Chapter 6

14-3-3 Positively regulates alkaline/neutral invertases

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Abstract
Plant photosynthesis produces carbohydrates necessary for growth and development, the main photosynthetic product being sucrose. Sucrose is transported from the photosynthetic areas (source) of the plant to non-photosynthetic areas (sink). Within sink tissue sucrose is converted to hexoses by two pathways in which: 1) sucrose is reversibly converted to fructose and UDP-glucose by sucrose synthases (SUS) or 2) irreversibly to fructose and glucose by invertases (INV). There are three types of invertases namely vacuolar (V-INV), cell wall (CW-INV) and cytosolic invertases (C-INV) or A/N invertases. Members of the cytosolic invertases clade are involved in overall plant development. In this study we have shown that 14-3-3 proteins physically interact with one member of the cytosolic invertases, CINV1. 14-3-3 Interaction occurs within the C-terminal region of the protein at S547, in a phosphorylation dependent manner. In vitro phosphorylation of S547 of recombinant AtCINV1 can be facilitated by CPK3, a calcium dependent kinase. Phosphorylation of S547 already has some stimulatory effect on the activity of recombinant CINV1. The subsequent binding of 14-3-3 to phosphorylated S547 further increases recombinant CINV1 activity. Our in planta data suggests that 14-3-3’s are positively regulating invertase activity as demonstrated by the 14-3-3 quadruple mutant klpe, which showed a reduction in the concentration of glucose and fructose and a reduction in total A/N invertase activity by 22% when compared to WT. These results highlight the importance of 14-3-3’s in sucrose conversion in Arabidopsis.
Introduction
Sugars are produced during photosynthesis through conversion of light energy into energy-rich carbon molecules (Rolland et al. 2006). These sugars are transported over a long distance from photosynthetic, source tissue, to non-photosynthetic, sink tissues. The major photosynthetically produced compound is sucrose (Rolland et al. 2006). Within sink tissue, sucrose can be cleaved reversibly by sucrose synthases (SUS) producing fructose and UDP-glucose, or irreversibly by invertases (INV) yielding glucose and fructose (Rolland et al. 2006; Vargas and Salerno 2010). The invertase family consists of two groups depending on their pH optimum: 1) the acid invertases (Ac-Inv) and 2) the cytosolic neutral/alkaline invertases (A/N-Inv or CINV). The vacuolar (V-INV) and extracellular or membrane bound (CW-INV) invertases belong to the Ac-INV group. The V-INV is involved in normal root growth and CW-INV is highly active after pathogen attacks and wounding (Barratt et al. 2009; Xiang et al. 2011).

The hydrolysis of sucrose by SUS requires less ATP compared to sucrose conversion by INV. However, it has recently become clear that A/N-Invertases affect Arabidopsis development more strongly compared to SUS (Barratt et al. 2009; Welham et al. 2009). A quadruple loss-of-function mutant of SUS (sus1/sus2/sus3/sus4) and the double mutant (sus5/sus6) did not show a developmental phenotype while mutating an a/n-invg (cinv1) showed a severe effect on root growth and a mild effect on leaf growth (Lou et al. 2007; Xiang et al. 2011). AtCINV1 transcripts are the most abundant A/N-Invertases in Arabidopsis (Xiang et al. 2011). The reduced root length phenotype of Atcinv1 is probably due to carbon starvation, since applying exogenous carbon improves the root elongation phenotype (Lou et al. 2007; Xiang et al. 2011).

CINV1 has been found in 14-3-3 interaction studies (Schoonheim et al. 2007; Chang et al. 2009; Swatek et al. 2011). In addition to the 14-3-3 interaction studies, the three 14-3-3 quadruple mutants and six 14-3-3 triple mutants showed reduced root growth which could indicate nutrient starvation (Chapter 3 of this thesis). The resemblance, although less severe, with the cinv1 reduced primary root phenotype and the potential CINV1–14-3-3 interaction, caused us to hypothesize that 14-3-3’s are required for CINV1 activity. To elucidate a potential interaction yeast-two-hybrid (Y2H), in vitro and in vivo pull-down assays were conducted. Measuring sugar metabolites in the 14-3-3 quadruple mutants revealed that the quadruple mutant klpc shows reduced glucose and fructose concentrations compared to WT and klun or unpc. Subsequently, we measured total A/N invertase activity in roots of WT, klp, klpc and cinv1 to unravel the outcome of the 14-3-3:CINV binding in planta.
Results

14-3-3 Can pull down endogenous CINV1

14-3-3 Interaction studies have shown an alkaline/neutral (A/N) invertase, CINV1, to be a putative 14-3-3 interactor (Schoonheim et al. 2007; Chang et al. 2009; Swatek et al. 2011). In addition, our mass spectrometry (MS) data of the 14-3-3 root interactome (Chapter 4 of this thesis) identified members of the A/N invertase family C-INVF and C-INVB in addition to the most abundant invertase, CINV1 (C-INVg). The MS data indicated that 14-3-3’s and A/N invertases are able to bind either directly or as part of a larger protein complex. To confirm our MS data, a 14-3-3 pull-down assay was conducted with Arabidopsis protein extract and At14-3-3PHI protein on nickel beads. After incubation and the washing of the beads, proteins bound specifically to the 14-3-3 protein were eluted with the R18 peptide, NIP peptide was used as control. R18 is a peptide that in vitro strongly competes with target proteins for binding within the 14-3-3 groove, whereas NIP peptide does not compete. To demonstrate the presence of A/N-Inv in the eluates, an antibody was used that specifically interacts with CINV1 (Lou et al. 2007). The western blot clearly demonstrated the presence of multiple CINV1 bands in the R18 eluate and lack of bands in the NIP eluate, indicating specific interaction between CINV1 and 14-3-3 protein (Fig. 1A).

