TWO β-AMYLASE- DERIVED TRANSCRIPTION FACTORS (BAM7 AND BAM8) INTERACT WITH OTHER SIGNALLING PATHWAYS

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Abstract

Plants are sessile organism, which obliges them to have sophisticated mechanisms of adaptation and tolerance to environmental conditions such as light, temperature, predators or drought. To be able to respond appropriately, the prevalent conditions must be sensed accurately, requiring specific sensing and signalling mechanisms. Transcription factors have the role to transduce and amplify a given (sensed) input signal. This implies that they perceive an upstream signal, either derived from a sensory unit (e.g. the light receptor phytochrome) or an upstream intermediary of a signal transduction pathway. Alternatively, the transcription factor might be the sensor itself. Two closely related transcription factors, BAM7 and BAM8, which belong to the family of starch degrading enzymes (β-amylases, BAMs), were suspected to have such sensory properties. In this study, their proposed role as sugar sensors was investigated along with the integration of their activity into light- and sugar-response pathways.

BAM7 and BAM8 differ from the starch-degrading family members (BAMs) since they cannot catalyse the hydrolysis of maltose from linear glucan chains. Nevertheless, the pseudo-enzymatic domain - the BAM-domain - is largely conserved. What makes BAM7 and BAM8 unique compared to the other BAMs is the additional transcription factor domain, which was acquired early during land plant evolution. Due to its homology to the brassinosteroid-responsive BZR1-type transcription factors, the N-terminal transcription factor domain is also called the BZR1-domain and collectively gives BAM7, BAM8 and their homologs in other species the name BZR1-BAMs. The BZR1-domain confers the ability to bind to DNA, with a preference for an 8-base-pair motif termed the BZR1-BAM Responsive Element (BBRE). BAM8 was shown to be a transcriptional activator, although no such activity could be demonstrated for BAM7. Nevertheless, genome-wide transcriptional changes and phenotypic characterisations of single-, double-mutant and wild-type plants suggest a redundant or supporting role of BAM7 and BAM8. A long-standing speculation is that BRZ1-BAMs can sense a sugar via the BAM-domain, which subsequently regulates their transcription factor activity. Since the BBRE contains a known light-regulated motif (the G-box) and a Brassinosteroid Responsive Element (the BRRE), an additional proposition is that BZR1-BAM-signalling coordinates with brassinosteroid- and light-signalling pathways through common target genes and/or via protein-protein interactions with light-signal transducing proteins.

At the start of my PhD, experimental evidence for a direct interaction of BZR1-BAMs with a sugar-ligand was missing. Furthermore, despite good evidence for an involvement of BZR1-BAMs in brassinosteroid signalling, there was no direct experimental data regarding an involvement of BZR1-BAMs in light signalling.

In this study, I aimed to identify the as-yet elusive sugar ligand and investigate its impact on BZR1-BAM’s activity. The acquired results identified trehalose 6-phosphate (Tre6P) as the most promising sugar
ligand of the BZR1-BAMs. Using Microscale Thermophoresis, I was able to show that Tre6P binds to BZR1-BAMs and, using Surface Plasmon Resonance, show that Tre6P reduces BZR1-BAMs affinity for the BBRE and lowers its DNA binding stability. Intriguingly, in vivo experiments with modified Tre6P concentrations indicated that reduced levels of Tre6P lower BAM8 activity, which was counterintuitive given its effect on DNA binding. Elevated Tre6P did not influence BAM8 activity in moderate to high light but enhanced BAM8 activity in low light. Comparisons of two already existing data sets comprising genome-wide transcriptional changes triggered by either BAM8 deregulation or elevated Tre6P provided preliminary evidence that there is a considerable overlap between BAM8 and Tre6P regulated genes and they predominantly respond in the same direction (up or down). Two other potential sugar ligands, sucrose 6-phosphate (Suc6P) and glucose 6-phosphate (Glc6P) were identified. However, their binding and effect on BRZ1-BAM was weaker and the significance of their interaction in vivo was not assessed.

To specify the BZR1-BAMs’ location of action in planta, hence their physiological role, the tissue specific expression patterns and subcellular localisation of BAM7 and BAM8 were rigorously investigated. BZR1-BAMs’s expression and protein localisation was mainly found in the nuclei of cells in developing tissue and substantiated the results of previous studies.

The possible involvement of BZR1-BAMs in light-signalling was addressed by looking for physiological evidence, such as altered response of plants with aberrant BZR1-BAM-levels (single-, double-mutant and overexpressing lines) to different light regimes. It was seen that ectopic expression of BAM8 (BAM8-ox) leads to stunted growth at the seedling stage in light but not in dark conditions. This reduced hypocotyl elongation was particularly evident upon shade treatment, to which BAM8-ox plants failed to respond. Transcriptional changes upon extended shade treatment supported the physiological responses, however the short-term transcriptional response to shade was not influenced by BZR1-BAMs. Protein-protein interaction studies with BZR1-BAMs and previously-identified putative interaction partners involved in light- or sugar-signalling were not conclusive and the mechanisms involved remain to be revealed.

In conclusion, I generated first experimental evidence that supports a role of the BZR1-BAMs as sugar sensors. Tre6P was seen to directly interact with BAM8 and to modulate its activity as a transcriptional activator. Convergence of BZR1-BAM-signalling with light-signalling pathways was supported by physiological experiments and long-term transcriptional changes of two shade-responsive genes. Yet molecular mechanisms for this interplay remain unclear. It is most plausible that the molecular interaction occurs indirectly via brassinosteroid-mediated signalling.
Zusammenfassung


BAM7 und BAM8 unterscheiden sich von den anderen stärkeabbauenden Familienmitgliedern dadurch, dass sie nicht katalytisch aktiv sind und daher lineare Zuckerketten der Stärke nicht zu Maltose hydrolysieren können. Interessanterweise ist die pseudo-enzymatische Domäne (auch BAM-domäne genannt) grösstenteils konserviert. Was BAM7 und BAM8 von den anderen BAMs unterscheidet und einzigartig macht, ist die N-terminale Transkriptionsfaktorendomäne, die sie im Laufe der erworben haben. Durch die Ähnlichkeit dieser Domäne zu den brassinosteroidregulierten Transkriptionsfaktoren des BZR1-types, wird sie auch BZR1-domäne genannt und gibt BAM7, BAM8 und ihren Homologen in anderen Pflanzenspezies die Bezeichnung der BZR1-BAMs. Dank der BZR1-Domäne können die BZR1-BAMs an DNA binden, wobei sie eine Präferenz für ein bestimmtes 8-nukleotid-langes Motiv haben, welches BBRE (steht für BZR1-BAM Responsive Element) genannt wird. Für BAM8 konnte eine Rolle als transkriptioneller Aktivator gezeigt werden, wohingegen BAM7s Rolle unbekannt ist. Anhand von genom-weiten transkriptionellen Veränderungen und den Phänotypen von einfach-, doppel-mutanten und wild-typ Pflanzen, werden redundante oder unterstützende Funktionen der BZR1-BAMs vermutet. Seit lange wurde spekuliert, dass die BZR1-BAMs Zucker via die BAM-domäne wahrnehmen könnten und dies ihre Aktivität als Transkriptionsfaktoren reguliert. Da das bevorzugte DNA-Bindemotiv (BBRE) auch ein bekanntes lichtreguliertes Motiv (die G-box) und ein Brassinosteroid Reguliertes Element (BRRE) beinhaltet, wurde vorgeschlagen, dass die Koordination des BZR1-BAM-, Licht- und Brassinosteroid-signalling durch gemeinsame Zielgene und / oder via Protein-Protein Interaktion mit Lichtsignalproteinen stattfindet.

Zu Beginn meiner Forschungsarbeit fehlte noch jeglicher experimentelle Nachweis, dass die BZR1-BAMs mit einem Zuckerliganden direkt interagieren können. Trotz guten Hinweisen, dass die BZR1-BAMs im
Brassinosteroid-signalling teilnehmen, bestanden keine konkreten Daten einer möglichen Teilnahme der BZR1-BAMs im Licht-signalling.

Das Ziel meiner Forschungsarbeit war es, den bis anhin unbekannten Zuckerliganden zu identifizieren und zu untersuchen, inwiefern dieser die Aktivität der BZR1-BAMs beeinflusst. Meine Resultate haben Trehalose 6-phosphat (Tre6P) als vielversprechendsten Liganden identifiziert. Mittels Microscale Thermophoresis konnte ich zeigen, dass Tre6P an die BZR1-BAMs bindet. Surface Plasmon Resonance ergab, dass Tre6P die Affinität der BZR1-BAMs für das BBRE-Motiv reduziert und die Bindungsstärke der BZR1-BAMs an die DNA verringert. Die Aktivität von BAM8 in vivo unter niedrigen Tre6P Konzentrationen geringer, was angesichts des Effekts auf die Bindungsstärke an DNA konter intuitiv ist. Bei erhöhten Tre6P Werten war die Aktivität von BAM8 unverändert (bei hohen Lichtintensitäten) oder erhöht (bei niedrigen Lichtintensitäten). Erste Hinweise, dass auch genomweit eine beträchtliche Anzahl an Genen durch BAM8 und Tre6P gemeinsam reguliert werden, stammte von einem Vergleich von zwei existierenden Datensätzen. Diese beinhalteten genomweite transkriptionelle Veränderung welche entweder durch BAM8 oder erhöhte Tre6P ausgelöst wurden. Zwei andere potentiellen Zuckerliganden, Saccharose 6-phosphate (Suc6P) und Glukose 6-phosphate (Glc6P), wurden identifiziert. Für ihre Interaktion mit BAM8 konnte bisher in vivo keine Funktion zugeschrieben werden.

Ein weiteres Ziel bestand darin, den Wirkungsort und damit die physiologische Rolle der BZR1-BAMs in planta genauer zu untersuchen. Es wurde die gewebespezifische Expression und die subzelluläre Lokalisierung von BAM7 und BAM8 betrachtet. Expressions-analysen und (Protein-)Lokalisierung haben ergeben, dass die BZR1-BAMs sich vornehmlich in den Zellkernen von sich entwickelndem Gewebe befinden. Dies bestätigte Ergebnisse von vorgängigen Untersuchungen.


Zusammenfassend kann gesagt werden, dass es mir gelungen ist, erstmals experimentelle Indizien zu schaffen, die die Hypothese einer Rolle der BZR1-BAMs als Zuckersensoren unterstützen. Das
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<td>2deoxy-Glc</td>
<td>2-deoxy-glucose</td>
</tr>
<tr>
<td>2deoxy-Glc6P</td>
<td>2-deoxy-glucose 6-phosphate</td>
</tr>
<tr>
<td>6deoxyGlc</td>
<td>6-deoxy-glucose</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
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<tr>
<td>AMY</td>
<td>Alpha-amylase</td>
</tr>
<tr>
<td>AS</td>
<td>Alternative splicing</td>
</tr>
<tr>
<td>AU</td>
<td>Air Unit</td>
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<td>B</td>
<td>Blue light</td>
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<td>BAM</td>
<td>Beta-amylase</td>
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<td>BBRE</td>
<td>BZR1-BAM Responsive Element</td>
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<tr>
<td>BE</td>
<td>BRANCHING ENZYME</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic Helix Loop Helix</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular Fluorescence Complementation</td>
</tr>
<tr>
<td>BR</td>
<td>Brassinosteroid</td>
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<td>BRRE</td>
<td>Brassinosteroid Responsive Element</td>
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<td>BRZ</td>
<td>Brassinosole</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>BUP</td>
<td>BZR1-BAM UPREGULATED</td>
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<td>Basic leucine zipper</td>
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<td>ds-BBRE</td>
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<td>ds-mBBRE</td>
<td>Double stranded mBBRE</td>
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<td>Far Red light</td>
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<td>FW</td>
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<td>G</td>
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<td>GA</td>
<td>Gibberellic Acid</td>
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<td>Genomic DNA</td>
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<tr>
<td>GeBPL</td>
<td>GLABROUS1 ENHANCER BINDING PROTEIN</td>
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<td>ISOAMYLASE1</td>
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<td>MALTOSE TRANSPORTER</td>
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<td>Microscale thermophoresis</td>
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<td>Nuclear Localisation Signal</td>
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<td>Shade Avoidance Syndrome</td>
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<td>Surface Plasmon Resonance</td>
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<td>Single stranded oligo AT</td>
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<td>TREHALOSE 6-PHOSPHATE PHOSPHATASE</td>
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<td>C-terminal half of YFP</td>
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<td>YFPn</td>
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1 Introduction

1.1 Starch metabolism

Starch is the main carbon storage compound of plants. It occurs in photosynthetic tissue to fuel energy demands during the dark period or unfavourable light conditions and, depending on the plant species, also in storage organs (e.g. potato tubers, cassava storage roots or wheat grains). Starch is composed of two main constituents, amylose and amylopectin, which both almost entirely consist of glucose (Glc) units. Glc units are covalently linked by α-1,4 or α-1,6 glycosidic bonds. Amylopectin has a tree-like structure with long linear chains, which are connected to shorter linear chains by α-1,6 branch points. Neighbouring linear chain segments can intertwine and form double helices that pack in a regular fashion, giving rise to the crystalline layers (lamellae) (Zeeman, Kossmann et al. 2010). Amylose consists of predominantly linear α-1,4 linked glucan chains with few or no branch points. It is synthesised within the framework of amylopectin. Synthesis and degradation of starch are well orchestrated processes and have been widely studied. Enzymes involved in starch synthesis are the starch synthases (SS), branching enzymes (BEs) and debranching enzymes (DBEs) (Zeeman, Smith et al. 2007). Starch degradation is initiated by starch phosphorylation by di-kinases (α-glucan, water dikinase, GWD; phosphoglucan, water dikinase, PWD) which subsequently allows degradation by α-, β-amyloses and DBEs. Furthermore, phosphatases, DBEs and disproportionation enzymes (DPEs) facilitate the complete degradation of the glucans down to exportable neutral sugars such as Glc and maltose (Mal) (Zeeman, Smith et al. 2007) (Fig. 1.1.1). As a precaution of running out of carbon reserves, plants have evolved mechanisms to adapt starch metabolism to environmental conditions (e.g. day length, light intensity and quality, temperature, biotic and abiotic stress, sugar status). Some mechanisms have been described, but more work is required to understand the control of starch metabolism (Streb and Zeeman 2012).

![Figure 1.1.1: Starch synthesis and degradation in Arabidopsis. Overview over components involved in transitory starch degradation in Arabidopsis. Starch gets phosphorylated by two kinases (GWD and PWD) to enhance accessibility for the degrading enzymes. The α- and β-amyloses hydrolyse the α-1,4 glycosidic-bonds, whereas the branch-points (α-1,6 glycosidic-bonds) are cleaved by DBEs (ISA3, LDA). The disproportionating enzyme (DPE1) recycles maltotriose to glucose and maltoheptaose, the latter of which is again substrate for β-amyloses. Maltose and glucose exit the chloroplast. The thickness of the arrows indicates the estimates of the respective fluxes. Adapted from Zeeman, Smith et al. 2007.](image-url)
1.2 The beta-amylase family

Beta-amylases (BAMs) constitute the main hydrolytic activity on linear glucan chains during transient starch degradation, whereas α-amylases are known to be the main drivers of other starch type degradation (e.g. cereal seed starch) (Wu, Ral et al. 2014). Alpha-amylases (AMYs), are endo-amylases acting on internal glycosidic-bonds, whereas β-amylases are exo-amylases that act on glucans from the non-reducing end of the chain. In photosynthetic tissue, Mal released by β-amylases is exported from the chloroplast to the cytosol and is subsequently available for further metabolism (Stitt and Zeeman 2012). Interestingly, it was not always evident that β-amylases are indeed important in starch degradation; early reports from Arabidopsis showed that the majority (80-90%) of β-amylase activity is localized outside the chloroplast; i.e. away from the starch. This extra-plastidial activity was assigned to the Arabidopsis BAM5 locus (Lin, Spilatro et al. 1988, Laby, Kim et al. 2001). The absence of BAM5 (bam5 mutants) did not affect leaf starch levels, further arguing against a role in starch degradation (Laby, Kim et al. 2001). Further investigations suggested that BAM5 localizes to phloem sieve elements (Wang, Monroe et al. 1995). In other species (pea, wheat and maize) β-amylases were reported to localize to the vacuole (Ziegler and Beck 1986, Datta, Selvi et al. 1999). The first convincing evidence for a key role in transitory starch degradation was presented by a study using an antisense construct against the chloroplastic β-amylase in potato. Plants with reduced β-amylase activity had a starch excess (Scheidig, Frohlich et al. 2002), which was later confirmed in Arabidopsis mutants (bam3 and others) (Kaplan and Guy 2005, Fulton, Stettler et al. 2008). Plasticity of starch metabolism in response to endogenous and environmental stimuli were shown in early studies investigating expressional and activity changes of β-amylase in different conditions. Those studies indicated that β-amylase expression and activity are regulated by various factors such as circadian clock (Chandler, Dalley et al. 2001), light (Sharma and Schopfer 1982), sugars (Mita, Suzuki-Fujii et al. 1995), water and salt stress (Datta, Selvi et al. 1999, Thalmann, Pazmino et al. 2016), cold stress (Kaplan and Guy 2004) or phytohormones (Wang, Lue et al. 1996). However, these studies were conducted in many different plant species (rice, maize, mustard) and were not all assigned to an Arabidopsis homologue, which makes it difficult to assign specific roles of different β-amylase isoforms in that model organism.

1.2.1 Members of the beta-amylase family in Arabidopsis

In Arabidopsis, the family of BAMs is composed of nine members, which can be grouped into four sub-families according to their sequence homology (Smith, Fulton et al. 2004, Fulton, Stettler et al. 2008). The subfamily I contains BAM5 and BAM6, which are likely paralogs after a recent segment duplication of the genome. Subfamily II is composed of BAM1 and BAM3 whereas BAM4 and BAM9 belong to subfamily III. BAM8, BAM7 and BAM2 all group into the subfamily IV, whereof BAM7 and BAM2 are
likely paralogs. Within the subfamilies, the exon structures are conserved, except for subfamily III; BAM4 and BAM9 are dissimilar from each other and from all other BAMs (Fig. 1.2.1). As mentioned before, not all BAMs are chloroplastic, suggesting that they fulfil additional roles other than starch degradation. According to in silico predictions of chloroplastic transit peptides, BAM1, BAM2, BAM3 and potentially BAM4, BAM8 are targeted to the chloroplast. In contrast BAM5, BAM6, BAM7, BAM9 were proposed to be extra-plastidial (Fulton, Stettler et al. 2008). For BAM1, BAM2, BAM3, BAM4 chloroplastic localisation was confirmed experimentally (Lao, Schoneveld et al. 1999, Sparla, Costa et al. 2006, Fulton, Stettler et al. 2008). Against the in silico prediction, a later study also found BAM6 and BAM9 to be located to the chloroplast (S.-K. Lee, M. Stettler unpublished). In vitro experiments showed BAM activity on amylopectin substrate for BAM1, BAM3 and low levels for BAM2, whereas BAM4 showed no hydrolytic activity (Fulton, Stettler et al. 2008). Additionally, BAM5 and BAM6 also showed catalytic activity (Laby, Kim et al. 2001, S.-K. Lee, M. Stettler unpublished). Further investigations on specific isoforms of the BAM family confirmed the heterogeneity of this family in respect to their functions. To date, the role of BAM3 as the major isoform responsible for leaf starch degradation at night is widely accepted. Out of all Arabidopsis BAM single mutants, bam3 has the strongest starch excess phenotype, which is further pronounced upon absence of BAM1 (in the double mutant bam1 bam3). Interestingly, even though most likely catalytically inactive, BAM4 also contributes to starch degradation. Its loss causes a starch excess phenotype and it amplifies the starch excess of bam1 bam3 in the triple mutant (bam1 bam3 bam4) that has an even higher starch accumulation than bam1 bam3. This proposes BAM4 to act as a regulator, possibly by sensing Mal and generating a feedback inhibition on starch degradation (Fulton, Stettler et al. 2008). The role of BAM1 has been widely studied, too. Besides its contribution to leaf starch degradation it was found more recently to play an important role in starch degradation in guard cells. BAM1 expression is elevated in guard-cell-enriched epidermal peels relative to the leaf mesophyll (Horrer, Flutsch et al. 2016). Its activity during the day is activated post-translationally by redox-regulation. Consequently, bam1 mutants have increased starch in illuminated guard cells and a reduced stomatal opening capacity compared to the wild-type, which renders bam1 plants more drought tolerant (Valerio, Costa et al. 2011, Prasch, Ott et al. 2015, Horrer, Flutsch et al. 2016). Besides the role in stomata opening, BAM1’s expression is also induced in mesophyll cells upon drought stress, most likely functioning in fuelling the production of drought-stress protectants (proline, soluble sugars) (Sparla, Costa et al. 2006, Valerio, Costa et al. 2011, Zanella, Borghi et al. 2016, Thalmann, Pazmino et al. 2016). BAM2 is less well characterised. Despite its low enzymatic activity and chloroplastic localisation, no function has been attributed to date. Loss of BAM2 in addition to BAM1 and BAM3 did not further enhance the bam1 bam3 starch excess phenotype (Fulton, Stettler et al. 2008). As mentioned before, the best characterised extra-plastidial BAM is BAM5, which localises to
the sieve elements and contributes to 80-90 % of total exo-amylase activity. Interestingly, it is induced by soluble sugars (Caspar, Lin et al. 1989, Mita, Suzuki-Fujii et al. 1995), which led to the proposed role to prevent the build-up of polymerised polysaccharides in sieve tubes under conditions of elevated sugars. However, to date there is no experimental evidence that BAM5 is required for normal rates of phloem transport (Laby, Kim et al. 2001). BAM6 and BAM9 are only scarcely characterised so far. BAM6’s physiological role is totally unclear. The loss of BAM9 in the bam4 background (bam4 bam9 double mutant) showed an amplification of the bam4 phenotype in respect to enhanced starch levels (M. Stettler, S.-K. Lee unpublished). Interestingly, BAM9’s expression is induced by two components involved in sugar-signalling, KIN10 and bZIP11 (for more details see section 1.3.1, 1.3.5), suggesting an involvement in carbon-stress signalling (Delatte, Sedijani et al. 2011). BAM7 and BAM8 differ from the other BAMs (also the closest homologue BAM2) by having an additional N-terminal domain. This two-domain structure of was identified in almost all (32 out of 33) vascular plants but absent in mosses and algae (Reinhold, Soyk et al. 2011). Furthermore, they were shown to be catalytically inactive but active as transcription factors (Reinhold, Soyk et al. 2011). These two BAMs are of particular interest for this study and are described in greater detail in the following section.

