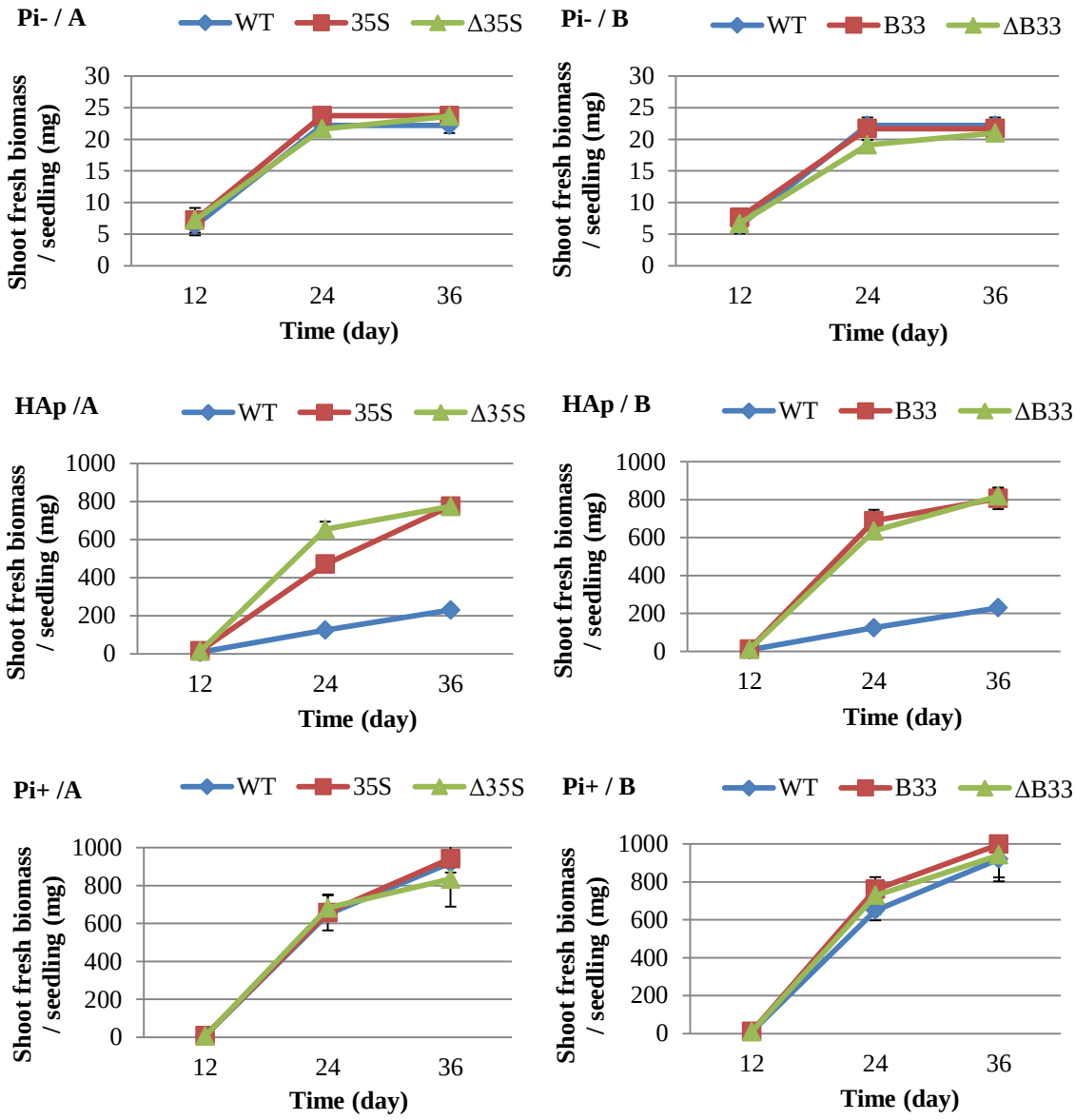
Figure 3.17: Root dry biomass (mg). Transgenic and wild-type seedlings grown for 12, 24 and 36 days on alkaline MS solid medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments, **A:** WT with 35S and Δ35S lines, **B:** WT with B33 group (B33-1, B33-2, B33-3) and ΔB33 group (ΔB33-1, ΔB33-2, ΔB33-3). The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** p ≤ 0001, ** p ≤ 0,001, * p ≤ 0, 01).

3.4.4.2. Shoot Fresh and Dry Biomass

A significant increase in shoot fresh (Fig. 3.18 HAp / A-B) and dry biomass (Fig. 3.19 HAp / A-B) of the transgenic seedlings grown in HAp was observed in all growth periods, compared with those the wild-type. When the seedlings were grown in Pi- (Fig. 3.18 and 3.19 Pi- / A-B) and Pi+ (Fig. 3.18 and 3.19 Pi+ / A-B), no significant difference in shoot fresh and dry biomass of the transgenic seedlings was observed compared with those the wild-type.

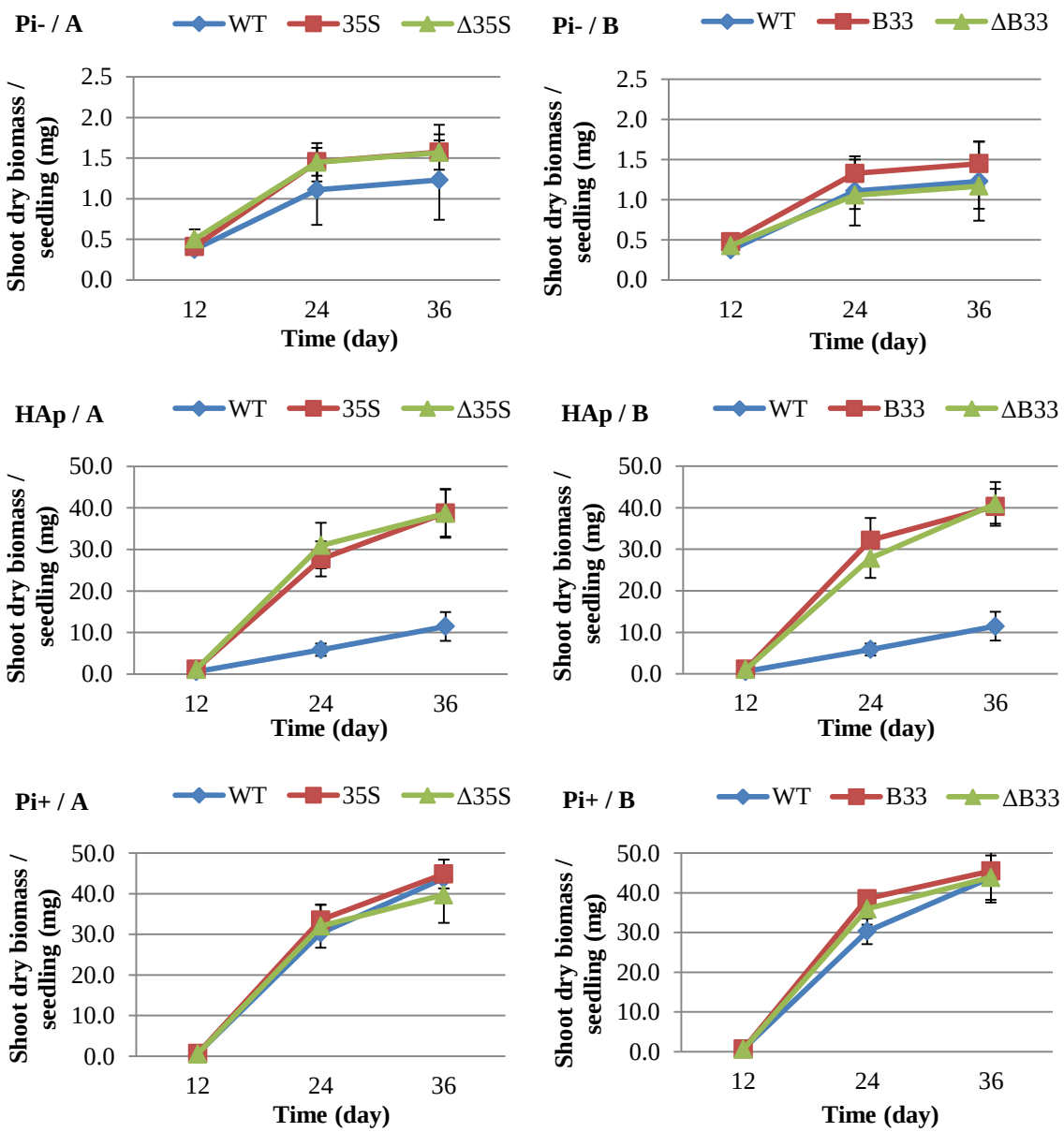
As is shown in (Fig. 3.18 and 3.19 Pi+ / A-B) the fresh and dry biomass of the shoots of transgenic and wild-type in Pi+ was higher than that in HAp (Fig. 3.18 and 3.19 HAp, Pi- / A-B) and it was lower in Pi- than that in HAp in all growth periods.

Again, the significant increase in the fresh and dry biomass of the shoot of the transgenic seedlings, shows the ability of the plants to use HAp for growth more efficiently than the wild-type. The increase in the biomass of seedlings in Pi+ clarifies the positive effect of the P availability in growth medium for the growth of the plants. In contrast, the high reduction in the seedlings biomass in Pi- shows the negative effect of P starvation in growth medium for the growth of the plants.



Time (d)	Pi-				HAp				Pi+			
	35S	Δ35S	B33	ΔB33	35S	Δ35S	B33	ΔB33	35S	Δ35S	B33	ΔB33
12												
24					***	***	***	***				
36					***	***	***	***				

Figure 3.18: Shoot fresh biomass (mg). Transgenic and wild-type seedlings grown for 12, 24 and 36 days on alkaline MS solid medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments, **A:** WT with 35S and Δ35S lines, **B:** WT with B33 group (B33-1, B33-2, B33-3) and ΔB33 group (ΔB33-1, ΔB33-2, ΔB33-3). The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** $p \leq 0,0001$).



Time (d)	Pi- 35S	Δ35S	B33	ΔB33	HAp 35S	Δ35S	B33	ΔB33	Pi+ 35S	Δ35S	B33	ΔB33
12												
24					***	***	***	***				
36					***	***	***	***				

Figure 3.19: Shoot dry biomass (mg). Transgenic and wild-type seedlings grown for 12, 24 and 36 days on alkaline MS solid medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments, **A:** WT with 35S and Δ35S lines, **B:** WT with B33 group (B33-1, B33-2, B33-3) and ΔB33 group (ΔB33-1, ΔB33-2, ΔB33-3). The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** $p \leq 0,0001$).

3.4.4.3. Influence of Sparingly Soluble P on the Root:Shoot Ratio of Transgenic Seedlings

A common response of the plants to low nutrient availability, particularly phosphate is represented by increasing the relative biomass allocation resulting in an increase in root:shoot ratio (Nielsen *et al.*, 2001; Agren and Franklin, 2003). The plant growth allocation may be due to, either the over stimulation of root growth at the expense of shoot growth or the opposite, with the increase of plant in age (Robinson *et al.*, 2010). To study the response of the plant root and shoot to different availabilities of P in alkaline nutrient medium, we determined the root:shoot biomass ratio of the transgenic and wild-type in three growth periods 12, 24 and 36 days.

As is shown in (Fig. 3.20), the root:shoot ratio of the transgenic seedlings in HAp was significantly less than that of the wild-type. While, no significant difference in root:shoot ratio was observed of the transgenic seedlings in Pi- and Pi+, compared with that of the wild-type in all growth periods. But there was an increase in the root:shoot ratio of both the transgenic and the wild-type in Pi- with the increase in age. Both the transgenic and the wild-type seedlings grown in HAp and Pi+ (Fig. 3.20 B-C), showed a significant reduction in their root: shoot ratio compared with Pi- in 24 and 36-day growth period.

The results suggest that the root:shoot ratio increases with the depletion of P in the growth medium or in the conditions of P starvation, and decreases with the increase of P concentration in the growth medium. Presumably, the decrease in root:shoot ratio of the transgenic seedlings in HAp treatment, compared with that of the wild-type is due to the ability of the transgenic seedlings to utilize HAp as a source of phosphate for plant growth more efficiently than the wild-type.

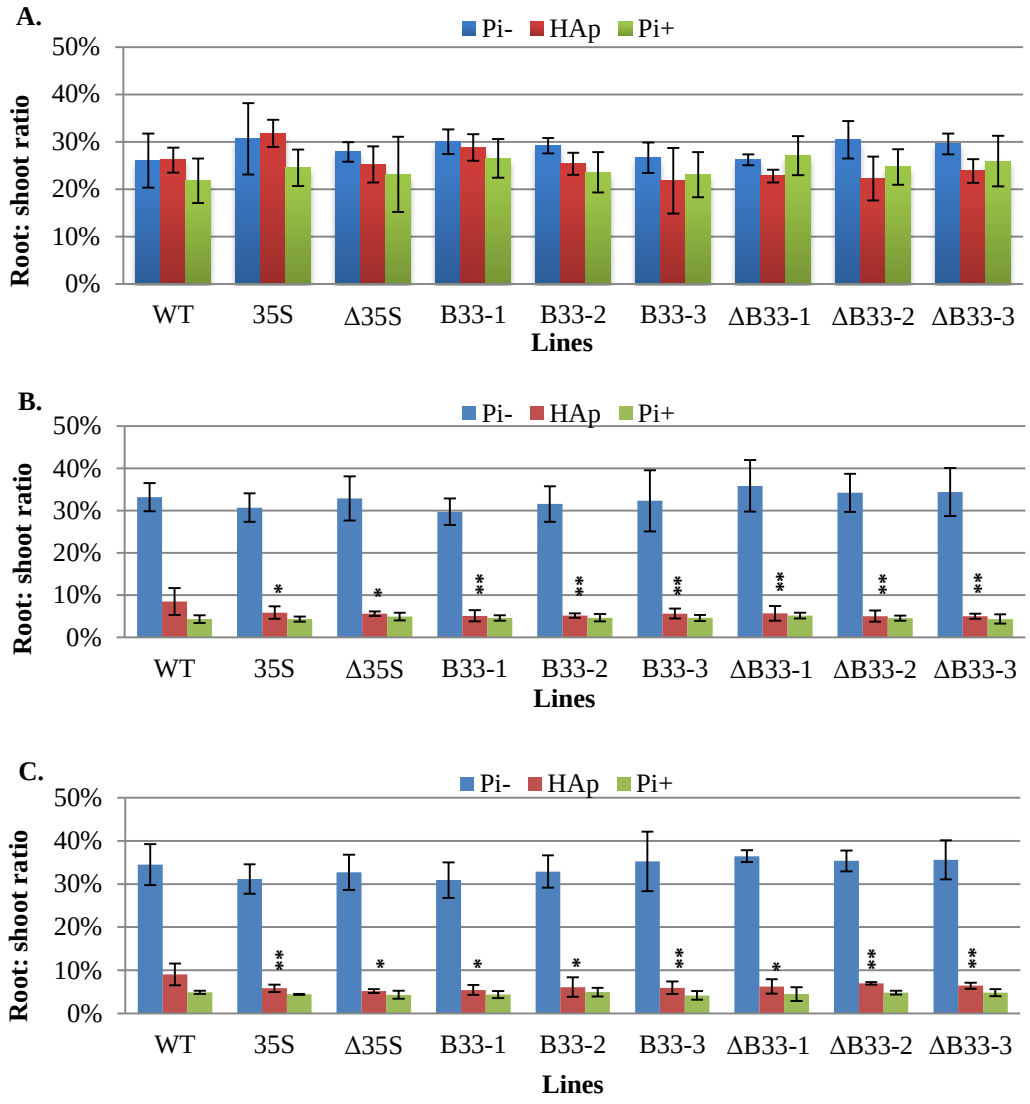


Figure 3.20: Root: shoot ratio. Transgenic and wild-type seedlings grown on alkaline MS solid medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp), or Pi-sufficient (Pi+) treatments. **A.** 12 days old seedlings, **B.** 24 days old seedlings, and **C.** 36 days old seedlings. The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: ** $p \leq 0, 001$).

3.4.4.4. Influence of Sparingly Soluble P in Shoot Content of P of the Transgenic Seedlings

The content of P in shoot was determined after 12, 24 and 36 days of seedlings grown in alkaline MS solid medium pH 8.0 in Pi-, HAp or Pi+ treatments. No significant difference in shoot content of P of the transgenic seedlings was observed, compared with the wild-type, when the seedlings were grown in Pi- and Pi+ in all growth periods. In contrast, shoot content of P of the transgenic seedlings showed a significant increase,

compared with the wild-type, when they were grown in HAp (Fig. 3.21 and 3.22). In addition, the transgenic and the wild-type seedlings showed a big decline in shoot content of P, when they were grown in Pi-, compared with those in HAp and Pi+. In contrast, the seedlings content of P in HAp was comparable to those in Pi+ in all growth periods.

The high content of P in shoot of the transgenic seedlings grown in HAp, could indicate, that the transgenic seedlings were able to use HAp as a source of P for growth more efficiently than the wild-type.

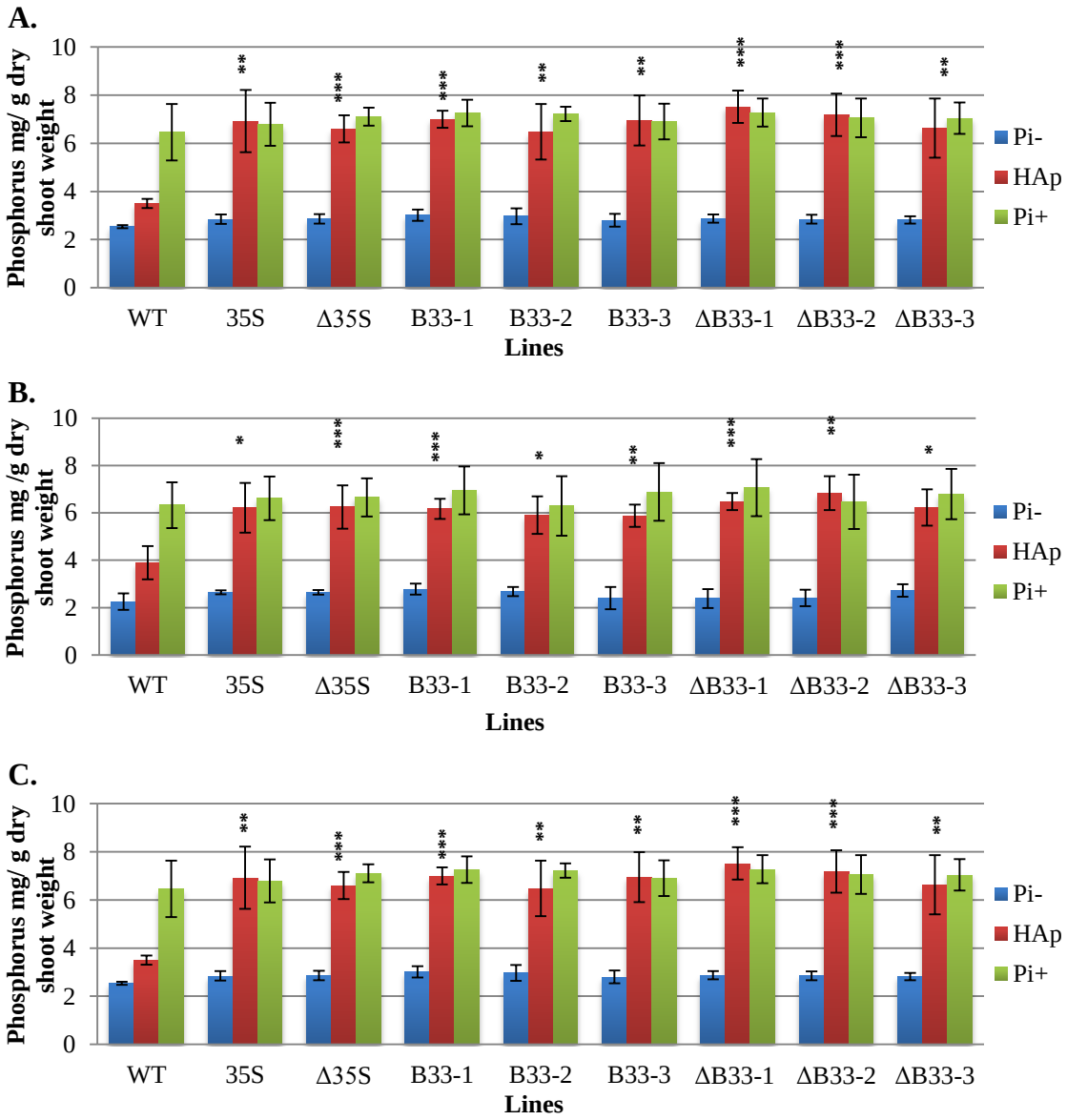


Figure 3.21: Shoot content of total P (mg/g DW). Transgenic and wild-type seedlings grown on alkaline MS solid medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp), or Pi-sufficient (Pi+) treatments. **A.** 12 days old seedlings, **B.** 24 days old seedlings, and **C.** 36 days old seedlings. The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** $p \leq 0,0001$; ** $p \leq 0,001$; * $p \leq 0,01$).

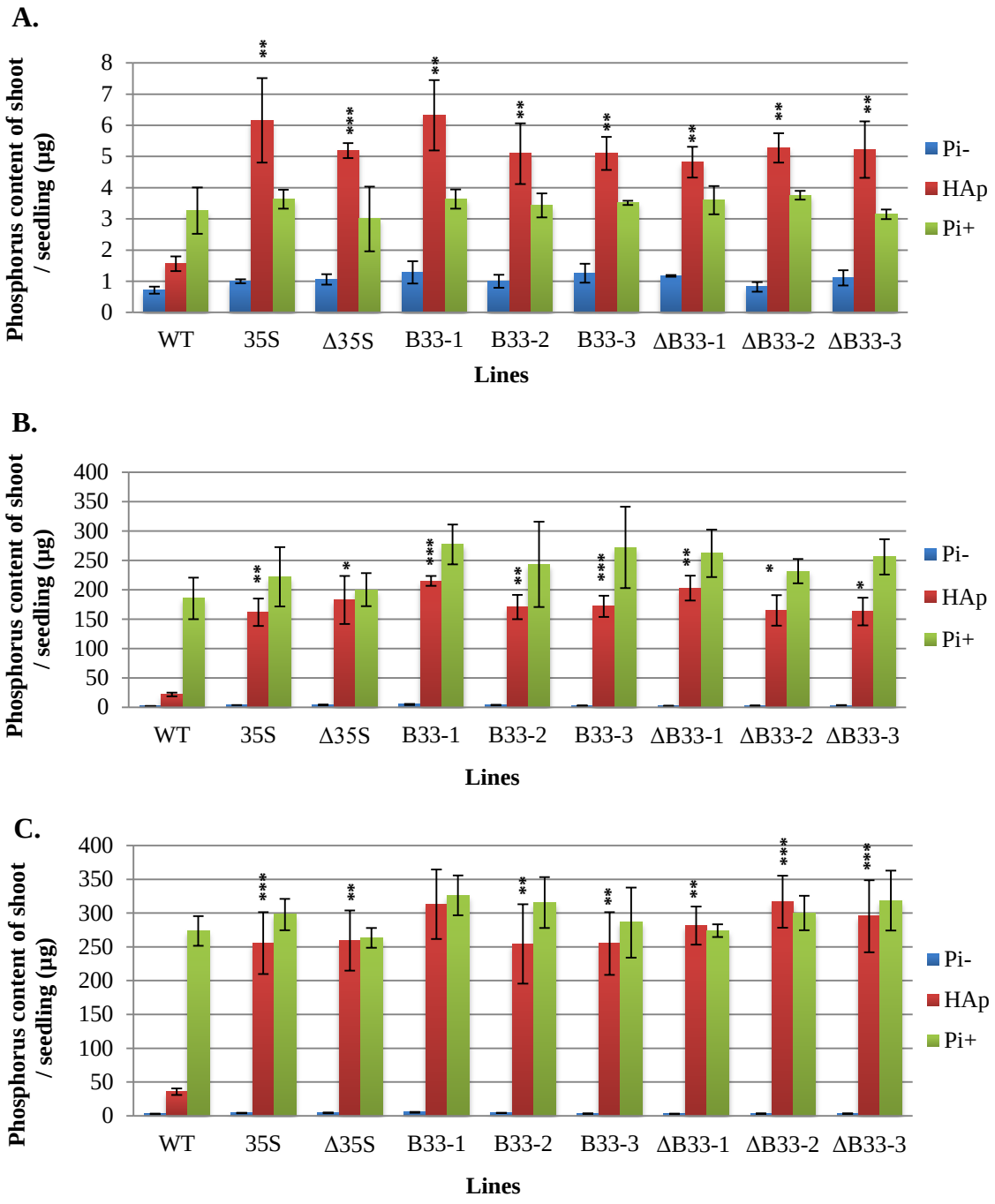


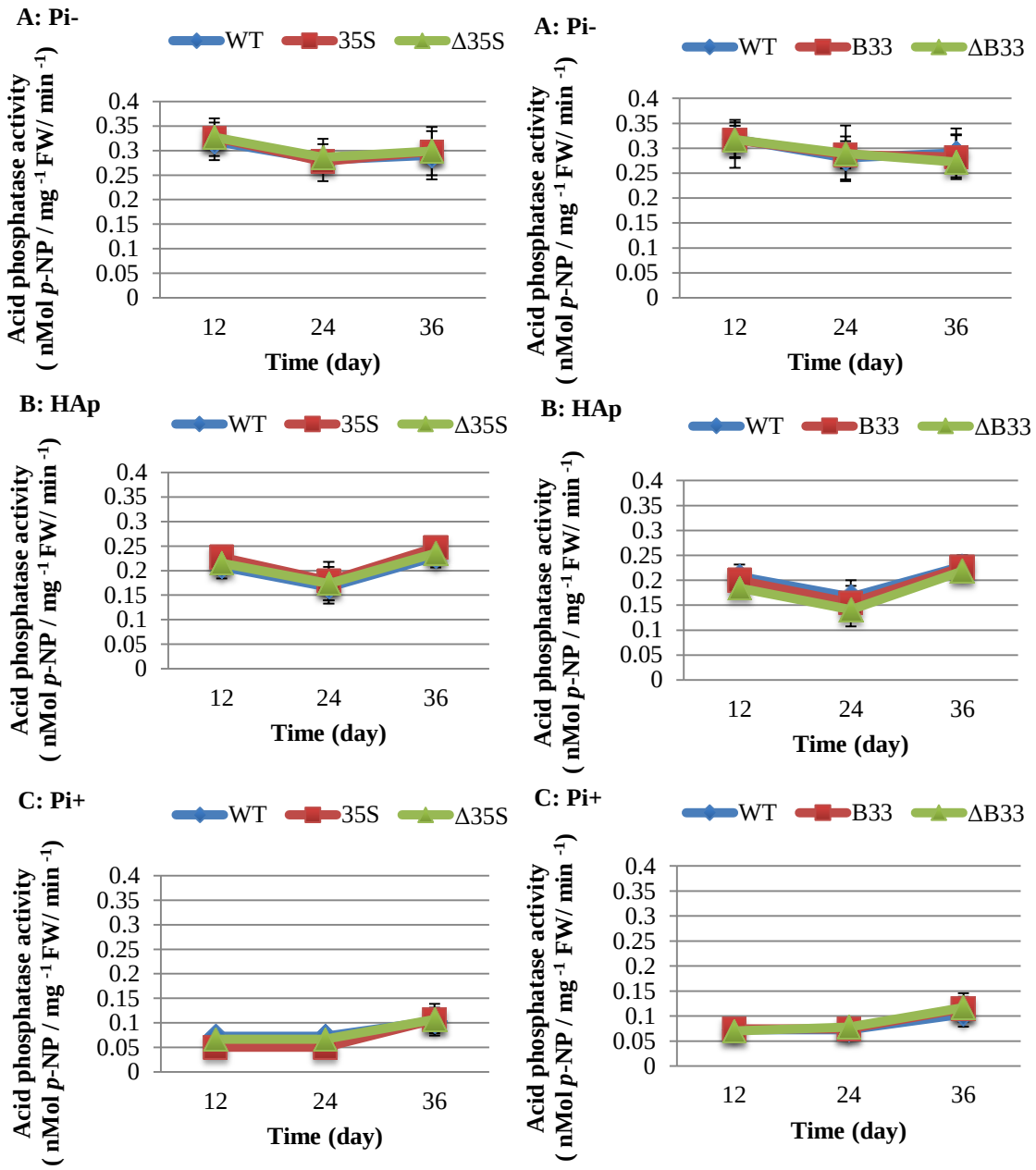
Figure 3.22: Shoot content of P per seedling (µg). Transgenic and wild-type seedlings grown on alkaline MS solid medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp), or Pi-sufficient (Pi+) treatments. **A.** 12 days old seedlings, **B.** 24 days old seedlings, and **C.** 36 days old seedlings. The data are from three independent experiments (rep.3, n=25 each, ±SD, t-test: *** $p \leq 0,0001$; ** $p \leq 0,001$; * $p \leq 0,01$).

3.4.4.5. Determination of Root-secretory Acid Phosphatase Activity

Acid phosphatase exists widely in the plant. This enzyme has intra and extra cellular activity (Ehsanpour and Amini, 2003), this activity is stimulated in the plant under low-P availability (Del Pozo *et al.*, 1999). The phosphatase produced by the plant is supposed to transform organic phosphorus into available Pi (Baldwin *et al.*, 2001). To determine the acid phosphatase activity of the transgenic seedlings under insoluble P treatment (HAp), we measured the root surface acid phosphatase activity.

As is shown in (Fig. 3.23) no significant difference in the activity of acid phosphatase was observed in the transgenic seedlings in all phosphate treatments, compared with that of the wild-type, of all growth periods. A great deal of acid phosphatase activity was observed in all tested seedlings in Pi- (Fig. 3.23A), compared with Pi+ (Fig. 3.23 B-C). Nevertheless, the activity of acid phosphatase in Pi- showed a significant decline with seedlings increase in age. The seedlings grown in HAp (Fig. 3.23B), showed a marked increase in the activity of acid phosphatase, compared with those grown in Pi+ (Fig. 3.23C), though, the increase was recorded less than those grown in Pi- (Fig.3.23A). Interestingly, the activity of acid phosphatase of the seedlings grown in HAp, showed a significant decrease in a 24-day growth period, and significantly increased in a 36-day growth period. Also, a significant increase in the activity of acid phosphatase was recorded in 36 days old seedlings grown in Pi+.

It can be observed that the activity of acid phosphatase patterns is essentially the same, both in the wild-type and the transgenic seedlings, in the same growth period and treatment of P. However, the decrease of the activity of acid phosphates of the 24 days old seedlings grown in HAp, might be referred to the increase of the available Pi in the growth medium. In other words, the roots were able to remobilize the insoluble phosphate (HAp) and use it for the growth of the plant. The increase of the activity of acid phosphatase of roots after 36 days of growth in HAp and Pi+, might be referred to the reduction of P concentration in the growth medium.