14-3-3 Physically interacts with CINV1 at phosphorylated S547

Most 14-3-3 target proteins interact through a so-called mode-I or mode-II motif (R/KXX(X)S/TXP) and in order to find a candidate site, CINV1 was analysed for the following three criteria (de Boer et al. 2013): Scansite score, disordered regions and identified phospho-sites. The site with the best Scansite (Mode-I) score was for S547 (score = 0.2885) at the very C-terminal end of the protein. This region is highly disordered (www.disprot.org/pondr-fit.php) and the S547 site has also been identified as a phospho-site by mass-spectrometry (van Bentem et al. 2008). To test direct interaction between At14-3-3’s and AtCINV1, a yeast-to-hybrid (Y2H) assay was performed with CINV1 fused to the activation domain of GAL4 (AD) and 10 Arabidopsis 14-3-3 genes fused to the GAL4 binding domain (BD). As shown in Fig. 1B, with the exception of MU, all 14-3-3 isoforms showed interaction with CINV1. In order to test if S547 is the 14-3-3 interaction site, the Y2H assay was repeated with a mutated form of CINV1 (CINV1-S547A) and as shown in Fig. 1B the point mutation annihilated the interaction with all 14-3-3 proteins.

The importance of phosphorylation of S547 was studied by means of competitive fluorescent anisotropy, as described by Wu et al. 2006. Briefly, the binding of the fluorescently labelled peptide FAM-SWTY to a 14-3-3 protein reduced the peptide mobility which resulted in an increase in the fluorescence anisotropy signal. As competing peptide the last 15 amino acids (AA) of the CINV1 protein (539KPVIKRASWPQL551) was used with non-phosphorylated and phosphorylated S547; dCINV and pCINV respectively. Whereas pCINV shows competition for 14-3-3 binding with FAM-SWTY seen as a decrease in the anisotropy signal, the non-phosphatepeptide dCINV does not (Fig. 1C). From this we conclude that the interaction between CINV1 and 14-3-3 proteins is phosphorylation dependent, and that the 14-3-3 binding motif is in the C-terminus of CINV1.
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Figure 1. 14-3-3 Binds CINV1 at S547.
A) In vivo pull-down using 14-3-3 to pull down endogenous CINV1 from Arabidopsis plant extract. 14-3-3 Bound proteins are eluted with a non-competitive peptide NIP (control) and a competitive peptide R18. Western blot is developed with AtCINV1 specific antibody and shows only for the competitive peptide R18 a CINV1 band. B) Yeast-two-hybrid between 14-3-3 and CINV1 WT and CINV1S547A and 14-3-3. Upper panel is DDO plates and shows yeast viability for all colonies and lower panel is QDO were colony growth indicate protein interaction. C) Competitive anisotropy measurements using the last 15 amino acids of AtCINV1 (KPVIKRSA539WPQL551) phosphorylated at S547 (pCINV1) or non-phosphorylated (dCINV1); no competition is seen with dCINV1, while competition is seen between 14-3-3 and AtCINV1 peptide phosphorylated at S547 (pCINV1).

S547 can be phosphorylated by CPK kinases
Recently it has been shown that CPK3 can phosphorylate the mode I 14-3-3 binding motif of the ion channel TPK1 (RRSRAP) (Latz et al. 2013). Since CINV1 has a similar motif (KRSASWP) we tested whether CPK3 is able to phosphorylate S547. For this HPLC C18 column chromatography was used to separate the phospho-Ser547 peptide and its parent non-phosphorylated peptide with a water/acetonitrile gradient. Fig. 2A shows that the retention time between pCINV1, which was eluted earlier, and dCINV1 differs. As shown in Fig. 2B CPK3 can phosphorylate the dCINV1 peptide.

To demonstrate that the CPK3 phosphorylated dCINV1 peptide has 14-3-3 binding capacity, a competition anisotropy measurement was performed. Fig. 2C shows that the CPK3 kinase phosphorylated dCINV1 peptide induces 14-3-3 binding by lowering the anisotropy.

To validate the Y2H assay and anisotropy measurements an in vitro pull-down assay was performed using full length recombinant His-14-3-3PHI, GST-CINV1 and GST-CINV1S547A proteins. GST-CINV1 and GST-CINV1S547A were both incubated with
CPK3 and 1 mM ATP or no ATP for 1 h at 30°C. After phosphorylation treatment, CINV1 and CINV1\textsuperscript{S547A} were allowed to bind to 14-3-3 coated beads. Fig. 2D shows that the CINV1\textsuperscript{S547A} mutant protein does not interact with 14-3-3. In addition, the NIP peptide does not elute GST-CINV1 WT. Only the R18 peptide eluted GST-CINV1 WT protein which showed that the CINV1/14-3-3 interaction is specific and phosphorylation dependent. From these experiments we conclude that CINV1 interacts with 14-3-3 proteins through phosphorylation of S\textsuperscript{547} in a canonical Mode-I/II 14-3-3 interaction motif (KRSASWP), and that S\textsuperscript{547} can be efficiently phosphorylated \textit{in vitro} by CPK3.