1.2.2 Structural features enabling hydrolysis reaction performed by BAMs

Structural evaluation on soybean and sweet potato BAMs revealed details about their catalytic mechanism, identifying the important amino acid residues involved (Mikami, Degano et al. 1994, Cheong, Eom et al. 1995). In soybean, it was shown that two highly conserved glutamates (E186 and E380) are crucial for catalysis by providing a proton to the glycosidic oxygen of the substrate (E186) and by activating the attacking water molecule (E380). Mutations in these two glutamates (E186Q, E380Q) revealed a great reduction in catalytic activity by 16,000- and 37,000-fold, respectively (Kang, Adachi et al. 2004). Crystallisation of the wild-type protein in presence (bound) or absence (unbound) of the product, Mal, revealed that the major structural differences between the two states are found in two
regions: the flexible loop (amino acids 96-103) and the inner loop (amino acids 340-346) (Mikami, Degano et al. 1994, Kang, Tanabe et al. 2005). In contrast, positions E186 and E380 remained unchanged upon Mal binding (Mikami, Degano et al. 1994). Both, flexible and inner loops, move closely toward the maltose molecules in the bound state (Kang, Tanabe et al. 2005). The importance of the translocation of the two loop regions for catalysis was revealed by mutations in the inner loop (T342V, T342S, T342A), which sowed a reduction in catalytic activity by approximately 13-fold compared to the wild-type protein (Kang, Tanabe et al. 2005).

1.2.3 BZR1-BAMs’ structure and function

BAM7 and BAM8, also called BZR1-BAMs, belong to the family of β-amylases (as mentioned in section 1.2.1) due to the presence of their (catalytically inactive) β-amylase domain (BAM-domain). However, they differ from all the other BAMs found in Arabidopsis by their N-terminal extension, defined as the BZR1-domain (Fig. 1.2.2-A). The BZR1-domain shows homology to the brassinosteroid-responsive (BZR1-type) transcription factors (Reinhold, Soyk et al. 2011). Comparisons between the protein sequence of BAM7 and BAM8 show that the biggest differences are found in the N-terminal part, upstream of the DNA-binding domain and in stretches of the BAM-domain. They have in common that both BAM-domains lack conservation of the inner loop. The outer (flexible) loop is poorly conserved in BAM8 only (Mikami, Degano et al. 1994, Soyk, Simkova et al. 2014). In Arabidopsis BZR1-BAMs, the previously mentioned glutamates important for catalysis (E186, E380 according to soybean nomenclature, corresponding to E422, E618 in BAM7 and E429, E623 in BAM8) are present (Soyk, Simkova et al. 2014) (Fig. 1.2.2-B). Nevertheless, comparisons including BZR1-BAMs of other plant species showed that while E186 is conserved throughout, E380 is substituted by other amino acids in some BAM7 and BAM8 isoforms (Soyk, Simkova et al. 2014). Furthermore, in silico alignments of the BAM8 to the solved structure of the enzymatically active soybean BAM (GmBMY1) revealed that the surface charge of the catalytic cleft of BAM8 is overall less electro-negative than GmBMY1, which would be expected to impact substrate binding and catalytic activity (Kang, Adachi et al. 2004, Soyk, Simkova et al. 2014). Measurements of the β-amylase activity of heterologously expressed BAM7 and BAM8 protein (in E. coli) suggested that they are catalytically inactive (Reinhold, Soyk et al. 2011, Soyk, Simkova et al. 2014).

Due to their relatively high degree in similarity to active BAMs, binding of an alternative carbohydrate other than linear glucan chains (e.g. malto-oligosaccharides) as a ligand was hypothesised. The prime candidate was Mal, but other soluble sugars were not excluded (Soyk, Simkova et al. 2014, Reinhold PhD Thesis). Acquisition of the N-terminal extension during evolution equipped BAM7 and BAM8 with additional putative features such as the abilities for dimerization, nuclear localisation and DNA-binding. The BZR1-domain of both proteins contains a putative bipartite nuclear localisation signal (NLS)
(Dingwall and Laskey 1991, Reinhold, Soyk et al. 2011), which is present within the basic domain of the basic helix-loop-helix-like motif (bHLH-like) that is also found in the BZR1-domain (Fig. 1.2.2-B). It was shown that the NLS is indeed required for nuclear localisation of BAM7 and BAM8. Amino acid substitutions within the conserved basic region of the NLS of BAM8 led to a cytosolic localisation when transiently expressed in tobacco leaves (Reinhold, Soyk et al. 2011).

DNA-binding ability and the nucleotide sequence of the preferred binding motif was determined by a random binding site selection (RBSS) experiment. The preferred binding motif was determined as an eight-letter motif (CACGTGTG), subsequently called BZR1-BAM Responsive Element (BBRE) (Fig. 1.2.2-C). Interestingly, the BBRE comprises two well-known target motifs; the brassinosteroid Responsive Element (BRRE) and the G-box (Reinhold, Soyk et al. 2011), which led to speculations about an involvement of BZR1-BAMs in the light- and hormone-signalling network (Soyk, Simkova et al. 2014). Amino acid substitution of the conserved E13 (equivalent in BAM7, E83; in BAM8, E100), known to be essential for DNA-binding of bHLH proteins, led to a great reduction in binding to the BBRE by BAM7 and BAM8 measured by DNA-Protein-Interaction Enzyme-Linked Immunosorbent Assay (DPI-ELISA) (Toledo-Ortiz, Huq et al. 2003, Brand, Kirchler et al. 2010, Soyk, Simkova et al. 2014). Besides DNA-binding, the bHLH(-like) domains are known to participate in dimerisation (Toledo-Ortiz, Huq et al. 2003). The two brassinosteroid-responsive transcription factors (BRASSINAZOLE-RESISTANT1, BZR1; BRI1-EMS-SUPPRESSOR 1, BES1) also belong to the bHLH-like proteins and for BES1, heterodimerization with the BES1-INTERACTING MYC-LIKE 1 (BIM1) was shown (Yin, Vafeados et al. 2005).

Indeed, BZR1-BAMs showed homo- and heterodimerisation with each other by Bimolecular Fluorescent Complementation (BiFC) (Soyk, Simkova et al. 2014). Furthermore, there was interaction of BAM7 and BAM8 with 14-3-3 proteins seen by co-immunoprecipitation, which for BAM8 was confirmed by BiFC and Microscale Thermophoresis (MST) (Soyk PhD Thesis).

**Figure 1.2.2: BZR1-BAMs in Arabidopsis.** (A) Schematic protein structure representation of BZR1-BAMs (BAM7, BAM8) compared to the enzymatically active BAM isoform (BAM3) and the BR-responsive transcription factor (BZR1). Light green, BZR1-domain; dark green, chloroplast transit peptide (cTP); blue, BAM-domain. (B) Different domains and motifs found in the BZR1-BAMs. Conserved residues important for catalysis (E186, E380), DNA binding (E13, E16) and dimerization (L27, hydrophobic residue) are indicated. Nomenclature according to Toledo-Ortiz, Huq et al. 2003. Bipartite nuclear localisation signal, NLS; basic helix loop helix-like domain, bHLH-like. (C) The preferred DNA binding motif BZR1-BAM Responsive Element (BBRE) contains the G-box and the Brassinosteroid Responsive Element (BRRE). Size of the letters (and percentages given below each letter) indicate the conservation of the respective nucleotide at the given position. Adapted from Reinhold, Soyk et al. 2011 (C).
1.2.4 BZR1-BAMs are (active) transcription factors

The *in vitro* DNA-binding, dimerization property and nuclear localisation of the BZR1-BAMs described above suggested their role as transcription factor. Indeed, *in vivo* and *in planta* experiments confirmed this hypothesis. Transient expression of BAM8 in mesophyll protoplasts showed an activation of the BBRE driven expression of the reporter gene, suggesting that BAM8 is a transcriptional activator under these conditions. In contrast, the presence of BAM7 did not result in reporter expression different to the no-effector control, while co-expression of BAM7 and BAM8 reduced transcriptional activation compared to BAM8 alone. This could be explained by a repressive role of BAM7 or by the competition of transcriptionally inactive BAM7 and transcriptionally active BAM8 for the BBRE motif (Soyk, Simkova et al. 2014). Possibly, BAM7 is not strictly inactive but its activity may rely on the presence of co-activating factors, which are limiting in this assay. Somewhat contradicting results, arguing for a redundant rather than differing roles for BAM7 and BAM8, were obtained from Arabidopsis knock out and stable overexpressor plants (*bam7.1, bam8.1, bam7 bam8.1, BAM7-ox, BAM8-ox*). In respect to the morphology of 4-weeks old Arabidopsis plants, *bam7.1, bam8.1* and *BAM7-ox* were indistinguishable from the wild-type. However, the *bam7 bam8* double mutant and *BAM8-ox* showed altered phenotypes; *bam7 bam8* plants had elongated petioles but wild-type like leaf shape, whereas *BAM8-ox* plants showed a stunted growth (petioles) with hyponastic leaves (Fig. 1.2.3) (Reinhold, Soyk et al. 2011). These findings were supported by genome-wide transcriptional profiling analysis. Only the absence of both BZR1-BAMs (*bam7 bam8*) or overexpression of BAM8 (*BAM8-ox*) led to significant transcriptional changes compared to the wild-type. Within the 1,000 bp promoter sequences of the 500 most up- and down-regulated genes, the BBRE motif was significantly overrepresented in the up-regulated genes in *BAM8-ox* and overrepresented in down-regulated genes in *bam7 bam8*, supporting the notion of redundant transcriptional activation activity (Reinhold, Soyk et al. 2011).
1.2.5 Differing functions of BAM7 and BAM8

Despite the high sequence similarity of BAM7 and BAM8, they possess different properties. Whereas BAM7 binds to DNA with low affinity and does not activate gene expression, BAM8 binds with high affinity and does activate expression. Further investigations by Soyk, Simkova et al. 2014 addressed these differences more in depth by generating different variants of BZR1-BAMs and evaluating DNA-binding, transcription factor activity on the BBRE in protoplasts, and the impact on genome-wide transcriptional output. The implication of DNA-binding on their specific behaviour as transcription factors was studied using plants overexpressing protein versions impaired in DNA-binding (BAM7-dna, BAM8-dna) or versions truncated to just the N-terminal part of the protein alone (BAM7-N, BAM8-N) in the respective single mutant background (Fig. 1.2.4). Impaired DNA-binding was achieved by mutating the conserved E13 (E83D in BAM7, E100D in BAM8), which led to reduced activity of BAM8-dna and a transcriptional profile similar to the bam8.1 mutant. BAM7-dna did not show any activity but interestingly had a transcriptional profile resembling bam7 bam8. It was suggested that BAM7 sequesters BAM8 away from the promoter and leads to reduced BAM8 DNA-binding and activity. Deleting the BAM-domains from the BZR1-BAMs (BAM7-N, BAM8-N) reduced the protein’s DNA-binding specificity; the set of deregulated target genes was much larger and broader than in BAM7-ox and BAM8-ox. Furthermore, the two N-terminal parts seemed to resemble each other in function; both plants (BAM7-N, BAM8-N) were stunted and showed similar transcriptional changes compared to the wild-type. Hence, the BAM-domain has a pivotal impact on transcriptional regulation, which supports the hypothesis of BZR1-BAMs function as transcriptional integrator of metabolic cues. To characterise this aspect in greater detail, chimeric constructs of BAM7 N-terminus fused to the BAM8 BAM-domain (BAM78) and vice versa (BAM87) were generated (Fig. 1.2.4). It became clear that the two BAM-domains conferred different properties to the proteins. Whereas the BAM-domain of BAM8 allowed transcriptional activation and DNA-binding, the BAM-domain of BAM7 reduced transcriptional activation and DNA-binding. Additionally, it was shown that the BAM-domain’s impact on transcription is independent from catalytic activity. To ensure that no putatively remaining catalytic activity was left, an amino acid exchange corresponding to the conserved E186 (previously shown to be crucial for hydrolytic activity in active BAMs, see section 1.2.2) to a glutamine (Q) was introduced. The overexpression of the resulting BAM7-cat (E422Q) and BAM8-cat (E429Q) (Fig. 1.2.4) led to expression profiles closely resembling those of BAM7-ox and BAM8-ox, respectively (Soyk, Simkova et al. 2014). Strikingly, the interference at the putative ligand binding site within the BAM-domain rendered the protein non-functional. Ligand binding was interfered with by substituting the E380 with the larger, oppositely charged amino acid arginine (R), corresponding to E618R in BAM7 (BAM7-lig) and E623R in BAM8 (BAM8-lig) (Fig. 1.2.4). BAM8-lig lost its DNA-binding affinity and transcriptional activator
Thus, it seems that the putative ligand might have a large impact on BZR1-BAMs activity. Further modes of regulation were proposed and may occur through phosphorylation, dimerization with each other (BAM7, BAM8 homo-/heterodimers) or other interaction partners.

### 1.2.6 Crosstalk of BZR1-BAMs with phytohormone- and light-signalling

The homology of the BZR1-BAM-domain to the BZR1-type transcription factors together with the preferred DNA-binding motif of the BZR1-BAMs (BBRE), which contains the BRRE and the G-box, made a (partial) convergent transcriptional response of BZR1-BAM-signals, brassinosteroid (BR) -signals and possibly light-signals likely. The convergence is supported by the high overlap of the BZR1-BAM- and brassinosteroid- (brassinolide, BL) responsive genome-wide transcriptome changes (Reinhold, Soyk et al. 2011). Among the overlapping genes, the number of BL repressed genes was bigger than the number of BL-induced genes. Furthermore, the BL repressed genes were enriched in BAM8-ox vs. wild-type up-regulated genes and enriched in bam7 bam8 vs. wild-type down-regulated genes. Altogether, this indicated that BR and BZR1-BAMs share some down-stream targets, which tend to be regulated in opposite directions (Reinhold, Soyk et al. 2011). The opposing action of BR and BZR1-BAMs on the BBRE was confirmed in a BBRE driven reporter assay performed in protoplasts where BES1 and BZR1 both had an inhibitory effect on BAM8 activating activity (Soyk, unpublished).

The convergence with light-signalling is mainly based on the G-box, which is a known light-responsive element. Besides containing the G-box in the BBRE, BZR1-BAMs also have a side specificity for the G-box (Soyk, Simkova et al. 2014). The light regulated transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and the BR regulated transcription factor BZR1 were shown to physically interact with each other and have a large set of commonly targeted promoters, in which the motif most enriched is the G-box (Oh, Zhu et al. 2012). Thus, a simultaneous interplay between BR, light and BZR1-BAMs may be envisioned. Apart from common target genes, BZR1-BAMs may also oppose BR action via reducing BR concentrations by downregulation BR biosynthesis genes (e.g. CPD, At5g05690). CPD was seen to be down-regulated in BAM8-ox seedlings (1.4 - 2-fold down, depending on the experimental conditions) but not significantly up-regulated in any conditions in the bam7 bam8 double mutants (Reinhold, Soyk
et al. 2011, Soyk, Simkova et al. 2014). BR levels have not been quantified in any of the BZR1-BAM mutants or overexpressors, hence this option is very speculative and given the modest change in CPD expression is considered as having a minor contribution. Irrespective of the mechanism, growth architecture also supports the idea of an antagonistic action of BZR1-BAMs and BR-signalling: constitutive BR-signalling mutants (bes1-D) and BR-insensitive mutants (bri1-5) have similar phenotypes to *bam7 bam8* (short roots) and *BAM8-ox* (low root branching), respectively (Wang, Nakano et al. 2002, Yin, Wang et al. 2002, Soyk, unpublished).

An overlap with auxin-signalling is less prominent than interactions with BR and probably indirect. Several auxin co-receptors INDOLE-3-ACETIC ACID INDUCIBLE (IAAs) are repressed in *BAM8-ox* seedlings, of which some were also direct targets of PIF4 and BZR1 (IAA5, IAA14, IAA2, IAA1, IAA29, IAA6, IAA19) or PIF4 and PIF5 (IAA29) (Keuskamp, Pöllmann et al. 2010, Oh, Zhu et al. 2012, 2015). Furthermore, some auxin-response factors (ARF2, ARF5, ARF11) are deregulated by BZR1-BAMs and are direct targets of BZR1 (Reinhold, Soyk et al. 2011). This again supports an involvement of BZR1-BAMs in the light- and hormone-signalling network. Indications for an overlap with abscisic acid (ABA) responses are rather scarce and indirect. Some SUCROSE NON-FERMENTING KINASES (SnRK2-9, SnRK3-10), which are known to be involved in ABA-signalling, are up-regulated in *BAM8-ox* in the dark and are therefore putative direct targets (Fujii, Chinnusamy et al. 2009, Cutler, Rodriguez et al. 2010).

### 1.2.7 The basic helix loop helix protein family

Basic helix loop helix (bHLH) proteins are a family of transcription factors that have the ability to dimerise and bind to specific DNA sequences. They have been well studied in eukaryotic systems. In Arabidopsis, 147 bHLH proteins were identified, which can be divided into subgroups. According to the basic domain, they are classified into DNA-binding and non-DNA-binding proteins. The DNA-binding proteins are further sub-divided into E-box (5’-CANNTG-3’) and non-E-box binding proteins. Within the E-box binders there is a further classification into the G-box (5’-CACGTG-3’) binding and those predicted to recognize other types of E-boxes (non-G-box binders). Crucial for E-box binding is the glutamate in position 13 (E13) and the aspartate in position 16 (R16). Three conserved amino acid residues are common to the G-box binding proteins: The histidine (H) or lysine (K) in position 9 (H9/K9), glutamate13 (E13) and aspartate17 (R17). According to that definition the BZR1-BAMs would classify as E-box binders (E13, R16 and R17 present but H/K9 absent) (Toledo-Ortiz, Huq et al. 2003). An earlier study in the mammalian system showed that mutations in the basic domain of the bHLH motif lead to impaired DNA-binding but do not impact dimerization (Voronova and Baltimore 1990). Residues important for dimerization of the bHLH proteins were determined to locate to the hydrophobic region (the helix domain) of the protein (Voronova and Baltimore 1990). Mutation in the conserved leucine27 (L27) abolished dimerization (Carretero-Paulet, Galstyan et al. 2010), whereas other hydrophobic residues
only prevent dimerization in combination with a second mutated conserved hydrophobic amino acid (Voronova and Baltimore 1990). A hydrophobic interface, interactions between charged amino acids and partner availability are further parameters that influence dimerization specificity. Overall it seems that DNA-binding is dependent on dimerization but dimerization is not dependent on DNA-binding (Voronova and Baltimore 1990).

1.3 Sugar sensing and signalling

Photosynthesis generates energy-rich sugar molecules. It is therefore not surprising that sugars not only have the crucial function as primary metabolites, but also play an important role as signalling molecules. As indicators of the energy and carbon nutritional status, they control a wide array of processes such as metabolism, stress resistance, growth and development in a range of organisms, such as bacteria, yeast, plants and animals. Failures in maintaining the nutrient and energy homeostasis in multi-cellular organisms lead to sever diseases, such as diabetes in mammals.