Lines	Pi-		HAp		Pi+	
	24-day	36-day	24-day	36-day	24-day	36-day
WT	*	*	***	*		**
35S	*	*	***	*		**
Δ35S	*	*	***	*		**
B33	*	**	***	*		**
ΔB33	**	**	***	*		**

Figure 3.23: Roots surface acid phosphatase activity. Transgenic and wild-type seedlings grown on alkaline MS solid medium pH 8.0, in Pi-starved (Pi-), hydroxyapatite (HAp), or Pi-sufficient (Pi+) treatments. **A.** Pi- treatment, **B.** HAp treatment, and **C.** Pi+ treatment. The data are from three independent experiments (rep.3, ±SD).

3.4.4.6. Scanning of the Root Surface Acid Phosphatase Activity

The BCIP-staining procedure was used to visualize the activity of acid phosphatase (APase) on the surface of the intact roots of the seedlings grown in Pi-, HAp or Pi+ on alkaline MS medium pH 8.0 (see 2.5). BCIP (5-bromo-4-chloro-3- indolyl-phosphate) is a colorless soluble substance, turns to blue and becomes an insoluble visible product, upon its hydrolysis by acid phosphatase (Goldstein *et al.*, 1988).

As is shown in (Fig. 3.24), blue color appeared on the root isolated from seedlings grown for 15 days in Pi-. No visible difference in the strength of root color among the transgenic and the wild-type was observed after 4 h of incubation. The color distribution was along the root surface, particularly the root hair and the root tip region. At the same time of incubation, no color was observed on root in HAp and Pi+. After 12 h of incubation (Fig. 3.25), blue color appeared on the regions covered by the root hair of the root isolated from the seedlings grown in HAp. While the strength of the root color in wild-type was markedly darker in HAp than the transgenic roots, no color was observed on the roots isolated from the seedlings grown in Pi+ at the same time of incubation. The roots isolated from the seedlings grown in Pi+ were colored in blue after 48 h of incubation (Fig. 3.26).

The result suggests that time variation and strength of the exhibited color on the root surface could be related to the variation of the activity of acid phosphatase. The earlier coloring in the roots in Pi- than that of HAp and Pi+, is due to the connection between the activity of acid phosphatase and the growth medium content of phosphate (Goldstein *et al.*, 1988). The variation of color strength between the transgenics (faint) and the wild-type (dark) in a 12 h incubation period is due to the ability of the transgenics to solubilize and utilize HAp better than wild-type.

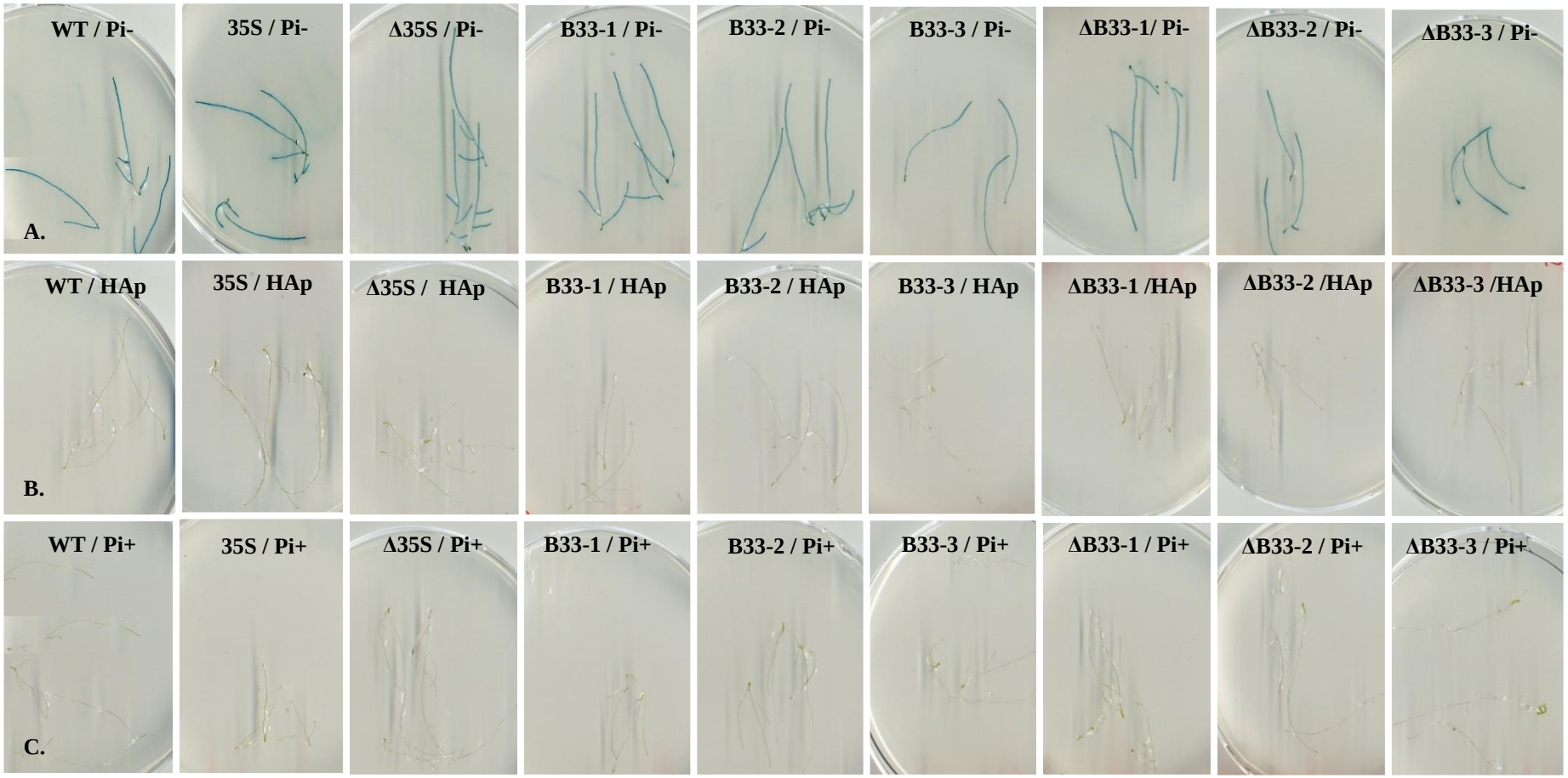


Figure 3.24: Root surface acid phosphatase activity scanning after 4 h of roots incubation with BCIP-agar in dark. Transgeinc and wild-type seedlings were grown for 15 days in alkaline MS solid medium at pH 8.0, in **A.** Pi-starved (Pi-), **B.** hydroxyapatite (HAp), or **C.** Pi-sufficient (Pi+) treatments.

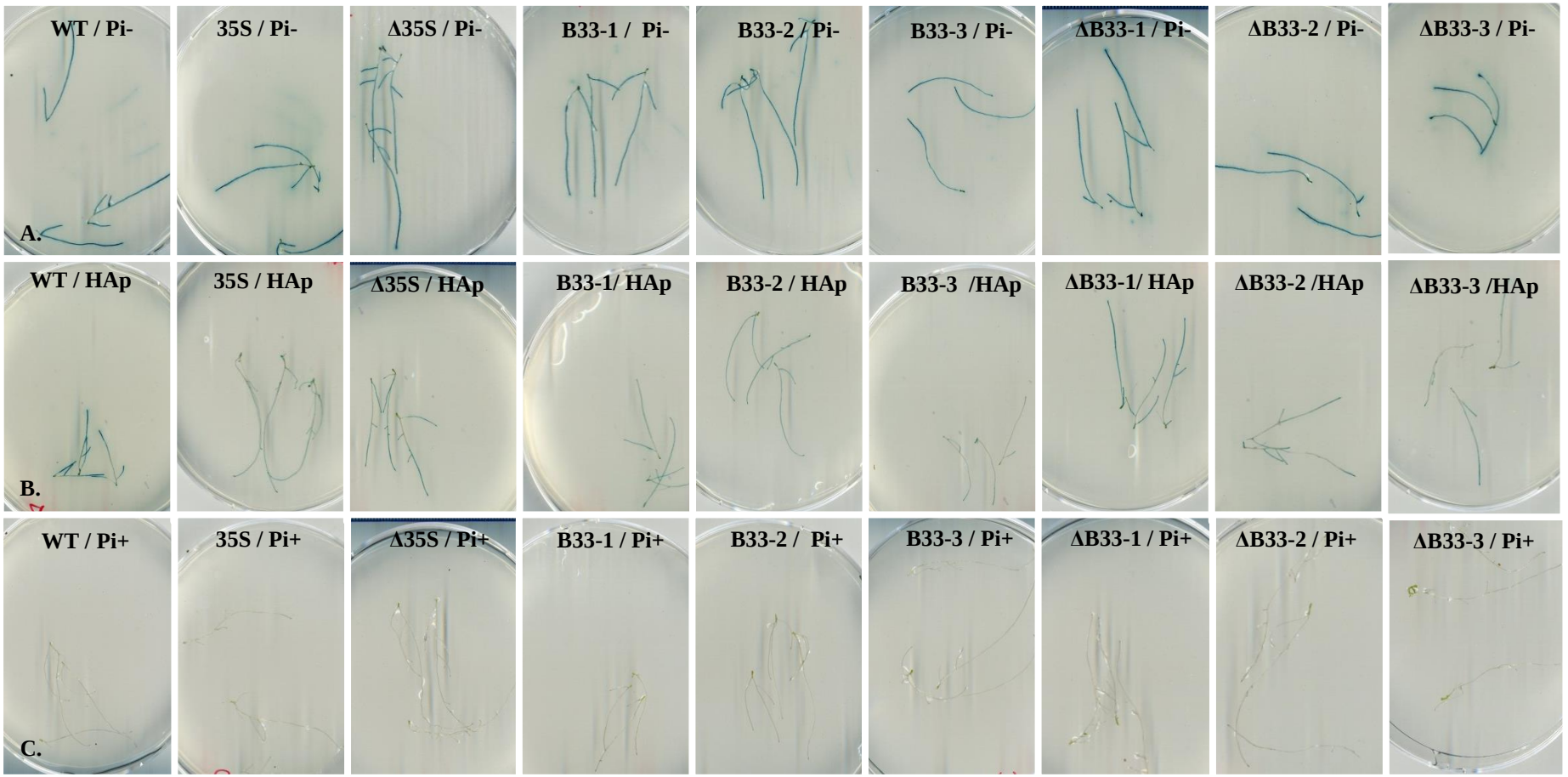


Figure 3.25: Root surface acid phosphatase activity scanning after 12 h of roots incubation with BCIP-agar in dark. Transgeinc and wild-type seedlings were grown for 15 days in alkaline MS solid medium at pH 8.0, in **A.** Pi-starved (Pi-), **B.** hydroxyapatite (HAp), or **C.** Pi-sufficient (Pi+) treatments.

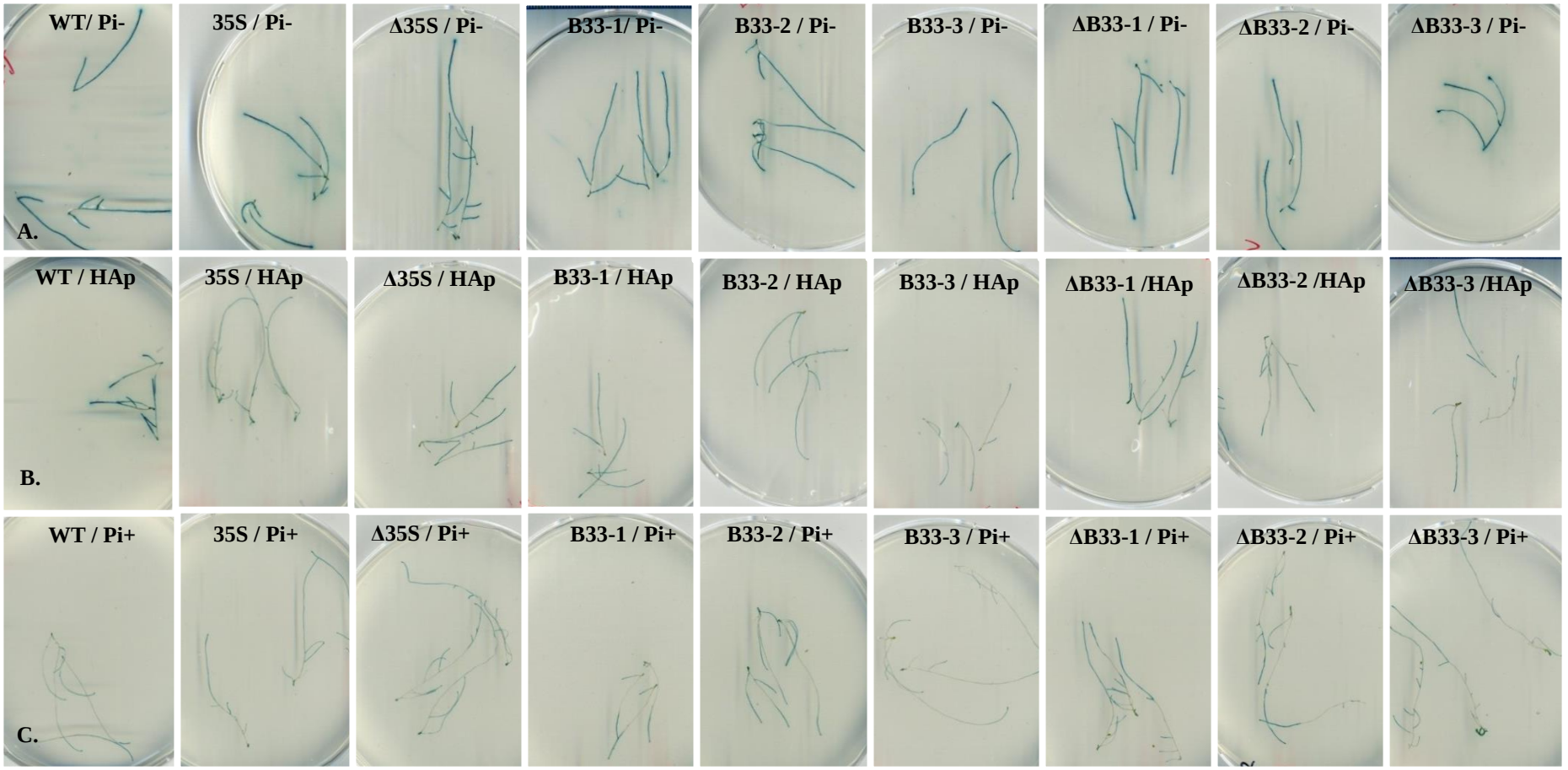


Figure 3.26: Root surface acid phosphatase activity scanning after 48 h of roots incubation with BCIP-agar in dark. Transgeinc and wild-type seedlings were grown for 15 days in alkaline MS solid medium at pH 8.0, in **A.** Pi-starved (Pi-), **B.** hydroxyapatite (HAp), or **C.** Pi-sufficient (Pi+) treatments.

3.4.4.7. Influence of Sparingly Soluble P on Acidifying Activity of Transgenic Root

The pH indicator, bromocresol purple (BCP) was used to indicate the change of pH in the zone of the root in Pi-, HAp, or Pi+ treatments. BCP (5', 5-dibromo-o-cresolsulfophthalein) is a non-toxic plant pH indicator with a color ranging from violet at pH 6.8 and above, to yellow at pH 5.2 and below (Gollany and Schumacher, 1993).

The 21-day old transgenic seedlings grown in HAp, showed a marked change in the color of the growth zone of the root after 72 h of incubation in BCP-agar medium. In contrast, no visible change in the color of incubation medium was observed in the root growth zone of the wild-type seedlings of the same age and treatment (Fig. 3.27B). Change of the medium color was particularly at the region of root hairs. The root acidification zone of the lines 35S, Δ35S, B33-1, B33-3 and ΔB33-3 was more visible than that in lines B33-2, ΔB33-1 and ΔB33-2. The transgenic and the wild-type seedlings grown in Pi-, showed less visible change in the color of the incubation medium than those in HAp (Fig. 3.27A). However, No change in the color of the root growth zone was observed for both the transgenic and wild-type seedlings grown in Pi+ (Fig.3.27C).

The result suggests that, the transgenic seedlings grown under HAp, were able to acidify the rhizosphere more efficiently than those of the wild-type. The high acidification ability of the transgenic seedlings, might be due to the seedlings increase in exuding of the organic acid under HAp. The increase in exuding of organic acid, plays a visible role in acidification of the root zone in HAp.

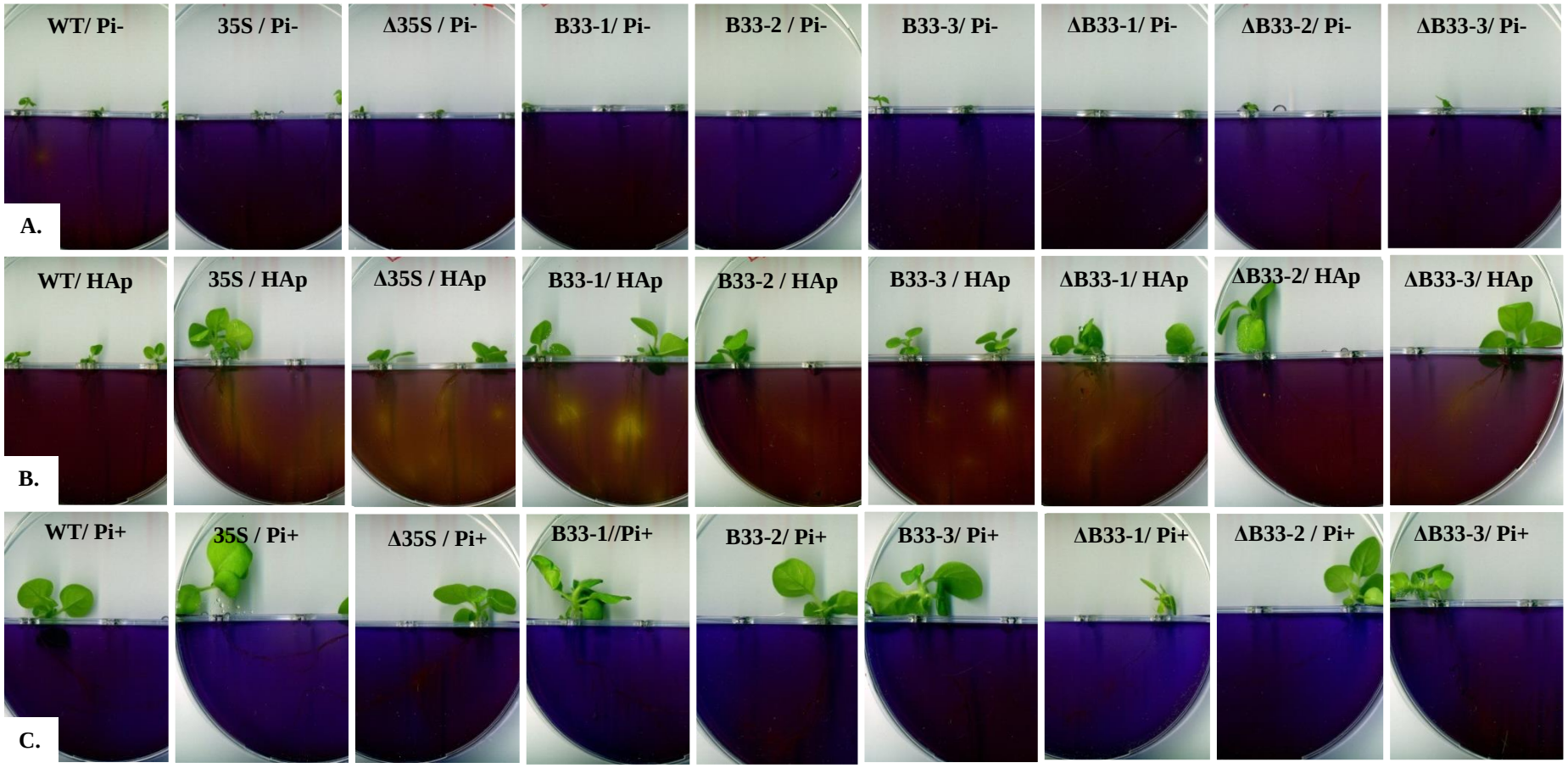


Figure 3.27: Root zone acidification activity scanning after 72 h of roots incubation with BCP (the BCP-medium with the root was covered with aluminum foil). Transgenic and wild-type seedlings were grown for 15 days in alkaline MS solid medium at pH 8.0, in **A.** Pi-starved (Pi-), **B.** hydroxyapatite (HAp), or **C.** Pi-sufficient (Pi+) treatments.

3.5. Influence of Soil Alkalinity on Growth and Yield of the Transgenic Plants

In order to investigate the ability of the plants to grow and develop under alkaline soil, a greenhouse experiment was conducted (see 2.7.1 and 2.7.2). Germination of the transgenic and wild-type plants started at the same time in day 5 of cultivation in all $CaCO_3$ combinations. Under the greenhouse conditions, the transgenics showed better growth in 256 g kg^{-1} and 512 g Kg^{-1} $CaCO_3$ treatments, compared with wild-type. The transgenic plants flowered 3 days in control soil, and 6-7 days in 256 g kg^{-1} $CaCO_3$ treatments before the wild-type. The transgenic plants grown in 512 g kg^{-1} $CaCO_3$ treatment reached the flowering stage 9-11 days after the flowering day of the transgenic plants grown in control soil treatment. In contrast, the wild-type plants in 512 g kg^{-1} $CaCO_3$ treatment failed to flower.

3.5.1. Tobacco Plant Fresh, Dry Biomass and Height Determination

After 12 weeks of growth, no significant increase in the fresh biomass of the transgenic plants in control soil and 256 g kg^{-1} $CaCO_3$ treatments was observed, compared with the wild-type (Fig. 3.28). The fresh biomass of the transgenic plants increased significantly in 512 g kg^{-1} $CaCO_3$ treatment, compared with that of the wild-type (Fig. 3.28). The increase in $CaCO_3$ concentration in soil has a negative effect on the growth of the plants, and their accumulation of fresh biomass for both the transgenic and the wild-type, especially in 512 g kg^{-1} $CaCO_3$ treatment. Thus, the effect of increasing the concentration of $CaCO_3$ in the soil on wild-type was greater than its effect on the transgenic plants. Therefore, around 50 % of the transgenic and 94 % of the wild-type, fresh biomass was lost, when the plants were grown in 512 g kg^{-1} $CaCO_3$ treatment.

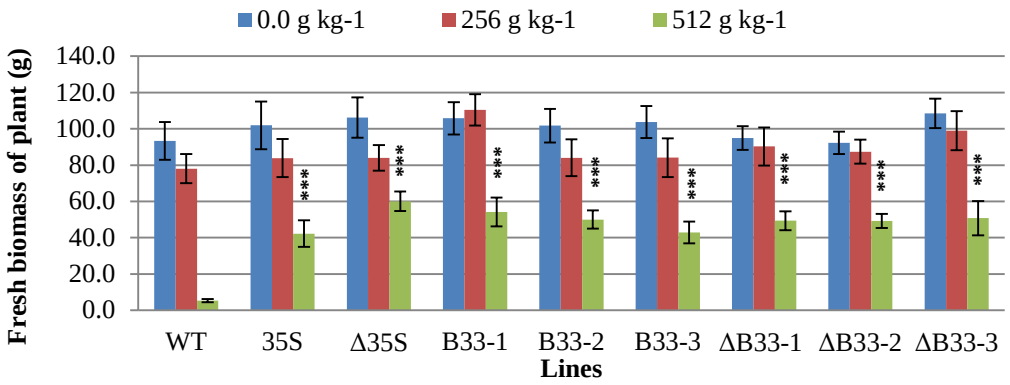


Figure 3.28: Fresh biomass of transgenic and wild-type plants grown for 12 weeks in 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ under greenhouse conditions. The data are for two independent experiments. (n=12 each, ±SD, t-test: *** p ≤ 0, 0001).

Dry biomass of the plants was determined after 96-120 h of drying the plants in oven. No significant difference in dry biomass among the transgenic plants and wild-type was observed in control soil, and 256 g kg^{-1} $CaCO^3$ treatments. The accumulated biomass of the transgenic plants increased significantly in 512 g kg^{-1} $CaCO_3$ treatment, compared with that of the wild-type (Fig. 3.29). The plants dry biomass reduced considerably, when $CaCO_3$ concentration was increased in the soil. Thus, in 512 g kg^{-1} $CaCO_3$ treatment, the increase of $CaCO_3$ in the soil severely affected the growth of wild-type, compared with the transgenic plants growth. The biomass reduction of the transgenic plants was around 52% and around 96 % of the wild-type (Fig. 3.29).

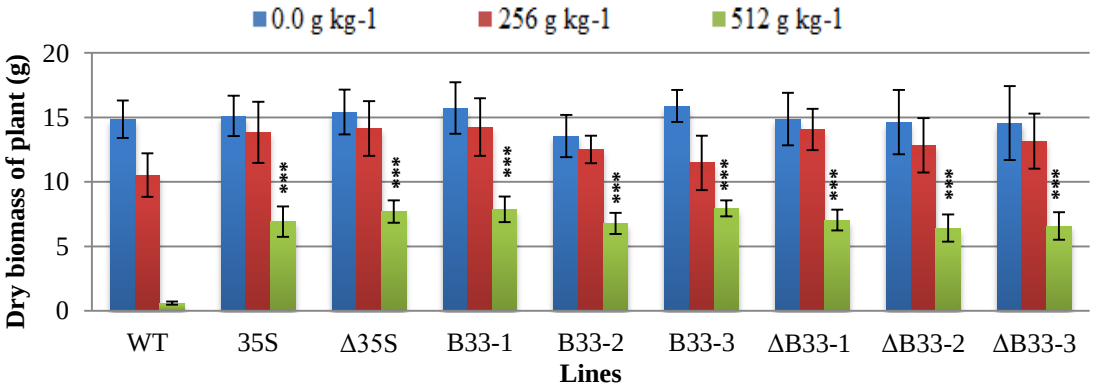


Figure 3.29: Dry biomass of transgenic and wild-type plants grown for 12 weeks in 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ under greenhouse conditions. The data are for two independent experiments. (n=12 each, ±SD, t-test: *** p ≤ 0, 0001).

The plant height was measured at the day of harvest. No significant increase in the height of the transgenic plants was observed, compared with the wild-type in control soil treatment (Fig. 3.30 and 3.31A 1-3). When the transgenic plants were grown in 256 g kg^{-1} $CaCO_3$ treatment, a significant increase in the height of the plant was observed, compared with the wild-type, except the lines B33-2 and B33-3 (Fig. 3.30 and 3.31B 1-3). All the transgenic plants grown in 512 g kg^{-1} $CaCO_3$, showed significant increase in their heights compared with the wild-type (Fig. 3.30 and 3.31C 1-3). The growth of the transgenic plants in 256 g kg^{-1} $CaCO_3$ treatment was uneven compared with control soil treatment. Some of the transgenic plants height was higher in control soil treatment than in 256 g kg^{-1} $CaCO_3$; others showed greater height in 256 g kg^{-1} $CaCO_3$ compared to control. The negative effect of $CaCO_3$ was observed clearly at 512 g kg^{-1} $CaCO_3$ that reduced the height of the plant appreciably. Consequently, the effect of the increase of $CaCO_3$ in soil was greater in wild-type plants, compared with the transgenic plants (Fig. 3.30 and 3.32) In general; the plant height reduction was around 50-55% in transgenic and 90% in wild-type plants in 512 g kg^{-1} $CaCO_3$.

It is apparent that elevation of $CaCO_3$ concentration in soil has a negative effect on plant growth. However, this effect seems to be less in the transgenic plants, compared with the wild-type. Therefore, it can be said, that the transgenic plants are more capable of uptake of phosphate (see 3.5.2), and grow better in alkaline soil than the wild-type.

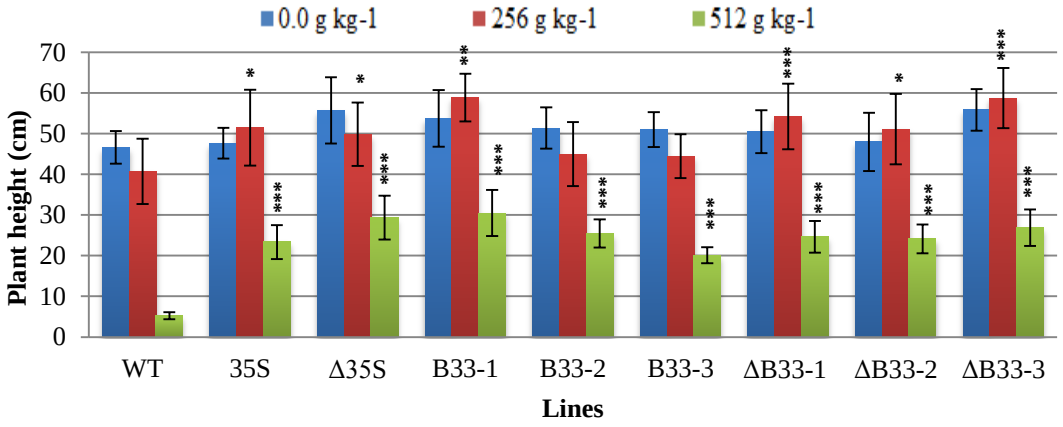


Figure 3.30: Transgenic and wild-type plants height after 12 weeks of growth in soil mixed with 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ under greenhouse conditions. The data are of two independent experiments. (n=12 each, ±SD, t-test: *** $p \leq 0,0001$; * $p \leq 0,01$).

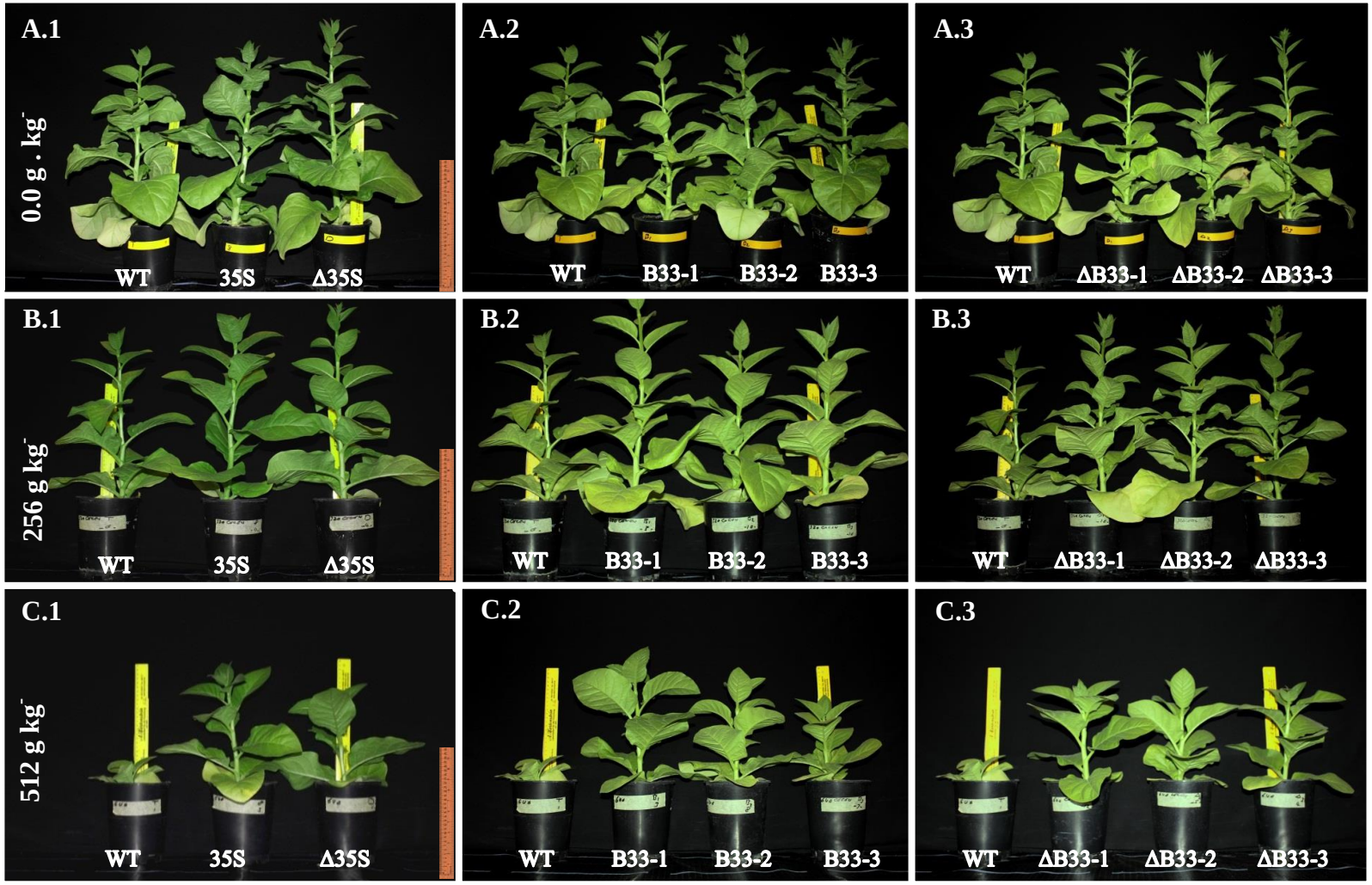


Figure 3.31: Tobacco plant growth. Transgenic and wild-type plants were grown for 12 weeks in alkaline soil/ $CaCO_3$ mixture, in 0.0 g kg^{-1} $CaCO_3$ (control treatmnt), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ treatments.