**Figure 2. AtCINV1 phosphorylation by CPK3 and subsequent 14-3-3 binding.**

A) Separation between the parent peptide dCINV1 (black) and the phosphorylated pCINV1 (S547) (red) using C18 reverse phase HPLC. The last 15 amino acids of AtCINV1 was used as peptide. A shift to the left indicates phosphorylation of the peptide. B) CPK3 is able to phosphorylate dCINV1 as seen in a shift to the left when ATP is added. C) 14-3-3 anisotropy measurements using dCINV1 peptide phosphorylated by CPK3 and 14-3-3 PHI. Reduction in anisotropy signal indicates 14-3-3 binding. D) \textit{In vitro} pull down assay using recombinant At14-3-3 PHI, CINV1 and CINV1\textsuperscript{S547A} in the presence of CPK3. First four lanes are eluted with a non-competitive peptide NIP and the last four are eluted with a competitive peptide R18. In the first four lanes no recombinant CINV1 protein band is found. In lane 5 WT CINV1 was not phosphorylated and shows no CINV1 band in the R18 elution. Lane 6 WT CINV1 is phosphorylated and was bound to 14-3-3. The S547A mutation abolishes 14-3-3 binding (lane 7-8). Western blot is developed with AtCINV1 specific antibody and shows only for the competitive peptide R18 a CINV1 band.
Recombinant invertase activity is enhanced by 14-3-3 binding

The previous experiments have shown that the 14-3-3 interaction motif is KRSAPSWP. This raises the question if phosphorylation and subsequent 14-3-3 binding to CINV1 affects the invertase activity. For instance, this is the case for the yeast neutral trehalase (Panni et al. 2008; Obsil and Obsilova 2011). To elucidate the function of CINV1/14-3-3 binding, alkaline invertase activity was measured in vitro. Since 14-3-3 binding to CINV1 is phosphorylation dependent, we first investigated phosphorylation dependent changes in invertase activity of recombinant CINV1. CINV1 phosphorylation by CPK3 enhanced the activity of wild-type CINV1 but not of CINV1S547A (Fig. 3A). This result indicates that CPK3 phosphorylation of S547 has an effect on recombinant CINV1 activity.

To investigate the effect of subsequent 14-3-3 binding to CINV1, GST-CINV1 was incubated with different concentrations of recombinant His-14-3-3PHI and CPK3 in the presence or absence of ATP, and changes in its invertase activity were measured. Fig. 3B shows that recombinant 14-3-3 in the presence of CPK3 had no effect on the mutant protein nor on the activity of CINV1 in the absence of ATP. However, in the presence of ATP and CPK3, the invertase activity was strongly stimulated in a concentration dependent manner. These results demonstrate that both phosphorylation and subsequent 14-3-3 binding enhance recombinant CINV1 activity in vitro.

The activity of endogenous alkaline invertase is enhanced by 14-3-3 binding

Our in vitro experiment showed that phosphorylation and 14-3-3 binding enhanced CINV1 activity. To test whether there is a similar effect on endogenous alkaline invertases, a pull-down assay was performed and the bound invertase was eluted with either buffer (control), R18(Lys) peptide or R18 peptide. R18(lys) is a mutated form of the R18 peptide that does not compete with 14-3-3 (Masters and Fu 2001). Next, both the amount of invertase (intensity measurements western blot (Fig. 3D)) and the invertase activity was measured on the differently treated 14-3-3 coated beads and in its elutions (Fig. 3E). The western blot in Fig. 3C shows that CINV1 is only eluted in the presence of R18. As shown in Fig. 3D, R18(lys) does not elute invertase activity nor does it affect the invertase activity that remains on the beads compared to buffer treated beads. Intriguingly, the R18 elutes 70% of the bound A/N invertase but the invertase activity in the eluate does not significantly differ from that of buffer treated beads. Since R18 does elute A/N invertase proteins as shown by Western blot (Fig. 3C), the reduced activity of A/N invertase in the R18 eluate indicates that 14-3-3’s are involved in either the activation of A/N invertases or the enhanced activation of A/N invertases.
Figure 3. Phosphorylation and 14-3-3 binding positively regulates WT CINV1 but not CINV1<sup>S547A</sup>. 
A) Recombinant invertase activity at 100 mM sucrose incubated with recombinant AtCPK3. Activation can only be seen in CINV1 WT with ATP. B) Recombinant invertase activity supplemented with different concentrations of recombinant At14-3-3 PHI. Only with phosphorylated WT CINV1 14-3-3 activation is seen. C) Representative western blot of a pull down assay with 14-3-3 PHI on beads and crude root protein extract. The first two lanes are empty beads. Lane 3-8 used 14-3-3 coated beads. Bf indicates buffer, R(L) is R18(Lys), a non-competing peptide, R is R18 a competing peptide. E indicates elution and B indicates beads. Western blot is developed with AtCINV1 specific antibody and shows AtCINV1 bands on the beads when 14-3-3 is present and in R18 elution. D) Intensity measurements of CINV1 Western blot bands of two independent pull downs. E) Invertase activity on the samples of D showing reduced activity in beads treated with R18.