Sugar sensing and signalling is particularly well studied in yeast (as a representative of the eukaryotic system). *Saccharomyces cerevisiae* is a facultative anaerobic organism, which prefers fermentation of sugars like glucose (Glc), sucrose (Suc) and fructose (Fru) even in the presence of oxygen. In yeast sugar sensing and signalling mechanisms mainly rely on Glc. The presence of Glc leads to repression of genes involved in respiration, gluconeogenesis and the uptake and metabolism of alternative carbon sources. Furthermore, Glc also triggers the expression of hexose transporters of either high or low affinity to increase the uptake capacity. Sensing of Glc occurs extracellularly via three plasma membrane localised proteins, namely the catalytically inactive hexose transporters (Snf3 and Rft2) and the G-protein coupled receptor (Gpr1). Intracellularly, Glc is sensed by the glycolytic enzyme Hexokinase2 (Hxk2). Besides Glc, Suc can also be sensed extracellularly by Gpr1. Metabolic intermediates such as glucose 6-phosphate (Glc6P) and trehalose 6-phosphate (Tre6P) exert regulatory functions via expression and allosteric regulation of glycolytic enzyme activities (Rolland, Baena-Gonzalez et al. 2006). Key down-stream components of sugar sensing are the cAMP-dependent Protein Kinase A (PKA) and the protein kinase Sucrose non fermenting1 (Snf1), of which the latter has homologues in plants (SNF1-RELATED KINASE1-3, SnRK1, SnRK2, SnRK3). Snf1 in yeast is activated upon glucose depletion, triggering expression and activity of metabolic enzymes, which are involved in the use of alternative carbon sources, gluconeogenesis and respiration (Hedbacker and Carlson 2008). PKA-signalling is involved in allowing a fast growth rate on fermentable sources (Rolland, Baena-Gonzalez et al. 2006).

1.3.1 Sugar sensing and signalling in plants

In plants, monitoring of the nutritional and energy status is particularly important due to their sessile, autotrophic life style. Thereby, sugars reflect the energy status and continuous sensing of sugar levels
are key to survival. Metabolic, environmental and developmental cues need to be integrated and an adaptive response triggered. It is therefore not surprising that plants’ sugar-signalling is coordinated with light-signals, the circadian clock and phytohormone-signalling (Lastdrager, Hanson et al. 2014), ultimately leading to modulation of investment into growth, development and photosynthetic activity. Adaptations in respect to primary carbon metabolism involve the expressional regulation of photosynthetic, starch synthesis and starch degradation genes (Krapp, Hofmann et al. 1993, Koch 1996) as well as the modulation of export into phloem depending on the sink strength (Vaughn, Harrington et al. 2002). In this context, ‘sink’ describes carbon importing, whereas ‘source’ carbon exporting tissues. In general, source activities are up-regulated under low sugar conditions, whereas sink activities are up-regulated when carbon sources are plentiful (Rolland, Baena-Gonzalez et al. 2006). Sugar signals arise either by pure carbohydrate concentrations, ratios with other nutrients such as nitrogen (C:N) (Palenchar, Kouranov et al. 2004) or fluxes through sugar sensors/transporters (Vaughn, Harrington et al. 2002, Eveland and Jackson 2012).

Mechanistically, sugar sensing and signalling is little understood in plants. The best characterised and confirmed sugar sensor is HEXOKINASE1 (HXK1), which is a metabolic enzyme catalysing Glc phosphorylation to glucose 6-phosphate (Glc6P) and has the additional function of a hexose sensor that transduces signals according to sugar availability, leading to transcriptional changes (Fig. 1.3.1). It was shown that the sensing function of HXK1 can be uncoupled from enzymatic activity (Moore, Zhou et al. 2003) (for further details see 1.3.2). In other organisms, there are more examples of metabolic enzymes ‘moonlighting’ as transcriptional regulators, including Arg5,6 in yeast (Hall, Zhu et al. 2004) and the glyceraldehyde-3-phosphate dehydrogenase in mammalian cells (Zheng, Roeder et al. 2003). In plants, several sugars have been recognised as singling compounds, namely Glc, Suc, Fru, Glc6P and Tre6P (Fig. 1.3.1). In general, hexoses tend to be more involved in organ growth promotion, whereas sucrose is more associated with differentiation and maturation (Eveland and Jackson 2012). However, with the exception of Glc, for none of the above-mentioned signalling compounds a sensor has really been established. Some were proposed but need further confirmation. In analogy to the yeast/mammalian system, a membrane bound G-protein was suggested to sense extracellular glucose (Chen and Zhang 2003, Chen and Jones 2004). A membrane bound sucrose transporter (SUT2), which does not have shown transport activity, was proposed as Suc sensor (Barker, Kuhn et al. 2000, Rolland, Baena-Gonzalez et al. 2006) and more recently, a putative cytosolic Fructose-1,6-bisphosphate (FRUCTOSE INSENSITIVE1, FIN1) was proposed as a fructose sensor (Cho and Yoo 2011). For Glc6P it was shown that its partitioning between the cytosol and the plastid established by a Glc6P/phosphate translocator (GTP2) impacts the plant’s response to exogenous sugars but is mediated through an as-yet unknown
sensor (Dyson, Webster et al. 2014). Tre6P was seen to be important in keeping cellular Suc levels in an optimal range (Figueroa, Feil et al. 2016), yet its sensor likewise still needs to be determined.

Apart from the sugar signals, there are other recognised components in sugar signal transduction in plants; TARGET OF RAMPAMYCIN (TOR) kinase, the SnRK1 and the C/S1-bZIP transcription factor network (Fig. 1.3.1). TOR is a master regulator, which is evolutionary conserved from yeasts to plants and human. In plants, TOR promotes growth in response to high sugar levels (Deprost, Yao et al. 2007). However, the underlying regulatory mechanisms remain still largely unknown, partially due to the lethality of Arabidopsis tor null mutants. TOR is predominantly stimulated by Glc through a HXK-independent Glc sensing pathway (Xiong, McCormack et al. 2013). In contrast, SnRK1 is activated upon sugar depletion and triggers repression of biosynthetic processes and growth (Baena-Gonzalez, Rolland et al. 2007). SnRK1 homologues are also found in yeast (Snf1) and mammals (AMP-activated kinase, AMPK). SnRK1’s activity is reduced by sugar-phosphates such as glucose 6-phosphate (Glc6P), glucose 1-phosphate (Glc1P) and trehalose 6-phosphate (Tre6P) (Nunes, O’Hara et al. 2013) (for further details see section 1.3.3). The S1- and C-class bZIP-family transcription factors form heterodimers and act as transcriptional activators positively influencing metabolic reprogramming and growth. Their sugar sensitivity occurs at the level of transcriptional regulation of the S-class bZIP family transcription factors; Suc arrests mRNA translation via ribosome stalling mechanisms in a concentration dependent manner (Smeekens, Ma et al. 2010). To conclude, there are growth promoting and growth inhibiting systems in response to sugar availability. To the former belong HXK1, T6P and TOR, whereas the latter are consisting of SnRK1 and the C/C1 bZIP transcriptional network (see Fig. 1.3.1).
1.3.2 HXK dependent glucose sensing and signalling

Hexokinase (HXK) is not only the best studied sugar sensor in plants, it is also considered as the most ancient and evolutionary conserved sugar sensor in E. coli, yeast, mammals and plants (Ramon, Rolland et al. 2008). First strong evidence for a role of HXK in plant sugar sensing was generated by a study investigating the physiological response of Arabidopsis plants with altered HXK levels to high (6 %, equivalent to 333 mM) exogenous Glc (Jang and Sheen 1997). Whereas HXK-overexpressing seedlings were hypersensitive to Glc and showed an even more severe phenotype (arrested growth and repression of cotyledon greening) than wild-types, HXK-antisense plants were more resistant. These results were confirmed by a mutant screen against resistance of Arabidopsis seedlings to high (6 %) exogenous Glc concentrations, where the mutation in glucose insensitive-2 (gin-2) was mapped to the HXK1 gene. Importantly, it was discovered that the enzymatic activity of HXK1 can be uncoupled from its sensing property. In other words, the catalytically inactive S177A and G104D mutants were able to complement the Glc sensitive phenotype in the gin-2 mutant background. Apart from the Glc resistance phenotype on high exogenous Glc supply, gin-2 shows a wild-type like phenotype under regular (light) growth conditions (80 µmol photons m$^{-2}$ s$^{-1}$) but has an increasingly stunted growth and darker green leaves with increasingly high light conditions (up to 320 µmol photons m$^{-2}$ s$^{-1}$), under which conditions photosynthesis is boosted leading to high intrinsic sugar levels (Moore, Zhou et al. 2003). These findings supported the hypothesis that HXK1 promotes growth under physiological conditions.

1.3.3 SnRK1-signalling pathways

The SnRK-family is composed of three types: SnRK1-, the SnRK2- and the SnRK3-type kinases. The latter two are plant specific and were found to be important in response to abiotic stress (particularly ABA response for SnRK2). Outside the catalytic-domain they have limited similarity to the evolutionary conserved SnRK1 and will not be discussed further here. As mentioned before SnRK1 has homologues in yeast and animals, which are all heterotrimeric serine/threonine kinases consisting of a catalytic $\alpha$-subunits and regulatory $\beta$- and $\gamma$-subunits, and are activated under limiting carbon- and energy conditions (Baena-Gonzalez, Rolland et al. 2007). The catalytic $\alpha$-subunits as well as regulatory $\beta$-subunits each have three homologues in plants. In contrast, the family of $\gamma$-subunits has numerous members. Intriguingly, plants encode a unique hybrid $\beta\gamma$-subunit, which also clusters together with the regular $\gamma$-subunits (Fig. 1.3.2). Actually, the plant SnRK1 heterotrimers rather contain the chimeric $\beta\gamma$-subunit than the conventional $\gamma$-subunits (Emanuelle, Hossain et al. 2015). Knock out of the $\beta\gamma$-subunit in Arabidopsis leads to lethality. Interestingly in respect to the involvement of SnRK1 in sugar-signalling, there is a carbohydrate binding module (CBM) in the $\beta$-subunit. In animals and yeast the $\beta$-subunit was shown to bind to glycogen in vitro (Wiatrowski, van Denderen et al. 2004, McBride, Ghilagaber et al. 2009). Plants contain a second CBM in the $\beta\gamma$-subunit, additional to the CBM in the $\beta$-subunit (Ball,
Assessment of the CBM’s function showed that the SnRK1 βγ–subunit CBM is not essential for complex formation but may be involved in facilitating other protein-protein interactions and allosteric regulation by a carbohydrate metabolite (Ramon, Ruelens et al. 2013, Emanuelle, Doblin et al. 2016). Possible candidates as metabolite ligands are sugar phosphates (Glc1P, Glc6P, Tre6P), which were all shown to be allosteric regulators of SnRK1 activity (Nunes, Primavesi et al. 2013, Broeckx, Hulsmans et al. 2016). The interplay between Tre6P and SnRK1 are described in more details later (see section 1.3.5). The subcellular localisation of SnRK1, which might give a hint about their exposure to particular metabolites showed ambiguous results: SnRK1α1 and SnRK1βγ subunits showed localisation in the cytoplasm, the nucleus, or both, depending on stable or transient expression and the tissue type. In general, SnRK1βγ mostly localised to the nucleus and the SnRK1α1 preferentially localised to the cytosol or a ring structure around the nucleus with the exception of meristematic guard cells, ovules and immature pollen where it was nuclear (Bitrian, Roodbarkelari et al. 2011, Williams, Rangarajan et al. 2014, Broeckx, Hulsmans et al. 2016). The tissue specific expression varied from the subunit isoform (e.g. SnRKα1 and SnRKα2). The most common form, SnRKα1, is expressed throughout development and SnRK1α1 and SnRK1βγ were both seen to be accumulating most in meristematic, elongating and differentiating zones of primary and lateral roots as well as in young leaf primorida (Bitrian, Roodbarkelari et al. 2011). Besides the allosteric inhibition by sugar-phosphates, the most important mode of post-translational regulation of SnRK1’s activity is phosphorylation and de-phosphorylation (Fig. 1.3.2). Phosphorylation within the T-loop of the α-subunit is essential for the activity of SnRK1.

**Figure 1.3.2: Regulatory role of SNF1-related kinase1 (SnRK1) in maintenance of energy homeostasis.** In general, SnRK1 is activated under low energy conditions and repressed in conditions of plentiful carbon availability. A major mechanism of regulation of the SnRK1 is by de-/phosphorylation by protein kinases (PK) and protein phosphatases (PP). Glc6P, Glc1P and Tre6P can act as allosteric inhibitors. Down-stream targets of SnRK1 are regulated via post-translational modification or (indirectly, mediated in part by bZIP TF) transcriptional regulation. SnRK1 is represented by its α-, β-, βγ-subunit. SPS, Sucrose Phosphate Synthase; NR, Nitrate Reductase; HMGR, 3-Hydroxy-3-Methylglutaryl-CoA reductase; bZIP TF, basic leucine zipper transcription factors; AMP, Adenosine monophosphate. Adapted from (Hulsmans, Rodriguez et al. 2016).
Myristoylation (Pierre, Traverso et al. 2007), sumoylation and interaction with regulatory proteins are further demonstrated regulatory mechanisms (Broeckx, Hulsmans et al. 2016, Crozet, Margalha et al. 2016). SnRK1 itself modulates down-stream target processes via post-translational modification (phosphorylation) leading to transcriptional reprogramming, changes in protein synthesis and enzyme activity (Broeckx, Hulsmans et al. 2016) (Fig. 1.3.2). Targets of post-translational modification are the \( 3\)-hydroxy-\( 3\)-methylglutaryl-CoA reductase (HMGR) (Sugden, Donaghy et al. 1999), Nitrate Reductase (NR) (Mackintosh, Douglas et al. 1995, Sugden, Donaghy et al. 1999), Sucrose-Phosphate Synthase (SPS) (Sugden, Donaghy et al. 1999), Fructose-2,6-bisphosphatase (F2KP) (Kulma, Villadsen et al. 2004) and class II TPS (see section 1.3.4) (Harthill, Meek et al. 2006, Nukarinen, Nagele et al. 2016). Transcriptional regulation by SRK1 is mainly to activate catabolic pathways and repress anabolic pathways and biogenesis of ribosomes. In conclusion, SnRK1 promotes energy generating processes and inhibits energy consuming processes (Broeckx, Hulsmans et al. 2016) (Fig. 1.3.2).

1.3.4 Trehalose 6-phosphate-signalling pathways

For a long time, trehalose (Tre) in plants was considered as conferring drought tolerance, as massive accumulation is seen in desiccation-tolerant resurrection plants (Adams, Kendall et al. 1990). Due to the little amounts found in flowering plants, its origin was initially assigned to microbial contamination (Kandler and Hopf 1980). The finding of 11 TREHALOSE 6-PHOSPHATE SYNTHASE (TPS) and 10 TREHALOSE 6-PHOSPHATE PHOSPHATASE (TPP) genes in Arabidopsis challenged this view (Leyman, Van Dijck et al. 2001). Nowadays TPS and TPP are presumed to be universal in the plant kingdom (Figueroa and Lunn 2016) and there has been a shift to seeing trehalose 6-phosphate (Tre6P) rather than Tre as the essential signalling compound. Both Tre and Tre6P levels in plants are very low. Cytosolic Tre6P concentrations of 5-week-old Arabidopsis rosettes were determined roughly 4 times lower than Glc6P (Tre6P 3.8–6.7 \( \mu \)M; Glc6P 13.9–19.8 \( \mu \)M, depending on growth regime) and about 10,000 times lower than Suc (Martins, Hejazi et al. 2013). However, one may take in consideration that these concentrations were determined from whole plants and particularly Tre6P may in reality cover a much broader concentration range if specific cell types at particular developmental stages would be measured.

Tre6P in plants is synthesised by TPS from UDP-glucose (UDP-Glc) and Glc6P and subsequently dephosphorylated by TPP to Tre. TREHALASE (TRE), a single copy gene, catalyses the cleavage of Tre into two Glc units (Fig. 1.3.3). The family of TPS genes is composed to two classes: Class I (TPS1-4) and class II (TPS5-11). Only class I contains members with shown catalytic activity (TPS1, TPS2, TPS4, while TPS3 is most likely a pseudogene) (Lunn 2007, Delorge, Figueroa et al. 2015). TPS1 seems to have a crucial role since tps1 mutants in Arabidopsis are essentially embryo lethal. Complementation of tps1 mutants using a seed specific promoter leads to a mature plant displaying stunted growth and delayed
or no flowering (Eastmond, van Dijken et al. 2002, Gomez, Gilday et al. 2010). TPS2 and TPS4 are expressed predominantly in developing seeds, siliques but not the embryo and are apparently not redundant to TPS1. The role of class II enzymes remains elusive. All the 10 members of the TPP gene family are catalytically active and, due to their diverging local and temporal expression, single proteins are proposed to play distinct, and others overlapping roles (Vandesteene, Lopez-Galvis et al. 2012). During the diel cycle Tre6P levels peak at the end of the day and fall quickly upon onset of the night. During the night, Tre6P levels recover to slightly higher amounts, stabilise and then start to decrease towards the end of the night. In fact, Tre6P levels track Suc levels particularly nicely during the day and the beginning of the night. Glc6P also follows a similar pattern to Suc (Martins, Hejazi et al. 2013).

Furthermore, it was observed that carbon (C)- starved seedlings contain extremely low levels of Tre6P which rose more than 26-times upon Suc supply, to levels higher than in the non-starved controls. Suc also reached concentrations substantially above the non-starved condition. In comparison, other neutral (Glc, Fru) and phosphorylated sugars (Glc1P, Glc6P, Fru6P) rose upon Suc re-addition but only to levels seen in control conditions (Lunn, Feil et al. 2006). Other experiments confirmed these findings and showed that re-addition of sugars to C-starved seedlings led to increases in Tre6P if the added sugar could be converted to Suc (e.g. Glc, Fru). The same was true in experiment with varying nutrient (nitrogen, phosphorus, sulphur) conditions – they led to changes in Tre6P content in cases where Suc levels were also modulated (Yadav, Ivakov et al. 2014). This led to the hypothesis that Tre6P is a proxy of Suc abundance and was further specified in the ‘Tre6P-Suc nexus model’. The model postulates that not only Suc levels control Tre6P abundance, but also Tre6P feed-back regulates Suc availability in order to keep Suc concentrations within a range that is optimal for the plant. Furthermore, this model would involve Tre6P independent Suc-signalling (Fig. 1.3.4). The ratio of Tre6P:Suc may vary between developmental stages, environmental conditions, tissues and plant species, in each case reflecting the specific need of the cells and tissues in the given circumstances (Yadav, Ivakov et al. 2014).

![Figure 1.3.3: Trehalose/ trehalose 6-phosphate metabolism in plants.](image)

**Figure 1.3.3: Trehalose/ trehalose 6-phosphate metabolism in plants.**

TPS, Trehalose 6-phosphate Synthase; TPP, Trehalose 6-Phosphate Phosphatase; TRE, Trehalase. Modified from Tsai and Gazzarrini 2014.
As mentioned earlier, Tre6P also has a significant impact on plant’s life; embryos impaired in Tre6P synthesis (tps1 mutants) only develop to the torpedo stage and carbon metabolism in these embryos is greatly affected (Gomez, Baud et al. 2006). The transition from pattern formation to seed maturation was proposed to be Tre6P dependent (Schlüpman, Pellny et al. 2003). In mature plants Tre6P also has an effect on carbon partitioning and carbon metabolism. Plants overexpressing the E. coli TPS gene (35S:otsA) have higher starch levels than wild-type, whereas overexpression of the E. coli TPP genes (35S:otsB) reduce the starch content (Kolbe, Tiessen et al. 2005). Initially it was proposed that starch levels are modulated by Tre6P indirectly via redox-activation of an enzyme critical in starch synthesis (ADP-Glc pyrophosphorylase, AGPase) (Kolbe, Tiessen et al. 2005). These results became controversial after it was found that the commercially available Tre6P at that time was by 30 – 40 % contaminated with a wide range of other compounds, including detergents (Lunn, Feil et al. 2006, Yadav, Ivakov et al. 2014). More recent studies found that inducible TPS lines (iTPS), induced at the beginning of the day, led to only small increase in starch content by the end of the day (but rapid Tre6P increase) (Martins 2013). Further investigating the impact of Tre6P on primary carbon metabolism (starch, soluble sugars) using transgenic plants carrying an inducible AtTPS1 showed, that there is only a small impact of Tre6P on starch synthesis (slightly promoted) but a bigger effect on starch degradation (reduced) (Fig. 1.3.4).