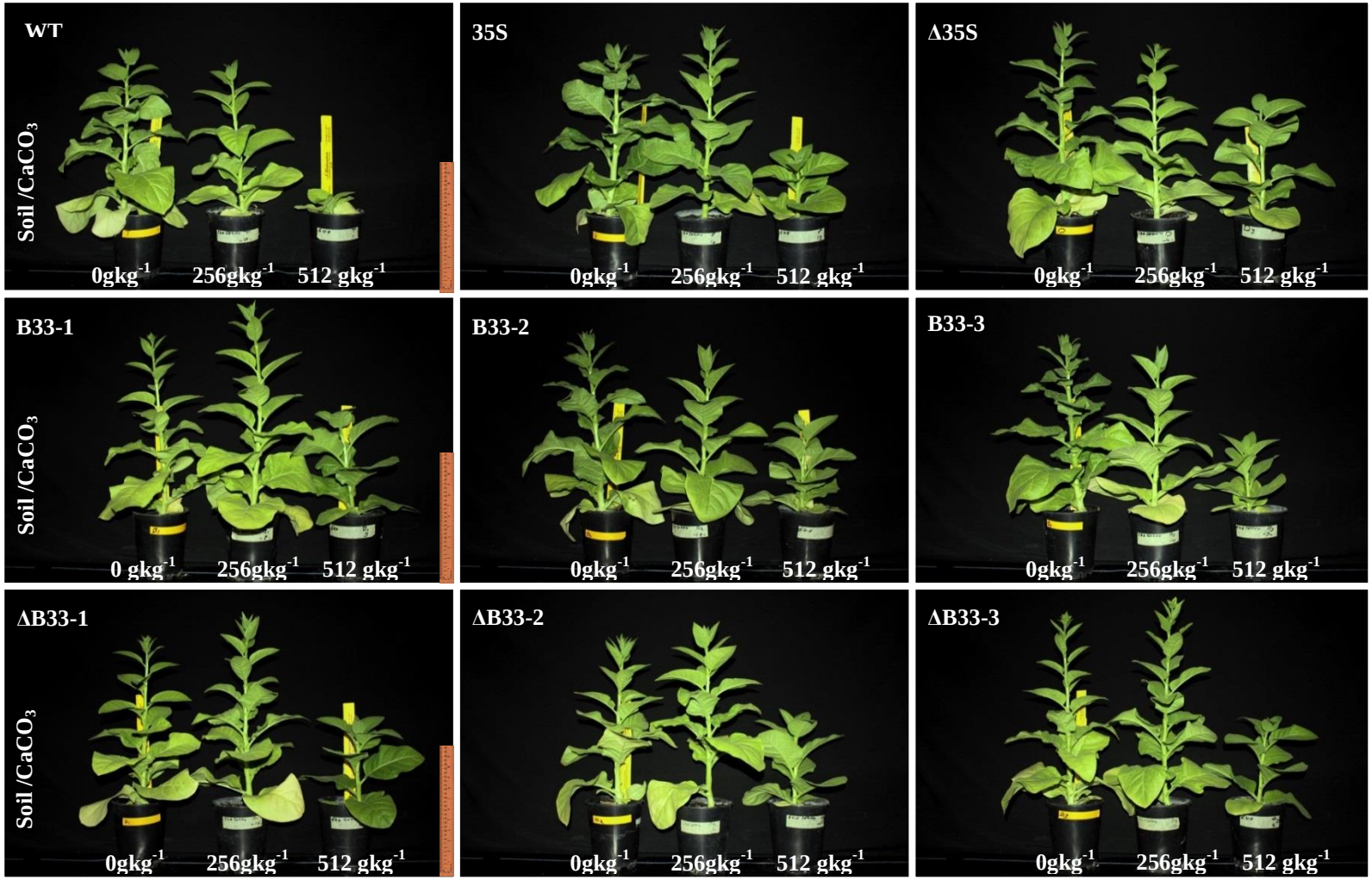


Figure 3.32: Tobacco plant height. The effect of $CaCO_3$ concentration on transgenic and wild-type plants grown for 12 weeks in alkaline soil/ $CaCO_3$ mixture, in 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ treatments.

3.5.2. Plant Content of Phosphorus

A visible decrease of total P content per plant was observed after 12 weeks of cultivation in 512 g kg^{-1} $CaCO_3$, compared with control soil and 256 g kg^{-1} $CaCO_3$ treatments. Furthermore, the wild-type plants were more affected by the increase of $CaCO_3$ concentration in soil than the transgenic plants. No significant difference was detected in the plant content of total P between the transgenic and the wild-type plants in control soil treatment. In contrast, a significant increase in P content of the transgenic plants in 256 and 512 g kg^{-1} $CaCO_3$ treatments was detected compared with the wild-type (Fig. 3.33). While, the highest percentage of the content of (P/g %) was in control soil treatment, the lowest percentage was in 512 g kg^{-1} $CaCO_3$ treatment (Fig. 3.34). The transgenic plants content of (P/g %) in treatment 256 g kg^{-1} $CaCO_3$ was comparable to that in the control soil treatment. In contrast, the wild-type content of (P/g %) in 256 g kg^{-1} $CaCO_3$ was less than that in control soil treatment. The transgenic plants content of (P/g %) in treatments 256 and 512 g kg^{-1} $CaCO_3$ was significantly higher than that of the wild-type (Fig. 3.34).

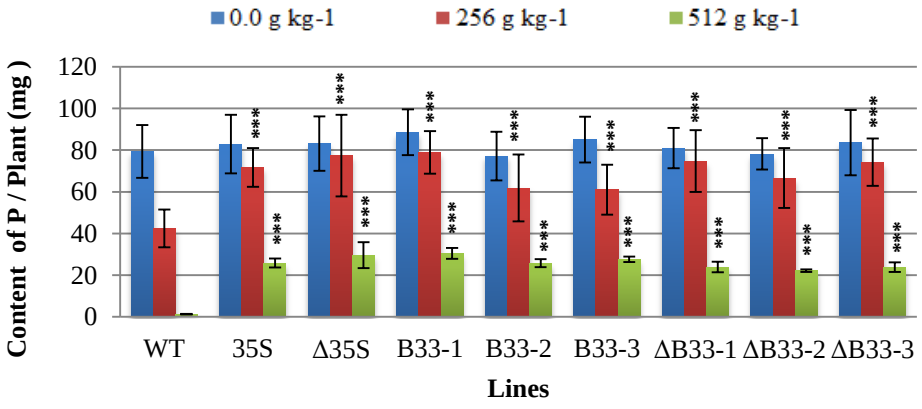


Figure 3.33: Content of phosphorus per plant (mg). Transgenic and wild-type plants gown for 12 weeks in 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ treatments under greenhouse conditions. The data are for two independent experiments. (n=12 each, ±SD, t-test: *** $p \leq 0, 0001$).

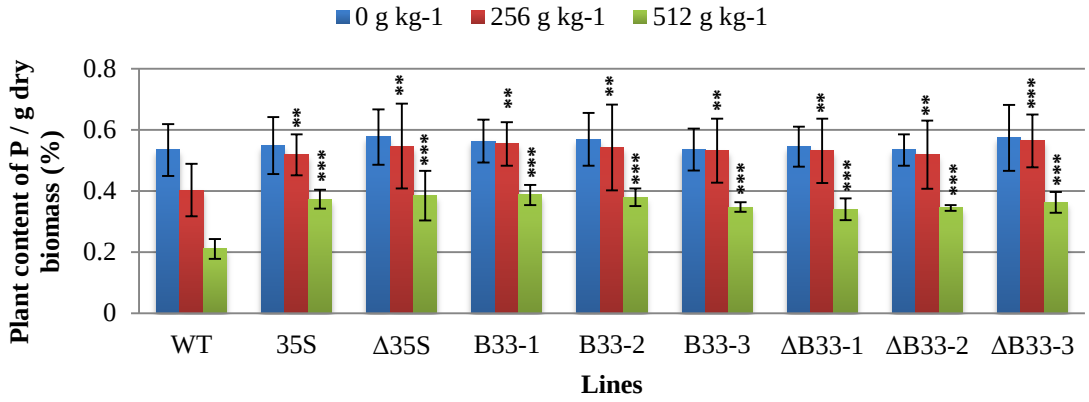


Figure 3.34: Plant content of phosphorus per gram dry biomass (%). Transgenic and wild-type plants gown for 12 weeks in 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ treatments under greenhouse conditions. The data are for two independent experiments. (n=12 each, ±SD, t-test: ** $p \leq 0{,}001$, *** $p \leq 0, 0001$).

3.5.3. Determination the Change of Soil pH

The soil pH was determined after the plant harvest. From the results shown in (Fig. 3.35), the transgenic plants acidification of soil was significantly higher than that of the wild-type. The transgenic plants in the treatment of 512 g kg^{-1} $CaCO_3$ were able to decrease the soil pH up to one unit.

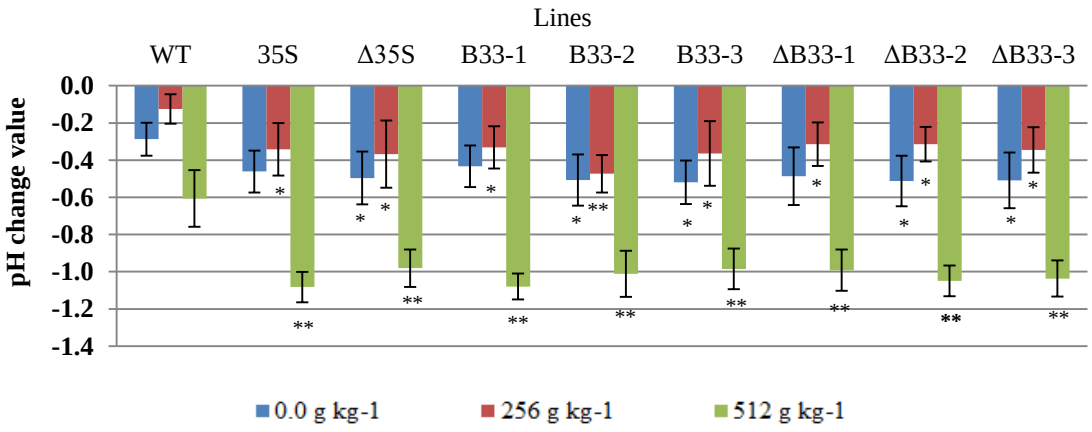


Figure 3.35: Reduction of soil pH by plants grown in soil of varying calcium carbonate content 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ during the trial period. The data represent the mean and standard error of twelve plants. The pH was determined before sowing and after harvest. The level of significance in relation to the wild-type is indicated (*t*-test: ** $p \leq 0.001$, * $p \leq 0.01$).

3.5.4. The Plant Yield of Seed Capsules

The yield of seed capsules decreased markedly with the increase of $CaCO_3$ soil content as shown in (Fig. 3.36). In other words, the yield of seed capsules was the highest averaging (18 capsules / plant) in transgenic plants and (15.9) in wild-type in control soil treatment. When the concentration of $CaCO_3$ was elevated in soil to 512 g kg^{-1}, the yield of capsules reduced by 58 % in the transgenic plants and 100 % in the wild-type (no capsules were produced). Thus, the transgenic plants ability of produce capsules was significantly less affected by the increase of $CaCO_3$ concentration compared with the wild-type.

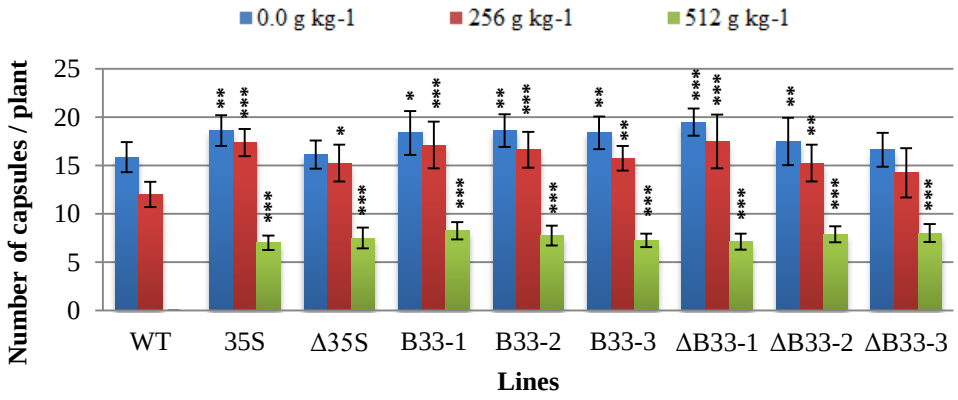


Figure 3.36: Seed capsules number per plant. Transgenic and wild-type plants grown for 12 weeks in 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ under greenhouse conditions. The data are for two independent experiments (n=12 each, ±SD, *t*-test: *** $p \leq 0.0001$, ** $p \leq 0.001$, * $p \leq 0.01$).

As is shown in (Fig. 3.37) the concentration of $CaCO_3$ in soil negatively affected the weight of the produced capsules, as it affected the number of the produced capsules (Fig. 3.36). For example, the weight of the produced capsules in transgenic and wild-type plants in control soil treatment was averaging 3.3 g, it reduced by 27 % in transgenic and 44 % in wild-type in 256 g kg^{-1} $CaCO_3$. When $CaCO_3$ was elevated to 512 g kg^{-1} the weight was reduced by 63 % in transgenic and 100 % in wild-type (no capsules were produced). Consequently, the weight of the transgenic plant capsules was less affected compared with the weight of the wild-type in all $CaCO_3$ treatments.

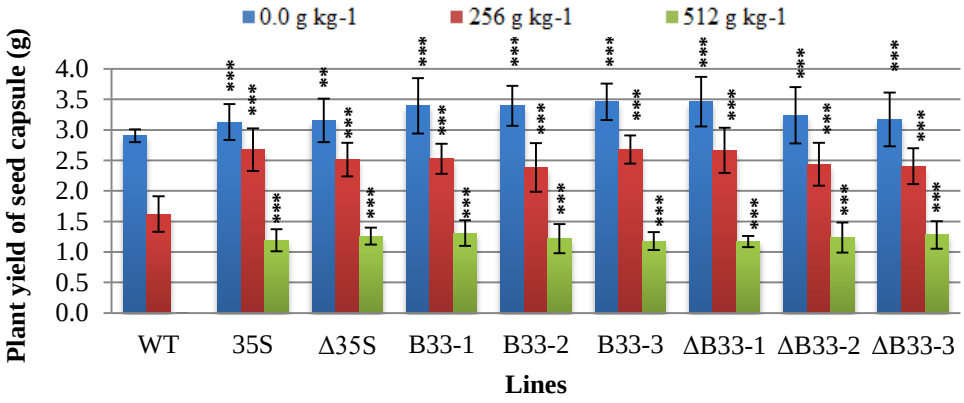


Figure 3.37: Cumulative capsule weight per plant (g). Transgenic and wild-type plants grown for 12 weeks in 0.0 g kg^{-1} (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ treatments under greenhouse conditions. The data are for two independent experiments (n=12 each, ±SD, t-test: *** $p \leq 0.0001$, ** $p \leq 0.001$).

Reduction in the weight of single capsule of the transgenic plants in 256 and 512 g kg^{-1} $CaCO_3$ was less affected compared with that of the wild-type. The wild-type failed to produce any capsules under 512 g kg^{-1} $CaCO_3$ treatment. However, the transgenic and the wild-type plants gained the same weight for capsule in soil control treatment (Fig. 3.38).

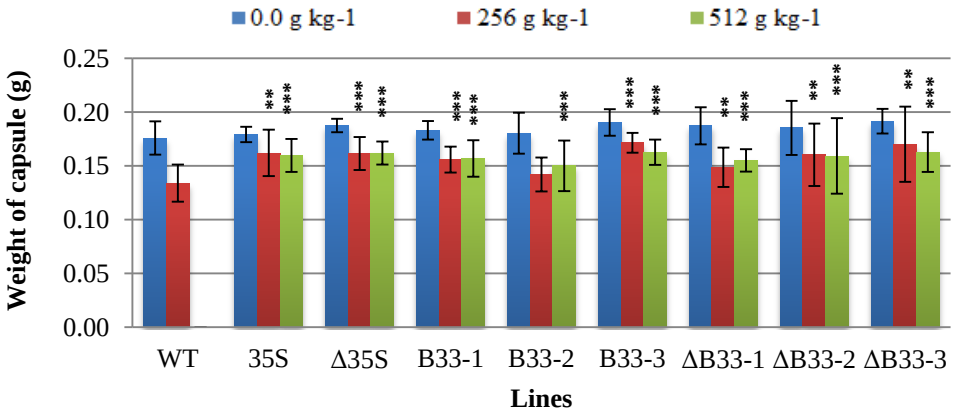


Figure 3.38: Single capsule weight per plant (g). Transgenic and wild-type plants grown for 12 weeks in 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg^{-1} $CaCO_3$ or 512 g kg^{-1} $CaCO_3$ under greenhouse conditions. The data are for two independent experiments (n=12 each, ±SD, t-test: *** $p \leq 0.0001$, ** $p \leq 0.001$,* $p \leq 0.01$).

Concerning the total weight of seed per plant, a significant difference was found in some of the transgenics, compared with the wild-type, when they were grown in control soil or in 256 g kg^{-1} $CaCO_3$ treatments. In contrast, in 512 g kg^{-1} $CaCO_3$ treatment the wild-type plants failed to produce seed, compared with the transgenic plants (Fig. 3.39).

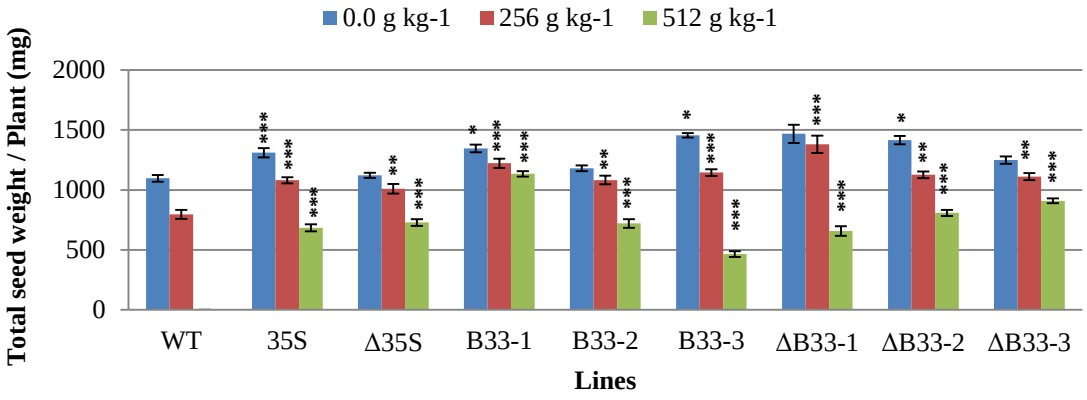


Figure 3.39: Total seed weight per plant (mg). Transgenic and wild-type plants grown for 12 weeks in 0.0 g kg^{-1} $CaCO_3$ (control treatment), 256 g kg-1 $CaCO_3$ or 512 g kg-1 $CaCO_3$ under greenhouse conditions. The data are for two independent experiments (n=12 each, ±SD, t-test: *** $p \leq 0.0001$, ** $p \leq 0.001$,* $p \leq 0.01$).

3.6. Metabolic Adaptation of the Transgenic Plant under HAp

Plants modify metabolic processes of adaptation to low phosphate conditions through giving priority for utilizing the internal inorganic Pi, and maximizing acquisition of external Pi (Huang *et al.*, 2008). In order to determine metabolic response of the transgenic lines to HAp, two-week old whole seedlings and three-week old shoots and roots from seedlings grown respectively in liquid and solid alkaline MS medium in Pi-, HAp and Pi+ were analyzed using GC-MS.

The content of organic acids, amino acids, sugars, phosphoric acid and nicotinic acid showed variations in seedlings. In general, an increase in the content of citric and malic acid was observed, in the seedlings grown in absence of phosphate (Pi-), (Fig. 3.40). Interestingly, most of the transgenic lines showed slightly less content of citric acid in HAp compared with wild-type. On the other hand, the seedlings content of fumaric acid in Pi+ showed an increase, compared with HAp and Pi- (see appendix 7.1.1.2). In addition, an increase of fumaric acid was found in transgenic seedlings grown in HAp.

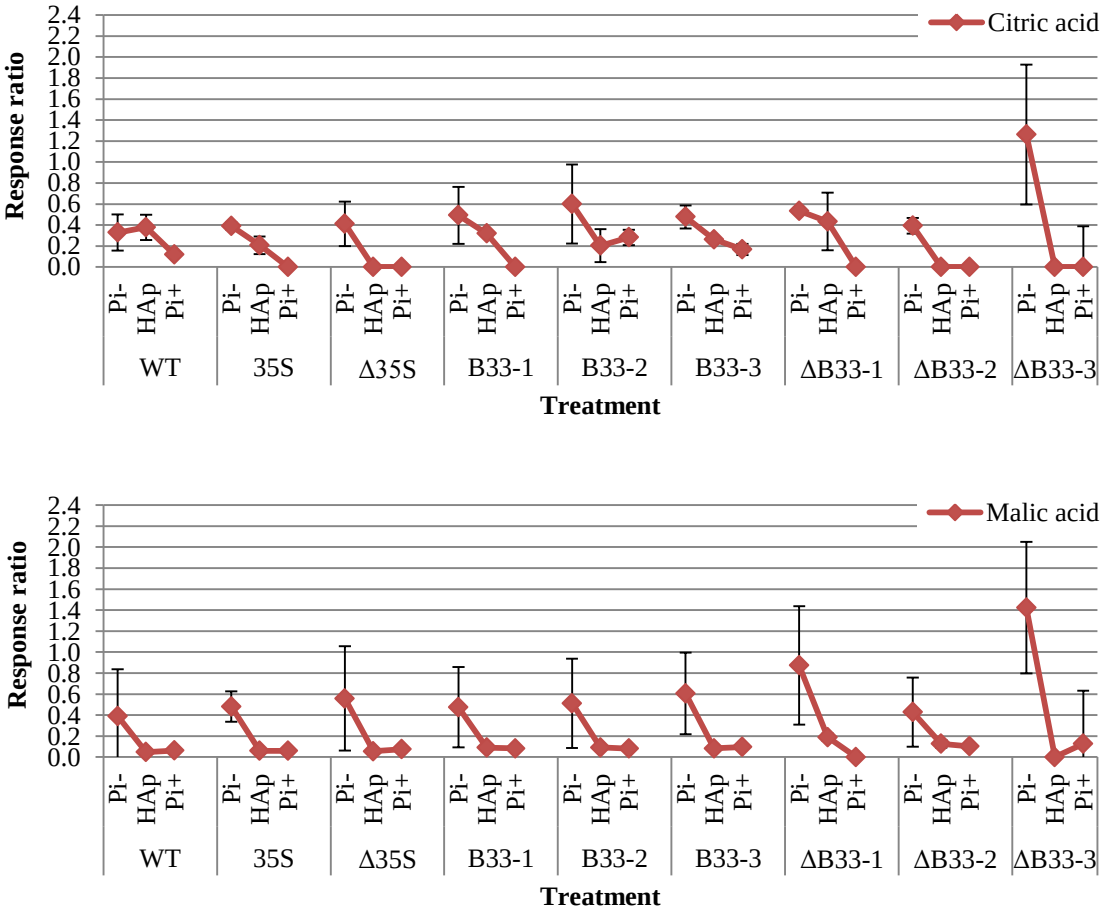


Figure 3.40: Citric and malic acid response ratio of two-week old transgenic and wild-type whole seedlings grown in MS alkaline liquid medium under Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments analyzed via GC-MS.

Transgenic and wild-type shoot samples of seedlings grown in solid Pi- medium, showed an increase in citric and malic acids compared with HAp and Pi+ treatments. An increase in malic acid in wild-type sample in HAp treatment was found compared with the transgenic sample. In addition, B33 transgenic group accumulated more citric acid in HAp treatment compared with the WT, 35S, Δ35S and ΔB33 groups of transgenic (Fig. 3.41). The shoots of wild-type and transgenic seedlings showed an increase in fumaric acid in HAp treatment, compared with Pi- and Pi+ treatments. Wild-type shoots content of fumaric acid in HAp showed an increase, compared with transgenic seedlings shoots (see appendix 7.1.2.2).

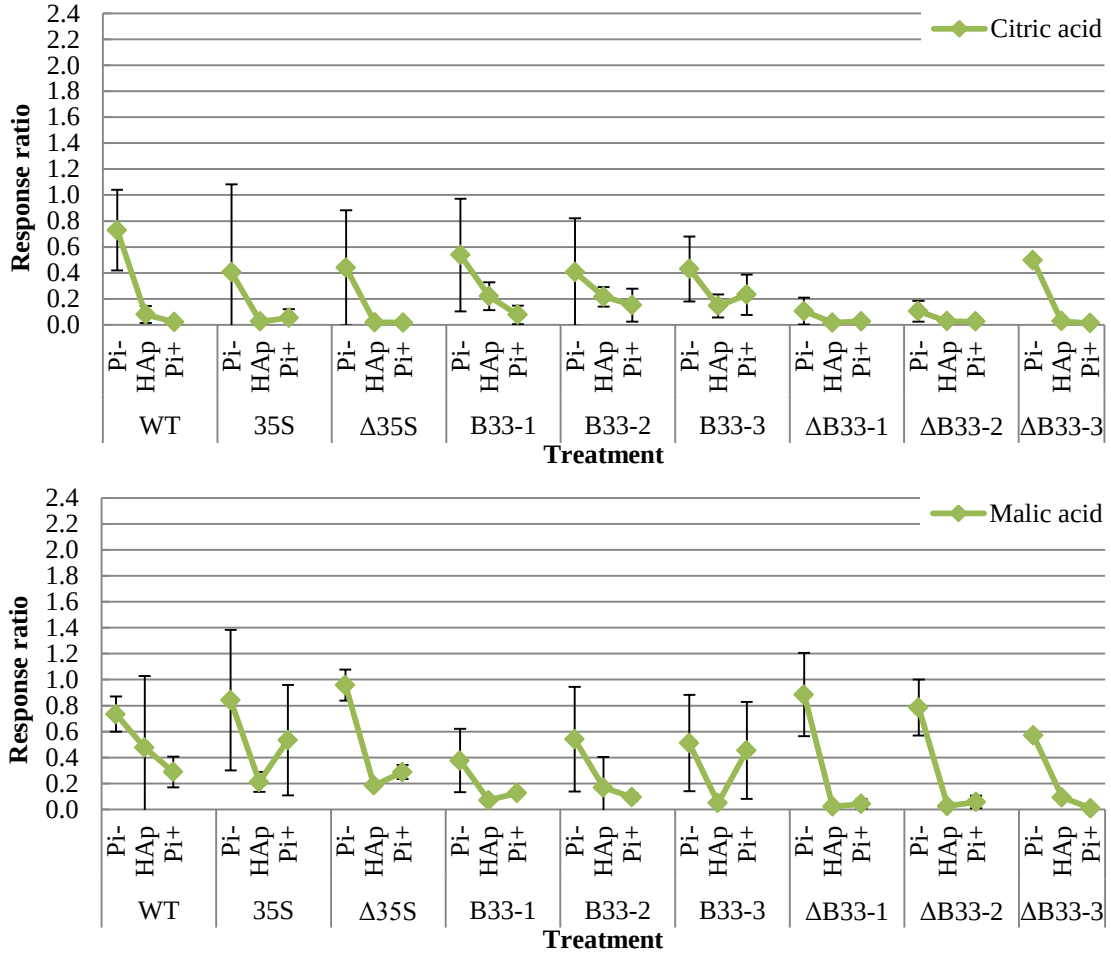


Figure 3.41: Citric and malic acid response ratio of three-week old transgenic and wild-type shoot of seedlings grown in MS alkaline solid medium under Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments analyzed via GC-MS.

In contrast, the transgenic and wild-type root samples grown in solid medium showed higher content of citric and malic acid in Pi+ treatment, compared with HAp and Pi- treatments. Furthermore, the transgenics showed a marked increase in their content of citric and malic acid in HAp treatment, compared with the wild-type (Fig. 3.42). No fumaric acid was detected in the root samples of all treatments.