**Fructose and glucose levels are reduced in a 14-3-3 quadruple mutant**

The next question we addressed was whether 14-3-3 mutants show a change in sugar metabolites due to changes in A/N-invertase activity in planta. If the A/N-invertases bind 14-3-3 proteins in vivo, then the absence of certain 14-3-3 isoforms is likely to affect the invertase activity and reduce the fructose and glucose concentrations in the root. Therefore, we measured the sugar content of root extracts of the three quadruple mutants (klun, klpc, unpc from Chapter 2) and compared the outcome with that of the wild-type content (Fig. 4A). Intriguingly, only the klpc mutant combination showed...
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strongly reduced levels of the invertase products, fructose and glucose. The sucrose concentration in the klpc roots was at the same level as those of the other genotypes (including WT), which may be due to the feed-back mechanism of sucrose transport to the roots as a sink. These results indicate that specific 14-3-3 isoforms are involved in sugar metabolism in planta.

**Total invertase activity is reduced in klpc but not in klp root extract**

Both klpc and cinv1 display a shorter primary root compared to WT, in which the phenotype of cinv1 is more severe. We therefore investigated the possible link between root growth and total invertase activity. The metabolomics study showed that klpc was reduced in invertase products glucose and fructose. The triple mutant klp was included in the total invertase activity analysis since this mutant appeared to be responsible for the klpc root phenotype. The total A/N invertase activity in the roots of the klpc mutant was significantly lower than that of the wild-type plants (reduction in B_max of 22%) (Fig. 4B). However, the total A/N invertase activity did not change in klp (Fig. 4C). The total A/N invertase activity of the cinv1 mutant in roots was 40% lower compared to wild-type (Fig. 4B). From these experiments we conclude that specific 14-3-3 proteins have a positive effect on the in planta activity of A/N invertases and that the activity is determined in an isoform specific manner.

**Shorter primary root phenotype of klpc could be due to carbon starvation**

A severely reduced primary root length is seen in cinv1 compared to WT, which can be complemented by germinating and growing cinv1 on 2% sucrose (Lou et al. 2007). Interestingly, this promoted root growth does not occur when the mutant is first grown on 0.5x MS plates without sucrose and after five days transferred to plates containing 2% sucrose (Fig. 4D). These results indicate that, sucrose deficient phenotype of the cinv1 mutant can only be complemented from germination onwards. Our experiments (Chapter 3) demonstrated that both the klpc and klp mutants exhibit a shorter primary root phenotype compared to WT, whereby klp appears to cause the klpc root phenotype since klc, kpc or lpc did not show a phenotype. We hypothesized that this phenotype could be caused by nutrient starvation. To investigate the possibility of sugar starvation, klpc and klp were germinated on plates without sucrose and after five days transferred to plates containing 2% sucrose. There was no significant difference in main root growth for klp on plates with and without 2% sucrose, indicating that the reduced primary root phenotype, like cinv1, could not be complemented by 2% sucrose (Fig. 4D). However, the main root growth of klpc on 2% sucrose was improved compared to control by +15% over a period of seven days (Fig. 4D). These results demonstrate that, at least in the case of klpc, primary root growth can be promoted by applying exogenous sucrose, indicating that the shorter root phenotype in klpc could be due to carbon starvation. Moreover, KAPPA, LAMBDA, PHI and CHI behave redundantly in main root growth on 2% sucrose since klp did not show a promoted root growth on 2% sucrose (Fig. 4D). Therefore, it can be concluded that although there is a reduced primary root growth in both klp and klpc the mechanism behind the root length reduction involves different pathways. In addition, total invertase activity is not the cause of the reduced primary root length.
Figure 4. Outcome of 14-3-3 interaction to CA/N invertases in planta.
A) Metabolomics study of fructose, glucose and sucrose in the 14-3-3 quadruple mutants klun, klpc and unpc (Chapter 2). The graph shows relative values whereby Sorbitol is used as internal standard. The mutant klpc shows reduced fructose and glucose concentrations. Statistical analysis student t-test * p < 0.05, ** p < 0.01, *** p < 0.005 error bars indicate SEM (n=5). B) Total root invertase activity for the Arabidopsis mutants klpc and cinv1. Both klpc and cinv1 show reduced total invertase activity (n=5). C) Total root invertase activity of the 14-3-3 mutant klp is indistinguishable from WT (n=3). D) Primary root growth assay on control and 2% sucrose plates between seven and 14 days after stratification (DAS) of klpc, klp and cinv1. Two WT and two mutant seedlings were grown vertically on plates under long day condition and root growth was measured as described in M&M. Figures show mutant root growth compared to WT on the same plate. Statistical analysis 2-way ANOVA, showing p value for interaction value * p < 0.05, ** p < 0.01, *** p < 0.005 error bars indicate SEM (n=3).
Discussion

The main purpose of photosynthesis is the production of sucrose. Sucrose is degraded reversibly by sucrose synthases (SUS) into UDP-glucose and fructose as well as irreversibly by invertases (INV) into glucose and fructose. In this Chapter we show that a member of the cytosolic invertase family, CINV1, physically interacts with 14-3-3 in a phosphorylation dependent manner with 14-3-3 positively regulating CINV1's activity.