An increase in phosphate content in starch upon induced AtTPS1 expression suggests impaired phosphorylation/de-phosphorylation processes during starch degradation, but an inhibition of β-amylase activity could not be fully excluded. In vitro assays with recombinant α-glucan, water dikinase (GWD), phosphoglucon, water dikinase (PWD), α-phosphoglucan phosphatases (SEX4) and β-amylase3 (BAM3) did not show a direct activity regulation by Tre6P (Martins, Hejazi et al. 2013). The soluble sugar content (Fru, Glc, Glc6P, Suc6P) was not markedly changed upon TPS1-induction (Martins, Hejazi

Figure 1.3.4: Tre6P-Suc nexus model in source leaves during the day and night. (A) During the day, Tre6P inhibits flux into sucrose synthesis but rather encourages investment into storage compounds such as amino- and organic acids. (B) At night, Tre6P reduces the energy remobilisation process of starch degradation. Phosphoenolpyruvate carboxylase (PEPC), Nitrate Reductase (NR), trehalose 6-phosphate (Tre6P), Trehalose 6-phosphate Phosphatase (TPP), Trehalose 6-phosphate synthase (TPS). Solid lines represent experimentally demonstrated interactions and dashed lines hypothetical interactions. Blue arrows indicate activation and red lines inhibition. Adapted from Figueroa and Lunn 2016.
et al. 2013). Overall it has become apparent that Tre6P regulates starch remobilisation at night, linking sink demand with starch mobilisation in source organs. During the day Tre6P may exert another regulatory function in respect to primary carbon metabolism: It is proposed to modulate carbon partitioning between Suc, organic acids and amino acids (Figueroa, Feil et al. 2016).

1.3.5 Convergence of SnRK1-, Tre6P-, Suc- and C/S1 bZIP transcription factor-signalling

The sugar-signalling components SnRK1, Tre6P, Suc and the C/S1 bZIP transcription factor network all act in response to C-availability and are interconnected at one point or another. The kinase SnRK1 is activated under conditions of low carbon availability and was shown to be inhibited by Tre6P (Nunes, Primavesi et al. 2013) (Fig. 1.3.2). To date, the precise mechanism of this inhibition is unclear. Interestingly, immuno-purified SnRK1 was not inhibited by Tre6P unless seedling extract was added back to the assay. In contrast, extracts of mature plants did not restore the inhibitory effect, indicating that there is an as-yet unknown intermediary factor (present in seedlings but not adult plants) required to enable the inhibition of SnRK1 by Tre6P (Zhang, Primavesi et al. 2009). In vivo data confirmed these opposing effects of Tre6P and SnRK1 by comparisons of expressional data of Tre6P and SnRK1 deregulated genes, which showed a more than 50 % overlap in commonly regulated genes of which the vast majority (97 %) were affected opposingly (as expected if Tre6P inhibits SnRK1). The former were determined in 35S:otsA expressing plants vs. wild-type (Zhang, Primavesi et al. 2009), whereas the latter were identified from transient expression of KIN10 (a catalytically active α-subunit of SnRK1) in protoplasts (Baena-Gonzalez, Rolland et al. 2007). Tre6P up-regulated genes were mainly identified as involved in energy consuming processes (amino acid, nucleotide, protein synthesis, TCA cycle and mitochondrial electron transport), whereas SnRK1 up-regulated genes were assigned to energy producing/remobilising processes (photosynthesis and protein degradation) (Zhang et al., 2009) (Fig. 1.3.2). These results initially led to the hypothesis that Tre6P promotes growth by inhibiting SnRK1, but considering results from more studies suggest a less linear, more complex Tre6P-SnRK1 relationship (Figueroa, Feil et al. 2016). A transcriptional regulation study with C-starved seedlings resupplied with Suc was indicative of a more complex response to C-status: They identified that the early starvation response was bZIP11 dependent and only during later stages of starvation did SnRK1 and Tre6P regulated genes play a bigger role (Cookson, Yadav et al. 2016). Interestingly, their promoter analysis showed an enrichment of known starvation-induced motifs (G-box and the ABA-responsive core element, ACGT) but also motifs associated with gibberellic acid (GA), light and the circadian clock (Cookson, Yadav et al. 2016). The interplay with the C/S1 bZIP transcription factor network with Tre6P and SnRK1 responses was further supported by the observation that growth arrest of seedlings caused by exposure to exogenously supplied Tre was reverted in KIN10 or bZIP11 overexpressing (KIN10-ox,
bZIP11-ox) plants. The cause for the growth arrest of wild-type plants grown on Tre was assigned to the secondary effect of elevated Tre6P levels which prevented SnRK1 activity to promote carbon allocation to heterotrophic tissue thereby ensuring fuel for growth. Constitutive expression of KIN10 (KIN10-ox) therefore helped to overcome this inhibitory effect. Since bZIP11 also positively impacts carbon utilisation for growth it was argued that overexpression of bZIP11 partially compensated for reduced SnRK1 activity and explains the rescuing effect of bZIP11-ox. The observed starch accumulation in wild-type plants grown on exogenous Tre was interpreted as a re-distribution or impaired utilisation of carbon resources (Delatte, Sedijani et al. 2011). Further studies using cell-specific and inducible expression systems, where metabolite levels, fluxes, protein phosphorylation patterns and transcript levels are analysed in one and the same tissue will help to further elaborate the crosstalk between SnRK1, Tre6P, Suc and bZIP11.

1.3.6 Sugar hormone interplay

1.3.6.1 Brassinosteroid- and sugar-signalling interplay
To date, there are only few reports describing the direct or indirect interaction of brassinosteroid- (BR) and sugar-signalling and the underlying mechanisms we only start to understand. From observations at the physiological level (root directional growth and lateral root formation), moderate concentrations of Glc (1-3 %, corresponding to 56 – 167 mM) and BR seem to act in a synergistic way, effects of Glc were enhanced by BR stimulation (Singh, Gupta et al. 2014, Gupta, Singh et al. 2015). To address the underlying molecular mechanisms, experiments using mutants affected in the HXK1-dependent (gin-2) and HXK-independent (G-protein; rgs1, thf1, gpa1) signalling pathways were performed. It was proposed that signalling occurs either via the HXK1-dependent (in case of lateral root formation) or via both, HXK-dependent and HXK-independent signalling (in case of root directional growth) whereby Glc-signalling happens upstream of BR-signalling. Interestingly, high light intensities triggered responses comparable to the Glc-induced response, suggesting that Glc represents environmental (light) conditions experienced by the source organs (Singh, Gupta et al. 2014). Quantification of gene expression changes further indicated that exogenous Glc supply significantly affects transcript levels of genes involved in BR biosynthesis, perception, signalling, and early response (Gupta, Singh et al. 2015). Glc was seen to induce transcription of BZR1 and to stabilise the BZR1 protein. Direct interaction of BZR1 and HXK1 could not be demonstrated. Nevertheless it was concluded that BZR1 is most likely the converging node between BR- and Glc-signalling, possibly also involving the interaction of BZR1 and PIF4 (Zhang and He 2015, Kuhn 2016).
Suc-induced physiological responses (hypocotyl elongation in the dark) at moderate Suc levels (90 mM) were seen to be BR dependent. Whereas expression of the BR-responsive transcription factors, BZR1
and BES1, were induced upon Suc treatment, BR biosynthesis was only marginally affected (Zhang, Liu et al. 2015). In contrast, a recent study investigating sugar- (Glc and Suc) induced hypocotyl elongation in the dark, concluded that the elongational response mostly occurs via the TOR-dependent and only marginally the HXK1-dependent pathway. In agreement with previous reports, sugars induced BR levels, stabilised the BZR1 protein but did not affect BR biosynthesis. Furthermore, under their experimental conditions PIF4 did not play an obvious role (Di, Wu et al. 2016).

In conclusion one can say that sugars (Glc, Suc) and BR seem to act synergistically and dependent on each other. How this is achieved mechanistically (via HXK-dependent/-independent pathways) is little understood. Depending on the system studied, the impact of sugars may enhance BR abundance, sensitivity or downstream signalling.

1.3.6.2 Abscisic acid- and sugar-signalling interplay

The existence of an overlap between abscisic acid- (ABA) and sugar-signalling has been long recognized. Nevertheless, mechanistic details are still scarce. Genetic evidence for a close overlap was obtained by four mutant screens for sugar insensitive mutants (sucrose uncoupled, sun; impaired sucrose induction, isi; glucose insensitive, gin; sugar insensitive, sis), which identified many ABA insensitive (abi) or ABA deficient mutants (aba) (Dekkers, Schuurmans et al. 2008). Furthermore, ABA and Glc share a number of commonly regulated genes. Motif searches showed that amongst others, the G-box was enriched in the deregulated genes (Li, Lee et al. 2006). HXK1-mediated Glc-signalling was shown to induce ABA biosynthesis, increase ABA levels, and ABA-signalling (Ramon, Rolland et al. 2008). More recently, an interplay between of ABA with Tre6P was also discovered. As for other sugar-signalling pathways, tissue type and developmental stage greatly determine the output for ABA- and sugar-signalling overlaps.

Germination is inhibited by ABA but can be counteracted by moderate levels of metabolisable sugars (Glc, Fru, Suc, 15 – 90 mM) (Finkelstein and Lynch 2000). A role of Tre6P/Tre in this context was shown in Arabidopsis mutants impaired in Tre or Tre6P metabolism which are resistant (tppg, tre1, TPS1-ox) or hypersensitive (weak alleles of TPS, tps1-11, tps1-12, tps1-13) to ABA-induced germination inhibition (Avonce, Leyman et al. 2004, Gomez, Gilday et al. 2010, Vandesteene, Lopez-Galvis et al. 2012).

At the seedling stage, exposure to high external Glc levels (> 300 mM) causes developmental arrest such as absence of cotyledon greening and leaf formation, which can either be overcome by gin, aba or some abi mutants (Dekkers, Schuurmans et al. 2008). Plants with elevated Tre6P (TPS1-ox) showed the same resistant phenotype to high Glc, which was likely due to reduced accumulation of ABA in TPS1-ox compared to the wild-type seedlings upon exposure to Glc (Avonce, Leyman et al. 2004). Contradictory to that, overexpression of E. coli TPS (otsA-ox) showed similar Glc sensitivity as the wild-type. Hence it was concluded, that the mechanism helping to overcome the sensitivity to high Glc levels may involve a signalling response exerted by AtTPS1 itself (but not otsA) and is not purely due to the
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Elevated Tre6P levels (Schluepmann, Pellny et al. 2003, Avonce, Leyman et al. 2004). However, reduced levels of Tre6P (overexpressing TpP of E. coli, ots8-ox) led to hypersensitivity to Glc (Schluepmann, Pellny et al. 2003). Apart from germination and seedling development, stomata conductance is another important point of action for ABA. In agreement with previous effects described, the tppg, tre1-1 and tre1-2 mutants have stomata which are unresponsive to ABA, thereby leading to increased drought susceptibility, whereas TRE1-ox plants are hypersensitive to exogenous ABA exposure (Vandesteene, Lopez-Galvis et al. 2012, Van Houtte, Vandesteene et al. 2013).

Altogether, the proposed signalling mechanism sets HXK1 upstream of TPS1, since in HXK1-antisense lines expression of TPS1 was absent. TPS1 itself down-regulates ABI4 upon exposure to Glc, thereby reducing ABA sensitivity (Avonce, Leyman et al. 2004). ABA most likely also acts downstream of Glc sensing (HXK1-dependent or independent), since addition of Glc not only increases expression of ABA synthesis and signalling genes but also raises ABA levels (Cheng, Endo et al. 2002, Rolland, Baena-Gonzalez et al. 2006).

For the interplay of ABA and Suc, it was observed that ABA enhances the induction of Suc-induced starch biosynthesis genes. Whether this response is directly triggered by Suc or a product of its metabolism remains unclear (Rook, Corke et al. 2001).

1.4 Light-signalling

Photoautotrophic organisms use light to fulfil their energy demand. Light is not a stable source but fluctuates over the day-night cycle, the season and other environmental influences such as shading. Therefore, it is crucial for survival and reproductive success to perceive, monitor and respond adequately to the prevalent light conditions. Plants have evolved numerous light-adaptive responses affecting various stages of their life cycle ranging from seed germination, de-etiolation, entrainment of the circadian clock, chloroplast movement, shade avoidance, stomatal opening, phototropism to flowering (Fig. 1.4.1). Light quality and quantity is perceived by at least five different photoreceptors, which are namely: Phytochromes (Rockwell, Su et al. 2006), cryptochromes (Lin and Shalitin 2003), phototropins (Christie 2007), members of the ZEITLUPE family (Somers, Schultz et al. 2000) and the UV-receptor UVR8 (Rizzini, Favory et al. 2011). Phytochromes sense mainly red (R) and far red (FR) light whereas cryptochromes, phototropins and members of the ZEITLUPE family predominantly perceive blue (B) and ultraviolet-A (UV-A) light. UV-B is sensed by a more recently discovered receptor UVR8 (Fig. 1.4.2). All photoreceptors, with the exception of the UVB receptor, are chromo-proteins, which are composed of an apo-protein and the chromophore (de Wit, Galvao et al. 2016). The UVR8 uses specific tryptophan residues from the protein itself to absorb the UVB-light (Jenkins 2014). The
chemical character of the two components (chromophore and apo-protein) determine the specific absorption spectra of the photoreceptor (Kami, Lorrain et al. 2010). Light perception by the chromophore leads to structural changes of the protein, initiating signal transduction (Harper, Neil et al. 2003, Pfeifer, Mathes et al. 2010). Upon light perception (e.g. transition from skotomorphogenic to photomorphogenic growth), early steps in light-signalling involve inactivation of negative regulators of light-induced growth such as PIFs, DE-ETIOLATED 1 (DET1), and COSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) (Duek and Fankhauser 2005, Lau and Deng 2012, Hartmann, Drewe-Boss et al. 2016). Subsequently, photomorphogenesis-promoting transcription factor activate down-stream transcriptional networks, leading to a wide range of gene-expression changes. Regulating the activity of transcription factors in response to light often involves basic post-translational mechanisms such as protein phosphorylation, thereby changing the affinity for DNA-binding or subcellular localisation, or ubiquitination, leading to degradation in the proteasome. Furthermore, the activity of a transcription factor can be modified by dimer-formation. The basic helix-loop-helix (bHLH) and basic leucine zipper (bZIP) protein families are typically regulated this way. Homo- and hetero-dimer formation modulates their activity (e.g. by changing DNA affinity) and enables them to interact in different transcriptional programs (Jiao, Lau et al. 2007, Hornitschek, Lorrain et al. 2009, Hao, Oh et al. 2012). The high degree of transcriptional regulation by light signals was shown in Arabidopsis plants, which were transferred from dark to light and showed a change in expression of 30 % of all genes of the genome (Ma, Li et al. 2001).
1.4.1 Characteristics of the different photoreceptors

Phytochromes (PHYA - PHYE) are involved in numerous light-regulated processes; in seed germination, shade avoidance, seedling development, shoot branching, circadian clock and flowering time. They are subdivided into type I (photo-labile) and type II (photo-stable) phytochromes. In Arabidopsis, all phytochromes except PHYA belong to the type II. Phytochrome-signalling mechanism is based on two different photo-convertible isoforms. One, the red absorbing Pr form is inactive and can be converted to the second active, far red absorbing Pfr form (Wang and Wang 2015, de Wit, Galvao et al. 2016). The subcellular localisation of phytochromes is dependent on the light quality. Continuous red light triggers PHYB - PHYE localisation to the nucleus, which can be reverted by far red light (Kircher, Kozma-Bognar et al. 1999, Gil, Kircher et al. 2000, Kircher, Gil et al. 2002). More recently it was shown that, for PHYB, dark to far red transition also leads to translocation to the nucleus (Zheng, Wu et al. 2013). Nuclear import of PHYA is induced by FR, R, B (Hisada, Hanzawa et al. 2000, Kircher, Gil et al. 2002).

The blue light receptors cryptochromes are important for entrainment of the circadian clock, photoperiodic induction of flowering and photomorphogenesis (Chaves, Pokorny et al. 2011). Out of the three members, two (CRY1, CRY2) have a known function, whereas CRY3, which is a more divergent family member, is of unknown function (Kleine, Lockhart et al. 2003, Chen, Chory et al. 2004). The photo-stable CRY1 is constitutively found in the nucleus, whereas the photo-labile CRY2 is only nuclear in the dark and largely cytoplasmic in the light. In contrast, CRY3 is present in mitochondria and chloroplasts (Kleine, Lockhart et al. 2003, Jiao, Lau et al. 2007). CRY1 and CRY2 both have a considerable indirect effect on transcriptional activation but do not bind DNA themselves (Yu, Liu et al. 2010, Chaves, Pokorny et al. 2011). Cryptochromes mediate the downstream transcriptional response via regulation of transcription factors (such as HY5 and HFR1) (Osterlund, Wei et al. 2000, Yang, Lin et al. 2005, Yu, Liu et al. 2010). Besides nuclear gene regulation there was also an impact on plastid genome transcription through regulation of expression of the nuclear-encoded plastid sigma factor genes SIG1 and SIG2 shown (Onda, Yagi et al. 2008).

![Figure 1.4.2: Photoreceptors found in plants. Each photoreceptor senses a particular part of the light spectrum and is either activated or inhibited upon perception. UVR8, UVB-receptor; CRY, cryptochromes; PHYB, phytochromes B; PHYA, phytochromes A; PHOT, phototropins; ZTL, ZEITLUPE family. Adapted from Casal 2013.](image)
The second type of blue light absorbing photo-receptors are the phototropins, which are involved in photo-movement responses such as phototropism, chloroplast movement and stomata opening. Two phototropins are known, PHOT1 and PHOT2, which are both largely associated with the plasma membrane. Upon activation by light, a fraction of PHOT1 is released to the cytoplasm. Phototropins have only a minor impact on transcriptional regulation and only a limited number of genes are under their control (Jiao, Lau et al. 2007). The third type of blue light receptors are the ZEITLUPE family (ZTL, FKF1 and LKP2), which have their role in entrainment of the circadian clock and the onset of flowering (Somers, Schultz et al. 2000). The photoreceptor UVR8 is also involved in phototropism although the precise mechanisms are unknown (Rizzini, Favory et al. 2011, Vandenbussche, Tilbrook et al. 2014).

1.4.2 Light- and sugar-signalling interplay

Light and sugars both represent an energy source and it is therefore not surprising that plants have found ways to sense and respond to both in a to coordinated way. There is a as-yet no established mechanism how these two signalling pathways converge. However, several lines of experimental evidence suggest they are interconnected, often also involving phytohormone signalling (e.g. GA, auxin). To date, coordination is primarily described between sugars and the light-regulated transcription factors PIFs, which are important for light-regulated elongation growth. It was reported that Suc induces hypocotyl elongation in the dark (but not light) in a PIF (PIF1, 3, 4,5) dependent manner. It was proposed that Suc induces elongation growth by increasing PIF expression and abundance. Furthermore, PIF activity was enhanced indirectly via increased GA levels, which released repression of DELLAs on the PIFs (Liu, Zhang et al. 2011).

Other studies have described the interaction of sugar and light-signalling in the context of auxin dependence. It was shown that Suc and Glc increase endogenous auxin level in a PIF-dependent way. Suc also induces auxin transport and signal transduction, altogether leading to enhanced hypocotyl growth (Lilley, Gee et al. 2012, Sairanen, Novak et al. 2012, Ljung 2013).

Furthermore, the circadian clock seems to be a linking point of sugar- and light-signalling, too. Carbohydrate metabolism is regulated by the circadian clock and conversely, some clock genes are expressed in a sugar-responsive manner (Haydon, Hearn et al. 2013). PIFs are expressed in a diurnal rhythm and their accumulation is (amongst other factors) clock regulated (Shin, Anwer et al. 2013). This indicates that PIF abundance is linked to sugars via the circadian clock genes. Another mode of regulating the transcriptional profile is by alternative splicing (AS). Investigations on AS events occurring upon different light treatment indicated that changes triggered by light were similar to changes upon exogenous sugar supply, which also argues for a coordination of sugar and light cues (Hartmann, Drewe-Boss et al. 2016). In conclusion, it seems that both sugar and light derived signals feed into a
larger network, which also includes other components crucial for plant fitness and survival, such as hormones and the circadian clock.

1.4.3 Shade avoidance syndrome (SAS)

To avoid losing their primary energy source (light), plants have evolved several mechanisms to respond to proximity of or shading by neighbours. Shade intolerant plants trigger an escape response, the so-called Shade Avoidance Syndrome (SAS). SAS is initiated primarily by sensed variation in light quality or quantity, leading to a complex network of changes involving hormonal responses and a wide range of downstream transcriptional adaptations. Ultimately, this results in different growth and morphological changes that help to compete with neighbouring plants. The morphological changes include increased leaf angles (hyponasty), elongation of hypocotyls, petioles, stems and internodes, reduced leaf-lamina expansion, reduced leaf thickness, reduced shoot branching, early flowering and adjustments of the photosynthetic apparatus in Arabidopsis (Franklin and Whitelam 2005, Casal 2012, Casal 2013). To date it is unclear if investments into elongation growth is fuelled by resource re-allocated from storage organs, as only indirect evidence exists so far. A few reports showed a negative impact of shade on yield (Libenson, Rodriguez et al. 2002, Ugarte, Trupkin et al. 2010). As previously mentioned, changes in light are the main driver of SAS. Plants generally absorb red (R) and blue (B) light to drive photosynthesis whereas green (G) and far red (FR) light is reflected or transmitted. Hence, overgrowth by another plant results in a reduction in overall light intensity (Photosynthetically Active Radiation, PAR) with a particular reduction in the R and B part of the spectra. In contrast, proximity of other plants is indicated by an enrichment of FR and G light without decreasing PAR. Apart from light, sensing volatiles of known (ethylene) and unknown nature (Pierik, Cuppens et al. 2004, Kegge, Weldegergis et al. 2013), as well as physical touch of leaf tips can also trigger a SAS response (de Wit, Kegge et al. 2012).