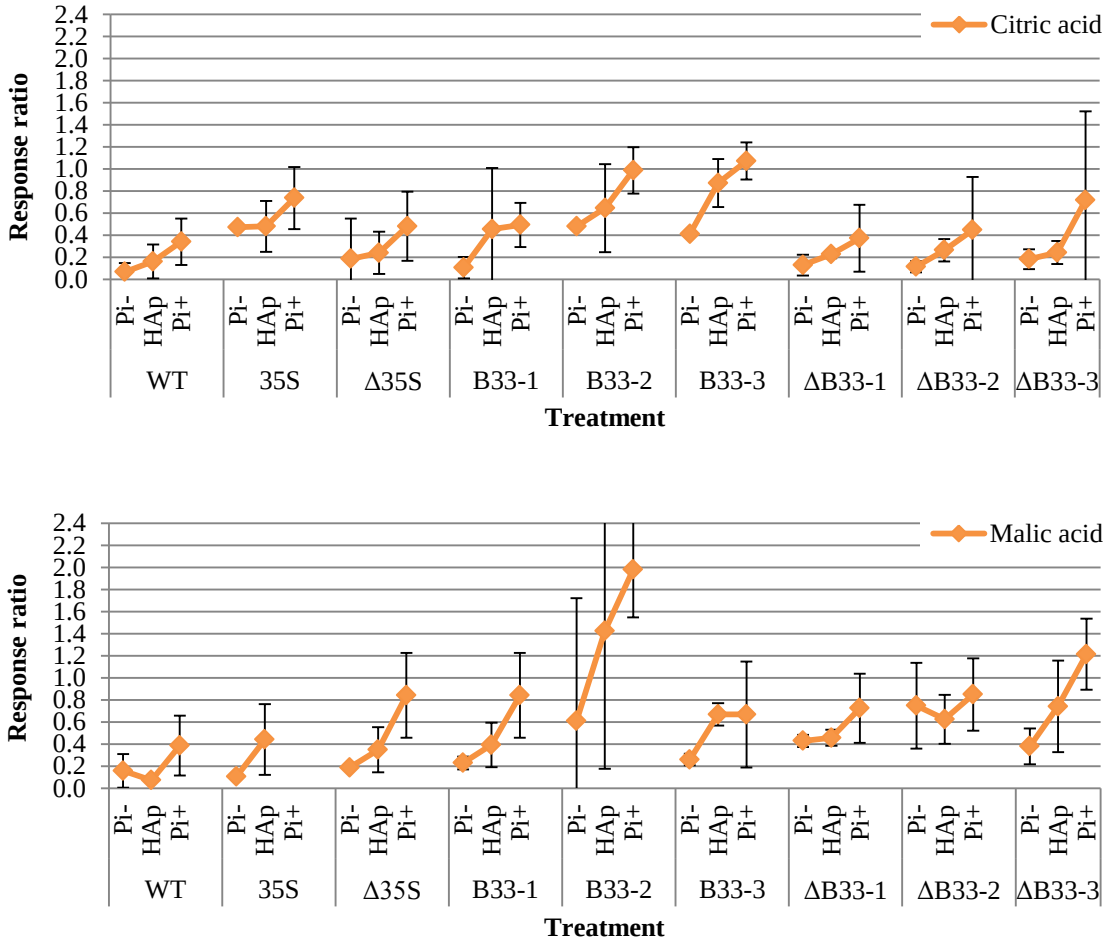


Figure 3.42: Citric and malic acid response ratio of three-week old transgenic and wild-type root of seedlings grown in MS alkaline solid medium under Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments analyzed via GC-MS.

As is shown in (Fig. 3.43), all the analyzed samples (whole seedling, shoot and root) showed an appreciable increase in their content of phosphoric acid in Pi+ treatment, compared with HAp and Pi- treatments. Moreover, the transgenics whole seedlings samples showed an increase in their content of phosphoric acid in HAp treatment, compared with the wild-type. In addition, an increase in phosphoric acid content of 35S, Δ35S and B33 groups of transgenic shoot samples was found in HAp treatment, compared with wild-type and ΔB33 group. The B33 and ΔB33 groups of transgenic root samples showed an increase in their content of phosphoric acid in HAp, compared with the wild-type, 35S and Δ35S. These results are consistent with the results obtained in (3.3.3 and 3.4.4.4), concerning the concentration of P in the plant material using the spectrometer.

The samples also showed differences in their content of amino acids in response to Pi-nutrition, and also between the transgenic and wild-type seedlings. All the samples for example showed an increase in their content of glutamic acid in Pi- treatment, compared with HAp and Pi+ treatments. Furthermore, the transgenic whole seedling and root samples showed a slight increase in their content of glutamic acid in HAp, compared with that of the wild-type. In contrast, the transgenic shoot samples showed a slight decrease in their content of glutamic acid in HAp, compared with the those of the wild-type (Fig. 3.44).

Concerning the content of sugars, most of the transgenics tended to increase their content of glucose in Pi-, compared with HAp and Pi+. The wild-type shoot and root samples showed a decrease in their content of glucose in Pi- and in Pi+ treatments, compared with HAp treatment. Interestingly the wild-type samples of all samples (whole seedling, shoot and root) showed higher content of glucose in HAp compared with transgenics (Fig. 3.45).

The complete list of the identified metabolites from different plant samples (whole seedling, root or shoot) grown in liquid or solid MS medium with different P treatments is shown in the appendix (7.1.1).

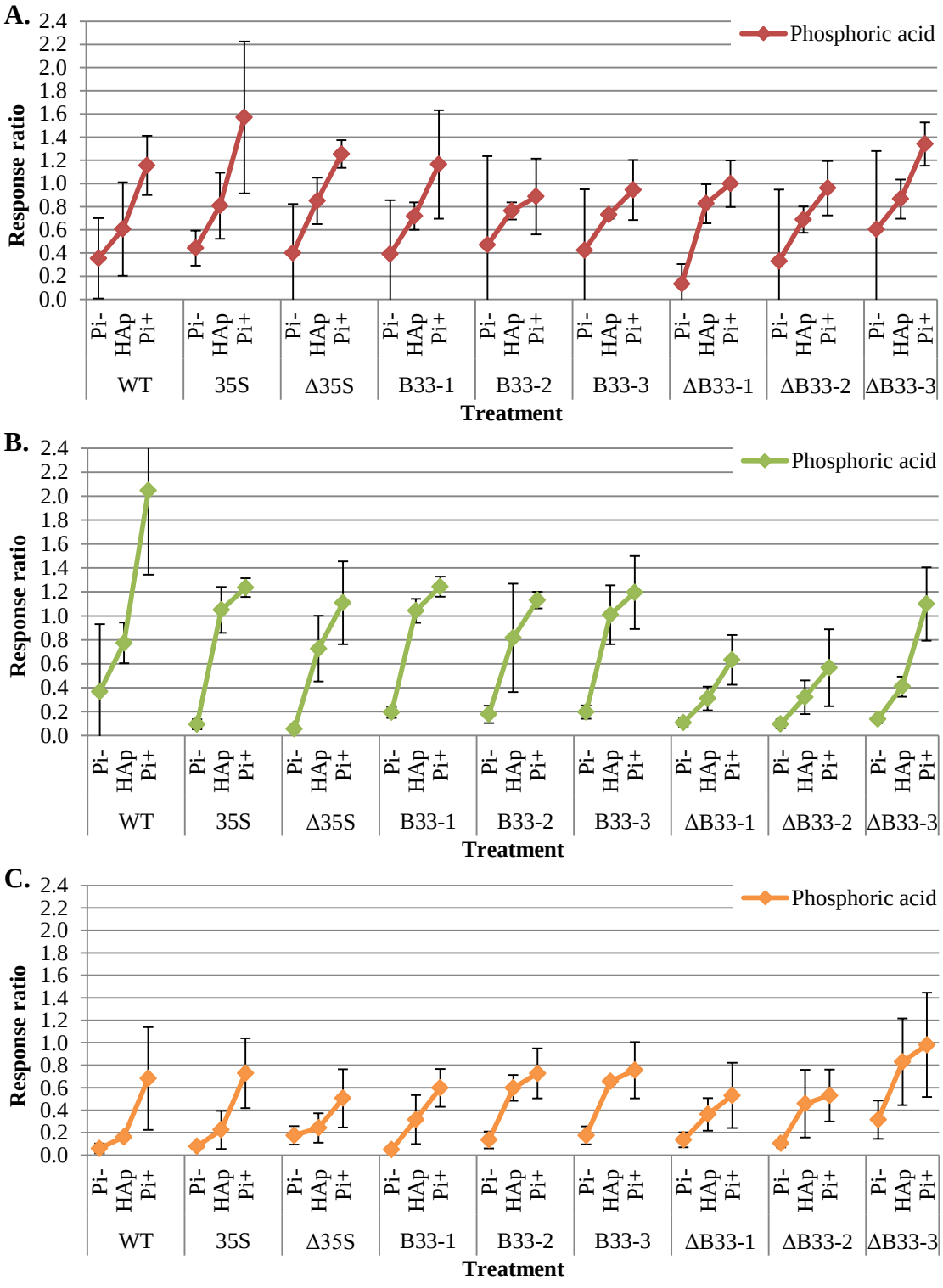


Figure 3.43: Phosphoric acid response ratio of transgenic and wild-type seedlings grown in MS alkaline medium under Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments analyzed via GC-MS. **A.** Whole seedling **B.** Shoot and **C.** Root**.**

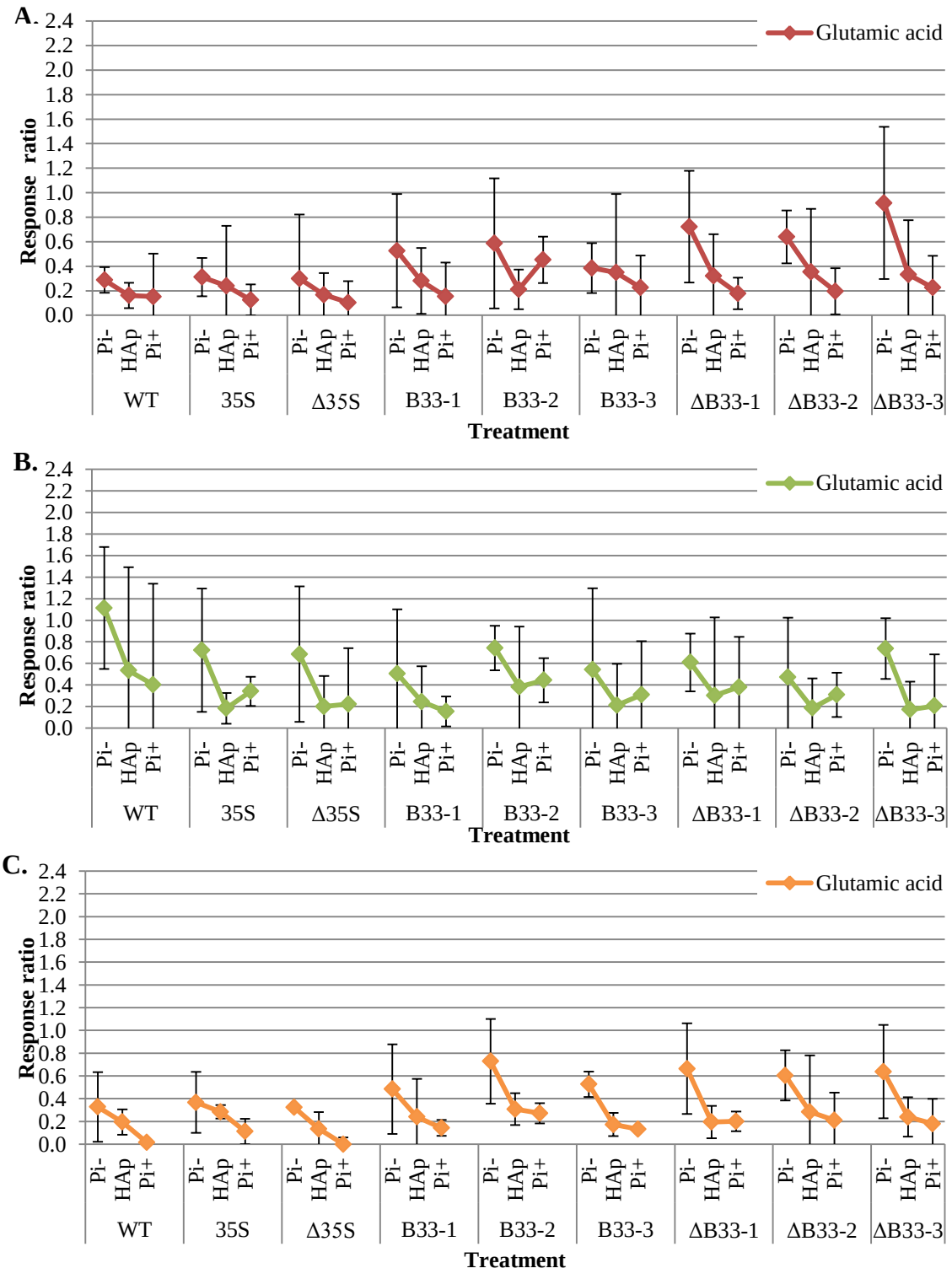


Figure 3.44: Glutamic acid response ratio of transgenic and wild-type seedlings grown in MS alkaline medium under Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments analyzed via GC-MS. **A.** Whole seedling **B.** Shoot and **C.** Root**.**

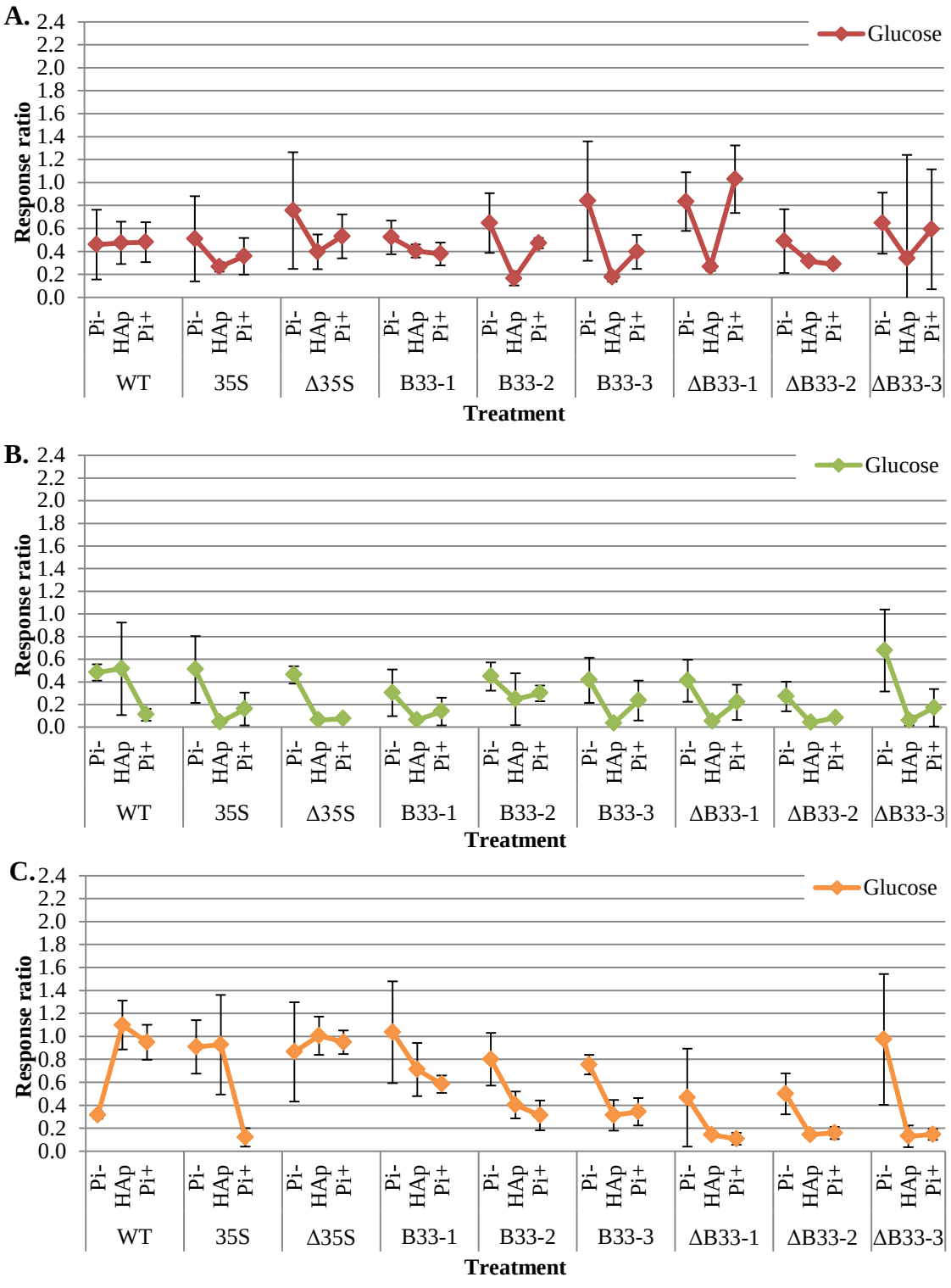


Figure 3.45: Glucose response ratio of transgenic and wild-type seedlings grown in MS alkaline medium under Pi-starved (Pi-), hydroxyapatite (HAp) or Pi-sufficient (Pi+) treatments analyzed via GC-MS. **A.** Whole seedling **B.** Shoot and **C.** Root.

4. Discussion

4.1. Germination of Transgenic Seed in Alkaline Condition

Prior work documented the effect of hydrogen-ion concentration (pH) on seed germination; Ghaderi-Far *et al.* (2010), Okay *et al.* (2011) and Tanveer *et al.* (2013) reported that pH values of 8 and above decreased the seed germination rate. Germination test is often conducted on seed before planting. Monitoring the time taken to germinate seed gives an indication of seed viability. In this work, in order to find out whether our transgenic and wild-type seeds germinate at the same time, we germinated them on alkaline solid MS medium at pH 8.0.

We found that germination of the transgenic and wild-type seeds (Fig. 3.1) started at the same time at day 4. Therefore, seedlings of the same age make comparisons of growth parameters between wild-type and mutants possible.

4.2. PPDK-overexpression in Tobacco Plant

Sheriff (1994) and Stenzel (1997) constructed the tobacco plants that overexpress the *Mesembryanthemum crystallinum* PPDK. Sheriff *et al.* (1998) found differences in free amino acids pattern of the transgenic seeds and leaves, compared with the wild-type. Trejo-Téllez *et al.* (2010) observed an increase of citric and malic acid exudation, when the transgenics roots were exposed to Al. The transgenic plants, which showed increase of citric, and malic acid exudation, showed increase in tolerance to the toxicity of Al in acid conditions.

To understand the role of Pyruvate, orthophosphate dikinase (PPDK) in tobacco plant under HAp in alkaline conditions, the first step was to make sure that the transgenic lines still keep the trait of PPDK overexpression, the PPDK protein was detected by SDS-PAGE and western blot. We used two of the transgenic lines made by Sheriff *et al.*, (1994), that overexpress the *M. crystallinum* PPDK under the control of 35S promoter and six other transgenic lines made by Stenzel (1997) that overexpress the *M.crystallinum* PPDK under the control of B33 promoter.

We found that the strength of protein bands of the transgenics on SDS-PAGE (Fig. 3.2A) was considerably higher than that of the wild-type. The western blot test (Fig. 3.2B) showed a substantial increase in strength of PPDK bands of the transgenics, compared with the faint band of the wild-type. This indicates that the transgenic plants still keep the trait of PPDK overexpression. This is consistent with the result of Trejo-Téllez *et al*., (2010) who found an increase in the band strength of the transgenic plants that overexpress the PPDK gene of *M.crystallinum*. After the existence of the trait was confirmed, we started studying the role of the overexpression of PPDK in plant growth and yield.

4.3. The Role of PPDK in C_3 plants

Overexpression of PPDK was achieved in many C_3 plants, for example, tobacco (Sheriff *et al.*, 1998), potato (Ishimaru *et al.*, 1998) and rice (Fukayama *et al.*, 2001). Sheriff *et al.* (1998) reported that the overexpression of the *M. crystallinum* PPDK gene, which targets the chloroplast of tobacco cells under the transcriptional control of 35S promoter (35S lines), increased the PPDK activity of tobacco leaves about 1.5-fold with the increase in number of seed per seed capsule, and weight of seed capsule. In contrast, transgenic tobacco plant produced less seed, or nearly no seed in the strongest phenotypes compared with wild-type, when the PPDK gene was expressed in the cytosol (Δ35S lines), (the plastidic signal sequence was deleted), (Sheriff *et al.*, 1998). Unlike the root-specific B33 promoter, that did not produce such phenotype, where both the transgenic lines that overexpress the cytosolic PPDK (ΔB33 lines) and the transgenic lines that overexpress the plastidic PPDK (B33 lines), showed a similar seed production (Trejo-Téllez *et al.*, 2010).

Trejo-Téllez *et al.* (2010) also reported that the transgenic tobacco plants that overexpress the *M. crystallinum* PPDK gene under the control of the root-specific B33 promoter, showed a different response between the PPDK (B33) and ΔPPDK (ΔB33) lines. This response is represented by increase the exudation of organic acid, and reduction in root accumulation of Al for the transgenic lines harboring the plastidic PPDK gene (PPDK lines), compared with those harboring the cytosolic PPDK gene (ΔPPDK lines) in the presence to Al stress. Moons *et al.* (1998) reported that the induction of PPDK in roots of rice plants under gradual drying, cold, salinity and mannitol, which indicates

water deficit. Also, the PPDK was induced in the roots of rice seedlings exposed to abscisic acid and low-oxygen atmosphere. This leads to the assumption that the cytosolic PPDK could be involved in non-photosynthetic CO_2 fixation in low-oxygen environment, and functions in an inducible metabolic response during low-oxygen and water deficit. Aoyagi and Bassham (1984) found during wheat seeds development that most of the metabolites resulted from the carboxylation of PEP and less from RuBisCO, which may suggest a role for PPDK in interconversion of amino acids.

PPDK reaction has a small free energy of activation (ΔG) of Keq ≈ 1 x 10^{2}, this means that the reaction of PPDK enzyme is readily reversible. The reaction favors formation of ATP and pyruvate from PEP, but it can be easily reversed to the direction of PEP formation through hydrolysis of PPi, catalyzed by pyrophosphatase. (Reeves *et al.*, 1968). In *Clostridium symbiosum,* PPDK functions as glycolytic enzyme and drives the reaction in the direction of ATP and Pyruvate synthesis (Mertens 1993), while in chloroplast, PPDK drives the reaction in the direction of PEP synthesis. This gluconeogenic direction is ensured by the high activity of pyrophosphatase in this compartment, preventing the reverse glycolytic reaction. In the cytosol, the pyrophosphate concentration is much higher due to the lack of pyrophosphatase (Weiner *et al*., 1987). The exudation of citric and malic acids under Al stress in both, the PPDK and the ΔPPDK groups of transgenic lines, indicates that the enzyme produces PEP as a precursor to the synthesis of organic acids in chloroplast, as well as in the cytosol (Trejo-Téllez *et al.*, 2010).

Remarkably, no significant differences between the PPDK and ΔPPDK lines were observed in all results that have been obtained in this study. These results are contradicted with the results obtained by (Trejo-Téllez *et al.*, 2010) in her study for the same lines, who found differences between the plastidic PPDK (B33) lines, that increased the organic acid exudation and decreased the accumulation of Al, and the cytosolic ΔPPDK (ΔB33) lines that exude less organic acid and accumulate more Al surrounding their roots tips. Also, contradict with the results of (Sheriff *et al.,* 1998) who found differences in the number of seed per capsule and seed capsule weigh in (35S and Δ35S) of PPDK lines. The 35S lines produced more seed and more seed capsules weight. In contrast the Δ35S produced less or nearly no seed. As mentioned above, PPDK generates PEP from pyruvate in chloroplast, which is secured by the high activity of

pyrophosphatase. As a consequence, the increased supply of PEP (substrate of PEPC) from the transgenic lines (PPDK overexpressing lines) could enhance the anaplerotic role of PEPC, thus ensures the continuation of the tricarboxylic acid cycle function through preventing the deprivation of its intermediates (citrate and malate), (Latzko and Kelly, 1983).

As is shown in Figure 4.1, PPDK generates PEP from pyruvate, PEP is a substrate for PEPC for the synthesis of OAA, that is reduced to malate via cytosolic malate dehydrogenase (MDH). Malate could then be transported to mitochondria via malate-oxaloacetate shuttle and converted to citrate by citrate-synthase (CS), (Doubnerova and Ryslava, 2011; Trejo-Téllez *et al.*, 2012)). Therefore, PPDK, precedes the metabolism of organic acids, and allows a significant increase in the exudation of organic acids that support the recovery of insoluble phosphate, by lowering the pH and complexation of calcium. Both processes make P more available to the plant, and significantly improve the growth of seedlings at alkaline pH in the presence of sparingly soluble phosphate (HAp). The ability of the transgenic plants to increase exudation of organic acids, leads to a significant growth in lime and phosphate-poor soils.

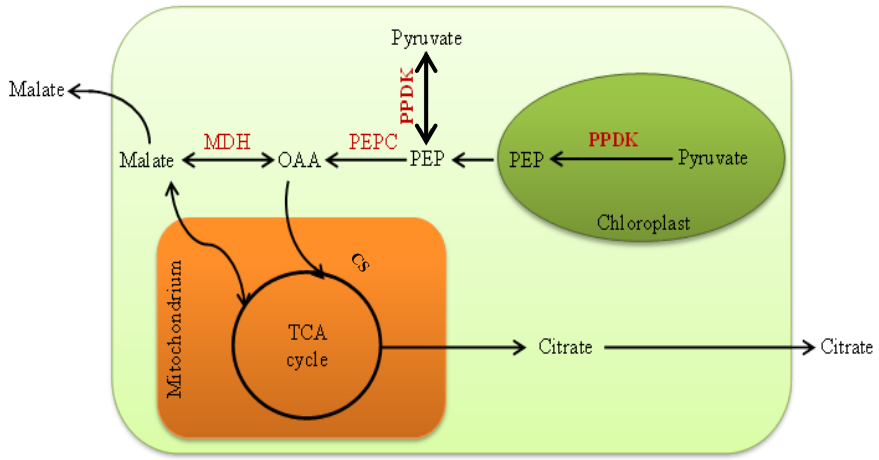


Figure 4.1: Scheme of some key metabolic pathways occurring in plant exposed to stress factors such as Pi-deficiency, like the anaplerotic function of PPDK and the enzymes PEPC and MDH, which are involved in organic acid (mainly citrate and malate) synthesis. PEP: phosphoenolpyruvate, **OAA:** oxaloacetate, **MDH:** Malate dehydrogenase, **PPDK:** pyruvate, orthophosphate dikinase, **TCA cycle:** tricarboxylic acid cycle, **CS:** citrate synthase, **PEPC:** phosphoenolpyruvate carboxylase.

(López-Bucio *et al.,* (2000) and Koyama *et al.,* (2000) found that the transgenic plants that overexpress citrate synthase (CS), showed a better growth and increase in seed yield. The improved growth of these plants was correlated with the increase in citrate exudation. However, Delhaize *et al*., (2001) did not find any role for the overexpression of citrate synthase in enhancing tolerance for aluminum, or improving the supply of phosphate. The reason for the different results could be the anaplerotic reaction of the TCA. An outflow of citrate from the cycle, caused by overexpression of citrate synthase can only be sustainable, if the cycle is replenished with C-skeletons, where PPDK has an active role in the process.

4.4. Influence of P-nutrition on Growth of Plants in Alkaline Condition

In alkaline soils, phosphorus forms insoluble compounds that are not available for plant uptake (Goldstein *et al.,* 1999; Lopez-Bucio *et al.,* 2000). Strategies are developed by the plant to cope with Pi-limitation and improving of its efficient acquisition. These strategies include root architecture, physiological and biochemical modifications (Raghothama, 1999; Dick *et al.,* 2011).

4.4.1. Influence of P-nutrition on Plants: Exudation of Organic Acid

Organic acids in the rhizosphere, such as citrate, malate, oxalate and fumarate, have been suggested to be involved in many processes, including nutrient acquisition and metal detoxification (Jones, 1998; Kamh *et al.,* 2001). Calcareous and alkaline soils are frequently characterized as Pi-deficient soils. Root exudation of organic acid plays an effective role in mobilizing insoluble P (Ström *et al.,* 2005). An increase in organic acid exudation, is reported in rape plant (*Brassica napus* L.) grown in calcareous soil (Zhang *et al.,* 1997). Furthermore, Dinkelaker *et al.* (1989) reported that white lupin (*Lupinus albus* L.) grown in Pi-deficient calcareous soil, released citrate as root exudate. The amount of the released citrate was about 23% of the total plant dry weight at harvest.

We found that the transgenic seedlings exposed to HAp, tended to increase the exudation of citric and malic acid, compared with the transgenics grown in Pi-starved (Pi-) and Pi-sufficient (Pi+) conditions (Fig. 3.7 and 3.8). The transgenics exudation of citric and malic acid in HAp was significantly higher than that of the wild-type. This result is

consistent with Zhang *et al.* (1997) who found that radish root exudation of organic acids increased between 15 times in succinic acid and 60 times in malic acid HAp. Trejo-Téllez *et al.* (2010) found that the transgenic plants that overexpress PPDK, increased the exudation of organic acid in acid growth conditions. Organic anions are commonly detected in the region of the rhizosphere, and their exudation from the plant root is associated with nutrient deficiency and inorganic ion stress (Ryan *et al.*, 2001).

The mechanism of organic acid mobilization of the insoluble P is represented by either, chelating of cations (Al^{3+}, Ca^{2+}, Mg^{2+}) surrounding the roots in the rhizosphere (Delhaize and Ryan, 1995), or acidifying the rhizosphere, (Geelhoed *et al.*, 1999; Hinsinger *et al.*, 2003). Organic acids can form stable chelate complex with Al^{3+} and other polyvalent cations like Ca^{2+} (Haynes and Mokolobate, 2001). In alkaline soils, P binds with Ca forming insoluble Ca-P complex. Citrate works as a chelating agent, it displaces P from the sparingly soluble Ca-P complex by forming Ca-citrate (Lopez-Bucio *et al.*, 2000). A role of citrate has been observed in Ca chelating from hydroxyapatite (HAp) particles of alkaline soil, citrate dissolves Ca-P efficiently by weakening the nanoparticle stability of HAp, through controlling the availability of free Ca; thereby, the nucleation rate (Martins *et al.*, 2008). Figure 4.2 shows the role of citrate as chelating agent

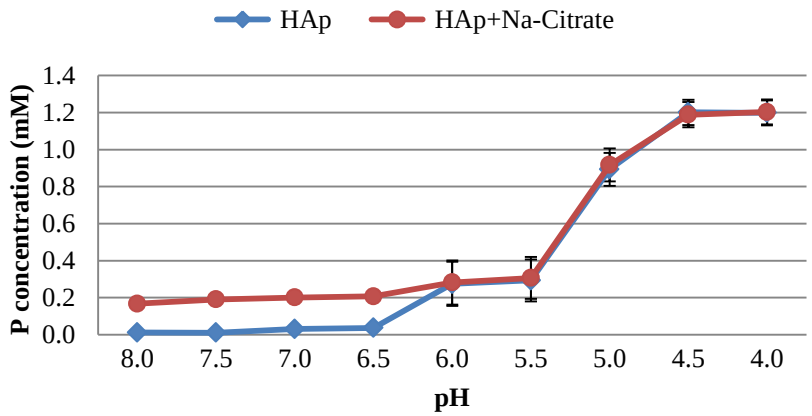


Figure 4.2: The role of citrate as chelating agent in P mobilizing from HAp. MS medium was prepared and divided in two parts. The first part was supplied with 600 mg /l HAp. The second part was supplied with 600 mg /l HAp and 1mM Na-Citrate. The medium pH was adjusted to pH 8.0 before adding HAp. 1N HCl was used to reduce the medium pH from pH 8.0 to pH 4.0. The samples were collected for each 0.5 pH unit and centrifuged 5 min, at 14000 rpm. The samples content of phosphorus was determined using the assay of (Bencini *et al.*, 1983).

Changing the rhizosphere pH could also be involved in P mobilizing process. We found a significant lowering of pH in growth solution of the transgenic seedlings in Pi- and HAp, compared with that of the wild-type (Fig. 3.3). The reduction of pH in the growth solution of both the transgenics and the wild-type was similar in Pi+, and it was significantly less from that in HAp and Pi-. These results are consistent with the results of (Dinkelaker *et al.*, 1989; Hoffland *et al.*, 1989). The significant lowering of pH in Pi- and HAp by the transgenics is correlated with their high exudation of citric and malic acid.