Phosphorylation of recombinant CINV1 enhances its activity and is further enhanced by 14-3-3

In the literature, metabolic enzymes have been reported to be regulated by 14-3-3 in a sucrose dependent manner (Bachmann et al. 1996; Moorhead et al. 1999; Weiner and Kaiser 1999; Cotelle et al. 2000). In Arabidopsis cells, 14-3-3’s have been found to bind and stabilize proteins that are involved in nutrient pathways such as nitrate reductase (NR), glyceraldehyde-3-phosphate dehydrogenase, a CDPK protein kinase, sucrose-phosphate synthase (SPS) and glutamyl-tRNA synthases. The binding with 14-3-3 was lost when cells were starved of sugars which resulted in degradation of the above-mentioned 14-3-3 targets. Sugar starved cells show a reduced nitrate assimilation and sugar synthesis, implying the role of 14-3-3’s in these pathways (Cotelle et al. 2000). Two of the aforementioned enzymes, NR and SPS, have been shown to be inhibited by 14-3-3 binding. At night, AtNR is down-regulated through phosphorylation by a CPK of S534 and subsequent binding of 14-3-3, causing inhibition of NR activity followed by NR degradation (Douglas et al. 1998; Weiner and Kaiser 1999). Another enzyme, AtSPS, is an enzyme catalysing the conversion from UDP-glucose and fructose-6-phosphate to sucrose-6-phosphate and sucrose that has been found to be inhibited by 14-3-3 after phosphorylation of S229 (Toroser et al. 1998). In this study, we show an additional 14-3-3 function in the sucrose metabolic pathway through the positive regulation of CINV1 in a phosphorylation dependent manner. In vitro phosphorylation of recombinant WT CINV1 by CPK3 enhanced its activity but not that of recombinant CINV1^{S547A} (Fig. 3A). This result indicates that phosphorylation of S547 is the first step in the enhancement of recombinant CINV1 activity. The difference in activity between WT CINV1 and CINV1^{S547A} could be due to conformational changes, resulting from its phosphorylation, which affects the invertase activity. The phosphorylation of S547 was CINV1 is essential for 14-3-3 binding as the binding of 14-3-3 enhanced recombinant CINV1 activity (Fig. 3B). In conclusion, recombinant CINV1 has invertase activity and its activity can be enhanced through CPK3 phosphorylation and further increased by 14-3-3 binding.

Endogenous A/N invertase shows activity in the presence of 14-3-3

Endogenous Arabidopsis A/N invertases, purified with 14-3-3 coated beads, showed reduced A/N invertase activity when the 14-3-3 binding was annihilated by R18 (Fig. 3E). As shown in Fig. 3C, R18 broke the binding between 14-3-3 and A/N invertases which resulted in a decrease of invertase activity on the beads (Fig. 3E). The invertase activity between the R18 elution and buffer elution does not differ and consequently the question arises if A/N invertases have activity in the absence of 14-3-3’s. Our results using recombinant CINV1 showed that recombinant CINV1 has invertase
activity in the absence of 14-3-3 and that 14-3-3 enhances invertase activity. The data from endogenous A/N activity on 14-3-3 coated beads shows that 14-3-3’s are either activating or enhancing the A/N invertase activity. A reason why recombinant invertase shows activity without 14-3-3 and endogenous A/N invertase without 14-3-3 reduced activity, could be that the concentration of recombinant protein used in this assay is higher compared to endogenous A/N invertases concentration and therefore exceeds the threshold of the activity assay. Since the buffer and R18 elution did not differ in invertase activity, there is a possibility that 14-3-3 is needed for activation in planta. Unfortunately this will be difficult to establish since the yeast-two-hybrid showed that all 14-3-3’s, except MU, could bind CINV1. A reduction in total invertase activity of 22% was seen in klpc (Fig. 4B) while klp (Fig. 4C) or klc (data not shown) did not show a reduction, which could indicate that removing more 14-3-3’s could result in lower total A/N invertase activity.

In addition to the question if 14-3-3’s are necessary for the activation of A/N invertases, there is the question whether stress could cause 14-3-3 binding to A/N invertases. It has been shown that overexpression of CINV1 results in transcriptional activation of oxidative stress defence genes (Xiang et al. 2011). Because 14-3-3’s are known to be involved in stress pathways it could well be that CINV1 activity is up-regulated through 14-3-3 binding under stress conditions. However, the phosphorylation of CINV1 is not stress related as in this study none of the plants were stressed before the pull-down assays or total invertase activity measurements. More research is needed to see if 14-3-3 is necessary for the basal activity of the A/N invertases or if 14-3-3 proteins bind under specific environmental conditions.

**14-3-3 Reduced root growth phenotype is independent of total invertase activity**

The loss of AtCINV1 in the atcinv1 mutant results in a reduction in main root growth by 30% compared to WT on control medium (Lou et al. 2007; Qi et al. 2007). Germinating and growing cinv1 seedlings on 2% sucrose plates improved primary root length, indicating that, in part, the cinv1 phenotype is due to carbon starvation (Lou et al. 2007). However, we show that when germinating cinv1 on plates without sucrose and transferring them to plates with sucrose, the phenotype is not restored. This result indicates that either during germination or just after germination the lack of CINV1 activity can be complemented by exogenous sucrose (Fig. 4D).