1.4.3.1 Response to enrichment in far red light and/or reduction in red light

There are grossly two different circumstances leading to a SAS in plants; either through direct shading by overgrowth or the proximity of neighbouring plants. True shade leads to a reduction in the overall light intensity whereas neighbour detection does not (Fig. 1.4.3 B-D). Both cases have in common that the ratio between R and FR light is reduced (low R/FR). Changes in the R to FR ratio is perceived by phytochromes. Their photo-reversible conformational change between the active Pfr and the inactive Pr forms is determined by the R/FR ratio (Holmes 1977, Smith, Casal et al. 1990, Martinez-Garcia, Gallemi et al. 2014). PHYB is the main perceiver of low R/FR signals, however other phytochromes act redundantly in certain low R/FR responses (e.g. PHYA in hypocotyl elongation; PHYD, PHYE in flowering time; PHYD, PHYE in petiole elongation; PHYE in internode elongation) (Franklin, Davis et al. 2003, Keuskamp, Sasidharan et al. 2010, Martinez-García 2010, Casal 2012, Ruberti, Sessa et al. 2012). The
photo-labile PHYA is mostly active in FR and is the only phytochrome to have an antagonistic (negative) role in SAS, inhibiting shade-induced hypocotyl elongation (Fairchild, Schumaker et al. 2000). The red light activated phytochromes (Pfr) translocate to the nucleus, thereby interacting with members of the bHLH family such as PIFs or PHYTOCHROME INTERACTING FACTOR LIKEs (PILs) (Duek and Fankhauser 2005). For SAS, particularly PIF4, PIF5 and PIF7 play important roles. Upon interaction with PHYB they become highly phosphorylated and are sent to the proteasome for degradation (Lorrain, Allen et al. 2008). This means that upon low R/FR, PHYB is inactivated, preventing phytochrome-mediated degradation of PIF4 and PIF5, thereby allowing their action in promoting elongation response (Duek and Fankhauser 2005). PIF7 seems to be activated via de-phosphorylation, whereupon it accumulates in the nucleus and allows shade-induced growth by inducing auxin biosynthesis (Li, Ljung et al. 2012). Transcriptional regulation by PIFs (PIF1, 3, 4, 5) is exerted through binding to their preferred target motif, the G-box (Zhang, Mayba et al. 2013). Low R/FR-induced rapid transcriptional changes can be reverted by high R/FR (Carabelli, Morelli et al. 1996, Salter, Franklin et al. 2003). Among the rapidly responding genes (PHYTOCHROME RAPIDLY REGULATED, PAR genes), which are postulated to be instrumental in implementing the SAS response (Steindler, Matteucci et al. 1999, Salter, Franklin et al. 2003, Roig-Villanova, Bou et al. 2006), are the homeodomain/leucine zipper (HD-ZIP) class II sub-family transcription factors ARABIDOPSIS THALIANA HOMEobox 2 (ATHB2), ATHB4, HOMEobox ARABIDOPSIS THALIANA 1 (HAT1), HAT2, HAT3 (Steindler, Matteucci et al. 1999, Ciarbelli, Ciolfi et al. 2008, Sorin, Salla-Martret et al. 2009), the bHLH proteins PIF3-LIKE1 (PIL1) (Salter, Franklin et al. 2003), PHYTOCHROME RAPIDLY REGULATED 1 (PAR1), PAR2 and LONG HYPOCOTYL IN FAR RED 1 (HFR1) (Sessa, Carabelli et al. 2005, Roig-Villanova, Bou-Torrent et al. 2007, Leivar, Tepperman et al. 2012). Among these PAR genes there are factors with demonstrated positive and negative roles in SAS. ATHB2 and PIL1 were both implicated in positively impacting elongation response, whereas the two atypical bHLH proteins HFR1 and PAR1 were shown to interact with PIFs, thereby preventing their binding to the promoter of target genes (Hornitschek, Lorrain et al. 2009, Hao, Oh et al. 2012). The shade-induced negative regulators of SAS are suggested to represent a negative feedback loop to prevent exaggerated elongation responses.

1.4.3.2 Response to low blue light

Under canopy shade, the blue part of the light spectra is reduced, too. Low B-light also triggers a shade avoidance response through pathways which have a limited overlap with those activated by low R/FR. This is supported by the observation that phyB-mutants grown under real canopies still show a robust SAS response, indicating that the low blue shade response compensates for the PHYB mediated SAS (Keller, Jaillais et al. 2011). The low B response is only triggered once true shading occurs (cannot detect neighbour proximity) and is therefore delayed compared to the low R/FR response, yet still important
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during later stages of competition (Fig. 1.4.3-D). In Arabidopsis, hyponasty, enhanced petiole and hypocotyl elongation are some of the described response to low B (Keller, Jaillais et al. 2011, Keuskamp, Sasidharan et al. 2011). Perception of the changing light conditions occurs via the cryptochromes and phototropins. Both cryptochromes (CRY1 and CRY2) were identified to regulate hypocotyl elongation (Pierik, Djakovic-Petrovic et al. 2009, Keller, Jaillais et al. 2011). CRY1 plays a role under high and low fluence, whereas CRY2’s role is limited to low fluence rates (Lin, Yang et al. 1998). Upon light perception, CRYs undergo structural changes that lead to interaction with CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and cause its translocation to the cytosol. This recruitment of COP1 by the CRYs prevents the interaction of COP1 with LONG HYPOCOTYL 5 (HY5), which otherwise would send HY5 to the proteasome for degradation. Since HY5 is a negative regulator of elongation growth (a positive regulator of photomorphogenesis) this means that under normal light conditions, HY5 is abundant in the nucleus and acts as a repressor, whereas in darkness or low B it is sent for degradation via COP1 and allows the shade response to occur (Osterlund, Hardtke et al. 2000).

Interestingly, CRY- and PHY-signalling have several points of convergence; they both interact with COP1, require PIFs to trigger shade-induced petiole and hypocotyl elongation (Yang, Tang et al. 2001, Seo, Watanabe et al. 2004, Keller, Jaillais et al. 2011, de Wit, Keuskamp et al. 2016) and many of their downstream regulators bind the G-box (PIFs, HY5) (Osterlund, Wei et al. 2000, Zhang, Mayba et al. 2013).

Phototropins contribute to the low B response mainly through modulating phototropic growth, which directs the plants towards gaps in the canopy (Ballare, Sanchez et al. 1987). The two phototropins (PHOT1, PHOT2) act redundantly under high blue light, whereas PHOT1 alone is important under low fluence rates (Sakai, Kagawa et al. 2001, Kong, Suzuki et al. 2006). Phototropins are located to the plasma membrane (Sakamoto and Briggs 2002, Kong, Suzuki et al. 2006) and interact with NONPHOTOTROPIC HYPOCOTYL 3 (NPH3), which in rice is known to be important in lateral auxin translocation, thereby allowing uni-lateral growth on the shaded side of the stem (Motchoulski and Liscum 1999, Haga, Takano et al. 2005). Another important player in phototropism is PHYTOCHROME KINASE SUBSTRATE 1 (PKS1). It was identified as being strongly up-regulated upon blue light illumination and later shown to interact with PHOT1 and NPH3, thereby playing a critical role for phototropism. Due to the fact that PKS1 was initially identified as a component of the phytochrome-signalling pathway, it was suggested that PKS1 represents a link between the phototropin- and phytochrome- (mainly PHYA) signalling in the phototrophic response of etiolated seedlings (Lariguet, Schepens et al. 2006). A recent study found that phototropism of green seedlings is enhanced in low R/FR, which occurs via PIF- triggered auxin biosynthesis, thereby supporting an interaction of phytochrome- and phototropin- mediated signalling (Goyal, Karayekov et al. 2016).
1.4.3.3 Response to low light intensities or enriched green light

Low overall light intensities represent a status of limited resources for the plant and needs to be overcome. Enrichment in green light indicates the presence of potential competitors (Fig. 1.4.3-C). Therefore, it seems sensible for the plant also to be responding to those two conditions. Indeed, reduced light intensity in the visible light spectra (PAR) or enrichment in green light also induces morphological changes similar to shade-induced adaptations. In low PAR these are namely petiole movement and hyponasty in Arabidopsis. Petiole movements are dependent on blue light and photosynthesis-derived signals, whereas hyponasty requires the photoreceptors PHYA, PHYB, CRY1 and CRY2 derived signals (Millenaar, van Zanten et al. 2009). Enrichment in green light results in elongation of petioles and upward bending of leaves. Originally, CRYs were proposed to be the responsible sensors by sensing the blue/green ratio (Sellaro, Crepy et al. 2010). A later, comparative transcriptional profile study suggested that the increased green light is most likely sensed by cryptochromes and an additional unknown light sensor (Sellaro, Crepy et al. 2010, Zhang, Maruhnich et al. 2011).

1.4.4 Shade – hormone interplay

Light-driven changes in morphology, growth and development are often accompanied and established by changes in hormone responses, which includes modulation of the hormone level, sensitivity or signalling. Phytohormones known to be involved in SAS are auxin, brassinosteroid (BR), ethylene and gibberellic acid (GA) (Kozuka, Kobayashi et al. 2010, de Wit and Pierik 2016). The shade – hormone
interplay for some phytohormones is better characterised than for others. While there is a lot of experimental evidence of concerted actions, mechanistically little is known. Most data pertain to auxin, BR and GA. The understanding for the ethylene interaction with shade-signalling is still very limited. What is known is that ethylene triggers shade related phenotypes and shade induces ethylene production. The best evidence for the ethylene – shade interplay comes from studies with tobacco (Pierik, Visser et al. 2003, Pierik, Cuppens et al. 2004). Findings about the interplay between BR, auxin and GA with shade are described in more detail in the following paragraphs.

1.4.4.1 Brassinosteroids
Brassinosteroids (BR) are powerful drivers of growth, also under shade conditions, which are involved in hypocotyl elongation, cotyledon and leaf expansion (Martine-Garcia 2010). There is a range of experimental data supporting this: The BR biosynthesis mutants dwarf1-101 (Luccioni, Oliverio et al. 2002), deetiolated2 (det2) (Martinez-Garcia, Galstyan et al. 2010), shade avoidance1 (sav1) (Tao, Ferrer et al. 2008) do not elongate in canopy shade, expression of the BR receptor BRI1 is rapidly up-regulated by a reduction of the R/FR ratio (Devlin, Yanovsky et al. 2003, Sorin, Salla-Martret et al. 2009) and seedlings are hypersensitive to BR (epibrassinolide, EBL) in low R/FR (Bou-Torrent, Galstyan et al. 2014)(Bou-Torrent 2014). Under low B light, SAS is reduced in the BR synthesis mutant rotundifolia 3 (rot3) (Kim, Tsukaya et al. 1998, Casal 2012), wild-type plants treated with the BR biosynthesis inhibitor brassinozole (BRZ) and mutants impaired in BR sensing (BR receptor mutants, bri-1) (Keuskamp, Sasidharan et al. 2011). It is to be noted that for all of the above mentioned low B light responses to be complete, a simultaneous blocking of auxin additionally to the BR pathways is needed (Keuskamp, Sasidharan et al. 2011, Casal 2012). Overall these findings indicate that BR abundance and sensitivity are important for full responses to low R/FR and low blue light.

To investigate how BR and shade expresional outputs converge, transcriptional changes of BZR1 were compared to expresional changes causes by PIF4. It became clear that BZR1 and PIF4 have a large overlap in common target genes (50 %). Furthermore, PIF4 and BZR1 interact physically and together bind to the promoter of target genes (Oh, Zhu et al. 2012, Oh, Zhu et al. 2014). Among the commonly regulated targets are some of the PAR genes such as ATHB2, ATHB4, HAT2, HAT3 and PAR1 (Sun 2010, Oh 2012). Interestingly, ATHB4 overexpression (ATHB4-ox) confers hypersensitivity to exogenously applied BR (epibrassinolide, EBL) in respect to hypocotyl elongation, while athb4 hat3 double mutants are less sensitive (Sorin, Salla-Martret et al. 2009). Conversely, ATHB4-ox and athb4 hat3 both show impaired hypocotyl elongation response to shade. These results indicated the role of ATHB4 and HAT3 in regulating SAS responses, likely integrating BR signals. Mechanistically it remains unclear how this is achieved, but possibly it occurs through regulation of other HD-Zip class II transcription factors, such as ATHB2, which is down-regulated in ATHB4-ox (Sorin, Salla-Martret et al. 2009). Further points of
convergence are the BRASSINOSTEROID-ENHANCED EXPRESSION (BEE) and BES1 INTERACTING MYC LIKE (BIM) proteins, which interact with the shade-regulated PAR1 gene (Cifuentes-Esquível, Bou-Torrent et al. 2013) (Fig. 1.4.4). BEEs and BIMs are both involved in BR-signalling, BEEs as functionally-redundant positive regulators of BR-signalling (Friedrichsen, Nemhauser et al. 2002) and BIM1 as an interacting partner of the BR-responsive transcription factor BES1 (Yin, Vafeados et al. 2005). Upon shade, the expression of BEE1, BEE2 and all BIMs is rapidly induced. Both triple mutants (bee1 bee2 bim3, bim1 bim2 bim3) show reduced hypocotyl elongation in response to shade (Friedrichsen, Nemhauser et al. 2002, Cifuentes-Esquível, Bou-Torrent et al. 2013). Thus, besides shade-regulated BR synthesis, BR perception and BZR1-PIF4 interaction, shade and BR responses are also co-ordinated through shade-induced transcriptional regulation of BR-signalling components (BEEs, BIMs) and the direct interaction of downstream light- and BR-signalling components (PAR1 with BEEs and BIMs).

1.4.4.2 Auxin

Among all phytohormones, auxin has emerged as a key player in mediating the elongation growth in response to shade, which is supported by the large overrepresentation of auxin regulated genes among the shade regulated genes (Hornitschek, Kohnen et al. 2012, de Wit, Galvao et al. 2016, Kohnen, Schmid-Siegert et al. 2016). The signalling pathway starting from shade perception to modulation of auxin responses and ultimately leading to the morphological changes is rather linear and well described. SAS responses triggered by low R/FR or low B both depend on modulated auxin abundance, spatial re-distribution and sensitivity to auxin (Tao, Ferrer et al. 2008, Hornitschek, Kohnen et al. 2012, Li, Ljung et al. 2012). Shade induces a rapid increase in auxin levels (Tao, Ferrer et al. 2008, Hornitschek, Kohnen et al. 2012, Li, Ljung et al. 2012, Tao, Ferrer et al. 2008). For hypocotyl elongation to occur, auxin
produced in the cotyledon seems to be required and sufficient (Kohnen, Schmid-Siebert et al. 2016). The importance of de novo auxin biosynthesis was supported by the observation that mutants with impaired auxin synthesis (shade avoidance3, sav3) showed a reduced SAS response in terms of hypocotyl length, petiole elongation and leaf angle (Tao, Ferrer et al. 2008). Later studies revealed that shade-activated PIF4, PIF5 and PIF7 directly bind to the promoters of a range of YUC genes (catalysing a rate-limiting step of auxin biosynthesis), thereby promoting auxin synthesis (Fig. 1.4.5). A multiple YUC mutant (yuc2589), which was insensitive to low R/FR, confirmed the significance of YUC dependent increase in auxin biosynthesis (Nozue, Tat et al. 2015, Muller-Moule, Nozue et al. 2016). Furthermore, treatment of wild-type plants with the auxin transport inhibitor naphthylphthalamic acid (NPA) led to reduced hypocotyl elongation upon shade mimicking conditions (Steindler, Matteucci et al. 1999). Besides promoting auxin synthesis, low R/FR also leads to lateral auxin distribution mediated by PIN-FORMED 3 (PIN3) (Keuskamp, Pollmann et al. 2010) and increased ATHB2 (a PAR gene) and IAA29 expression (Kunihiro, Yamashino et al. 2011). ATHB2, IAA19 and IAA29 were identified as direct targets of PIF5 and most likely alter the responsiveness to auxin (Fig. 1.4.5). ATHB2-ox has long hypocotyls even in high R/FR, which can be reverted by NPA (Steindler, Matteucci et al. 1999, Sawa, Ohgishi et al. 2002, Hornitschek, Kohnen et al. 2012).

Large-scale transcriptional analysis supported the interconnectivity of BR- and auxin-signalling in respect to shade avoidance. Expressional changes upon end-of-day far red treatments (EOD-FR), mimicking low R/FR shade conditions, showed an over-representation of auxin- and BR-related genes (Kozuka, Kobayashi et al. 2010). Furthermore, petiole elongation in EOD-FR and hypocotyl elongation in low B are reduced in auxin- and BR-deficient conditions (Kozuka, Kobayashi et al. 2010, Keuskamp, Sasidharan et al. 2011). Altogether, this shows that auxin and BR are coordinated in respect to SAS response and may act interdependently as in other growth conditions (Nemhauser, Mockler et al. 2004, Kozuka, Kobayashi et al. 2010, de Wit and Pierik 2016). It was proposed that one mechanism of auxin and BR integration is via the AUXIN RESPONSE FACTOR 2 (ARF2), which is phosphorylated by BRASSINOSTEROID INSENSITIVE 2 (BIN2). BIN2 activity is modulated by BR and ARF2 is a repressor, which competes with activator ARFs for DNA-binding. Phosphorylation of ARF2 by BIN2 leads to...
reduced DNA-binding affinity of ARF2 and hence a release of transcriptional repression, thus promoting the expression of auxin-response genes (Vert, Walcher et al. 2008, Casal 2012). Another point of convergence is through the direct interaction of BZR1 and ARF6 at the promoters of common target genes (Oh, Zhu et al. 2014).

1.4.4.3 Gibberellic acid
GA is involved in many growth-related processes including germination, hypocotyl elongation, leaf expansion, petiole elongation and flowering. Depending on the species investigated, the importance of changes in GA levels (tobacco, sorghum, potato and others) or GA sensitivity (Arabidopsis, cucumber, pea) in respect to shade responsiveness varies (Martínez-García 2010). GA perception leads to degradation of DELLA proteins, which are negative regulators of GA-signalling, thereby releasing repression on GA regulated genes and allowing growth (Fleet and Sun 2005, de Wit and Pierik 2016). In low R/FR conditions as well as in CRY1 mutants, GA related genes are up-regulated. This is in agreement with the observation that both low R/FR and low B trigger a reduction of the DELLA proteins, thereby allowing elongation growth (hypocotyl, petiole elongation) (Djakovic-Petrovic, de Wit et al. 2007). Furthermore, it was discovered that the DELLA protein RGA interacts with PIF4 and PIFS, thereby preventing their action as positive regulator of shade (de Lucas, Daviere et al. 2008). This means that not only active phytochromes (Pfr), but also DELLAs inhibit shade responses, which is released upon inactivation of phytochromes (e.g. by shade) or perception of GA (de Wit and Pierik 2016).

1.4.4.4 Light, auxin, gibberellic acid and brassinosteroid interplay
Recently, a model integrating the PIF4 – BZR1, the DELLA –PIF4 (mentioned earlier) and the BZR1-DELLA interaction (Bai, Shang et al. 2012) was proposed (Jaillais and Vert 2012). It suggests that under skotomorphogenic conditions, phytochromes are inactivated and the GA-signalling is active, leading to DELLA degradation. This results in released repression on PIFs and allows the interaction of BZR1 with PIF4 and transcription of their common target genes. Among the genes up-regulated via this process are the PRE-family proteins, which are known to be involved in elongation growth. This mechanism for skotomorphogenesis may also apply for shade-signalling (Fig. 1.4.6). Another study extended the interaction further to auxin-signalling in respect to hypocotyl elongation. It was observed that PIF4 directly interacts with the auxin response factor (ARF6) on a large set of common direct target genes. Furthermore, ARF6, PIF4 and BZR1 were seen to share direct targets and act cooperatively. In contrast, the GA-responsive DELLA protein RGA inhibits the action of all other three components (Oh, Zhu et al. 2014). Both proposed mechanisms would not only allow to integrate light, BR, GA and auxin signals but also consider ethylene, abscisic acid and jasmonate derived signals, since these hormones were all shown to also modulate DELLA abundance (Sun, Fan et al. 2010, Leivar and Quail 2011, Jaillais and Vert
The convergence of all these signalling pathways on DELLA abundance would add another level of plasticity and coordination to the system. Nevertheless, further studies investigating the molecular mechanisms of these interactions also at different developmental stages are needed to complete the picture.