Through using the bromocresol purple (BCP), the transgenic seedlings showed high efficiency in acidifying the root zone from pH 8.0 "Violet color zone" up to pH 5.0 "yellow color zone" (Fig. 3.27). The ability of transgenic roots to induce change in rhizosphere pH was due to their exudation of significant amount of organic acid, when the seedlings were exposed to HAp (Neumann and Martinoia, 2002; Hinsinger *et al*., 2003; Diatloff *et al*., 2004). Therefore, the high exudation of organic acids of the transgenics could be the reason for the significant acidification of the rhizosphere under Pi-stress more than the wild-type.

The efflux of citric acid through the root membrane and rhizosphere acidification, is probably mediated by the activity of a unique membrane anion channels (Diatloff *et al.*, 2004). Plasma membrane H^+-pumping ATPase also is proposed to be involved in plant adaptation to Pi-deficiency (Yan *et al.*, 2002) as shown in Figure 4.3.

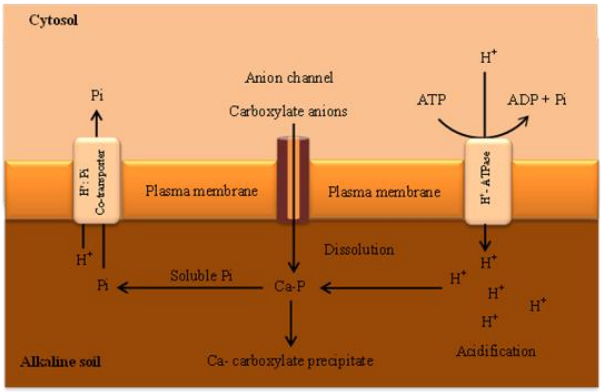


Figure 4.3: Model for root-induced chemical phosphate mobilization in the rhizosphere by exuding of carboxylates. **protons H+:** hydrogen ion (proton), **Pi:** inorganic phosphate, **ATP:** adenosine triphosphate, **ADP:** adenosine diphosphate, **Ca-P:** calcium phosphate complex (Neumann and Martinoia, 2002).

The increase of both, exudation of organic acid, and pH lowering of the transgenics in HAp, was correlated with the improved growth, compared with the wild-type (Fig. 3.4). The biomass (Fig. 3.5A and 3.5B) and total P (Fig. 3.6) of the transgenics increased significantly in HAp. Interestingly, the biomass and total P content of the transgenics in HAp were comparable to those in Pi+. In contrast, the biomass and the total P content of both the transgenic and wild-type were similar in Pi-, which are consistent with the results of (Wang *et al.*, 2010; Lopez-Arredondo and Herrera-Estrella, 2012; Lü *et al.*, 2012; Siane, 2012). Consequently, the increase of biomass and P content of transgenics is closely correlated with the increase of organic acid exudation in HAp treatment. Lopez-Bucio *et al.* (2000), found that tobacco overproducing citrate synthase, increased efflux of citrate, accumulated more of P and yielded more biomass of leaves and fruit, under Pi-limiting conditions, and required less P fertilizer to achieve optimal growth. Jones and Darrah (1994) found that citrate was capable of mobilizing P from soil that possessed large Ca-P fraction. This mobilization of P was both due to the complexation of the citrate anion, and the dissolution properties of the protons released from citric acid. This proves that the transgenic plants were able to provide adequate amount of P for plant uptake from HAp. In other words, the increase of production and exudation of organic acids, especially citrate was effective in P mobilizing from HAp through chelating and acidification processes. So, organic acids can be expected to be of little consequence in P mobilization from alkaline soils.

4.4.2. Influence of P-nutrition on Root Architecture Modifications and Plant Growth

Modification of root growth is an adaptive strategy, to provide the nutrients for growth under low nutrient available soil (Al-Ghazi *et al.*, 2003). Plant-induced modification in root architecture under Pi-stress was reported in many plant species like common bean (Ho *et al.*, 2005; Nord *et al.*, 2011), Arabidopsis (Bates and Lynch, 1996, 2000), and white lupin (Shen *et al.*, 2005). In this work, to investigate the response of transgenic seedlings to HAp, length of primary and secondary root, biomass of root and shoot, and root: shoot ratio, were measured after three growth periods (12, 24 and 36 days).

In general, absence of P or presence of HAp in the growth medium, made both the transgenic and wild-type seedlings produce longer primary roots than that in the presence of P, with the seedlings increase in age (Fig. 3.9), (Li *et al.*, 2012). Growing of the transgenic seedlings for 24 to 36 days in HAp and Pi-sufficient conditions stimulated them to produce significantly more secondary roots, both in number and length than the wild-type (Fig. 3.11B-C, 3.12B-C, 3.14B-C and 3.15B-C). However, Pi-starved conditions made the transgenic and wild-type seedlings grow similar for the same length of time (24 to 36 days), (Fig. 3.11B-C, 3.12B-C, 3.14A and 3.15A). Growing of both the transgenic and the wild-type for a short term (12 days) in Pi-starved and HAp treatments, did not stimulate them to a better produce of the secondary roots than that in Pi-sufficient treatment (Fig. 3.11A, 3.12A, 3.14A and 3.15A). Interestingly, the growth of the secondary roots of the transgenic seedlings in HAp treatment was higher than that in Pi-starved and Pi-sufficient treatments. This increase in growth of the secondary roots for the transgenics, both in number and length could be related to a better use of the sparingly soluble HAp as a source of P for growth by these plants (Shen *et al.*, 2011; Niu *et al.*, 2012).

Root hair length and density were highly controlled by P-availability in growth medium. Pi-starvation and HAp stimulated the seedlings to produce long and dense root hair, compared with the seedlings grown under Pi-sufficiency (Fig. 3.10). Furthermore, the distribution of root hair on roots among the tested lines was similar in both primary and secondary roots. This increase in root hair elongation and density is commonly observed in Pi-deficient Arabidopsis (Bates and Lynch, 1996, 2000; Ma *et al.*, 2001).

Interestingly, the transgenic and wild-type seedlings length average of the secondary root was less than that of the primary root, in all treatments and in all growth periods (12, 24 and 36 days). This result contradicts with what was mentioned in several studies, that low-P availability increases the length and number of the secondary roots of Arabidopsis, and reduces the primary root growth by reducing the cell elongation (Williamson *et al.*, 2001; Sanchez-Calderon *et al.*, 2005; Bai *et al.*, 2013). Ward *et al.* (2008), attributed this inhibition in Arabidopsis primary root elongation to iron (Fe) toxicity. He found that, the reduction of Fe concentration without any increase of P in Pi-deficient growth medium induces the primary root elongation.

The increase in the secondary root growth in HAp is associated with the marked increase in shoot growth and leaves sizes (Fig. 3.13, 3.14 and 3.15). In Pi-, the 24 and 36 days old seedling showed shortage in the secondary root growth and number, with severe reduction in the growth of shoots and leaves. Fredeen *et al.* (1989) found that low P treatment reduced significantly soybean shoots. The most reduction was in the leaf, which diminished around 85%. Johnson *et al.* (1996); Abdolzadeh *et al.* (2010), found that the plant Pi-nutrition has a visible influence in the growth of both root and shoot. These architectural modifications in the plant roots are a response to Pi-stress, enhancing the total surface area available for soil exploration and acquisition of Pi. The more proliferated root system enables the plant to exploit more soil Pi (Ramaekers *et al.*, 2010). The accumulated fresh and dry biomass of root (Fig. 3.16 and 3.17), and shoot (Fig. 3.18 and 3.19) in Pi- was reduced enormously in the transgenic and the wild-type seedlings in 24 and 36 days old seedlings. In contrast, a significant increase in the accumulated fresh and dry biomass in root and shoot of the transgenics and wild-type was found in Pi+ in the seedlings from the same age (Colomb *et al.*, 2000). No significant difference in the accumulated biomass was observed among the transgenic and wild-type seedlings in Pi- and Pi+. The increase in root:shoot ratio, is considered as a common response to Pi-deficiency. The relative increase of biomass allocation to root, results in an increase in the root:shoot ratio, and this enhances phosphate acquisition (Nielsen *et al.*, 2001; Agren and Franklin, 2003; Péret *et al.*, 2011). Therefore, P absence (Pi-) in growth medium controls shoot and root growth, through increasing the root growth over shoot growth (Fig. 3.20). In Pi+ medium the response of seedlings to the availability of P was to increase the shoot growth over the root growth (Fig. 3.20), (Li *et al.*, 2009). The root:shoot ratio of the transgenic and wild-type was similar in Pi- and Pi+. The transgenic seedlings grown in HAp, significantly produced less root:shoot growth compared with those of the wild-type, which is consistent with the results of (Chiangmai and Yodmingkhwan, 2011).

Phosphate availability in the growth medium clearly affected the internal content of P in seedlings. In both Pi+ and HAp seedlings content of total P per g dry biomass (Fig. 3.21) was comparable and markedly higher than that in Pi-. In addition, the transgenic seedlings accumulated a significant higher amount of total P per g dry biomass than the wild-type. The content of total P in seedlings (Fig. 3.22) was also very high in Pi+ and HAp compared with Pi- and significantly higher in the transgenic seedlings than that in those of the wild-type in all growth periods. These results are consistent with the results

of (Lopez-Bucio *et al.*, 2000) who found an increase in P content of the transgenics that showed an increase in citrate exudation. The transgenic seedlings high content of P and their significant increase in shoot and root biomass in HAp, indicate the ability of the transgenics to a better acquire of P from the sparingly soluble HAp treatment.

4.4.3. Influence of P-nutrition on Plant: Root Surface Acid Phosphatase Activity

Among the wide set of responses to cope with P-limiting, the plants increase their level of intracellular and extracellular acid phosphatase (APase), which helps to catalyze inorganic phosphate (Pi) hydrolysis from its organic form (Tian *et al.*, 2012; Żebrowska *et al.*, 2012). As part of our study about the influence of Pi-nutrition on the transgenics, we measured the activity of acid phosphatase of the root surface. Two experiments were set up to measure APase in the intact root segments removed from the primary roots. *P*-NPP was used in the first experiment to measure the root surface APase activity in different seedlings ages (12, 24 and 36 days) grown in Pi-, HAp and Pi+. The second experiment was performed to observe APase activity, through watching time duration of root staining and scanning the obtained color formed from the hydrolysis of BCIP by APase.

We found that Pi-starvation, leads to increase the activity of APase of both the transgenics and wild-type (Fig. 3.23 A). In contrast, HAp and Pi-sufficiency, lead to decrease the activity of APase of both the transgenics and wild-type (Fig. 3.23 B-C). However, the activity of APase under HAp was less than that in Pi-starved. No significant differences in APase activity were found among the transgenics and wild-type in all (P) treatments. This result is consistent with Goldstein *et al.* (1988) and Tomscha *et al.* (2004), who found that Pi-stress induced the excretion of acid phosphatase. The significant increase in APase activity observed in seedlings grown for 36 days in Pi+, could be related to the decrease of P content in growth medium. Where we observed that the seedlings grown in liquid medium for 3 weeks, used about 75% of the medium content of P. Whereas, the significant decrease of APase activity for seedlings grown for 24 days in HAp, could be correlated with the increase of organic acid efflux from the roots to the growth medium. Consequently, increase of Pi content in the medium. Goldstein *et al.*,

(1988) found that the level of APase excretion from the root surface is inversely proportional to the level of exogenous orthophosphate (Pi) in the growth medium.

To visualize the APase activity in the transgenic seedlings in response to Pi-nutrition, BCIP-staining technique was used (Bozzo *et al.,* 2006). We found that the blue-color on the root surface of Pi-starved seedlings was apparent in 4h after incubation with BCIP-agar overlay (Fig. 3.24). In contrast, the time taken by the Pi-sufficient seedlings to exhibit the blue-color was 48h (Fig. 3.26). A distinct difference in root surface staining was visualized between the transgenics and wild-type, under HAp after 12 h of incubation (Fig. 3.25). This suggests that root surface APase activity is controlled by the level of available phosphate (Pi) in the growth medium (Goldstein *et al.,* 1988; Del Pozo *et al.,* 1999), The faint staining of the transgenic roots, compared with the dark-staining of the wild-type roots, is due to their low excretion of APase under HAp. This low excretion of APase is a consequence of the ability of these seedlings to exude significantly higher amount of citrate and malate, which play a major role in increasing the growth medium content of available P.

4.5. Growth and Yield of Transgenic Plant in Alkaline Soil

Alkaline soil is characterized as a low-P soil. Therefore, many plant species are unable to grow in such soil (Shenoy and Kalagudi, 2005; Ström *et al.*, 2005). In order to study the transgenic plants growth and productivity in alkaline soil, three combinations of soil / calcium carbonate were used. The combinations are 0.0g kg^{-1}, 256g kg^{-1} and 512 g kg^{-1} $CaCO_3$. The plants were grown under greenhouse conditions and harvested after three months.

Transgenic plants showed better growth than wild-type with the increase of $CaCO_3$ concentration in the soil. They were higher (Fig. 3.30, 3.31 and 3.32), accumulated more biomass (Fig. 3.28 and 3.29) and their content of total P (Fig. 3.33 and 3.34) was significantly higher than that of the wild-type in 512 g kg^{-1} $CaCO_3$ treatment. The wild-type growth was severely affected in 512 g kg^{-1} $CaCO_3$ treatment. This growth was directly correlated with the ability of the transgenic plants to acidify the soil significantly better than the wild-type (Fig. 3.35). Furthermore, the transgenic plants flowered and fruited successfully, when they were grown in high $CaCO_3$ soil content up to 512 g kg^{-1}, in

contrast the wild-type failed to do so. López-Bucio *et al.* (2000) found that, tobacco overexpress citrate synthase was able to grow better and increased seed yield under alkaline growth conditions. A reduction in the transgenic plant yield of capsules (Fig. 3.36, 3.37) was observed in 256 and 512 g kg^{-1} $CaCO_3$ compared with control treatment (0.0 g kg^{-1}). However, it was less than that of the wild-type. The seed capsule (Fig. 3.38) produced by transgenic plants under alkaline stress were comparable to control treatment, and significantly higher than those of the wild-type. The total seed weight per plant of the transgenic lines showed reduction in 256 and 512 g kg^{-1} $CaCO_3$ treatments. However, this reduction was less than that of the wild-type at the same conditions of soil alkalinity (Fig. 3. 39). This suggests that, the ability of the transgenic plants (PPDK overexpression lines) to grow and yield under alkaline soil is significantly higher than the wild-type.

4.6. Influence of P-nutrition on Transgenic Plant Metabolism

Phosphorus (P) has multiple effects on plant metabolism. Its deficiency is a major limitation for plant growth and yield (Warren, 2011). Plants can respond defensively to this stress, modifying their metabolic pathways for adaptation to low-P conditions (Li *et al.*, 2007; Huang *et al.*, 2008). In order to determine metabolic response of the transgenic and wild-type seedlings to Pi-nutrition, plant samples from liquid and solid alkaline MS medium, supplied with different P supplements were analyzed using GC-MS.

Some of the detected metabolites increased in Pi-starved treatment, others decreased, and some others did not change. We found an increase in the content of organic acids, especially (citric and malic) of whole seedlings and shoot samples in Pi-starved treatment (Fig. 3.40 and 3.41), compared with that in HAp and Pi-sufficient treatments. Many studies reported a decrease in both root and shoot content of organic acids under P-stressed conditions, which is inconsistent with our results (Achituv and Bar-Akiva, 1978; Hernandez *et al.*, 2007). Hoverer, this increase in the whole seedling and shoot samples content of organic acid may be due to the plant strategy of improving the plant ability of adaptation to low P conditions, that proposes involvement in altering the partitioning of carbohydrates into organic acids and amino acids to enable more efficient utilise of C in P-stressed plants (Huang *et al.*, 2008). In contrast, a decrease in the content of organic acids of root samples in Pi-starved and HAp was found, (Fig. 3.42) compared with Pi-sufficient root samples. This decrease of organic acid in roots is attributed to the

increase of root exudation of organic acid to root space under Pi-stressed condition, to enhance the soil availability of P (Neumann and Römheld, 1999; Pearse *et al.*, 2007; Lin *et al.*, 2011; Oburger *et al.*, 2011; Siane, 2012).

All the analyzed samples of Pi-sufficient treatment showed an increase in phosphoric acid level. In contrast, they showed sharp reduction of phosphoric acid level in Pi-starved treatment (Fig. 3.43). This results is matched with the results obtained in this study through measuring the plant sample content of P using spectrometer. Also it is consistent with the results of Huang *et al.* (2008), who found an increase in the content of phosphoric acid for the plants grown in the presence of P, and found a reduction in the content of phosphoric acids for the plants grown under Pi-deficient conditions. Interestingly, the whole seedlings and roots samples of transgenics in HAp treatment showed an increase in their phosphoric acid level, compared with those of the wild-type. In general, Pi-starved and HAp samples showed an increase in their amino acids levels, compared with those of Pi-sufficient samples (Fig. 3.44). The increased levels of amino acids in shoot and root of Pi-starved and HAp plants are likely to result from the increase of protein degradation and repression of protein synthesis (Wu, 2003; Hernandez *et al.*, 2007; Huang *et al.*, 2008).

Sugars work as substrates for energy metabolism, and biosynthesis of complex carbohydrates, providing sink tissues with a necessary source for growth. Sugars also work as secondary messengers, with the ability of regulating the plant growth in response to biotic and abiotic stresses. Pi-stress leads to an increase of sugars in both shoots and roots (Fig. 3.45). These results are consistent with (Hernandez *et al.*, 2007; Nilsson *et al.*, 2007), who reported that Pi-stress led to an increase in the level of sugars in common beans and Arabidopsis. This increase suggests that glycolysis might be hindered in both shoots and roots of Pi-stressed plants. In contrast, stimulation in glycolysis was reported in lupin and rice under Pi-stress condition (Uhde-Stone *et al.*, 2003; Wasaki *et al.*, 2006). Glycolysis is necessary to maintain high level of organic acid secretion in proteoid roots of lupin (Pearse *et al.*, 2006). Also, the increase of sugars in sink tissues (root) under Pi-starvation conditions, plays a role in relocation of carbon source to the roots, which increases their relative biomass to shoots (Hammond and White, 2008).

4.7. Conclusions

From above mentioned, it could be suggested that, the increase exudation of citrate and malate of the transgenic seedlings, played a central role in alleviation of P stress in growth medium, through remobilization of HAp and releasing of Pi. In addition, it played an appreciable role in lowering of pH in alkaline soil, consequently, increasing the availability of P for plant uptake. This leads to a better growth and yield for the transgenics in alkaline conditions. So, we can conclude that overexpression of PPDK in tobacco plants, significantly enhances the ability of plants in using the insoluble P compounds from lime and alkaline soil, and facilitates its uptake through increasing exudation of organic acids. P-stress, induces modification in the root architecture like increasing the primary and secondary root growth, also it increases the formation of root hairs, root:shoot ratio and induces change in the level of the plant metabolites. Direct effect for the overexpression of PPDK was found in the transgenic lines content of metabolites in different P-nutrition. Acid phosphatase excretion to the root surface is inversely proportional with the level of the growth medium content of Pi.

4.8. Area of Further Studies

Though, a considerable progress has been achieved in understanding the role of PPDK in replenishing the TCA cycle intermediates, and its role in the mechanisms of Al-tolerance and enhancing uptake of P from the lime soil, based on organic acid efflux. Much is still to be studied on the molecular mechanisms underlying the activation of anion-channels by HAp and /or Al toxicity. So, we need a better understanding of the processes involved in how the plant cells sense the deficiency of P and Al stress, that leads to channel gating and organic acid efflux. In addition, the genes encoding these anions-channels need to be cloned. Also, studying the effect of PPDK overexpression on the tolerance of tobacco plants to Al toxicity in alkaline conditions, and the effect of PPDK gene knockout on the cell metabolism of organic acids in tobacco plant under metal and Pi-deficiency.

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6. Zusammenfassung

Phosphor (P) ist ein wichtiger Nährstoff für Pflanzen und essentiell für Wachstum und Reproduktion. Er ist Bestandteil von verschiedenen Molekülen wie DNA, RNA, Phospholipiden und ATP. Phosphormangel führt zu Problemen in der landwirtschaftlichen Produktion. Im Vergleich zu anderen Nährstoffen ist die P-Konzentration im Boden niedrig. In alkalischen Böden bildet P Verbindungen, die nicht pflanzenverfügbar sind, weshalb jährlich in der Landwirtschaft Millionen Tonnen Phosphor-Dünger ausgebracht werden. Bis zu 80% des P geht verloren, da er nicht mehr für die Aufnahme durch Pflanzen verfügbar ist. Daher ist die Entwicklung von Pflanzensorten mit effizienterer P-Aufnahme die beste Alternative um den Einsatz von Phosphor-Düngemitteln zu reduzieren und eine nachhaltigere Landwirtschaft zu gewehrleisten.

Pyruvat, Orthophosphat Dikinase (PPDK) ist ein Enzym, das die Umwandlung von ATP, Pi und Pyruvat zu AMP, PPi und Phosphoenolpyruvat (PEP) katalysiert. Einige Studien zeigten auf, dass es einen Anstieg in der Aktivität von PPDK in Pflanzen unter abiotischen Stressbedingungen wie Abwesenheit von P. gibt. In anderen wurde berichtetet, dass die Überexpression von PPDK zu einer erhöhten Exsudation organischer Säuren und somit zu gesteigerter Toleranz der Pflanzen gegen Al-Stress führt. Organische Säuren spielen eine wichtige Rolle bei der Mobilisierung des unlöslichen P und somit dessen Zugänglichkeit für Pflanzen.

Es wurden transgene Tabakpflanzen untersucht, die plastidäre (PPDK) oder cytosolische (ΔPPDK) M. crystallinum PPDK unter der Kontrolle von B33- und CaMV-35S-Promotoren exprimieren. Um die Fähigkeit der effizienten Nutzung von schwerlöslichen P durch PPDK-überexprimierende Pflanzen zu untersuchen, erfolgte die Anzucht in alkalischen MS-Medien (pH 8,0) ohne P (Pi-), mit P (Pi+) und mit HAp sowie in alkalischen Böden mit unterschiedlichen Mengen an $CaCO_3$ (0,0 g kg^{-1}, 256 g kg^{-1} und 512 g kg^{-1}). Als lösliche P-Quelle wurde dem Wachstumsmedium 1 mM KH_2PO_4 hinzugefügt und als unlösliche P-Quelle das schwer lösliche Hydroxylapatit (HAp) entsprechend 1 mM P.

Es wurden mehrere physiologische Parameter gemessen. Die transgenen Pflanzen und der Wildtyp haben sich sowohl in Anwesenheit (Pi+) als auch in Abwesenheit (Pi-) von löslichem P gleich verhalten. Interessanterweise fanden wir deutliche Unterschiede in

den meisten der gesammelten Daten zur HAp-Behandlung beim Vergleich der transgenen Pflanzen und des Wildtyps. In HAp war das Wachstum der transgenen Pflanzen besser als das des Wildtyps. Die transgenen Pflanzen hatten eine erhöhte Exsudation von organischen Säuren, akkumulierten mehr Biomasse und ihr Gehalt an P war signifikant höher als im Wildtyp. Die transgenen Keimlinge, die in festem MS-Medium mit HAp gewachsen sind, hatten ein deutlich geringeres Wurzel-Sprössling-Verhältnis als die Keimlinge des Wildtyps. Die Aktivität der sauren Phosphatase der Wurzeloberfläche war bei den transgenen und bei den Wildtyp-Keimlingen bei allen P-Behandlungen während aller Wachstumsphasen gleich. Transgene Sprösslinge haben in Gegenwart von HAp das Wachstumsmedium besser angesäuert als die des Wildtyps. Darüber hinaus war die Anzahl und Länge der sekundären Wurzeln bei den transgenen Keimlingen in HAp höher als beim Wildtyp. Überraschenderweise war der P-Gehalt in den transgenen Keimlingen in HAp und Pi+ ähnlich und deutlich höher als beim Wildtyp.

Das Wachstum von transgenen Pflanzen in alkalischen Böden wurde weiter untersucht. Sie haben den pH-Wert des Bodens stark verringert, wuchsen deutlich besser und haben Blüten gebildet. Im Gegensatz dazu zeigte der Wildtyp, der in dem gleichen Boden wuchs, nur eine geringe Versauerung und das Wachstum war stark eingeschränkt. Darüber hinaus gab es keine Blütenbildung. Proben der Jungpflanzen und Sprösslinge sowohl von den transgenen Pflanzen als auch vom Wildtyp zeigten eine Zunahme in ihrem Gehalt an Zitronen-und Äpfelsäure in Pi-im Vergleich zu denen in HAp und Pi +. Interessanterweise zeigten Wurzelproben von transgenen Pflanzen und vom Wildtyp einen Rückgang von Zitronen-und Äpfelsäure in Pi-und HAp im Vergleich zu Pi +. Allgemein war der Aminosäuregehalt aller Proben auf Pi- hoch im Vergleich zu HAp und Pi+. Die Jungpflanzen-, Wurzel- und Sprossproben sowohl der transgenen Pflanzen als auch des Wildtyps zeigten unterschiedliche Zuckerzusammensetzungen unter P-Behandlungen.

PPDK spielt eine Rolle in der anaplerotischen Aufstockung der Intermediate des Citratzyklus. Die Überexpression von PPDK führt zu einer Verbesserung des Stoffwechsels organischer Säuren. Das bessere Wachstum transgener Pflanzen, die PPDK überexprimieren hängt mit ihrer Eigenschaft zusammen, große Mengen organischer Säuren in den Boden abgeben zu können, was die Verfügbarkeit von P für die Pflanze erhöht.

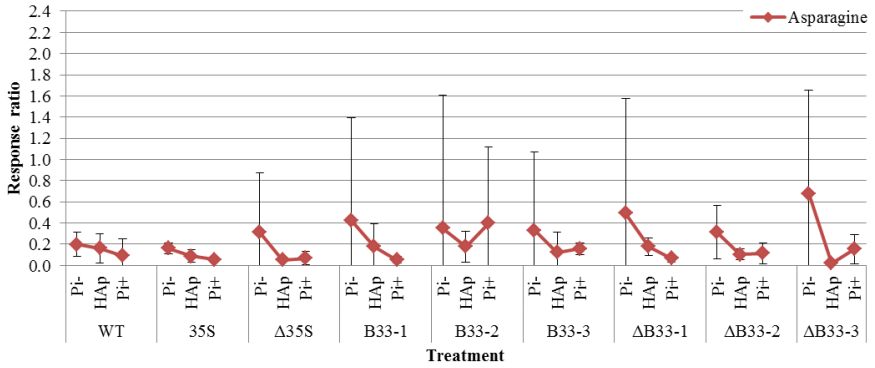
7. Appendix

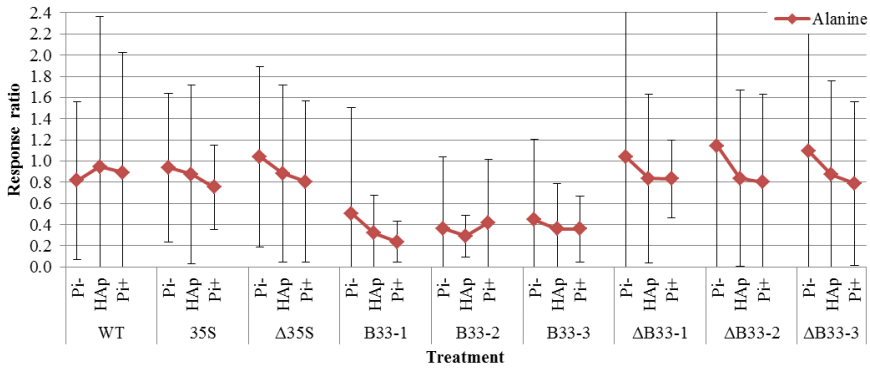
7.1. List of the Identified Metabolites

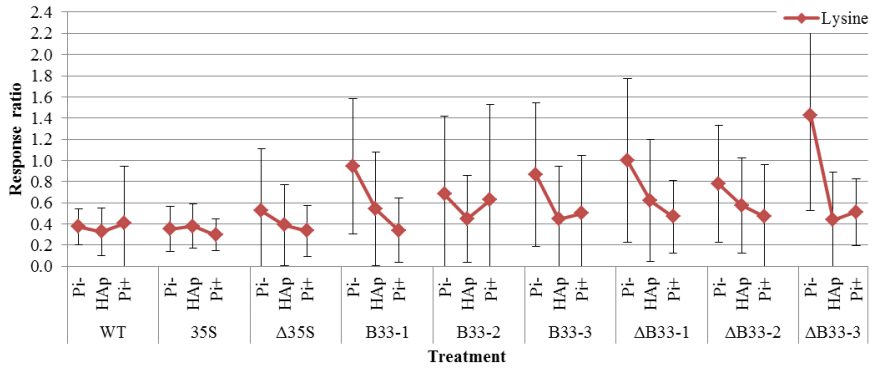
The identified metabolites in whole seedling, shoot and root samples of seedlings grown in alkaline MS medium supplied with different P treatments analyzed via GC-MS.