Like cinv1, although less severely, the 14-3-3 triple mutant klp and the quadruple mutant klpc both show a reduced primary root phenotype. This primary root phenotype could be due to sugar starvation, since the klpc mutant showed reduced glucose and fructose concentrations in roots (Fig. 4A). In this study, we investigated if sucrose is a determinant for the reduced root phenotypes (Fig. 4D). The reduced root growth phenotype of klp and cinv1 was not complemented by adding 2% sucrose to the medium, four days after stratification. However, the reduced root phenotype of klpc was rescued by this treatment. Both cinv1 and klpc showed reduced total invertase activity demonstrating that the reduced root phenotype is not due to reduced invertase activity, since klp showed a reduced root phenotype but did not show a reduced total invertase activity. Moreover, there was redundancy between KAPPA, LAMBDA, PHI and CHI for invertase activity. Although the reduced main root phenotypes between klp and klpc
were similar, they seemed to originate from different pathways. In the case of *klp* but not for *klpc* the reduced root phenotype can be restored by 2% sucrose. Whether the reduced root phenotype seen in other 14-3-3 higher order mutants (Chapter 3) can be restored by 2% sucrose needs further investigation.

In conclusion, 14-3-3 proteins are involved in sucrose hydrolysis through positive regulation of the activity of CINV1. The association between 14-3-3 and CINV1 occurs at KRSApS\(^547\)WP whereby *in vitro* S547 can be phosphorylated by CPK3. We have shown that KAPPA, LAMBDA, PHI and CHI behave redundantly in invertase activity since *klp* does not show reduced invertase activity and *klpc* does. In addition, the reduced main root phenotype of *klpc* can be rescued when transferring plants five days after stratification to 2% sucrose plates whereas *cinv1* and *klp* are not rescued. These results indicate that reduced invertase activity is not involved in the reduced root growth phenotype of *cinv1* but could be involved in the reduced root phenotype of *klpc*. The reduced main root phenotype in *klpc* and *klp* results from two distinct pathways. Intriguingly, the concentration of the invertase products, glucose and fructose, is lower in *klpc* than in WT, *klun* and *unpc* and this phenomenon provides additional proof of the involvement of 14-3-3 proteins in sucrose metabolism.

**Material and Methods**

**Plant growth and material**

All plants used are in the *Arabidopsis thaliana* Columbia ecotype (Col-0) background, for the 14-3-3 mutants see Chapter 2, *cinv1* (SALK_095807) a kind gift from Prof. Dr Hong-Wei Xue. Plants were either grown on ½ strength Hoagland solution (3 mM KNO\(_3\), 2 mM Ca(NO\(_3\))\(_2\), 1 mM NH\(_4\)H\(_2\)PO\(_4\), 20 \(\mu\)M Fe-EDTA, 0.5 mM MgSO\(_4\), 1 mM KCl, 25 \(\mu\)M H\(_3\)BO\(_3\), 2 \(\mu\)M MnSO\(_4\), 2 \(\mu\)M ZnSO\(_4\), 0.1 \(\mu\)M CuSO\(_4\), 0.1 \(\mu\)M (NH\(_4\))\(_6\)Mo\(_7\)O\(_24\)) or on 0.5x MS medium (pH 5.8) solidified with 12 g L\(^{-1}\) of plant agar (Sigma A1296). For plants grown on plate, seeds were surface sterilized by rinsing the seeds in 70% ethanol (10 min), followed by 10 min 25% bleach + 0.1% Tween-20. Thereafter, the seeds were washed 3 times with sterilized MQ and resuspended in 0.1% sterile agarose. Seeds were plated on 0.5 x MS medium (pH 5.8) and stratified for 3 days at 4°C. For germination, plates were placed vertically in a growth chamber with 14 hours light (22°C)/ 10 hours dark (18°C), 170 \(\mu\)mol.m\(^{-2}\).sec\(^{-1}\).

**Root growth assay**

Seeds were sterilized as mentioned above. Four days after stratification (DAS), 2 seedlings of WT and 2 seedlings of mutant plants were transferred to 120 mm x 120 mm Petri dishes containing 0.5xMS medium (pH 5.8) solidified with 12 g L\(^{-1}\) of plant agar (Sigma A1296) with or without 2% sucrose (=day 4). Plates were scanned using a flatbed scanner at day 7, 9, 11 and 14 after transfer. Root phenotypes were analysed using ImageJ (Schneider *et al.* 2012). Statistical analysis was performed in SPSS (version 21).