Figure 1.4.6: Interaction of brassinosteroid-, light- and gibberelic acid-signalling with flexibility for inputs of ethylene, auxin, abscisic acid and jasmonate derived signals. Under photomorphogenic conditions, phytochromes and DELLA driven inhibition of PIFs, BZR1 and ARF6 inhibits elongation growth. In shade the skotomorphogenic model may also apply, whereby common targets of PIF, BZR1 and ARF6 allow longitude growth to occur. Adapted from Jaillais and Vert 2012.
2 Research objective

The primary aim of this study was to identify the as-yet elusive sugar ligand and investigate its impact on BZR1-BAM transcription factor activity in vitro and in vivo. To determine if the protein-sugar interaction was BAM-domain dependent, a BAM8 version with proposed inability to bind to the putative ligand (due to steric hindrance in the binding pocket) was included. A potential involvement of the BZR1-BAMs in sugar-signalling in planta was studied by growing BZR1-BAM mutants and overexpressing plants on high glucose and high sucrose medium, an approach that has often been employed to probe sugar-signalling networks. Nevertheless, applying exogenous sugars to plants is non-physiological, therefore an alternative approach was to use mutants in primary carbon metabolism (sugar mutants), which suffered from impaired growth. It was suspected that one cause of the reduced growth was due to altered soluble sugar levels, which were sensed and triggered a growth inhibiting response. Hence, I hypothesized that this growth defect would be at least partially compensated in the absence of the sensor (e.g. BZR1-BAMs). BZR1-BAM mutant and overexpressing plants were therefore crossed to these sugar mutants and the growth phenotypes of the resulting genotypes was investigated. A third approach to unravel the putative involvement in sugar-signalling was by assessing protein-protein interactions with candidates known to have a link to sugar-signalling.

Another aim of this study was to explore the role of BZR1-BAMs in planta in more details by looking at the plant-wide expression pattern and the subcellular localisation of the BZR1-BAMs. For that purpose, transcriptional and translational fusion constructs, including as much as possible of the potential native regulatory elements, were generated and stably expressed in Arabidopsis.

A previous study (Reinhold, Soyk et al. 2011) identified a resemblance of the BZR1-BAM double mutant (bam7 bam8) plants to shade-grown plants. Later it was proposed that the BZR1-BAMs might act as integrators of a metabolic signal into light-signalling pathways by modulating light (e.g. shade) triggered responses depending on the prevalent carbon status (Soyk, Simkova et al. 2014). Hence, a convergence of BZR1-BAM-, light- and brassinosteroid-signalling was suspected. I aimed at identifying the genome wide direct chromatin binding sites of BZR1-BAMs. In order to compare BZR1-BAM direct targets with direct targets of transcription factors known to play key roles in brassinosteroid (BZR1) and light-signalling (PIF4) pathways. Furthermore, physiological responses to different light treatments were addressed. For the most promising one (shade treatment), the BZR1-BAM-dependent transcriptional changes of known shade induced genes upon shading was quantified.
3 Material and Methods

3.1 Plant methods

3.1.1 Plant material

*Arabidopsis thaliana* mutant or stably overexpressing (*-ox*) plants of *bam7.1, bam8.1, bam7 bam8, BAM8-ox* (*bam8.1* background) and *BAM7-ox* (wild-type background) genotype were all created in the Columbia background and genotyped as described previously (Reinhold, Soyk et al. 2011). Plants constitutively overexpressing the BAM8-lig protein were in the *bam8.1* (Columbia) background and selected by BASTA resistance (Soyk, Simkova et al. 2014). For light response experiments *pif4 pif5* (Lorrain, Allen et al. 2008), *cry1* (T-DNA insertion line, GABI_091D11), *phyB-9* (Reed, Nagpal et al. 1993, Neff, Neff et al. 1998), *hf1-101* (Duek and Fankhauser 2003) and *phyA-211* (Reed, Nagatani et al. 1994) were used. All mutant alleles were in the Columbia ecotype, except for the *phyA-211*, which is in Landsberg erecta ecotype. Mutant combinations of *bam7 bam8* and *BAM8-ox* were created by crossing these to *mex1-1* (Niittyla, Messerli et al. 2004), *dpe2-5* (SALK_73273)(Chia, Thorneycroft et al. 2004), *isa1-1* (SALK_042704)(Delatte, Trevisan et al. 2005), *pgm1* (Caspar, Huber et al. 1985), *hxk1-c* (SALK_070739)(Lee, Yoon et al. 2012), which were all of Columbia ecotype.

3.1.2 Plant growth on soil and agar medium

*Arabidopsis thaliana* and *Nicotiana benthamiana* were grown on nutrient rich soil (D. Kistner, BP-Substrat 2) compost in a Percival AR95 growth chambers (CLF Plant Climatics) at a constant temperature of 20 °C and 70 % humidity. If not mentioned differently, light periods were set to 12 h light/ 12 h dark (12/12 light/dark cycle). Standard light intensity was set to 150 µmol photons m⁻² s⁻¹. For physiological assays and localisation studies, plants were grown under sterile conditions on solid half-strength Murashige Skoog (MS) medium including MES and vitamins (Duchefa, M0255.0010) at pH 5.7 and 0.8 % [w/v] phytoagar (Duchefa, P1001.1000). Seeds were surface-sterilized by washing twice in 500 µl 80 % [v/v] ethanol followed by rinsing with sterile water. If not mentioned differently, the light period was set to 12/12 light/dark cycle.

3.1.3 Shade avoidance response (true shade and neighbour detection)

Seeds were grown on solid medium (as above but 1.6 % [w/v] phytoagar). Plated seeds were kept at 4 °C in the dark for 3 d. Subsequently seedlings were grown vertically at 21 °C for 4 d in white light (high red to far red ratio, high R/FR) conditions and then either transferred for another 3 d to low R/FR light or kept in the high R/FR. Light intensities of photosynthetically active radiation (PAR, 400-700 nm) were set to 120 µmol photons m⁻² s⁻¹ for neighbour detection experiments or 40 µmol photons m⁻² s⁻¹ for true shade experiments. The day/night cycle was set to 16h/8h. High R/FR indicates a red (640-700 nm) to...
far red (700-760 nm) ratio of 1.0 and low R/FR was set to 0.2.

3.1.4 Pure red, blue and far red fluence response experiment
Seeds were plated onto solid half-strength MS medium and kept at 4 °C in the dark for 3 d. To initiate germination, seeds were exposed to white light for 6 h and subsequently transferred back to darkness at 20 °C for 18 h. Exposure to the respective continuous pure light (red (R), blue (B) or far red (FR)) occurred for 5 d. The light sources were LED lamps (CLF Plant Climatics). Fluence rates were reduced by 1-3 layers of neutral density filters (Lee Filters, B209, Menzi ebz AG, Horgen). As a control, seeds were kept in the dark instead of exposing them to light. On average the hypocotyl lengths of 21 seedlings were analysed.

3.1.5 Physiological assay on sugar containing plates
Seeds were plated onto solid half-strength MS medium supplemented with or without 2 % [w/v] sucrose (Suc) or 2 % [w/v] glucose (Glc) and kept at 4 °C in the dark for 3 d. Subsequently they were exposed to 4.5 d of high R/FR (PAR, 95 µmol photons m⁻² s⁻¹, R/FR = 3.0), which was followed by a low R/FR (R/FR= 0.03) treatment for 5 d. Control plants were kept in high PAR, for 9.5 d. There were two different sets of experiments; in the first, the low R/FR treatment was performed under low PAR (17 µmol photons m⁻² s⁻¹), whereas in the second, the low R/FR treatment was performed under high PAR (95 µmol photons m⁻² s⁻¹). The control conditions were identical for both experiments. On average, the hypocotyl lengths of 19 seedlings were analysed. R=655-665 nm, FR=725-735 nm, PAR=400-700 nm

3.1.6 Transient transformation of Nicotiana benthamiana by leaf infiltration
To study subcellular localisation or protein-protein interactions by Bimolecular Fluorescence Complementation (BiFC), proteins were transiently expressed in epidermal cells of Nicotiana benthamiana leaves as described before (Sparkes, Runions et al. 2006). Single colonies of Agrobacterium tumefaciens (GV3101 or ALG-O), which carried the binary plasmids containing the gene of interest, were transferred to 25 ml LB medium (Brunschwig, 244610) supplemented with antibiotics (50 µg/ml rifampicin, vector resistance and 50 µg/ml gentamicin for GV3101) and incubated shaking (220 rpm) at 28 °C for 48 h. Cells were spun down at 3,000 g for 10 min at 20 °C and washed once in infiltration buffer without acetosyringone (50 mM MES, 2 mM NaH₂PO₄, 0.5 % [w/v] glucose). Subsequently, cells were resuspended in infiltration buffer containing 100 µM acetosyringone to an OD₆₀₀nm of 0.5 - 1. For co-expression of constructs, bacterial strains were mixed before infiltration to a final OD₆₀₀nm of 0.1 for each construct and incubated shaking at 20 °C for at least 1 h. The silencing suppressor p19 was constitutively co-expressed for BiFC assays (Lindbo 2007). Bacteria were infiltrated with a 1ml syringe into the lower epidermis. After infiltration, plants were incubated for 3 d before
fluorescence was imaged using a confocal Zeiss LSM780 microscope (Zeiss, Jena, Germany) (see section 3.4.2, 3.4.3).

3.1.7 Stable transformation of *Arabidopsis thaliana*

*Arabidopsis* were stably transformed with *Agrobacterium tumefaciens* strain GV3101 as described previously (Clough and Bent 1998). Selection of transformed lines was performed by spraying with a 0.1 % [v/v] BASTA (glufosinate-ammonium) solution or growing seedlings on soil soaked with 0.1 % [v/v] BASTA. For each construct, between 10 and 20 primary transformants (T1) were selected and analysed for protein abundance by Western blot (see section 3.2.2). Over the next generations, plants were sprayed with or grown on BASTA to obtain stable transgenic lines. The T2 generation was tested for single insertions of the transgene by counting the ratio of BASTA resistant/susceptible among a population of 100 seedlings. In the T3 generation, homozygous lines were selected.

3.1.8 Transactivation Assay in protoplasts of *Arabidopsis thaliana* mesophyll cells

Transactivation assays in protoplasts of Arabidopsis mesophyll cells were performed as described earlier (Yoo, Cho et al. 2007, Reinhold, Soyk et al. 2011) with minor modifications. As a reporter construct, a pUC18 vector carrying the LUC gene under the control of twelve repetitions of the BBRE (CACGTGTG) upstream of the minimal 35S promoter was used (Reinhold, Soyk et al. 2011). As the effector plasmids, a pEarleyGate201 construct carrying the BAM8 coding sequence was used. The empty pEarleyGate201 served as a no-effector control. To correct for variable transfection rates, a Ubq10:GUS:nosT construct (Yoo, Cho et al. 2007) was used as transfection control. To modify endogenous Tre6P concentrations, pGreen vectors carrying the *E. coli TPS* (*otsA*) or *TPP* (*otsA*) genes (Yadav, Ivakov et al. 2014), or the pEarleyGate201 carrying the rice OsTPP1 were co-expressed. Fully-grown leaves (leaf number 5-7) of 4-week old plants grown in 12/12 day/night cycle were harvested and digested with Maceroenzyme R-10 (Serva) and cellulase Onozuka R-10 (Serva) during 3-4 h in the dark. After digestion, the same volume (50 ml) of W5 solution (4 mM MES pH 5.7, 0.5 M mannitol, 20 mM KCl) was added. Protoplasts were filtered through four layers of nylon mesh (100 µm) and pelleted at 100 g for 3 min. Supernatant was removed, protoplasts were gently resuspended in 10 ml of W5 solution and rested on ice for 30 min. Subsequently, protoplasts were resuspended in MMg solution (0.4 M mannitol, 15 mM MgCl2, 4 mM MES pH 5.7) to a final concentration of 5*10^5 cells/ml. Protoplasts were transformed with 25 µg DNA of effector, reporter, transfection control and Tre6P modifying enzymes constructs in the following ratios: 10: 8: 2: 5. After transformation, protoplasts were incubated for 2, 6 or 16 h in WI solution (4 mM MES, pH 5.7, 0.5 M mannitol, and 20 mM KCl) supplemented with or without 15 mM sucrose in continuous light. Light conditions were either set to moderate (50 µmol photons m^{-2} s^{-1}) or high light (120 µmol photons m^{-2} s^{-1}) intensities. Enzymatic
assays were performed as described previously (Yoo, Cho et al. 2007). Three technical replicates were processed per plasmid combination. The high light experiments were repeated twice and the moderate light experiment three times. The expression of effector proteins was checked by immunoblotting using polyclonal anti-BAM8 antibodies, anti-HA antibody, anti-otsA antibody or anti-otsB antibody (Yadav, Ivakov et al. 2014) (for details see Table 3.1). Tre6P was quantified as described before (Lunn, Feil et al. 2006) with minor modifications. The protoplast aliquots were mixed with ice cold methanol/chloroform (7:3, [v/v]) and incubated at -20 °C for 2 h. After adding double the volume of ice-cold water, samples were centrifuged at 13,000 g at 4 °C for 10 min and the water fraction collected. The extraction step was repeated once more and water soluble fraction was removed and added to the first aliquot. Subsequently, samples were dried using a centrifugal vacuum dryer at 30 °C and analysed by LC-MS/MS, which was kindly performed by Dr. John Lunn, Max Planck Institute in Potsdam-Golm.

### 3.2 Biochemical methods

#### 3.2.1 Production of recombinant proteins and protein purification

For production of recombinant proteins, HIS-tagged versions (BAM7, BAM8, BAM8-dna, BAM8-lig) were overexpressed in *E. coli* BL21 (Stratagene) using the pET21a(+) vector (Novagene). For that purpose, the respective *E. coli* strains were grown in liquid LB medium (Brunschwig, 244610) containing the selective antibiotic at 37 °C with shaking (220 rpm) 16 h to an OD₆₀₀ of 0.6 - 0.9. To induce protein expression, 1 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) was added and cultures were further incubated at 16 °C for 20 h. Cells were harvested by centrifugation at 5,000 g, at 4 °C for 20 min and resuspended in Binding Buffer (50 mM HEPES, pH 7.5, 0.5 M NaCl, 10 % [v/v] glycerol, and 15 mM imidazole) and broken using a M-110P microfluidizer processor (Microfluidics, Lampertheim, Germany) until suspension became more transparent. Cell debris were separated from the soluble components by centrifugation for 15 min at 20,000 g. The supernatant was filtered through a 0.45 µm sterile filter and subsequently added to the pre-washed Ni-NTA resin (MN). His-tagged protein was allowed to bind to the Ni-NTA resin by incubation at 4 ° for 2 h under gentle agitation. Resin was sedimented by centrifugation at 200 g for 3 min at 4 °C and the supernatant was discarded. Protein bound resin was resuspended in Binding Buffer containing protease inhibitor (EDTA free, Roche), transferred to empty spin-columns and sedimented once more. Resin was washed five times in Triton Buffer 50 mM HEPES, pH 8.0, 0.5 M NaCl, 10 % [v/v] glycerol, 40 mM imidazole, 0.5 % [v/v] Triton-X 100) and five times with Wash Buffer (50 mM HEPES, pH 7.5, 0.5 M NaCl, 10 % [v/v] glycerol, and 40 mM imidazole), each by a centrifugation step of 2 min at 200 g at 4 °C. For the last wash, the supernatant was allowed to drip out by gravity flow. Elution of the proteins from the column was achieved by adding 10 ml Elution Buffer (50 mM HEPES, pH 7.5, 0.5 M NaCl, 10 % [v/v] glycerol, and 250 mM imidazole) to the column and
collecting fractions every 500 µl. The elution fractions containing the highest protein concentration of the best purity were determined by spectrosocopically measuring protein concentration (NanoDrop, Thermo Scientific) and by SDS-PAGE followed by Coomassie staining of the gel. Parameters used for the absorbance measurements were the following: Molar weight of 77.76 kDa (BAM7) and 77.16 kDa (BAM8) and molar extinction coefficient of 104.29*10^3 (BAM7) and 113.2*10^3 (BAM8). The five chosen elution fractions were pooled and a buffer exchange into Storage Buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10 % [v/v] glycerol, 0.05 % [v/v] Tween) was performed by using Nap-25 columns (Illustra NAP-25 Columns, Fisher Scientific) according to manufacturer’s instructions. To reduce the volume and thereby increase the protein concentration, buffer-exchanged protein solution was added to the prewashed Amicon 50 column (Amicon Ultra-4, 50 kDa, Millipore). Prewashing of the Amicon 50 was achieved by washing once with water and once with storage buffer. The volume of the protein solution was reduced by one or more centrifugation steps at 3,500 g at 4 °C, each for 5-10 min. Between centrifugation steps, the solution was gently pipetted up and down to prevent precipitation of protein at the filter surface. Centrifugation steps were repeated until the desired concentration and volume was achieved. Protein was aliquoted, immediately flash frozen in liquid nitrogen and stored at -80 °C until further use.

3.2.2 SDS-PAGE and Immuno-blotting

Frozen plant material was homogenized using a mix-mill and glass beads. One-hundred mg tissue powder were extracted in 1 ml Laemmli buffer (63 mM Tris-HCl pH 6.8, 15 % [v/v] glycerol, 2% [w/v] Sodium Dodecyl Sulfate (SDS, Sigma 71725), 0.15 % [w/v] bromophenol blue, 7 mM DTT). Samples were suspended by vortexing, followed by heating to 95 °C for 5 min and repeated vigorous vortexing. Samples were centrifuged at 15,000 g for 2 min and loaded on 10 % [w/v] (for BAM7, BAM8, otsA) or 12 % [w/v] (for otsB, OsTPP) SDS-PAGE gels prepared with the Biorad mini-PROTEAN system (Biorad, Cressier, France) according to the manufactures instruction. Gels were run at constant 20 mA per gel. Proteins were blotted onto Immobilon-P Transfer Membrane (Sigma) for chemiluminescence detection or Immobilon-FL PVDF (Merck&Cie) for ODYSSEY detection. Blotting was performed at 100 V for 90 min at 4 °C in Blotting Buffer (25 mM Tris-HCl, 192 mM glycine, 20 % [v/v] ethanol). For chemiluminescence detection, membranes were blocked in TBST buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05 % [v/v] Tween-20) supplemented with 5 % [w/v] milk powder for 1 h at 20 °C, gentle agitation. For ODYSSEY detection membrane was subjected to a drying step of 40 min at 20 °C, a short washing for 20 s in 100 % [v/v] methanol and a rinsing step in TBST buffer before blocking in TBST buffer supplemented by 5 % [w/v] milk powder. After blocking, membranes were incubated in the primary antibody solution of 1 or 5 % [w/v] milk powder in TBST supplemented with the according antibody dilution (see table 3.1)
overnight at 4 °C under gentle shaking. After 16 h, membranes were washed in TBST for four times 15 min at 20 °C under shaking. Secondary antibodies conjugated with horseradish peroxidase (chemiluminescence) or conjugated to red or green fluorophore (ODYSSEY) were diluted 1:10,000 in TBST (chemiluminescence) or 1:20,000 (ODYSSEY) TBST containing 0.01 % [w/v] SDS, in both cases supplemented with 1 % [w/v] milk powder, and incubated with the membrane for 1 h at 20 °C. Membranes were washed for four times for 15 min in TBST with agitation and developed with WesternBright ECL chemiluminescence substrate (Witec ag, Littau) according to the manufactures instruction. For the ODYSSEY technique, membranes were imaged on the Odyssey Imaging System (Li-Cor, Homburg, Germany).