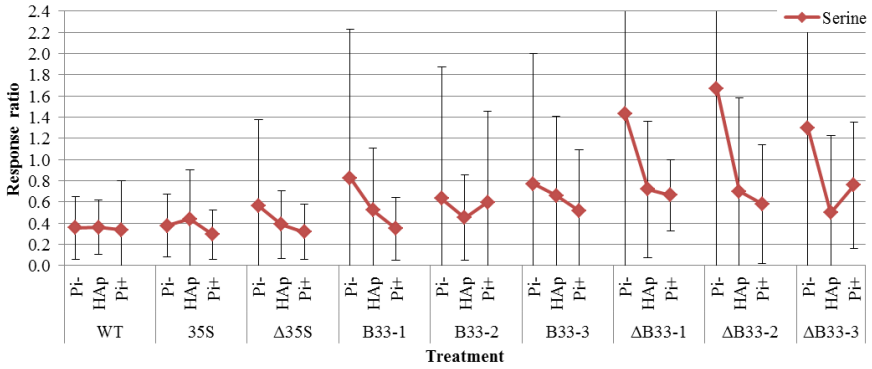
7.1.1. The Identified Metabolites of Whole Seedling

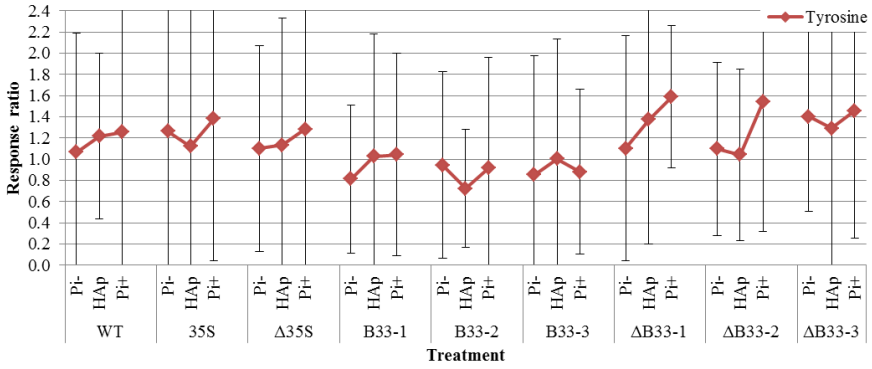
7.1.1.1. Amino Acid

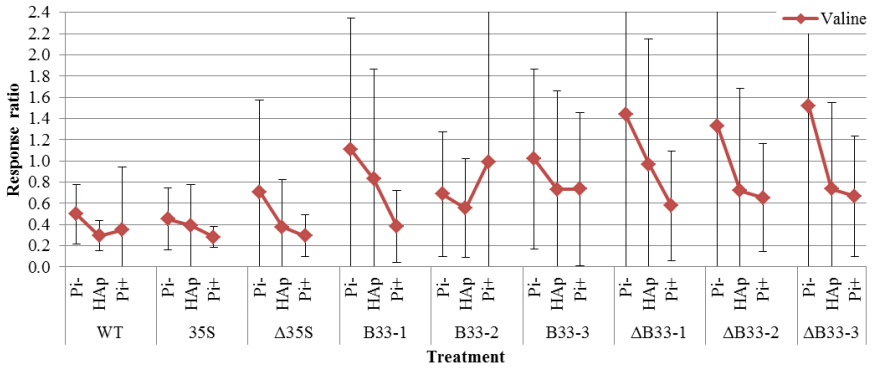


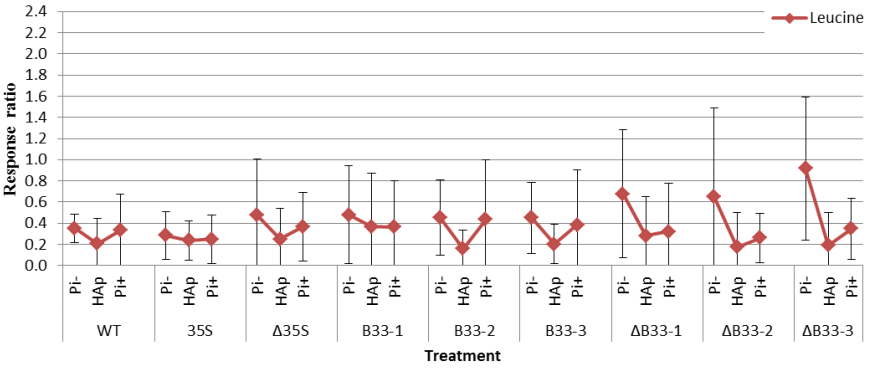





Serine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Tyrosine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Valine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment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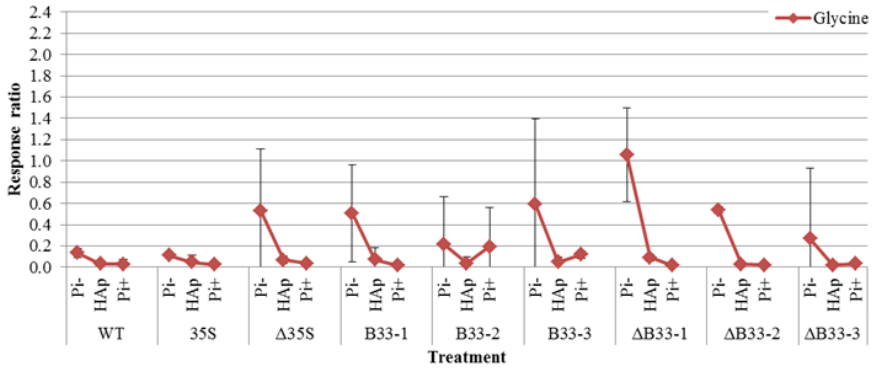
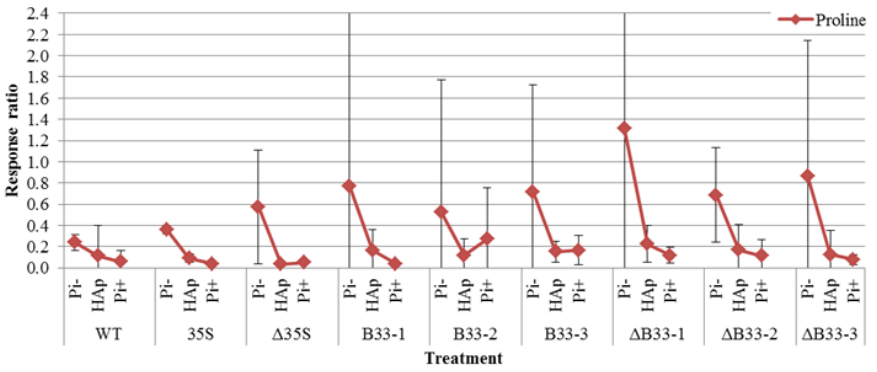
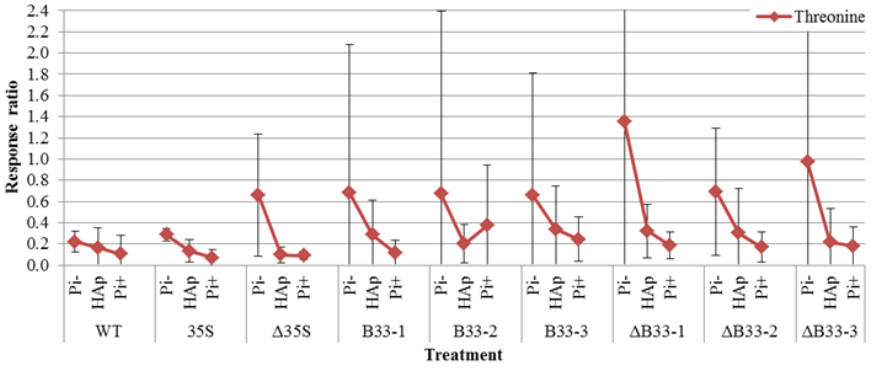
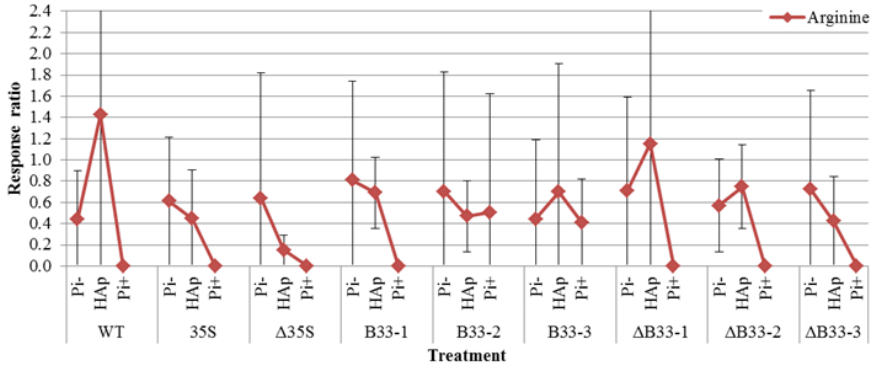
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Glycine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Proline
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Threonine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment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Response ratio
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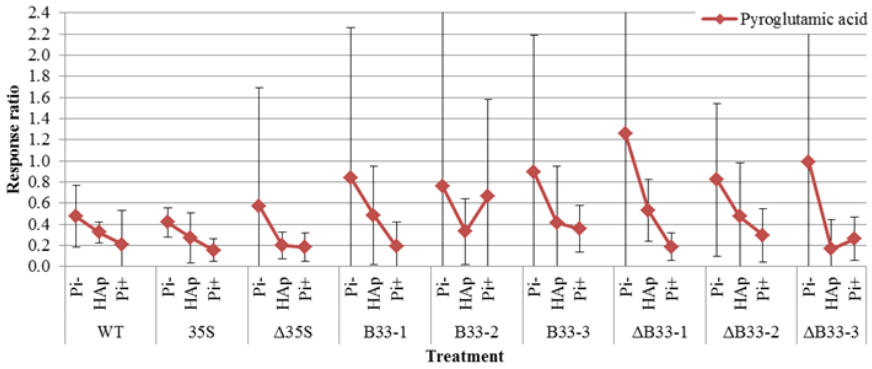
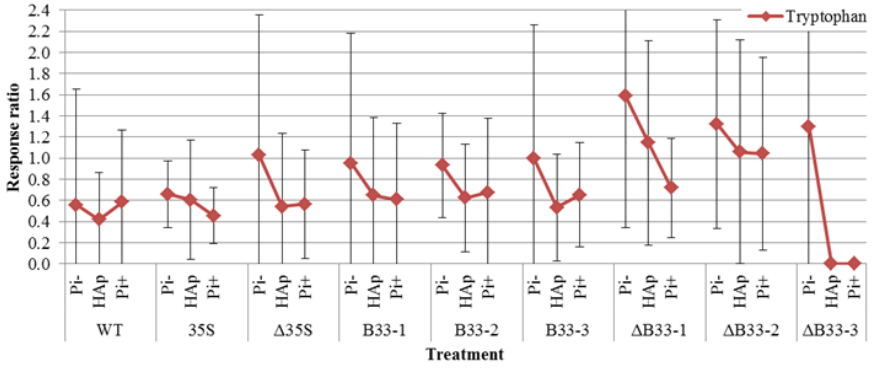
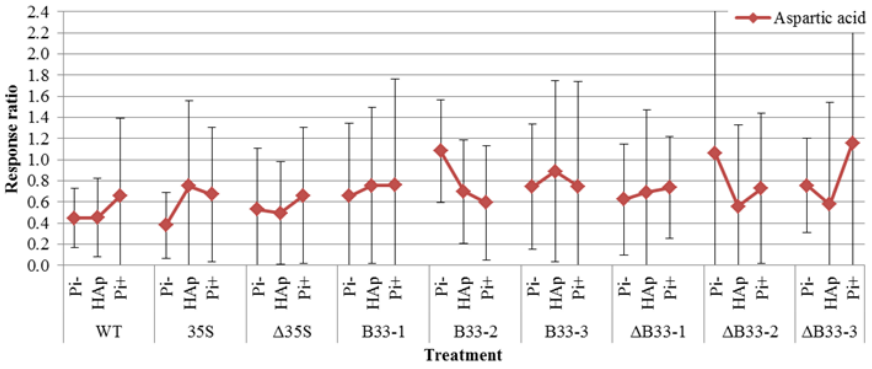
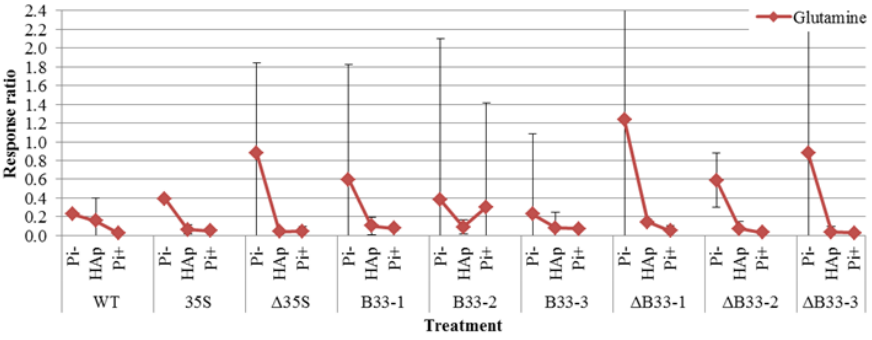
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

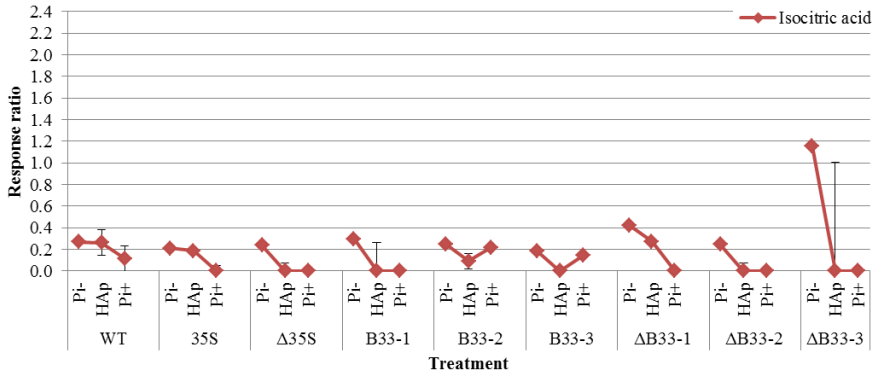

Pyroglutamic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

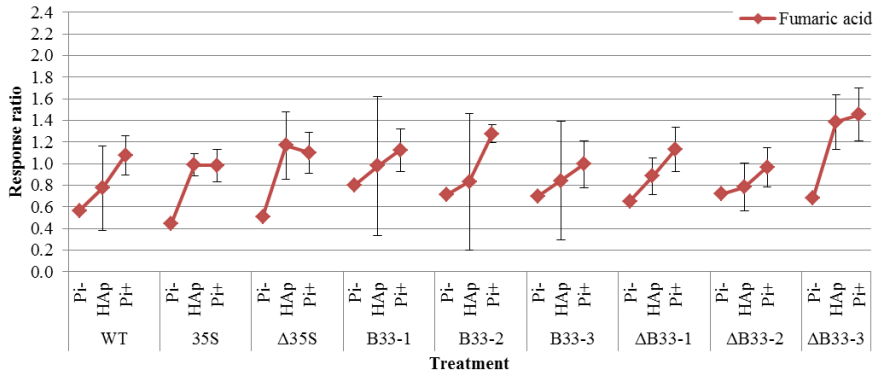

Tryptophan
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

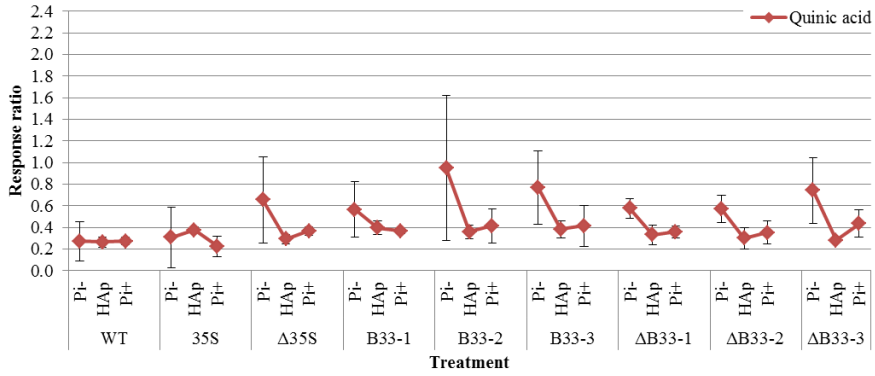

Aspartic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

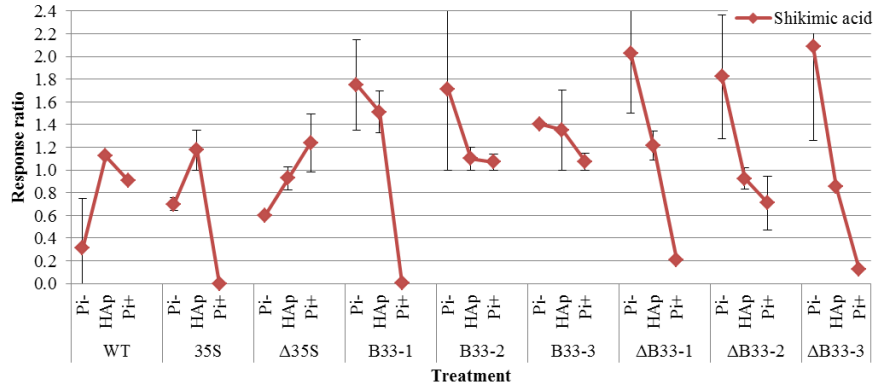

Glutamine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

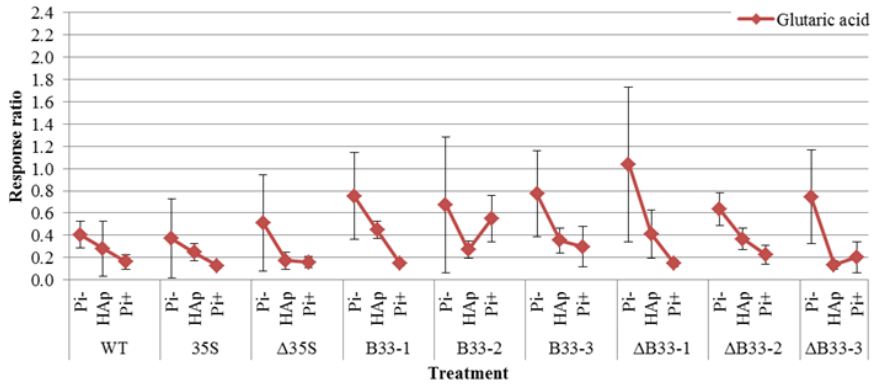
7.1.1.2. Organic Acid

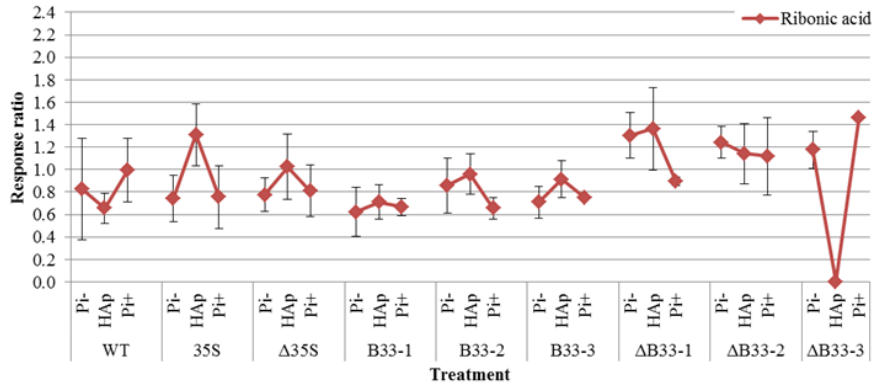


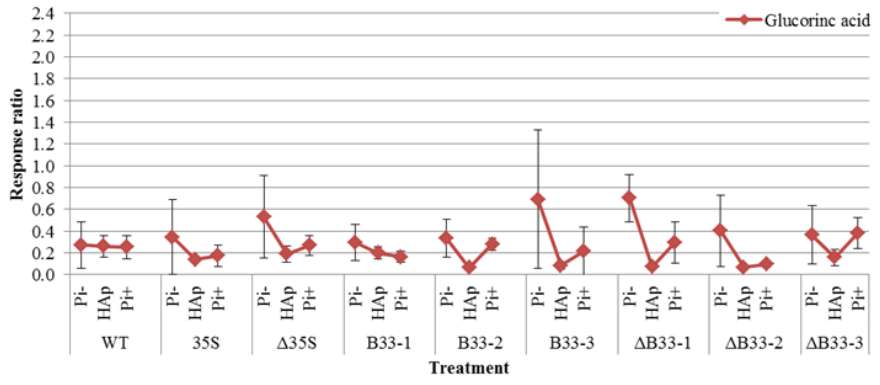


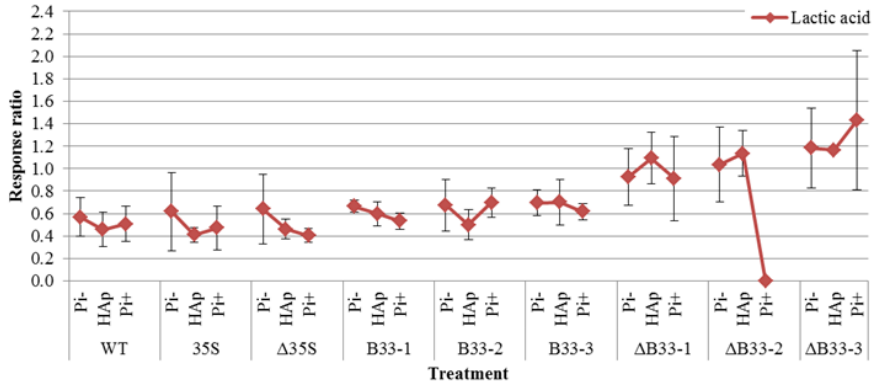




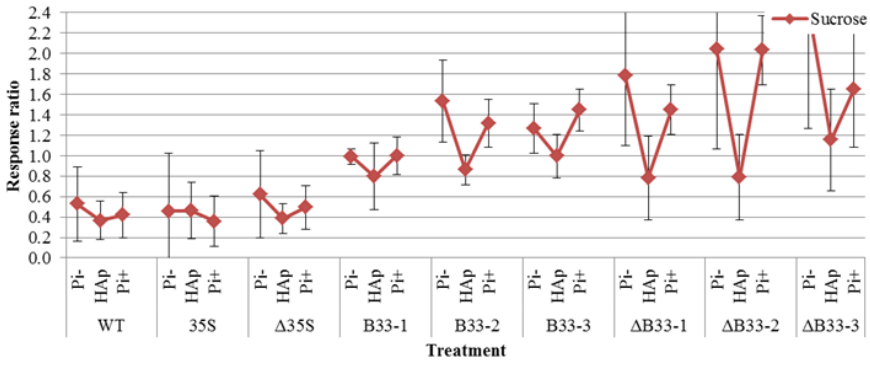

Glutaric acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

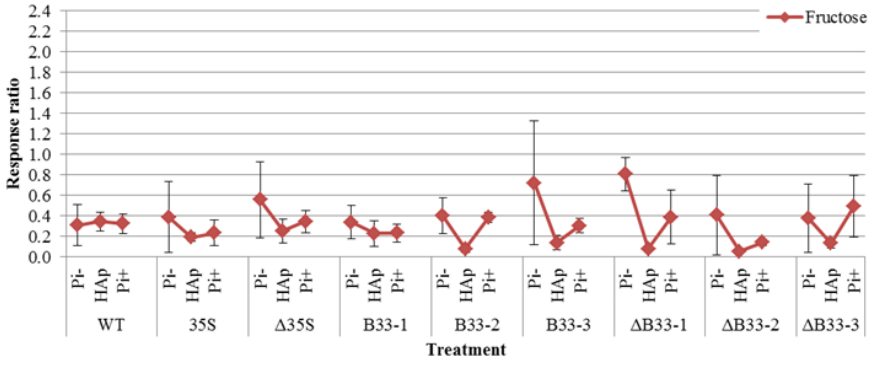

Ribonic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

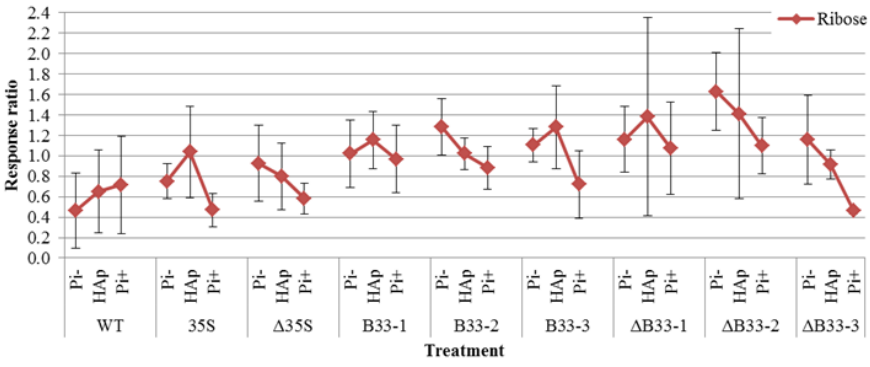

Glucorinc acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

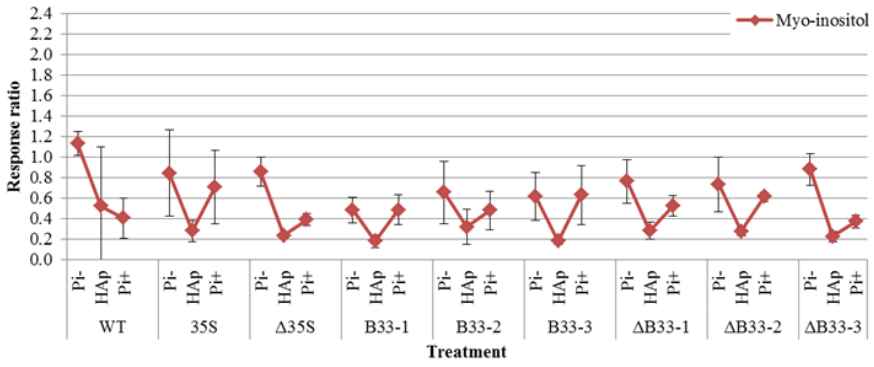

Lactic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

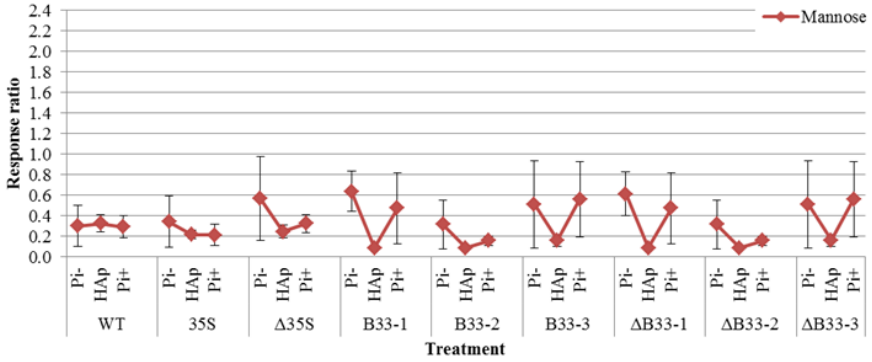
7.1.1.3. Sugars

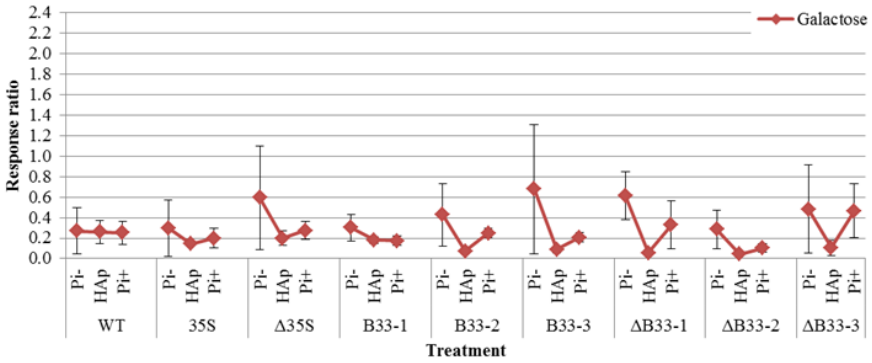

Sucrose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

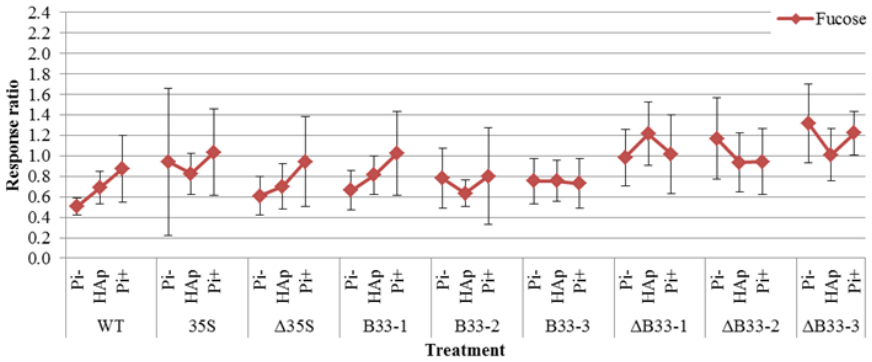

Fructose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

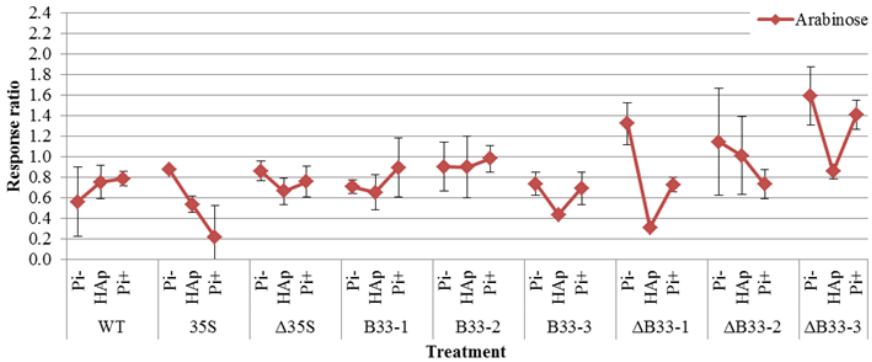

Ribose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Myo-inositol
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

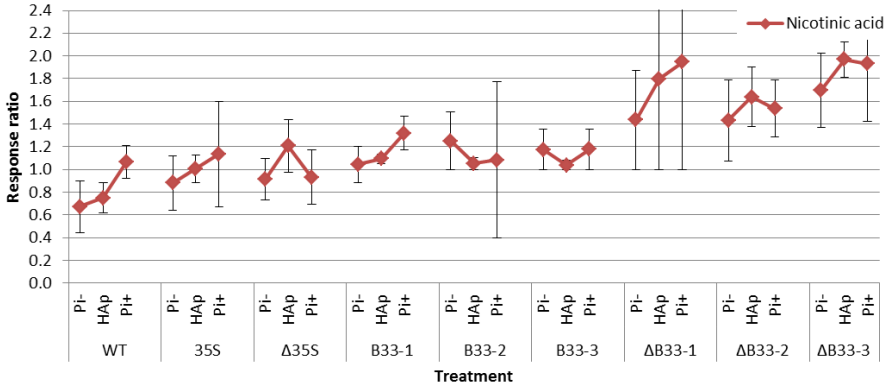

Mannose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Galactose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Fucose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