**Yeast-two-hybrid assay**

Both full length AtCINV1 and AtCINV\(_{547A}\) were cloned into pGADT7 vectors (Clonetech). At14-3-3 in the pGBK7T (Clonetech) vector were provided by Prof. Dr. C. Oecking. Yeast two hybrid transformations were performed using the LiAc method. In short, PJ694A yeast strain was selectively grown overnight in Dropout supplement-Lys (MP Biomedicals) at 30°C, 220 rpm, after which a 1:500 dilution was used to grow yeast in YAPD medium o/n at 30°C, 220 rpm. An 0.5 ml aliquot per transformation was transferred into an eppendorf and supernatant was removed and 2.5 \(\mu\)l of pGADT7, 2.5 \(\mu\)l of pGADT7 and 5 \(\mu\)l of boiled and sonicated salmon sperm (Stratagene) were added and resuspended. Next, 100 \(\mu\)l 40% PEG-4000/0.2M LiAc was added and vortexed. Thereafter, samples were placed at 30°C, 220 rpm for 30 min followed by 15 min incubation at 42°C. Samples were plated on selective SD-DDO (−LW) medium and placed in a 30°C incubator for 3-5 days. Four colonies were resuspended in 50 \(\mu\)l MQ of which 5 \(\mu\)l was spotted on SD-DDO, SD-TDO (−LWH medium supplemented with 5 mM 3-AT) and
SD-QDO (-LHWA). Plates were incubated for 1-3 days after which viability was checked. At least three independent colonies had to show viability to consider interaction.

**Phosphorylated peptide separation by Reverse phase HPLC**

Phospho-peptide pCINV1Ser547 (\(^{13}KKVIKRASWPQL\)) and its parent dephosphorylated form dCINV1 Ser547 were synthesized by GL Biochem (Shanghai). Phosphorylation of peptide was performed by incubating 1 µM peptide for 1 h at 30°C together with 0.2 µM of recombinant kinase in the presence of 1 mM ATP in phosphorylation reaction buffer: 20 mM HEPES-KOH (pH 7.4), 20 mM MgCl\(_2\), 1 mM DTT, 25 mM β-glycerophosphate and 100 µM CaCl\(_2\), final volume 100 µl. For separation of the phosphorylated and non-phosphorylated peptides a Shimadzu HPLC with a C18 column (250×4.60 mm, 5 micron, Phenomenex) and a water/acetonitrile gradient from 0 to 10% for 5 min, from 10 to 40% for 30 min was used. The flow rate was 1ml/min and peptides were detected at 220 nm.

**Anisotropy**

Each sample contained 100 nM FAM-SWpTY (kind gift from Dr. M. Li, Baltimore, MD, USA), different concentrations of recombinant His-14-3-3 protein and various concentrations of the last 15 AA of phosphorylated (pCINV1) or non-phosphorylated (dCINV1) CINV1 peptide in a final volume of 200 µl (PBS). Samples were incubated for 30 min before their anisotropy was measured with the Cary Eclips fluorescence spectrophotometer (Varian). The phosphorylation of CINV1 peptide was performed by incubating 100 µM dCINV1 peptide for 2 hours at 30°C together with 0.2 µM CPK3 in presence of 1 mM ATP in a final volume of 100 µl, using phosphorylation reaction buffer (20 mM HEPES-KOH (pH 7.4), 20 mM MgCl\(_2\), 1mM DTT, 25 mM β-glycerophosphate and 100 µM CaCl\(_2\)). The reaction mixture was incubated for 30 min at RT and the anisotropy was measured with Cary Eclips fluorescence spectrophotometer (Varian, USA). All anisotropy values were corrected for background FAM-SWpTY.

**Protein expression and purification**

GST-CPK3 and GST-CPK21 were a kind gift from Dr. D. Geiger (Univ. Würzburg, Germany). Full length CINV1 and CINV1S\(^{547}\)A were cloned into the recombinant expression vector pGEX6 while full length Arabidopsis 14-3-3PHI and UPSILON were cloned into the N-terminal His-vector pRSETC. Both the GST- and His-vectors were transformed into the E. coli strain BL21 (DE3) cells. The transformed bacteria were then grown in LB-medium with ampicillin at 37°C to an optical density of 0.6-0.8 at 600 nm. Glutathione S-transferase (GST)-tagged protein expression was induced by adding 0.5 mM isopropylthio-β-galactoside (IPTG) and growth continued for 4-5 hours at 30°C. Cells transformed with the His-constructs were grown for 4-5 hours at 25°C after induction. GST-protein containing cells were harvested and crushed by French Press in 30 ml of phosphate-buffered saline (PBS) contained 1× protease inhibitor cocktail (Roche). GST-fusion proteins were subjected to column purification at RT using glutathione sepharose (GSTrap™ FF columns, 1 ml, GE healthcare). 50 mM Tris-HCl with 10 mM reduced glutathione was used for protein elution, then protein containing fractions were pooled and desalted with 50 mM HEPES; pH 8.0. Cells expressing the His-tagged proteins were harvested and lysed by French Press in 30 ml of binding/wash buffer (300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole, 1× protease inhibitor cocktail (Roche); pH 7.4). Lysates were centrifuged and the His-fusion protein was bound to Nickel sepharose (HisTrap™ HP columns, 1 ml, GE healthcare). The His-tagged protein was eluted with 300 mM imidazole in binding/wash buffer. Protein containing fractions were pooled and desalted with 50 mM HEPES (pH 8.0). Protein concentrations were determined by Bradford micro-assay (Bio-Rad) using BSA as a standard.