### 3.2.3 Surface Plasmon Resonance (SPR)

All SPR experiments were performed on a Biacore T100 biosensor (GE Healthcare) device on a HC-1000M chip (XanTec bioanalytics GmbH, Düsseldorf). All processes were performed in 1x HBS running buffer (20xHBS-EP+ Buffer, pH 7.6, H8022, Teknova) at 20 °C. Biotin-coupled oligonucleotides were immobilised on the chip surface through its stable binding to streptavidin, which was covalently immobilised to the chip surface. The biotin coupled oligonucleotides were namely double stranded BBRE (ds-BBRE), mutated BBRE (ds-mBBRE) or single stranded oligoAT (ss-oligoAT) (Table 3.2). Single stranded biotin coupled forward BBRE, mBBRE (fw) and reverse (rv) oligonucleotides as well as the biotin coupled oligoAT were synthesised and HPCL purified by Microsynth. Annealing of the fw and rv nucleotide was achieved as described before (Brand, Kirchler et al. 2010). To covalently immobilise streptavidin, the surface was activated by a mixture of 0.005 M NHS/0.2 M EDC (N-hydroxysuccinimide/ N-ethyl-N’-(3-diethylaminopropyl) carbodiimide) at a flow rate of 5 μl/min. Streptavidin was diluted in the running buffer to 0.1 mg/ml and injected over the activated flow cell for 360 s until the surface was
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### Oligonucleotide Sequence (5'-3')

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')</th>
<th>Provider/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBRE fw</td>
<td>GTAAAATACAATCGATCACGTAGATAGACGC</td>
<td>Reinhold et al. 2011</td>
</tr>
<tr>
<td>BBRE rv</td>
<td>GCCGTGTTCCTACGATCGATCGATTCTTTTAC</td>
<td>Reinhold et al. 2011</td>
</tr>
<tr>
<td>mBBRE fw</td>
<td>GTAAAATACAATCGATACTTGTGATCGATTCTTTTAC</td>
<td>Reinhold et al. 2011</td>
</tr>
<tr>
<td>mBBRE rv</td>
<td>GCCGTGTTCCTACGATCGATCGATTCTTTTAC</td>
<td>Reinhold et al. 2011</td>
</tr>
<tr>
<td>oligo-AT</td>
<td>ATA ATA AAT AAT AAT</td>
<td>Microsynth</td>
</tr>
</tbody>
</table>

**Table 3.2: List of nucleotides used for the SPR experiments**

Covered by approximately 7,000 Response Units (RU). The amine-coupled surface was subsequently blocked with ethanolamine (1M, pH 8.5) for 360 s. To further prepare the surfaces for the experiment, the reaction cell was exposed to biotinylated ds-BBRE or biotinylated ds-mBBRE (100 nM in HBS) for 120 s to a coverage of roughly 500 RU. To block the reference surface and non-occupied streptavidin on the reaction surface, both cells were exposed to biotinylated single stranded AT-oligo nucleotide ss-oligoAT (5 µM) for 700 s. At this point both chip cells were ready for use. An alternative blocking method (only if specifically indicated) was achieved by injecting biotinylated Bovine Serum Albumin (BSA, 0.1 mg/ml) instead of ss-oligoAT. The drawback of BSA blocking is that it differs more in chemical property from the reaction surface (ds-BBRE) than ss-oligoAT.

Analytes used in all SPR experiments were diluted in SPR binding buffer, vortexed (sugars) or mixed by flicking at the tube (proteins). All proteins used for the SPR experiments were 6xHis-tagged, expressed in E. coli and purified by Ni-NTA and stored at -80 °C in storage buffer (as described in section 3.2.1). Before use, proteins were thawed on ice and spun at 20,000 g at 4 °C for 15 min to eliminate precipitates. Sugars used for SPR are listed in Table 3.3. There were in principle two different modes of experiments performed; association experiments and binding experiments. If not stated differently, they were performed as follows: For the ‘Association Experiment’, 7 start up cycles were performed before the actual experiment to allow the system to equilibrate. For association to occur, the analyte (protein with or without sugar) was injected for 200 s at a flow rate of 5 µl/min. During the following dissociation phase (120 s, same flow rate), the system came into equilibrium or dissociation was allowed to occur. Regeneration was achieved by injection of NaCl (1 M) for 60 s at a flow of 30 µl/min. Subsequently, the cycle was repeated with the next analyte. For the ‘Binding Experiment’, 7 start up cycles preceded the actual experiment. The sample contact (usually 5 mM or 0 mM sugar) was set to 45 s with a dissociation time of 60 s, both at a flow rate of 30 µl/min. In the first regeneration step, 1 M NaCl was injected for 60 s. In the second regeneration step, protein (if not stated differentially BAM8 37.5 nM, BAM7 50 nM) was injected for 200 s with a stabilisation period of 30 s, both at a flow rate of 5 µl/min.
All SPR results were analysed with the Biacore provided Biacore T100 evaluation software 2.0.2. Results were doubly referenced by subtracting the reference cell from the reaction cell and normalising to the no-analyte (buffer) injection.

<table>
<thead>
<tr>
<th>Compound</th>
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<th>Article nr.</th>
<th>Lot nr.</th>
<th>Purity (%)</th>
<th>Mw (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose 6-phosphate sodium salt</td>
<td>Sigma</td>
<td>G7879</td>
<td>DLBL0293V</td>
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<td>282.12</td>
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<tr>
<td>D-Glucose1-phosphate disodium salt hydrate</td>
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<td>SLBK3097V</td>
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<td>304.1</td>
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<td>Sucrose 6-monophosphate dipotassium salt</td>
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<td>041H3840V</td>
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<td>BCBN2971V</td>
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<td>SLBB8739V</td>
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<td>Maltose-1 phosphate (α and β)</td>
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<td>D(+)-Maltose monohydrate</td>
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<td>137064 21408105</td>
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<td>2-Deoxy-D-glucose 6-phosphate sodium salt</td>
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<td>6-deoxy-D-glucose</td>
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<td>083M4142V</td>
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<td>U4625</td>
<td>SLBN1993V</td>
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<td>610.27</td>
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</table>

Table 3.3: List of sugars used for SPR experiments

3.2.4 Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) was performed using the ‘LowCell#ChIP kit protein A’ (Diagenode) following manufacturer’s instructions with some adaptations for plant samples. Above-ground tissue of 14-d-old seedlings grown on soil in 12/12 h day/night cycle were harvested 4 h into the light and washed once in cold water. Subsequently, tissue was fixed in 1 % [v/v] formaldehyde for 13 min and quenched with glycerine (125 mM) for 5 min (both steps under vacuum infiltration). Tissue was dried on paper towel, frozen in liquid nitrogen and homogenised by mortar and stored at -80 °C until further use. To isolate chromatin, homogenised tissue was gently suspended in freshly prepared MEB (20 mM PIPES-KOH pH 7.6, 1 M hexylene glycol, 10 mM MgCl₂, 0.1 mM EGTA pH8.0, 60 mM KCl, 0.5 % [v/v] Triton-X 100, 5 mM β-mercaptoethanol and EDTA-free protease inhibitor cocktail (Roche)) and incubated on a rotating wheel for 15 min at 4 °C. Suspension was filtered through a 50 µm filter (Cell Trics, LabForce) and centrifuged for 10 min at 1,500 g. The chromatin-containing pellet was resuspended in kit-provided buffer B and subjected to 16 cycles of 30 s on/ 30 s off sonication at high power (Bioruptor, Diagenode) to produce fragments peaking at 350 bp. An aliquot of chromatin was kept for non-sonication control prior to sonication. In two centrifugation steps (15,000 g, 1 min at 4
°C) the chromatin containing supernatant was separated from the cell debris (pellet). The supernatant was diluted in kit-provided complete buffer A and 1/25 volume of prewashed protein A-coated paramagnetic beads were added for preclearing on the rotating wheel for 1 h at 4 °C. Subsequently, magnetic beads were removed and chromatin split into the different aliquots for each of the immune precipitation (IP) samples and smaller volumes for input and sonication controls. The latter two were kept at 4 °C until further use. The according antibodies were added to the IP samples (anti-HA, Abcam ab9110; anti-H3, Abcam ab1971; IgG, kit-provided) at a concentration of 10 µg/ml and incubated for 16 h on the rotating wheel at 4 °C. The anti-H3 antibody and IgG served as positive and negative controls, respectively. For the pull-down, 1/10 volume of Protein A-coated paramagnetic beads were added to the chromatin sample and incubated for 1 h at 4 °C on the rotating wheel. Immuno-complex bound paramagnetic beads were washed five times in complete buffer A as follows: a soft spin was followed by 1 min incubation on the magnetic rack, where after the supernatant was discarded and beads were resuspended in complete buffer A. After the last wash, beads were resuspended in in Buffer C and immediately mounted onto the magnetic rack. Elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1 % [w/v] SDS) was added to the collected beads and the input and sonication control samples (mentioned before). Crosslinking of chromatin to proteins was reversed by adding NaCl (200 mM) followed by an incubation at 65 °C for 16 h. To dilute the SDS concentration, an equal volume of TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA) was added. Subsequent RNase A (0.2 mg/ml) treatment was performed at 37 °C for 2 h, followed by a Proteinase K treatment at 45 °C for 2 h. Chromatin was recovered by phenol/chloroform precipitation. In brief, an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) (77617, Sigma) was added to the samples and mixed thoroughly by vortexing. Phase separation was achieved by centrifugation at 20,000 g for 2 min. To precipitate DNA, sodium acetate pH 5.2 (300 mM), 20 µl/ul glycogen and ethanol (100 % [v/v]) was added to the aqueous phase, incubated at -80 °C for 1 h and centrifuged at 20,000 g for 25 min at 4 °C. The recovered and air-dried pellet was subsequently resuspended in water and ready for further analysis. Shearing efficiency was assessed by separating the ‘sonication control’ samples on a 1.5 % [w/v] agarose gel. Relative enrichment in the IP samples was quantified by quantitative PCR (qPCR). Results were normalised to the input and expressed relative to the wild-type IP. Primers used for qPCR were designed to span the BBRE in the promoter of BUP1 and BUP2 (see Table 3.6).

3.2.5 Microscale thermophoresis (MST)
Microscale thermophoresis (MST) was performed with Nickel-NTA purified recombinant 6xHis-BAM8 proteins stored in storage buffer at -80 °C (see section 3.2.1). Prior to use, protein was labelled according to Monolith NT™ Protein Labelling Kit GREEN – NHS (NanoTemper) with the NT-547 dye. After the labelling procedure, NT-547 labelled 6xHis-BAM8 protein were separated from unreacted free
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dye according to the kit’s instructions and eluted in 600 µl storage buffer. Concentrations of fluorescently labelled 6xHis-BAM8 (NT-547-BAM8) were determined by the absorbance spectra at 550 nm and was measured to be 1.2 µM. For MST measurements, fluorescently labelled 6xHis-BAM8 stock was diluted in 1x HBS buffer (H8022, Teknova) and kept constant at a final concentration of 6.45 nM. A dilution series with sugars was made by serial 1:1 dilution, starting from an initial sugar concentration of 250 mM sugar. At the end, 16 working solutions containing 6.45 nM NT-547-BAM8 and 250 mM - 7.63 µM of the respective sugar were obtained and loaded onto the Standard Treated Capillaries (NanoTemper). Sugars investigated were trehalose 6-phosphate (Tre6P), trehalose (Tre), glucose 1-phosphate (Glc1P), glucose 6-phosphate (Glc6P), glucose (Glc), maltose 1-phosphate (Mal1P), maltose (Malt), mannose 6-phosphate (Man6P), mannose (Man), fructose 6-phosphate (Fru6P), sucrose 6-phosphate (Suc6P) and sucrose (Suc) (for details see Table 3.3). Measurements were subsequently performed in the Monolith NT.115 instrument (NanoTemper), exciting with the green LED1 at 557 nm. The recorded fluorescence was normalized and analysed with the built-in Monolith software. The normalised fluorescence ($F_{\text{norm}}$) represents the ratio between the initial fluorescence before heating ($F_{\text{initial}}$) and the fluorescence after a defined time period of exposure to heat ($F_{\text{hot}}$). For the analysis, both thermophoresis and T-jump were considered.

3.3 Molecular biology method

3.3.1 Genotyping of mutant alleles

Arabidopsis mutant alleles were confirmed by PCR. For that purpose, genomic DNA was extracted from plant material as described previously (Edwards, Johnstone et al. 1991) and dissolved in water. Primer combinations used for PCR are listed in Table 3.4. DreamTaq Green DNA Polymerase (Thermo Fischer Scientific, Reinach) was used according to manufacturers’ instructions. PCR conditions were the following: Initial DNA denaturation was allowed at 94 °C for 2 min, followed by 35 cycles of denaturation for 15 s at 94 °C, annealing for 15 s at a primer specific temperature and elongation at 72 °C for the time suitable for fragment length (1 min/ kb). The final extension was allowed at 72 °C for 7 min.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Line name</th>
<th>Gene ID</th>
<th>fw primer (5'-3')</th>
<th>rv primer (5'-3')</th>
<th>Transgene primer (5'-3')</th>
<th>AT (°C)</th>
<th>Enzyme</th>
<th>WT (bp)</th>
<th>Mutant (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bam7-1</td>
<td>SALK_000892</td>
<td>At1g45100</td>
<td>AAGAATTTCTGGAAGG</td>
<td>TGCTTTGTTGTTGTTG</td>
<td>TGGTTCAGTTASTGGG</td>
<td>56</td>
<td>BccI</td>
<td>~750</td>
<td>~700</td>
<td>Reinhold, Soyk et al. 2011</td>
</tr>
<tr>
<td>bam8-1</td>
<td>SALK_070279</td>
<td>At5g45300</td>
<td>GGACAGCCTATGTGA</td>
<td>TCTGCC</td>
<td>GCGTGGACCGCTTG</td>
<td>56</td>
<td>~750</td>
<td>~750</td>
<td>~700</td>
<td>Reinhold, Soyk et al. 2011</td>
</tr>
<tr>
<td>hxl-1</td>
<td>SALK_042572</td>
<td>At1g29130</td>
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<td>TGCTTTGTTGTTGTTG</td>
<td>TGGTTCAGTTASTGGG</td>
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<td>~750</td>
<td>~750</td>
<td>~700</td>
<td>Lee, Yuan et al. 2012</td>
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<td>At1g29130</td>
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<td>TGCTTTGTTGTTGTTG</td>
<td>TGGTTCAGTTASTGGG</td>
<td>56</td>
<td>~750</td>
<td>~750</td>
<td>~700</td>
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<tr>
<td>pgm1</td>
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<td>55</td>
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<td>1200/300</td>
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</tr>
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<td>SALK_72733</td>
<td>At1g29130</td>
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<td>TGGTTCAGTTASTGGG</td>
<td>55</td>
<td>~750</td>
<td>~750</td>
<td>~700</td>
<td>Nishida, Maier et al. 2004</td>
</tr>
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<td>dpe2-5</td>
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<td>55</td>
<td>~750</td>
<td>~750</td>
<td>~700</td>
<td>Chia, Thornton et al. 2004</td>
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</tbody>
</table>

57
Table 3.4: Genotyping of the mutant lines used in this study. PCR products were amplified with the forward (fw) and reverse (rv) primer from genomic DNA to obtain the wild-type (WT) product or with fw or rv and the transgene primer to generate the mutant product. In case of point mutations, the wild-type (WT) product was digested with the enzymes mentioned in the table, according instructions of the provider of the enzyme. The annealing temperature (AT) of the primers is given in °C and the length of the expected amplified fragment is indicated in base pairs (bp).

3.3.2 Colony PCR
Positive clones were selected by PCR, using a gene specific and a vector specific primer. PCR was performed using the DreamTaq Green DNA Polymerase (Thermo Fischer Scientific, Reinach), following manufacturers’ instructions. The PCR program was the same as described in 3.3.1, except for the initial denaturation step, which was prolonged to 5 min.

3.3.3 Molecular cloning
All cloning procedures were carried out with Gateway compatible vectors (Invitrogen, Basel). In brief, attB-flanked fragments were generated by amplification from cDNA or genomic DNA (gDNA) with primers containing the att-overhang and a gene specific part using iProof High-Fidelity DNA Polymerase (Bio Rad, Cressier). The attB-flanked fragments were inserted into the attP-containing donor vector to generate the entry clone by the BP reaction. The expression clone was generated by the LR reaction of the destination vector with the entry clone. Entry clones and expression clones were amplified by transformation into E. coli (DH5α), selection on the LB medium containing the appropriate selective antibiotic and purification by the NucleoSpin Plasmid kit (Macherey-Nagel, Oensingen). Positive clones were confirmed by colony PCR. If the entry clone and the destination vector contained the same selective antibiotic resistance, the entry clone was digested before LR reaction or the BP fragment was amplified from the entry clone by PCR using the iProof High-Fidelity DNA Polymerase with the M13 fw and M13 rv primers. Primers used for the cloning are listed in Table 3.5. For the transcriptional reporter construct, the attB-flanked fragment was amplified from gDNA and introduced into the pCR8/GW/TOPO vector (Thermo Fischer) generating the entry clone. Subsequently, the gene fragment was introduced into the pMDC163 destination vector (Curtis and Grossniklaus 2003), which contained the GusA gene and a nos terminator (performed by Klara Simkova, unpublished). For the translational reporter construct the MultiSite Gateway Three-Fragment Vector Construction Kit (Invitrogen) was used. The attB-flanked fragments were all amplified from gDNA and introduced into the pDONR™ P4-P1R (promoter+5’SUTR region), the pDONR™221(gene without STOP) and the pDONR™P2R-P3 (tag) (Invitrogen). The GFP-tag was amplified from another entry clone, whereas the HA-tag was generated by primer annealing. All three fragments were simultaneously inserted into the pB7m34GW.0 destination vector (Karimi, De Meyer et al. 2005) by the LR reaction. For the BiFC constructs with MYC2, TPS1 or GeBPL, the attB-flanked fragments were amplified from cDNA. They were first introduced into the pDONR™221, which was then used as template to amplify the gene
containing region with the M13 fw and M13 rv primers. The purified PCR product (DNA Clean & Concentrator, Lucerna Chem) was subsequently inserted into the pSPYCE-35S GW (YFPn) or pSPYNE-35S GW (YFPC) (Schutze, Harter et al. 2009), which led to C-terminally fluorescently tagged proteins. The entry clone carrying the BAM7 V114D I115E and BAM8 V131D I132E were generated by using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Basel), which introduced the nucleotide changes leading to the desired amino acid changes (primer used see Table 3.5). As templates for the mutagenesis kit, pDONR™-221 carrying BAM7 or BAM8 coding sequence (CDS) was used. To finally generate the BiFC construct, the same procedure as described above for MYC2, TPS1 and GeBPL was followed. The rice TPP1 gene was synthesized by the company Biomatik, which provided the lyophilized in pBSK(+) vector carrying the OsTPP1 gene. The BP reaction was directly performed between the OsTPP1 carrying pBSK(+) vector and the pDONR™-221. The same resistance of the entry clone and the destination clone made a digestion step with NheI (Thermo Fischer Scientific) necessary. The purified OsTPP1-containing fragment (purified by DNA Clean & Concentrator, Lucerna Chem) was introduced into the pEarleyGate201 (pEG201) vector (Earley, Haag et al. 2006). The expression clone pGreen containing the 35S:otsA or the 35S:otsB construct (Yadav, Ivakov et al. 2014) were kindly provide by Dr. J. Lunn (Max Planck Institut in Potsdam-Golm). To test expression of the correct protein, pGreen 35S:otsA, pGreen 35S:otsB and pEG201 OsTPP1 were transformed into A. tumefaciens, transiently expressed in N. benthamiana and visualised by immunoblotting. Both, pGreen 35S:otsA and pGreen 35S:otsB required a co-transformation with pSoup. His-tagged BAM8 was expressed from the pET21a(+) vector (Novagen, Merck, Darmstadt, Germany), its cloning has been previously described (Soyk, Simkova et al. 2014).

<table>
<thead>
<tr>
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<th>pDestination</th>
<th>Tag/marker</th>
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<td>pMDC163</td>
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### Material and Methods

#### Translational reporter construct (pB7:B7-GFP/-HA, pB8:B8-GFP/-HA)

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<td>BAM7_A_attB1_F</td>
<td>GGGGACAGTTTGGTACAAAAAAGGAGCTTAAATGGCG</td>
<td>pDONR</td>
<td>HA/GFP</td>
</tr>
<tr>
<td>BAM7_A_attB2_R</td>
<td>GGGGACACCTTTGTACAAAAACTGGGTCAAACCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAM8_A_attB4_F</td>
<td>GGGGACAGTTTGGTACAAAAAAGGAGCTTAAATGGCG</td>
<td>pDONR</td>
<td>HA/GFP</td>
</tr>
<tr>
<td>BAM8_A_attB1r_R</td>
<td>GGGGACACCTTTGTACAAAAACTGGGTCAAACCTTG</td>
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<tr>
<td>BAM8_A_attB1_F</td>
<td>GGGGACACTTGTACAAAAACTGGGTCAAACCTTG</td>
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<tr>
<td>BAM8_A_attB2_R</td>
<td>GGGGACACCTTTGTACAAAAACTGGGTCAAACCTTG</td>
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<tr>
<td>HAtag_attB2_F</td>
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<tr>
<td>HAtag_attB3_R</td>
<td>GGGGACACACTTTGTACAAAAAAGGAGCTTAAATGGCG</td>
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<tr>
<td>BiFC (MYC2-YFPn/-YFPc, TPS1-YFPn/-YFPc, GeBPL-YFPn/-YFPc)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer name</td>
<td>Sequence (5'-3')</td>
<td>GenBank Accession</td>
<td>Type</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------</td>
<td>------------------</td>
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<tr>
<td>MYC2_fw</td>
<td>GGGGACAAGTTTGGTACAAAAAAGGAGCTTAAATGGCG</td>
<td>pDONR</td>
<td>YFPn/ YFPC</td>
</tr>
<tr>
<td>MYC2_rv</td>
<td>GGGGACACCTTTGTACAAAAACTGGGTCAAACCTTG</td>
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<td></td>
</tr>
<tr>
<td>TPS1_fw</td>
<td>GGGGACAAGTTTGGTACAAAAAAGGAGCTTAAATGGCG</td>
<td>pDONR</td>
<td>YFPn/ YFPC</td>
</tr>
<tr>
<td>TPS1_rv</td>
<td>GGGGACACCTTTGTACAAAAAAGGAGCTTAAATGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GeBPL_fw</td>
<td>GGGGACAAGTTTGGTACAAAAAAGGAGCTTAAATGGCG</td>
<td>pDONR</td>
<td>YFPn/ YFPC</td>
</tr>
<tr>
<td>GeBPL_rv</td>
<td>GGGGACACCTTTGTACAAAAAAGGAGCTTAAATGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimerisation mutants (QuikChange) (BAM7V114D115E-YFPn/-YFPc, BAM8V131D132E-YFPn/-YFPc)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer name</td>
<td>Sequence (5'-3')</td>
<td>GenBank Accession</td>
<td>Type</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------</td>
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<tr>
<td>BAM7_V114D115E_fw</td>
<td>GATATTAACGATGATGAGGGCGCTTGGGCA</td>
<td>pDONR Tm</td>
<td>YFPn/ YFPC</td>
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<tr>
<td>BAM7_V114D115E_rv</td>
<td>TGCCAAAGCGCGCTCAGATGATGAGGGCG</td>
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<tr>
<td>BAM8_V131D132E_fw</td>
<td>GATATGAATGACGATGAGGGCGCTTGGGCA</td>
<td>pDONR</td>
<td>YFPn/ YFPC</td>
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<tr>
<td>BAM8_V131D132E_rv</td>
<td>AGCCCAAGCGCGCTCAGATGATGAGGGCG</td>
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</tr>
<tr>
<td>M13_fwd</td>
<td>GTAAGACGCGGCGCAGC</td>
<td>pDONR</td>
<td>YFPn/ YFPC</td>
</tr>
<tr>
<td>M13_rev</td>
<td>GGAACACGAGCCAGGACGATG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.5: Constructs generated in this study. Fw, forward primer; rv, reverse primer; V, Valine; I, Isoleucine; D, Aspartic acid; E, Glutamic acid; YFPn, N-terminal part of YFP; YFPc, C-terminal part of YFP. References for the vectors are found in the main text.