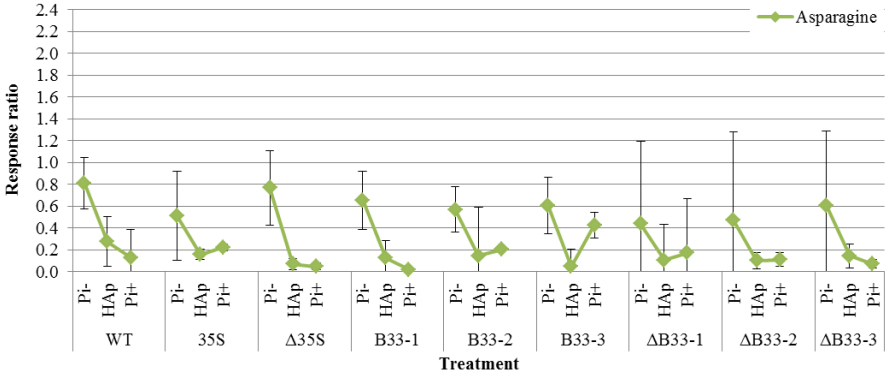

Arabinose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

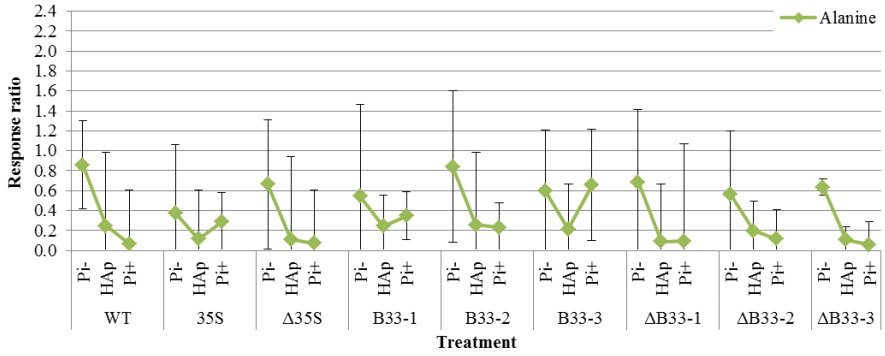
7.1.1.4. Nicotinic Acid

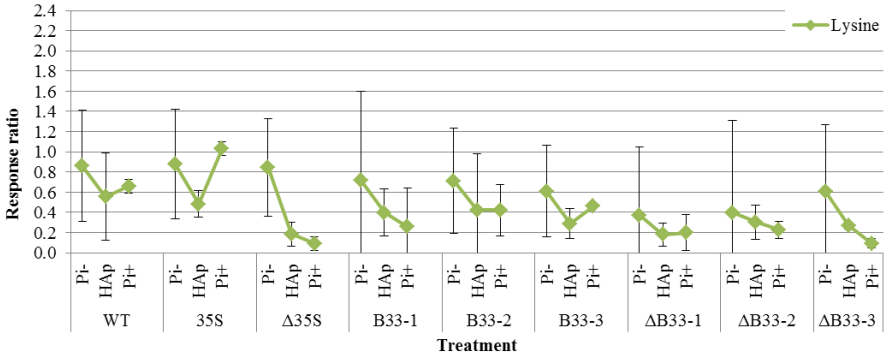


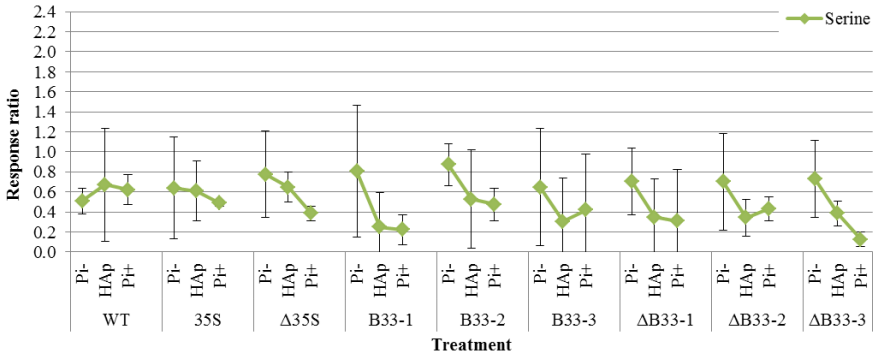
7.1.2. The Identified Metabolites of Shoot

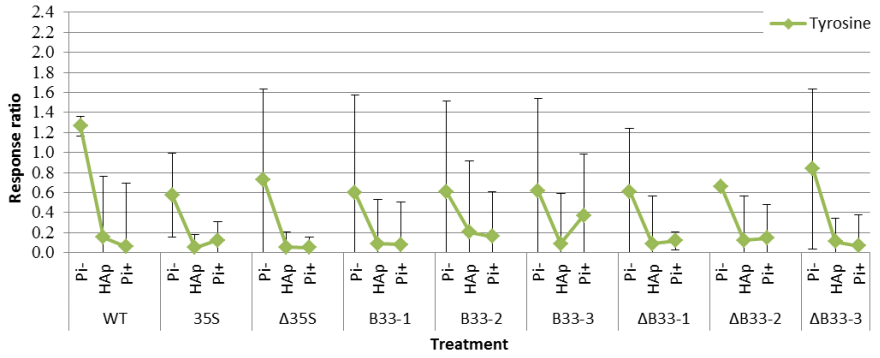
7.1.2.1. Amino Acid

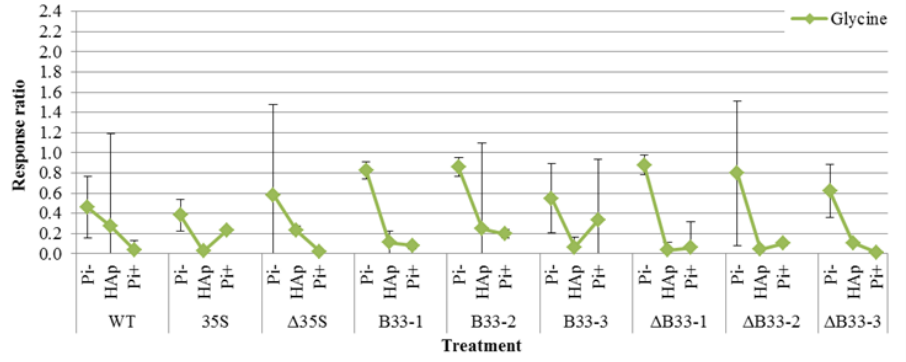


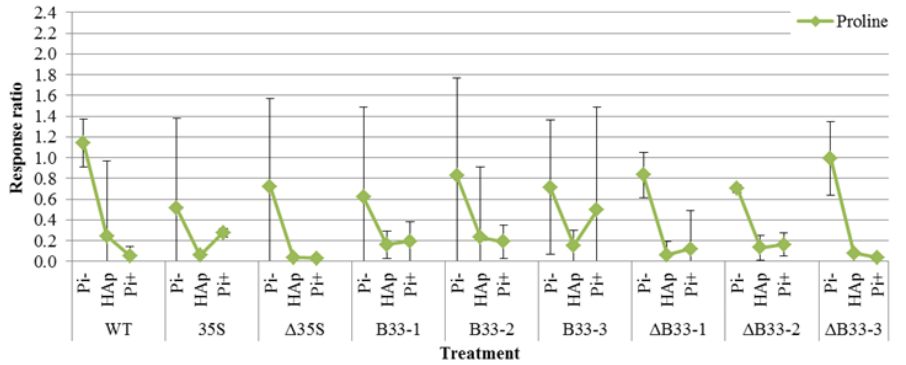

Alanine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

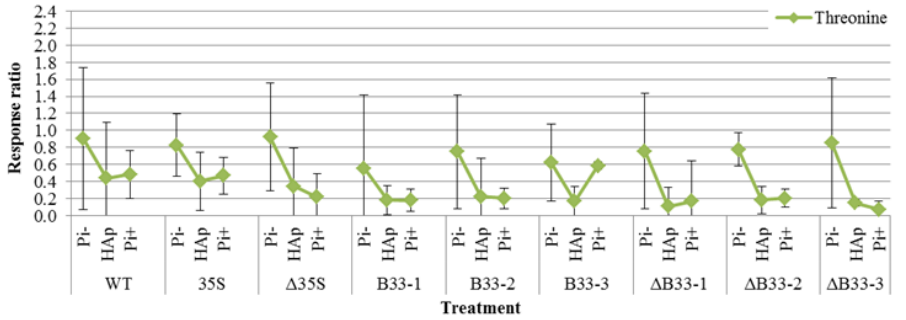

Lysine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

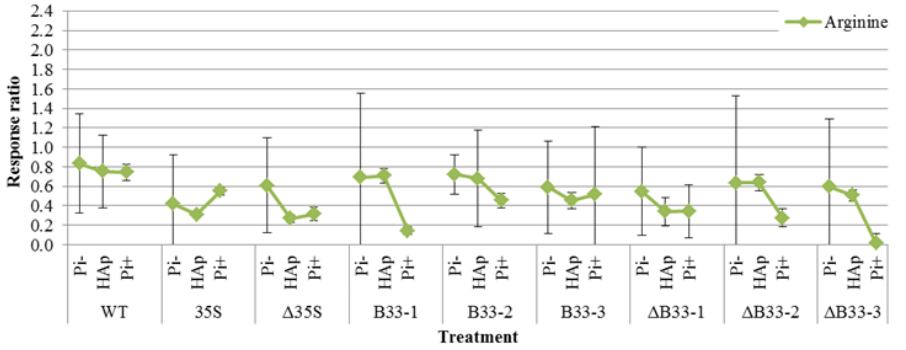

Serine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

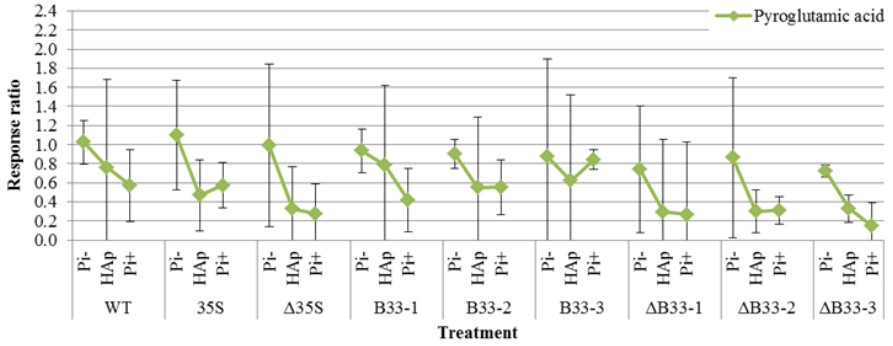

Tyrosine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

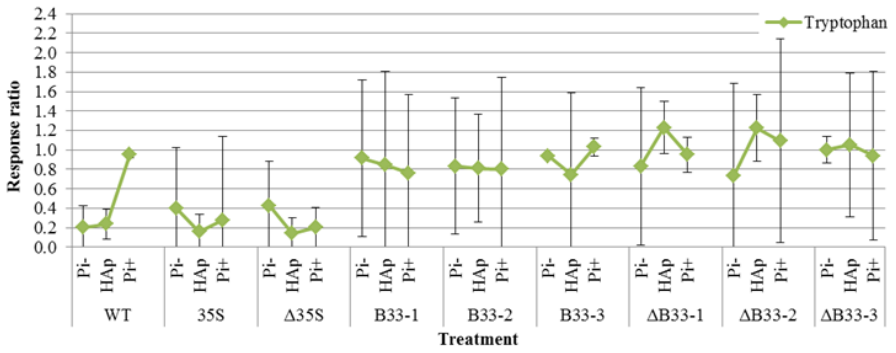

Glycine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

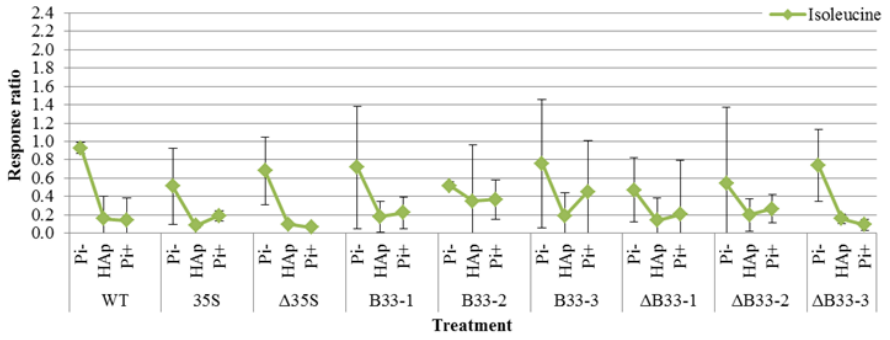

Proline
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

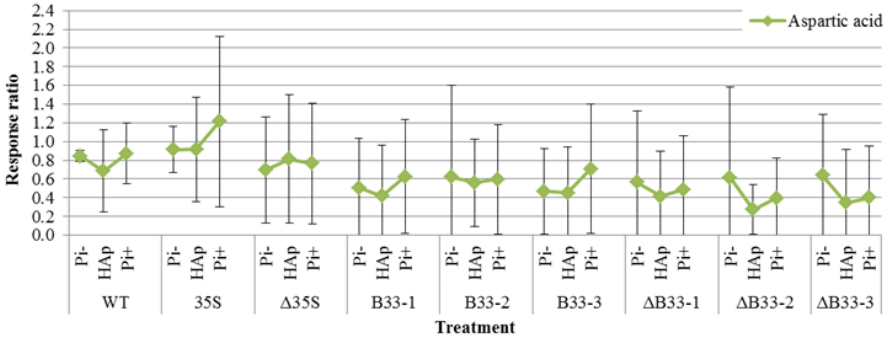

Threonine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

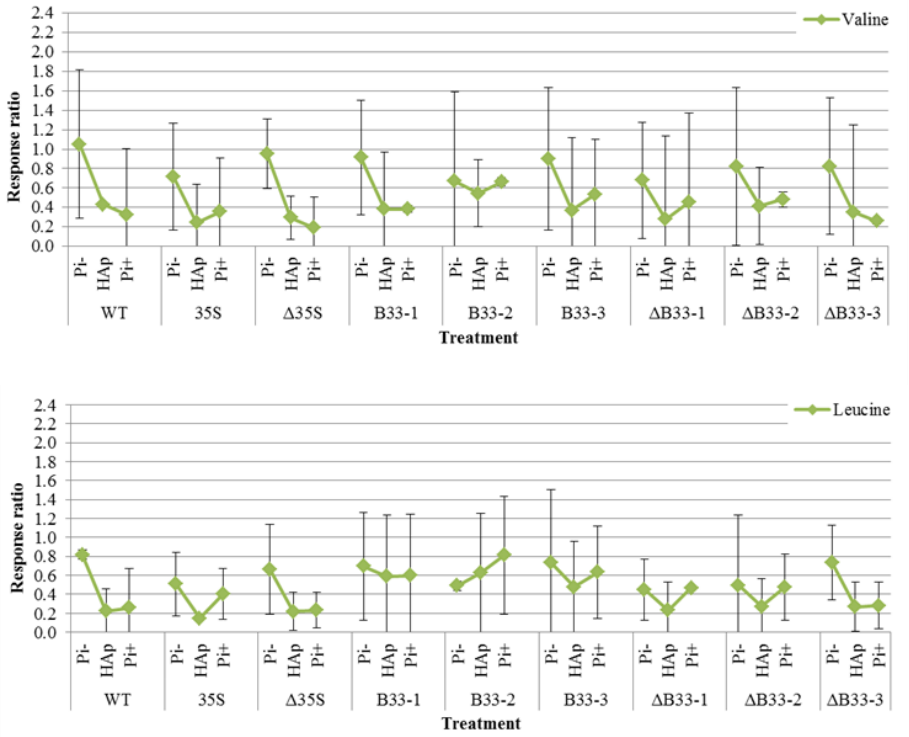

Arginine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Pyroglutamic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

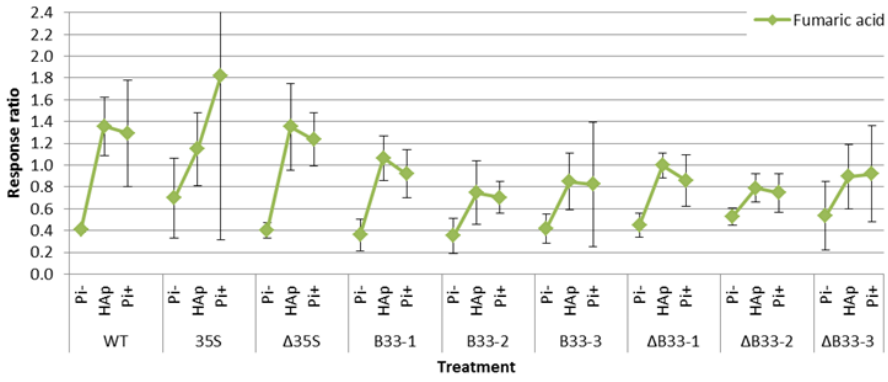

Tryptophan
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

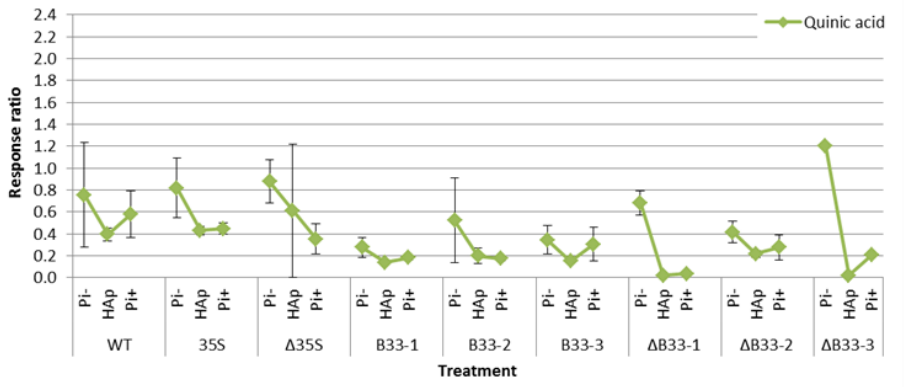

Isoleucine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

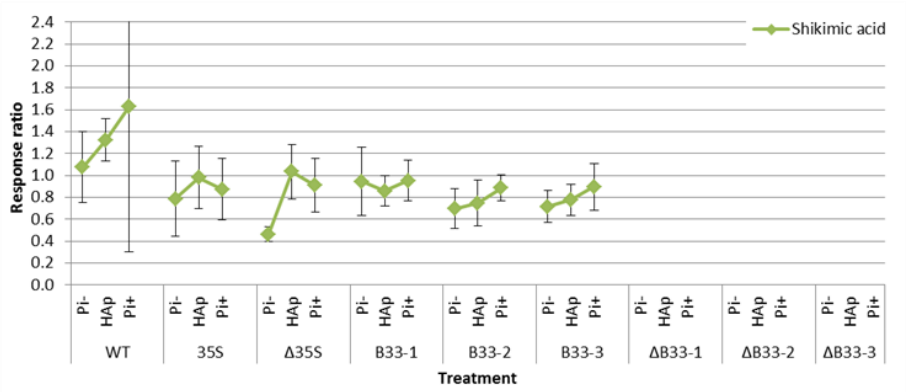

Aspartic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

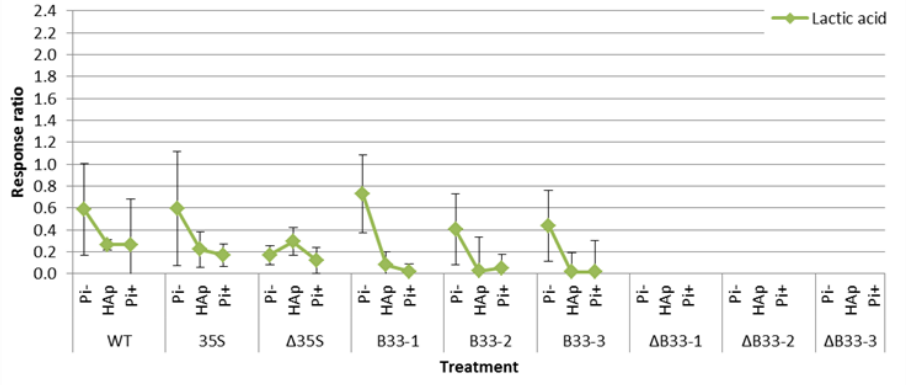


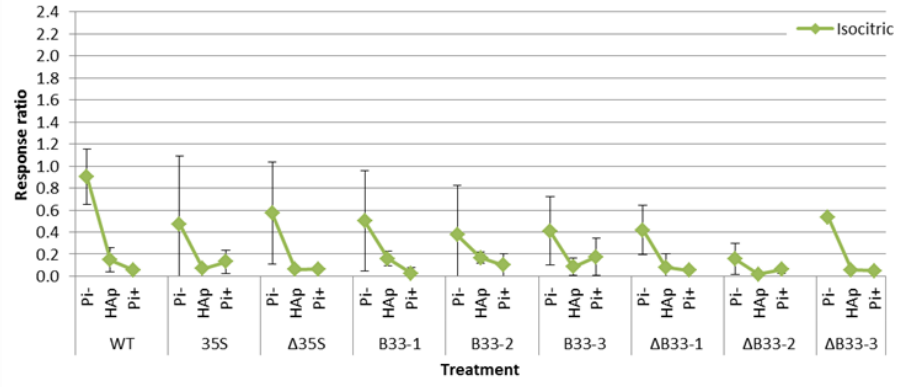
7.1.2.2. Organic Acid

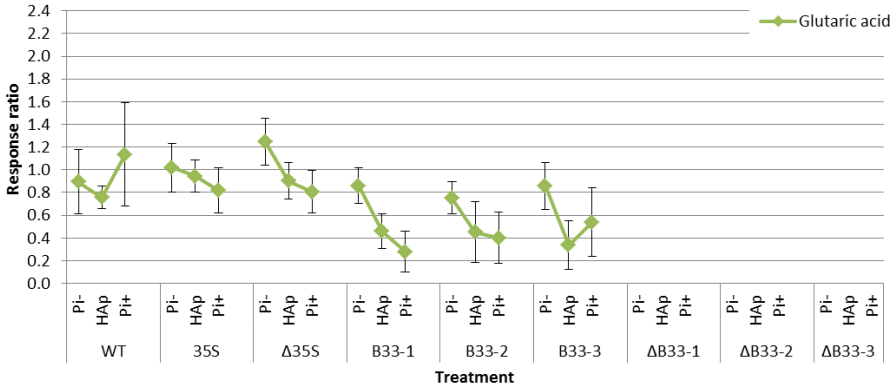


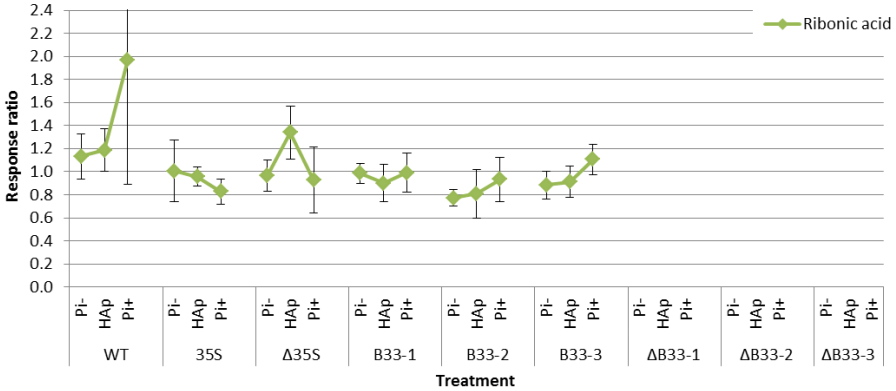

Quinic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Shikimic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

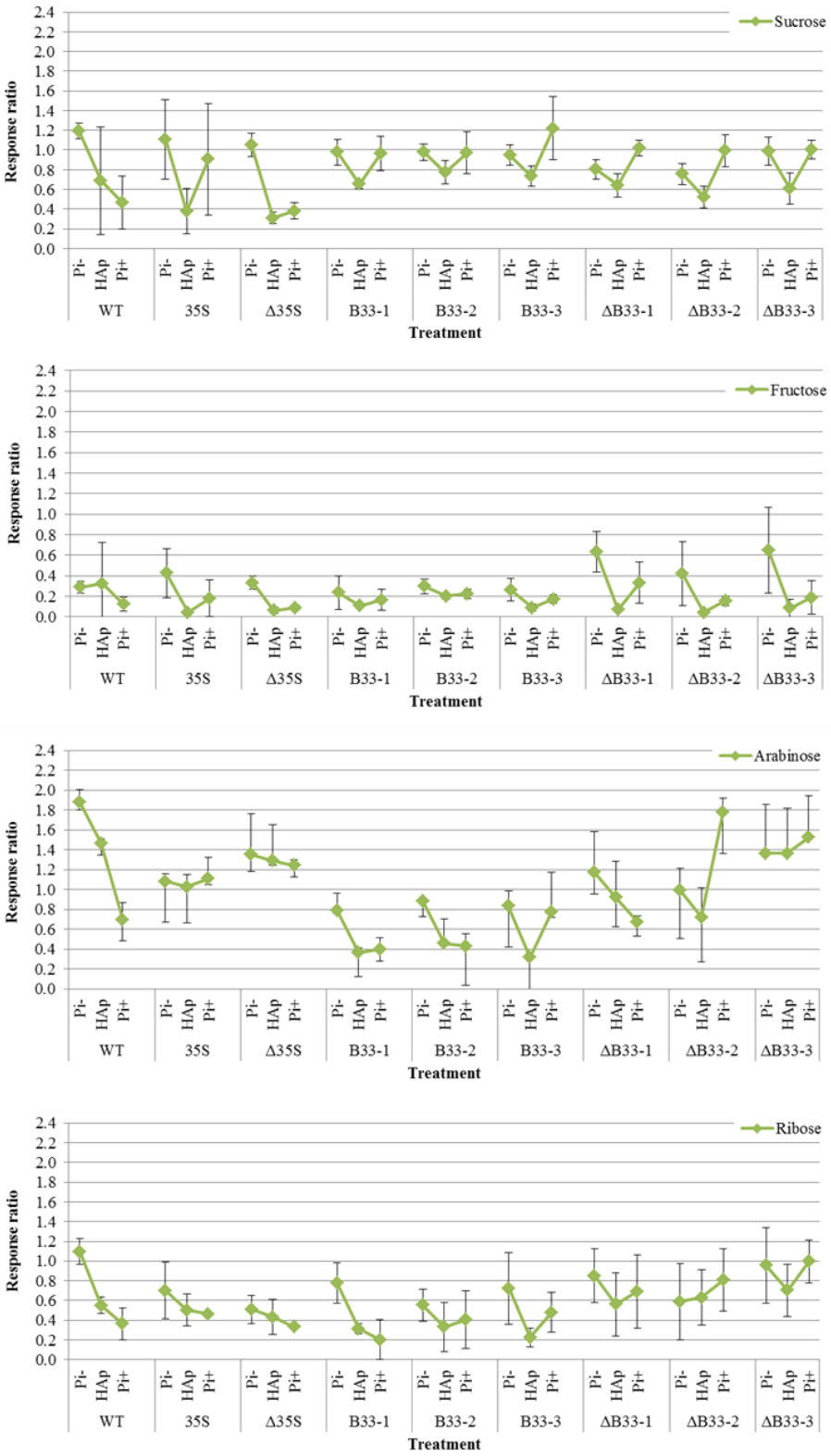

Lactic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

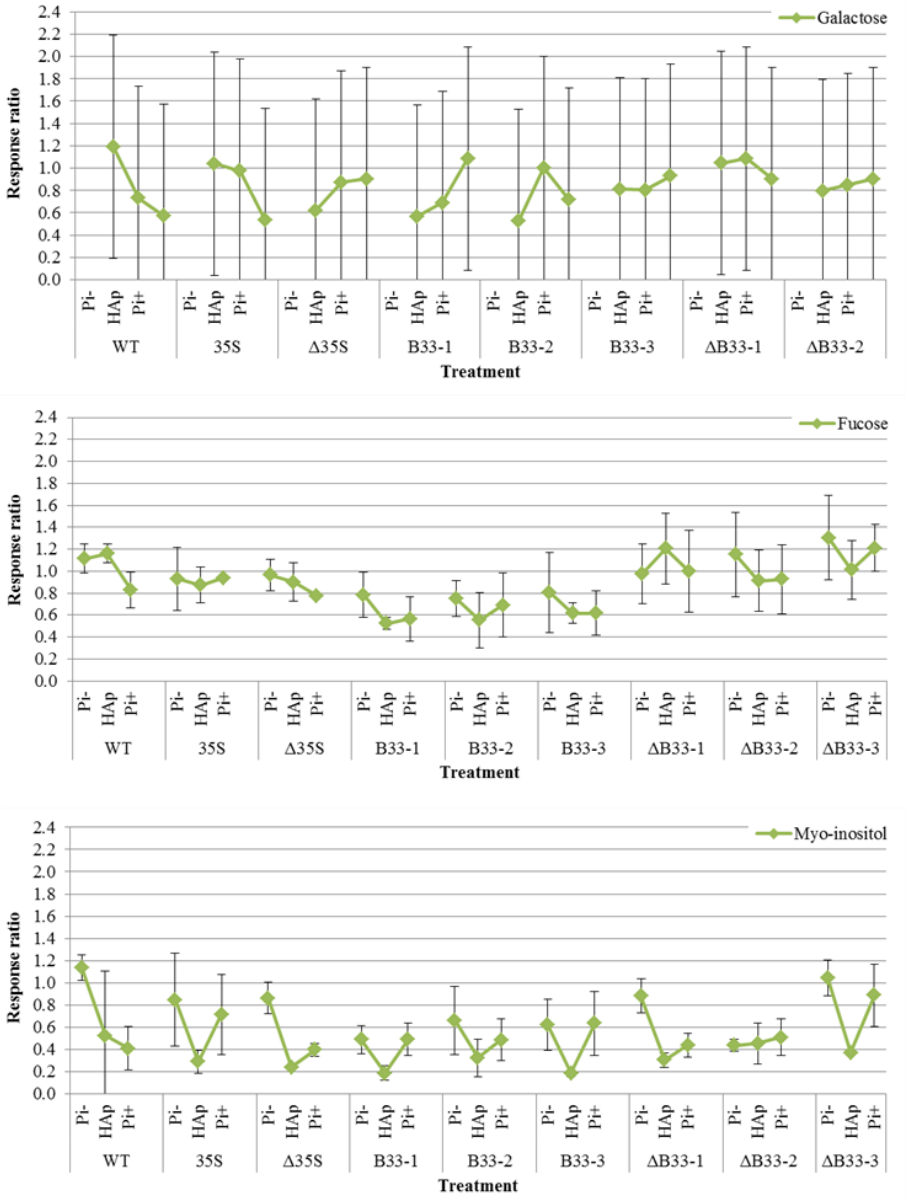

Isocitric
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Glutaric acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

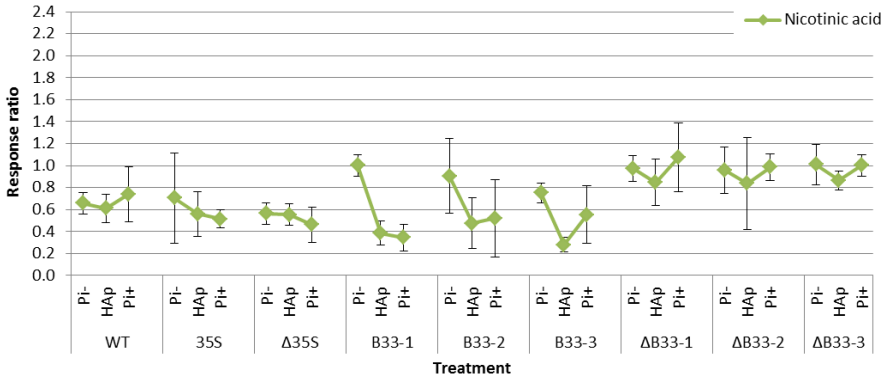

Ribonic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

7.1.2.3. Sugars


Sucrose
Fructose
Arabinose
Ribose
Response ratio
Treatment
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3

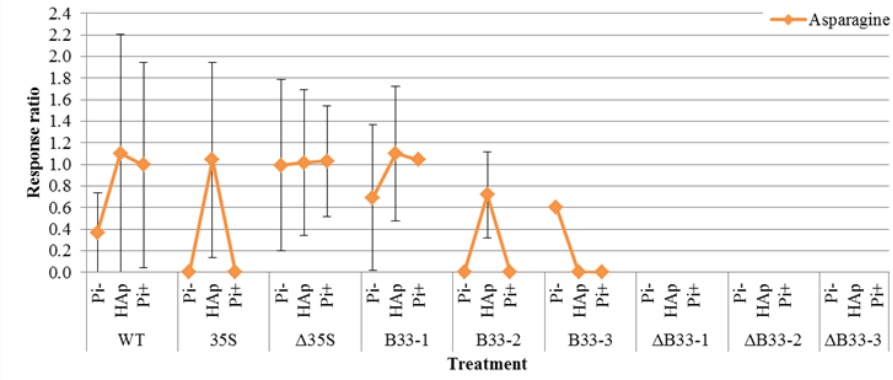

Galactose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
Treatment
Fucose
Response ratio
ΔB33-3
Treatment
Myo-inositol
Response ratio
ΔB33-3
Treatment