**In vitro pull-down assay**

50 µg of His-14-3-3 PHI was coated to 100 µl nickel bead suspension and extensively washed with binding buffer (300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole, pH 8). 10 µg of purified GST-CINV1 was incubated with GST-CPK3 (1µg) in phosphorylation buffer described above in a total reaction volume of 100 µl at 30°C for 1 hour. Following phosphorylation, the mixture was submitted to the 14-3-3 coated beads and incubated for 1 hour at RT. Beads were extensively washed (5 times, 1 ml for 1 min) with binding buffer. Bound proteins were eluted with 100 µl of 100 mM R18lys (14-3-3 non-interacting peptide, PHCVPRLDSWKLKANMCLP), followed by an R18 (14-3-3 interacting peptide, PHCVPRLDSWLDLEANMCLPP) elution. Beads were separated from supernatant by placing the tube into the Magnetic Stand. The eluted protein were separated by 10% SDS-PAGE, transferred to a PVDF
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14-3-3 pull-down using whole plant protein extract

Fully developed leaves and roots of 4-5 week-old plants were ground with mortar and pestal in liquid nitrogen and extracted with extract buffer; 50 mM HEPES-NaOH (pH 7), 10 mM MgCl₂, 1 mM Na₂EDTA, 2 mM DTT, 10% ethylene glycol, 0.02% Triton, 1× complete protease inhibitor (Roche) and 1× phosphoSTOP (Roche). Protein extracts were centrifuged twice at 20,000g for 15 min. After coating 50 µg of His-14-3-3 PHI to 100µl nickel beads and extensive washing with binding buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 8), 2 mg of protein extract was added to the empty beads and to the pre-coated His-14-3-3 PHI beads and incubated overnight at 4 °C. Beads were extensively washed (5 times, 1 ml for 1 min) with binding buffer. For competition experiments, beads were incubated for 20 minutes with 100 µl of 100 µM R18 or NIP peptide (ERYMGICMRKQNNFVPVCLRS) in 50mM HEPES-KOH (pH 8.0). Beads were separated from supernatant by placing the tube into the Magnetic Stand, and resuspended in 100 µl of 50 mM HEPES-KOH (pH 8.0). 20 µl supernatant and beads were used to measure alkaline invertase activity or to detect CINV1 by Western blot. Each experiment was repeated at least 2 times.

Total A/N invertase activity measurements in crude root extracts

Plant material was ground with liquid nitrogen after which 1:2 volumes of extraction buffer was added (50 mM HEPES-NaOH (pH7), 10 mM MgCl₂, 1 mM Na₂EDTA, 2 mM DTT, 10% ethylene glycol, 0.02% Triton, complete protease inhibitor (Roche) and PhosSTOP (Roche) according to manufactures guidelines. After two times centrifugation (15 min, max, 4°C), supernatant was transferred to Macrocon tubes (3 kD, Millipore) to concentrate the sample and the buffer was exchanged to 50 mM HEPES-NaOH pH 8.0, 1 mM Na₂EDTA. Protein concentration was measured using Bradford. For competition experiments, samples were incubated for 10 minutes with 50 µg of total protein was incubated with different concentrations of sucrose at 30° C for 15 min, where after proteins were denatured by boiling for 10 minutes. Samples were centrifuged max for 1 min and glucose measuring buffer was added to the supernatant (50 mM HEPES-NaOH (pH7.0), 2 mM MgCl₂, 1 mM Na₂EDTA, 1 mM ATP, 1 mM DTT, 0.4 mM NADP, 2U G-6-P, 4.2U hexokinase). The samples were incubated at RT for 15 minutes after which the glucose concentration was measured at OD340 nm.

Metabolomics of 14-3-3 quadruple mutants and WT

Plants were grown in half stress Hoagland (3 mM KNO₃, 2 mM Ca(NO₃)₂, 1 mM NH₄H₂PO₄, 20 µM Fe-EDTA, 0.5 mM MgSO₄, 1 µM KCl, 25 µM H₂BO₃, 2 µM MnSO₄, 2 µM ZnSO₄, 0.1 µM CuSO₄, 0.1 µM (NH₄)₂MoO₄₇) in a growth chamber at 14/10h day/night regime, 22/18°C day/night temperature and a photon flux density of 170 µmol.m⁻².s⁻¹. Fully developed roots of 22 day-old plants were harvested for metabolite extraction. Metabolite profiling by GC-time of flight (TOF)-MS was performed as described previously (Lisec et al. 2006; Erban et al. 2007). Around 50 mg of frozen ground material was homogenized in 300 µL of methanol at 70°C for 15 min and then 200 µL of chloroform at 37°C for 5 min. The polar fraction was prepared by liquid partitioning into 400 µL of water. The polar fraction was derivatized by methoxyamination and subsequent trimethylsilylation. Samples were analysed using GC-TOF-MS (ChromaTOF software, Pegasus driver 1.61; LECO). The chromatograms and mass spectra were evaluated using TagFinder software (Luedemann et al. 2008) and NIST05 software (http://www.nist.gov/srd/mlst.cfm). Metabolite identification was manually supervised using the mass spectral and retention index collection of the Golm Metabolome Database (Kopka et al. 2005; Hummel et al. 2010). Peak heights of the mass fragments were normalized on the basis of the fresh weight of the sample and the added amount of an internal standard ([¹³C₆]-sorbitol) (Watanabe et al. 2013).

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References


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