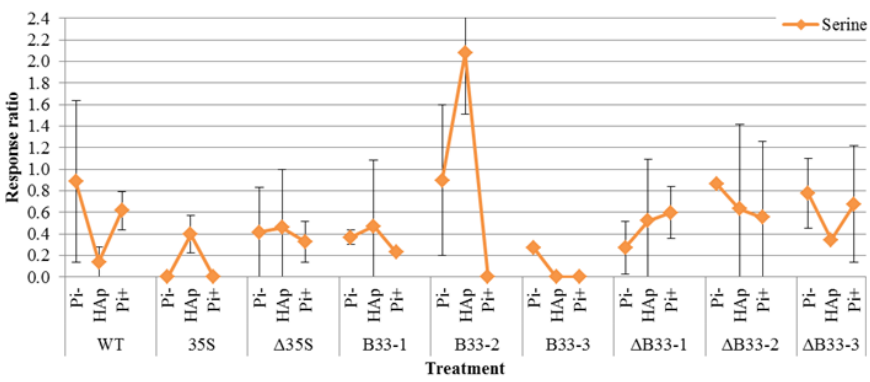
7.1.2.4. Nicotinic Acid

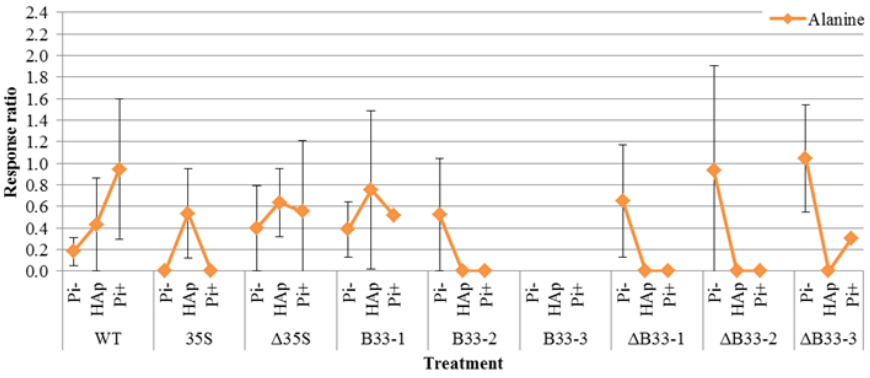


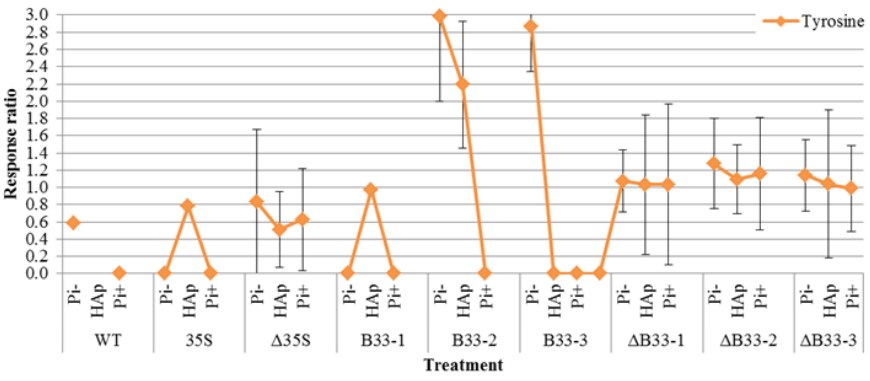
7.1.3. The Identified Metabolites of Root

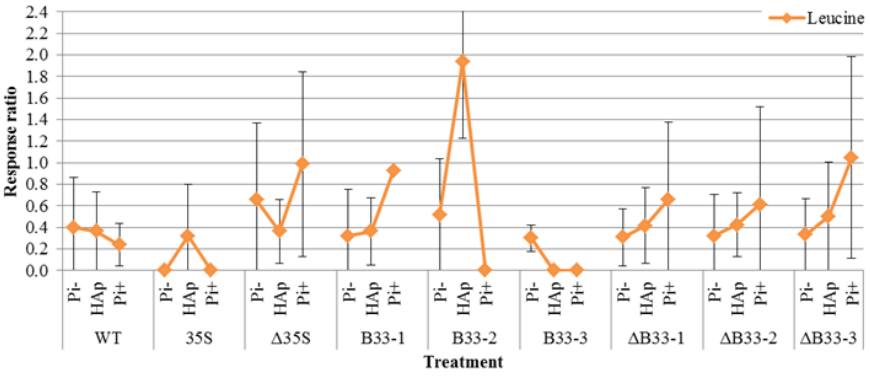
7.1.3.1. Amino Acid

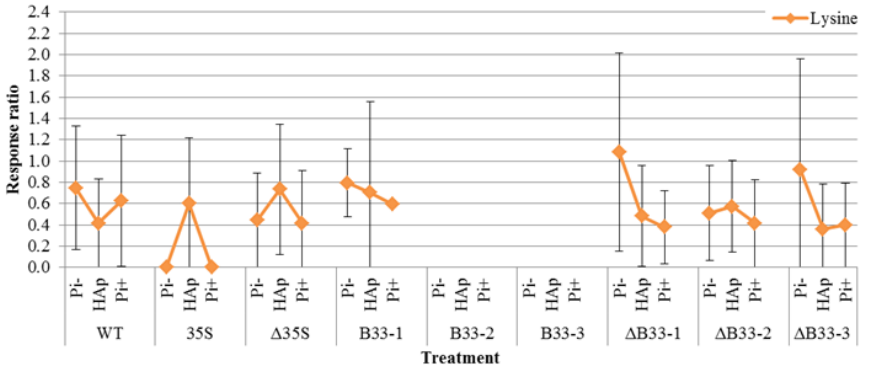


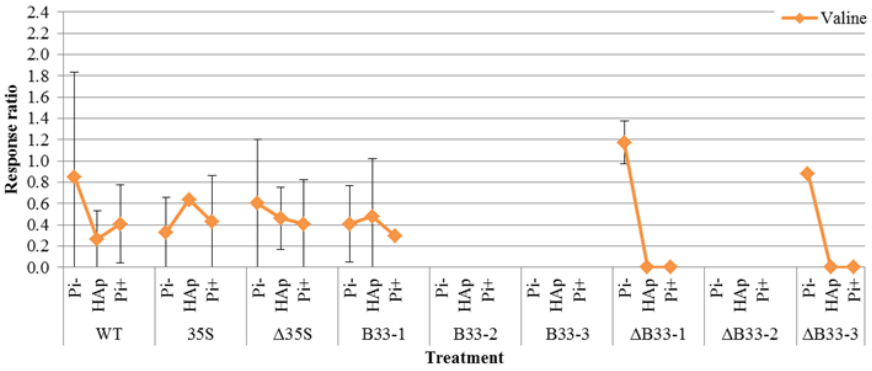

Serine
Response ratio
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

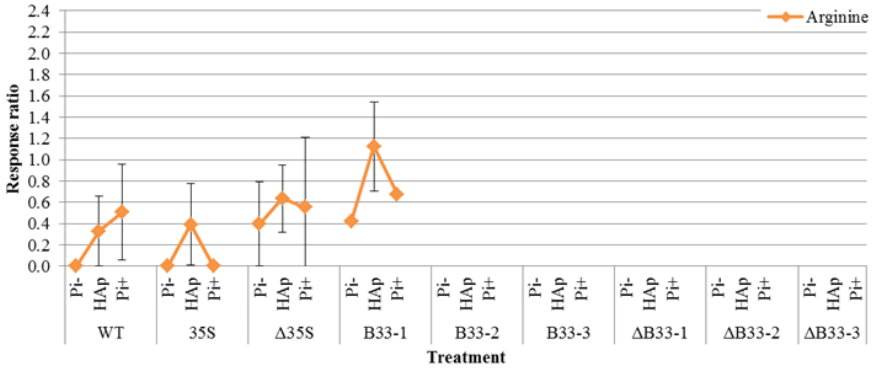

Alanine
Response ratio
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

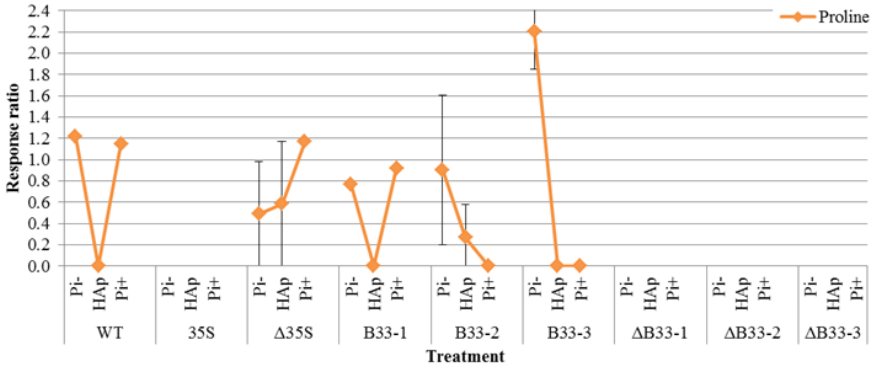

Tyrosine
Response ratio
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

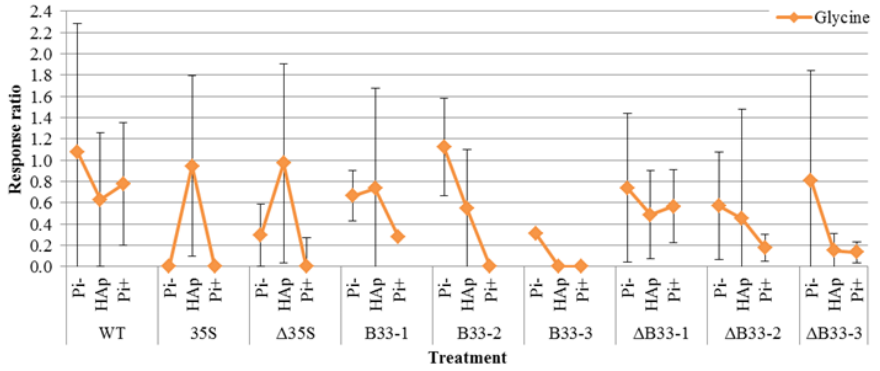

Leucine
Response ratio
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

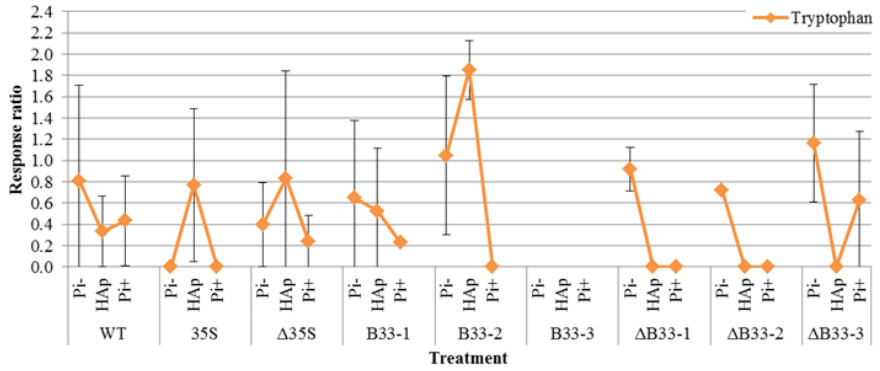

Lysine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

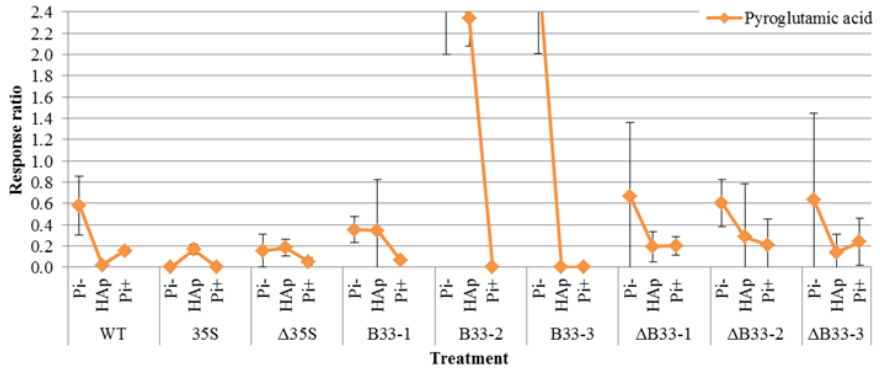

Valine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

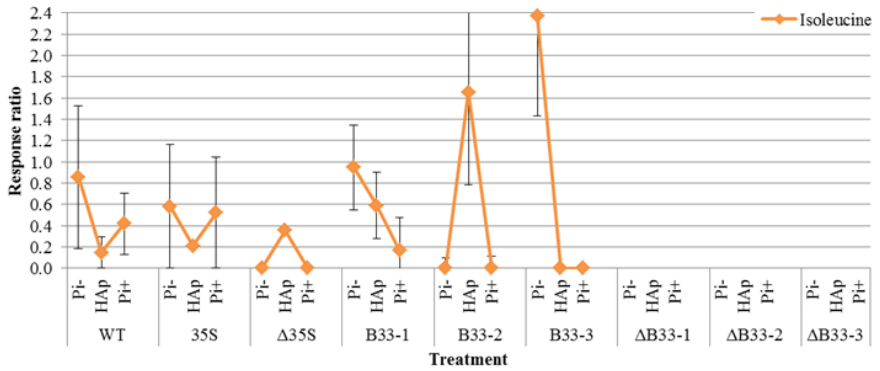

Arginine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

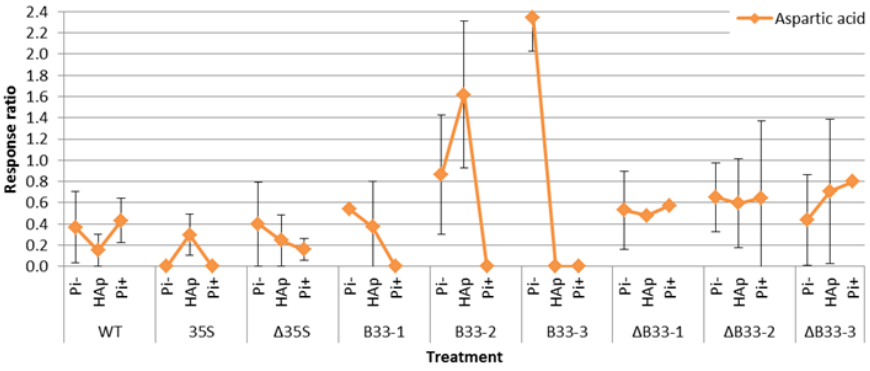

Proline
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

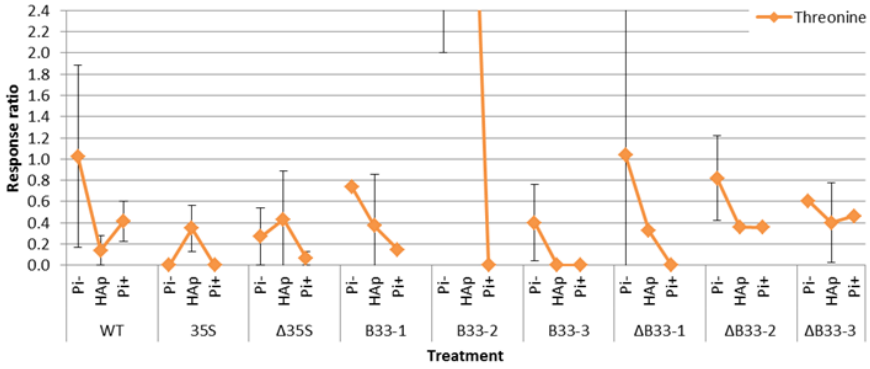

Glycine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Tryptophan
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

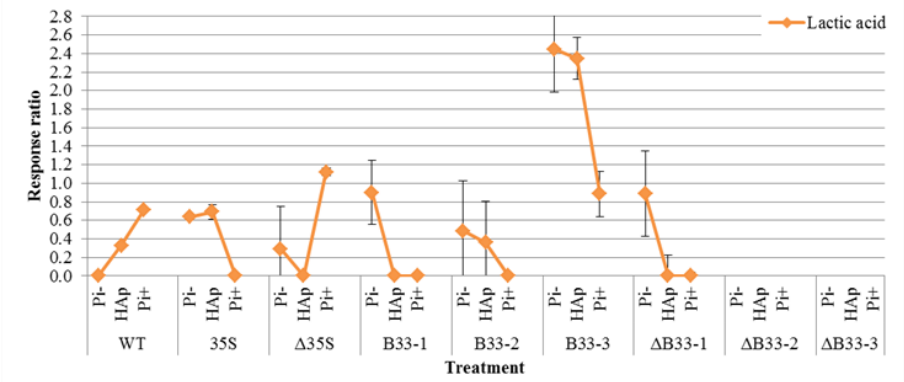

Pyroglutamic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

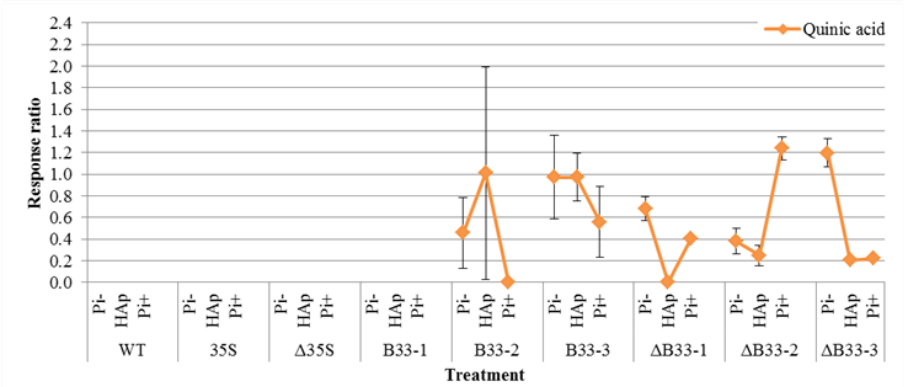

Isoleucine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

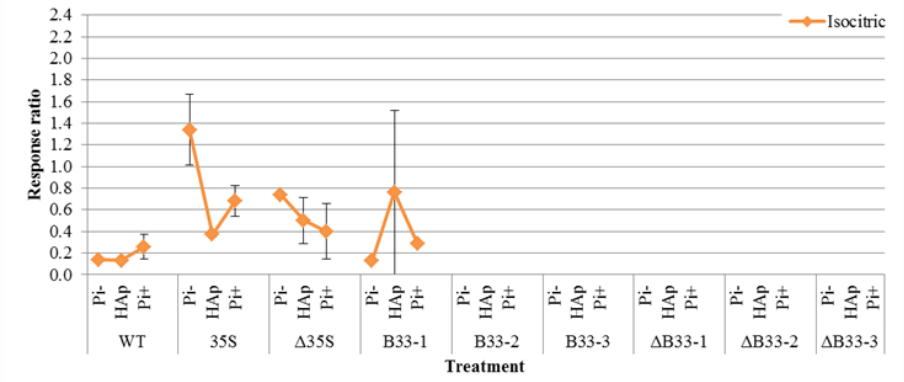

Aspartic acid
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

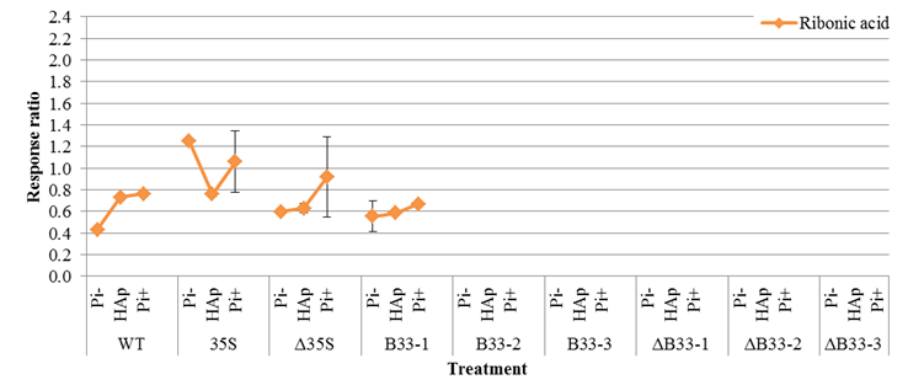

Threonine
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

7.1.3.2. Organic Acid

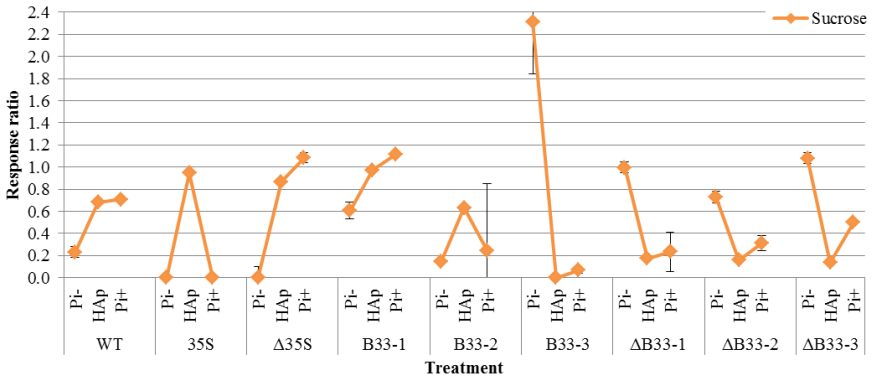


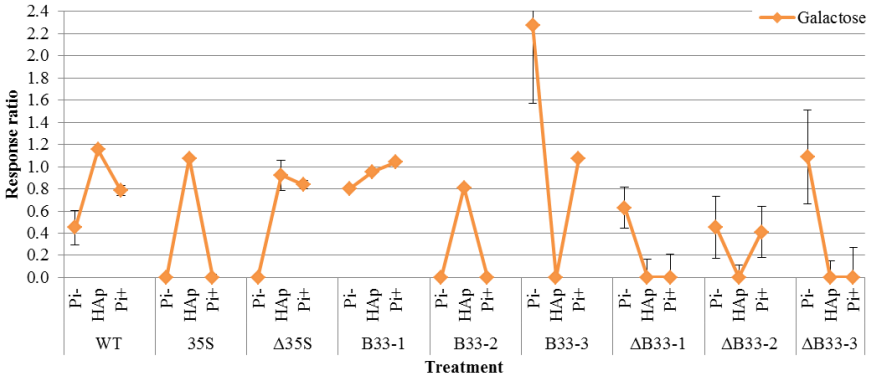


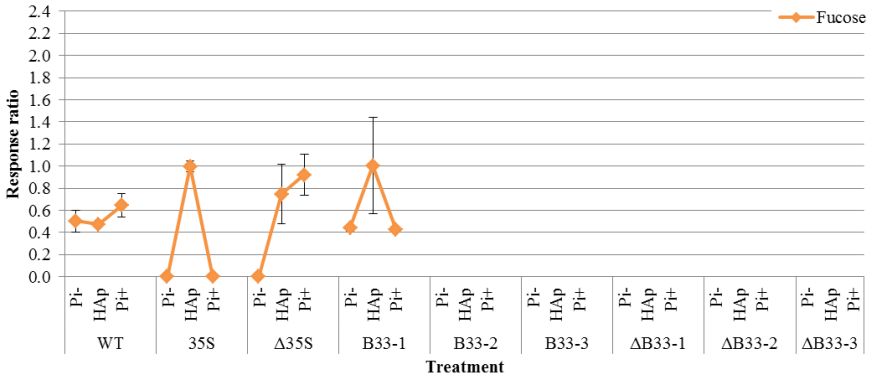


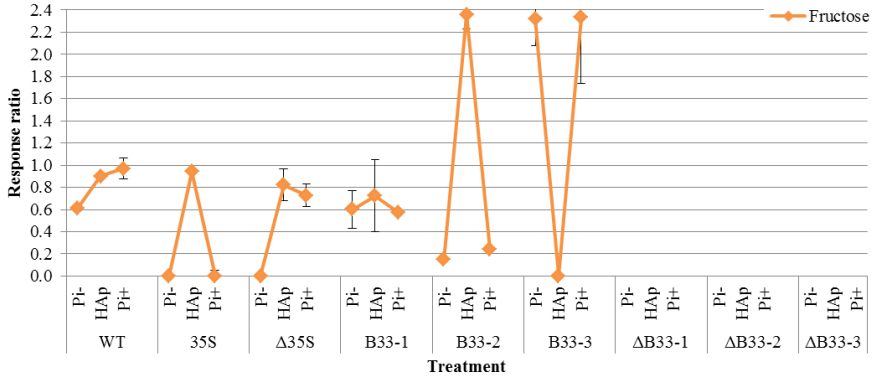


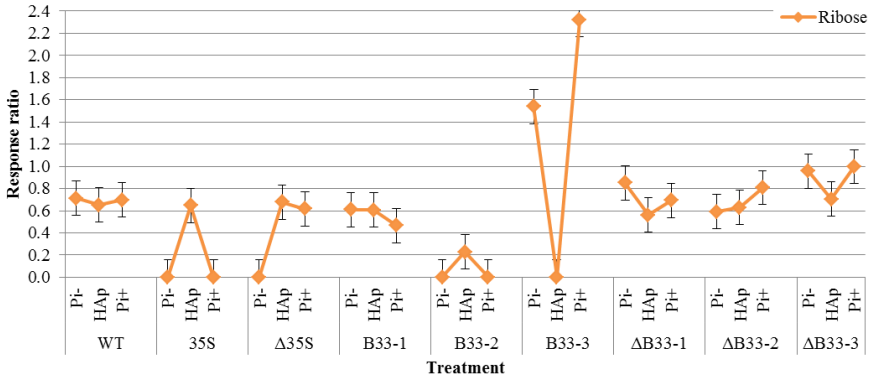
7.1.3.3. Sugars

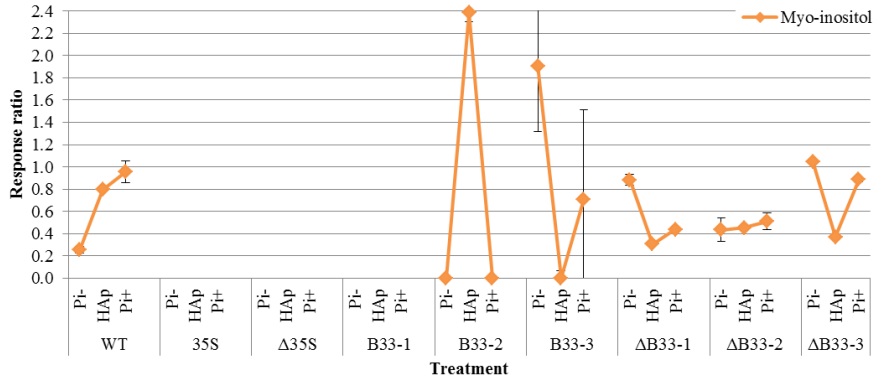

Sucrose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

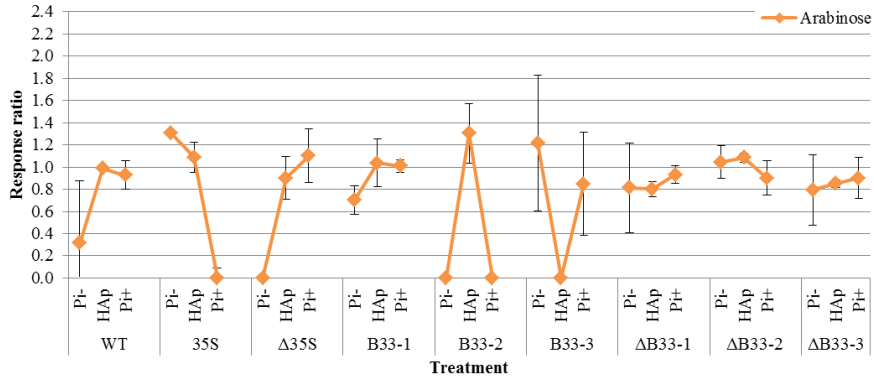

Galactose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

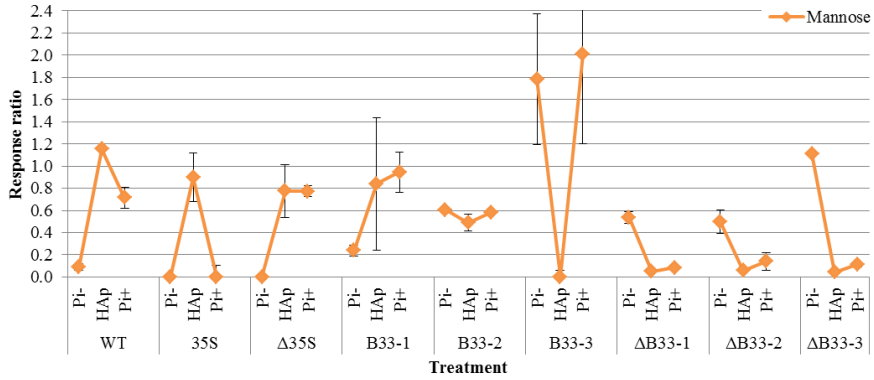

Fucose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Fructose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

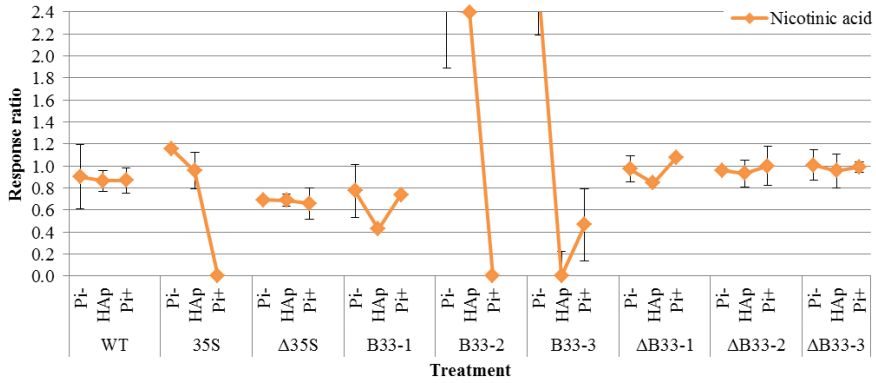

Ribose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Myo-inositol
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Arabinose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment


Mannose
Response ratio
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi-
HAp
Pi+
WT
35S
Δ35S
B33-1
B33-2
B33-3
ΔB33-1
ΔB33-2
ΔB33-3
Treatment

7.1.2.4. Nicotinic Acid



7.2. Declaration of honor

I hereby declare that I have made the dissertation presented itself without unauthorized aid. I have not used any other sources than the specified ones. Furthermore, I declare that I have not filed this dissertation in any other faculty.

7.3. Curriculum vitae

For the reasons of data protection, the CV is not published in the online version of the doctoral thesis.