3.3.4 RNA extraction and reverse transcription

Plant material (approximately 100 mg if not mentioned differently) was harvested and immediately frozen in liquid nitrogen. Homogenisation of the plant tissue was achieved using a mixer mill and glass beads. After adding 1 ml of TRIZOL reagent (Invitrogen, Zug, Switzerland) samples were allowed to thaw and were briefly vortexed before incubation at 30 °C for 6 min. Chloroform (0.2 ml) was added, samples mixed by vigorous shaking by hand for 15 s and incubated for 3 min at 20 °C. Phase separation was achieved by centrifugation at 13,800 g for 15 min at 4 °C. The upper aqueous phase was transferred into a fresh 1.5-ml tube and mixed with 0.5 ml isopropanol. Incubation for 10 min at 20 °C and subsequent centrifugation at 13,800 g for 10 min at 4 °C led to precipitation of RNA. Removal of the supernatant was followed by washing of the RNA with 80 % [v/v] ethanol and centrifugation at 10,000 g for 5 min at 4 °C. The supernatant was discarded, the pellet air-dried for 5 - 10 min at 20 °C and subsequently dissolved in 50 µl of H₂O by pipetting. Incubation for 10 min at 55 °C allowed good dissolving of RNA. To eliminate contaminations of genomic DNA, RNA was treated with recombinant DNaseI (Roche, Basel, Switzerland) in incubation buffer provided by the manufacturer, in presence of RNase inhibitor Ribolock (Thermo Fischer Scientific, Reinach) for 1 - 2 h at 37 °C. DNaseI treated RNA was purified using phenol/chloroform (Roti-Aqua-Phenol/Chloroform/Isoamylacohol, Roth, Reinach), which was added to the sample and led to phase separation after vigorous vortexing (15 s) followed by centrifugation at 12,000 g for 1 min at 4 °C. The upper aqueous phase was transferred to a fresh tube containing 5 µl sodium acetate, pH 5.2 and 125 µl 100 % [v/v] ethanol. Precipitation was achieved by incubation at -20 °C for 10 min, followed by centrifugation for 15 min at 12,000 g, 4 °C. The pellet was washed once with 75 % [v/v] ethanol, subjected to centrifugation for 15 min at 12,000 g, 4 °C and dried for 5 min at 37 °C. RNA was dissolved in 11 µl RNase free water by pipetting and stored at -80 °C until further use. Integrity of the RNA was tested on a freshly prepared 2 % [w/v] agarose gel. Synthesis of cDNA from 3 μg DNasel treated RNA was conducted with the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific AG, Reinach) according to the manufacturer’s instructions using oligo (dT)₁₈ primer for random amplification. Quality of cDNA in respect to contamination with gDNA was tested by PCR with PP2A primers (see Table 3.6) which bind gDNA as well as cDNA, leading to fragments of different sizes (342 bp and 128 bp, respectively).

3.3.5 Quantitative PCR

For high numbers of samples, quantitative PCR (qPCR) was conducted with 1 µl of cDNA (1:5 diluted), 5 µl of KAPA SYBR FAST Master Mix (Kapa Biosystems), 1 µl of primer mix (5 µM of each forward and
reverse primer) and 3 µl water. PCR reactions were run on the Light Cycler 480 II (Roche) and analyzed in the according software. For lower (<96) number of samples, quantitative PCR (qPCR) was conducted with 2 µl of cDNA, 10 µl of Fast SYBR® Green Master (Thermo Fisher Scientific AG, Reinach), 2 µl of primer mix (5 µM of each forward and reverse primer) and 6 µl water. PCR reactions were run and analyzed in a 7500 Fast Real-Time PCR system (Applied Biosystems, Rotkreuz). Primers used for qPCR are listed in Table 3.6. Expression levels were normalised to PP2A (AT1G13320) in the corresponding sample and expressed relative to the control condition (usually a wild-type sample).

<table>
<thead>
<tr>
<th>Gene/ Primer name</th>
<th>Gene ID</th>
<th>fw primer (5'-3')</th>
<th>rv primer (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUP2_upstream BBRE</td>
<td>At5g57785</td>
<td>CCGCTAATCGATGATGAGCC</td>
<td>ATCCACGGTTCCAAGGCTCG</td>
<td></td>
</tr>
<tr>
<td>BUP2_BBRE spanning</td>
<td>At5g57785</td>
<td>CATTTCCGCGCAATGACAGA</td>
<td>ATGAATTCTCCACAGCTTT</td>
<td></td>
</tr>
<tr>
<td>BUP1_upstream BBRE</td>
<td>At5g22580</td>
<td>AAGGCTAGAAAGAGCTACTGT</td>
<td>CTGTTCAACTATTCAACTCT</td>
<td></td>
</tr>
<tr>
<td>BUP1_BBRE spanning</td>
<td>At5g22580</td>
<td>CAGATTGTCAGAGCACAGAC</td>
<td>CGTTGAAAATGTACACGGT</td>
<td></td>
</tr>
<tr>
<td>ATHB2</td>
<td>AT4G16780</td>
<td>TCAGTAGGATGAGATGAGTGT</td>
<td>TGCTTCTCCGAGGTAGAG</td>
<td></td>
</tr>
<tr>
<td>ATHB4</td>
<td>At2g44910</td>
<td>TTCAACACATGCAACAATCAG</td>
<td>TGTACGTTTATCCACAGAG</td>
<td></td>
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<tr>
<td>PAR1</td>
<td>AT2G42870</td>
<td>CACGCTAGCTGCTACGCA</td>
<td>TCGGTCTTCAGTACCCCGTG</td>
<td>Reinhold, PhD Thesis</td>
</tr>
<tr>
<td>HFR1</td>
<td>AT1G02340</td>
<td>GATGCGTAAGTACACGAACTCG</td>
<td>AGAACGAAACCTTGGTCCGTG</td>
<td>Cifuentes-Esquivel et al., 2013</td>
</tr>
<tr>
<td>PRE1</td>
<td>ATSG39860</td>
<td>CTGATAAGGACTCAGGCTCCG</td>
<td>TTCATCGACAGATTGAGAGAAG</td>
<td>Cifuentes-Esquivel et al., 2014</td>
</tr>
<tr>
<td>IAA19</td>
<td>AT3G15540</td>
<td>GACAACTGCGAATACGTTACC</td>
<td>TCTCAACCTCCTGGATGACCT</td>
<td></td>
</tr>
<tr>
<td>BUP1</td>
<td>At5g22580</td>
<td>AGTTCAGGGGCAAATACCC</td>
<td>GAATGTGAGCGGTAGG</td>
<td></td>
</tr>
<tr>
<td>CPD</td>
<td>AT5G05690</td>
<td>GCTCAACCTAGAGAGAGAGAGGA</td>
<td>CTTGTAGGCGGTCTATACACCA</td>
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<td>LCD1</td>
<td>AT2G37860</td>
<td>AAGGAGACTGAGAGAGAGAG</td>
<td>TGGTAGCTTGGCTGGCAATCC</td>
<td></td>
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<tr>
<td>BAM8</td>
<td>At5g45300</td>
<td>GGTTGAGATTGGTGCTTGGAG</td>
<td>TTCTAACAATGACTGAACTC</td>
<td></td>
</tr>
<tr>
<td>PP2A</td>
<td>At1g13320</td>
<td>CAACCCACACTATCTATATCG</td>
<td>TTAGATGCGATTACCCGCA</td>
<td>G</td>
</tr>
</tbody>
</table>

**Table 3.6: Primers used for quantitative real time PCR (qPCR).** All primers were newly designed, except for HFR1, PAR1 (Cifuentes-Esquivel, Bou-Torrent et al., 2013) and ATHB-4 (Reinhold, PhD Thesis).

### 3.3.6 Plasmid DNA preparation for Transactivation Assay

For plasmid isolation, a E. coli culture was grown for 16 h at 28 °C, 220 rpm shaking in 5 ml LB medium containing the according antibiotic. Subsequently, it was transferred into 500 ml fresh, pre-warmed LB medium supplemented with antibiotics and grown for 16 h at 28 °C, 220 rpm shaking. Cells were harvested by centrifugation at 5,000 g for 15 min at 4 °C, and pellet stored at -20 °C until further use. Cells were re-suspended in 15 ml of Solution I (10 mM Tris-HCl, pH 8, 1 mM EDTA) by pipetting and lysed by addition of 50 ml fresh Solution II (0.1 M NaOH, 1 % [w/v] SDS). The cell suspension was mixed by inverting and incubated for 10 min at 20 °C. After addition of 25 ml Solution III (3 M potassium
acetate, 5 M acetic acid) the suspension was again mixed by inverting and incubated on ice for 10 min. After centrifugation for 5 min at 10,000 g, the supernatant was filtered through a 100 µm nylon mesh and DNA was precipitated by addition of 54 ml isopropanol. DNA was pelleted by centrifugation at 10,000 g for 30 min at 4 °C and the supernatant discarded. The pellet was rinsed with 95 % [v/v] ethanol and air-dried. Pellets were re-suspended in 3.5 ml Solution I with 7 µl (10 mg/ml) of RNase H (Invitrogen, Zug, Switzerland) and incubated for 20 min at 37 °C. After transfer to a fresh 15 ml tube, 1 ml Roti-Aqua-Phenol (Roth, Germany) was added, mixed by vortexing and centrifuged for 5 min at 4,000 g at 20 °C. The supernatant was transferred to a new 15 ml tube containing 5.5 g CsCl₂. After adding 6 ml of Solution I, CsCl₂ was allowed to dissolve on a turning wheel for 75 min. Once dissolution was complete, 0.1 ml ethidium bromide (EtBr; 10 mg/ml; Sigma-Aldrich, Buchs, Switzerland) was added and the solution was mixed by vortexing. After centrifugation for 5 min at 5,000 g, the red floating disc was discarded. Samples were transferred to Quick seal ultracentrifugation tubes (Beckmann Coulter, Nyon, Switzerland) and centrifuged at 53,000 rpm for 18.5 h in a NVT65.2 rotor (Beckmann Coulter, Nyon, Switzerland). A hole was pinched into the centrifugation tube with a capillary needle just below the red ring formed. The red-stained DNA band was isolated with a syringe. After addition of 1 volume of water, EtBr was extracted twice with n-butanol (saturated with 1M NaCl). In specific, n-butanol was added and the solution mixed by inverting. Subsequently, the two phases were allowed to separate and then the upper phase was discarded. DNA was precipitated with three volumes of 95 % [v/v] ethanol, collected by centrifugation at 4,600 g for 5 min and washed once with 70 % [v/v] ethanol. The DNA was dissolved in 1 ml water by pipetting.

3.4 Microscopic methods

3.4.1 Staining methods

3.4.1.1 GUS staining

Plant tissue was directly harvested into 90 % [v/v] acetone on ice, vacuum infiltrated for 10 min at 20 °C and incubated at 20 °C for another 30 min. The acetone solution was replaced by staining buffer without X-Gluc (0.2 % [v/v] Triton-X 100, 50 mM NaPO₄ buffer, 2 mM potassium ferrocyanide (Sigma P9387, Buchs), 2 mM potassium ferricyanide (Sigma 244023, Buchs), and vacuum infiltrated for 10 min at 20 °C. Subsequently, staining buffer without X-Gluc was replaced with staining buffer including 2 mM X-Gluc (4-Methylumbelliferyl-β-D-glucuronide hydrate, Sigma, Buchs), infiltrated under vacuum for 15 min on ice for a total of three times (releasing the vacuum in between) and incubated at 37 °C, for 16 h to allow GUS staining to occur. De-staining (chlorophyll) of the tissue was achieved through a successive ethanol series, with incubations steps of 30 min at 20 °C going from 20 %, 35 % to 50 % [v/v] ethanol. Fixation was performed by incubation in FAA solution (50 % [v/v] ethanol, 3.7 % [w/v]
formaldehyde, 5 % [v/v] acetic acid) for 30 min at 20 °C. For final storage, FAA was replaced by 70 % [v/v] ethanol.

3.4.1.2  Propidium Iodide
A propidium iodine working solution was prepared by diluting the stock solution (1 mg/ml, Sigma P4864, Buchs) by 1:1,000 in water. Roots were incubated in the working solution for 1 min (or less) and directly moved to the microscopy slide and assessed on the Confocal Laser Scanning Microscope (CLSM).

3.4.2  Light field microscopy
Light field microscopy was performed on a Zeiss Axio Zoom (Carl Zeiss GmbH, Jena, Germany).

3.4.3  Fluorescence microscopy with Confocal Laser Scanning Microscopy (CLSM)
Sub-cellular localization studies and BiFC analysis were conducted using a Zeiss LSM 780 equipped with an argon laser (Carl Zeiss GmbH, Jena, Germany). For BiFC, YFP fluorescence was excited with the 514 nm argon laser line and detected between 518 nm and 557 nm. Auto-fluorescence of chloroplasts was excited with the same argon laser line (514 nm) and recorded between 661 nm and 721 nm, both with an Air Unit (AU) of 1.1. For translational fusion constructs (pB7: B7-GFP, pB8: B8-GFP) in A. thaliana or N. benthamiana, eGFP fluorescence was excited with the 488 nm argon laser line and detected between 493 and 572 nm. Auto-fluorescence of chloroplasts was excited with the 633 nm argon laser line and recorded between 645 nm and 722 nm (AU=2.6). In case of imaging of roots, the auto-fluorescence was replaced by PI staining, which was excited with the 488 nm argon laser line and recorded between 638 nm and 707 nm (AU=1.1). For translational fusion constructs, which were crossed to lines expressing nuclear RFP-tagged proteins, eGFP fluorescence was excited with the 488 nm argon laser line and detected between 493 and 572 nm and mRFP fluorescence was excited with the 594 nm argon laser line and detected between 599 and 645 nm. Both dyes were excited and their emission recorded simultaneously, thus both on Track1 (AU=2.6). To avoid cross reaction with the RFP signal, the chlorophyll auto-fluorescence was excited (633 nm argon laser line) and detected (between 647 and 722 nm) sequentially to mRFP/eGFP, thus on a separate track (Track2). Image processing was done using ZEN 2011 software (Carl Zeiss GmbH, Jena, Germany) and ImageJ software.

3.5  Bioinformatics

3.5.1  Database mining
Genevestigator (https://genevestigator.com) was consulted to investigate if BZR1-BAM upregulated 1 (BUP1, At5g22580), BZR1-BAM upregulated 2 (BUP2, At5g57785), BAM7 (At2g45880) and BAM8 (At5g45300) are responsive to Glc or Suc treatment or light treatment. For the former (Glc, Suc
response), all treatments available in Genevestigator which included Glc or Suc supplemented to wild-type plants were included. In total, there were 5 studies meeting these criteria available. For the latter (light treatment), all light perturbation studies on wild-type plants were included. Furthermore, Genevestigator and the eFP Browser, (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) were used to compare expression levels of BZR1-BAMs with outputs of the transcriptional- and translational fusion constructs.

3.5.2 Statistics for shade treatment
To evaluate if hypocotyl length significantly differed in different treatments and genotypes, a two-way ANOVA in combination with a Tukey's range test was performed. Different letters indicate significant difference ($p<0.01$).
4 Results and Discussion

4.1 Role of BZR1-BAMs involved in Tre6P sensing

The two-domain structure of the BZR1-BAMs, which includes the N-terminal transcription factor domain and the enzymatically inactive β-amylase domain (Reinhold, Soyk et al. 2011) suggests a role as a metabolic sensor, integrating a metabolic signal into a transcriptional response (see sections 1.2.3 to 1.2.5). However, to date there is plenty experimental evidence for DNA binding and transcription factor activity but limited evidence supporting the hypothesis of a direct protein-sugar interaction. Even though the importance of the BAM-domain in transcriptional regulation has been shown previously (Soyk, Simkova et al. 2014), the direct link to a metabolic signal is still elusive. To further explore the sugar sensing hypothesis, I employed two different in vitro techniques, Surface Plasmon Resonance (SPR) and Microscale Thermophoresis (MST), to find and characterise putative protein-sugar interactions. Through MST, I addressed the physical interaction of BAM8 and different sugars. SPR was used to look at the impact a range of sugars have on BAM8 binding activity to DNA. From the large number of metabolites occurring in biological samples, I narrowed down the list to the most likely candidates. Out of 13 candidates tested, the most promising ligand from both methods was trehalose 6-phosphate (Tre6P), a known signal metabolite. Subsequently, I investigated the effect Tre6P has on BAM8’s in vivo transcription factor activity and compared genome wide transcriptional data of plants with unnaturally high/low Tre6P concentrations with genome wide BZR1-BAM deregulated genes. To extend the study to the whole plant level, growth experiments on high Glc and Suc containing media addressed the question if overexpressing BAM8-lig, which is suggested to be incapable of binding the putative ligand (Soyk, Simkova et al. 2014) (for details see section 1.2.5), led to the same physiological Glc-insensitive response as triggered in BAM8-ox. Mutant combinations with the known sugar sensor HXK1 were created with the intention of testing at the physiological level if the two pathways are more likely to act in concerted or separate manner.

4.1.1 Phosphorylated-sugars interact with BAM8

The direct interaction of BAM8 protein with a number of sugars was tested by Microscale Thermophoresis (MST). MST is an in-solution technique, which relies on the characteristic migration property of a given molecule (e.g. protein) within a micro-thermal gradient. Migration depends on the mass, charge and hydration shell of the investigated molecule. Upon interaction with one or more interaction partners at least one of the three parameters changes, leading to a detectable deviation in migration behaviour compared to the unbound state. In our case, His-tagged BAM8 (6xHis-BAM8) was expressed in E. coli, purified by Ni-affinity chromatography (Fig. 4.1.1-A), fluorescently labelled at primary amines (K residues) with the green light absorbing NT-547 dye (Nano Temper) and exposed to
Results and Discussion

Different sugars in the MST. Putative interaction partners, namely: glucose (Glc), glucose-6 phosphate (Glc6P), glucose-1 phosphate (Glc1P), fructose (Fru), fructose-6 phosphate (Fru6P), sucrose (Suc), sucrose-6 phosphate (Suc6P), mannose (Man), mannose-6 phosphate (Man6P), trehalose (Tre), trehalose 6-phosphate (Tre6P), maltose (Mal) and maltose-1 phosphate (Mal1P). Analysis was

![Image of results and discussion](image-url)
Results and Discussion

performed by including the temperature-jump (T-jump) as well as the thermophoresis. The temperature-jump describes the rapid temperature dependent change in fluorescence of the fluorophore - an inherent property that also depends on the local environment of the fluorophore. Thermophoresis is a much slower process and is limited by the protein’s diffusion rate.

Of the chosen potential interaction partners, only Tre6P and Glc6P triggered consistent changes in NT-547-BAM8 mobility, hence binding curve. From at least three different independent experiments, a dissociation constant ($K_D$) in the low milli-molar range was determined (see Table 4.1). These two interactions seem to be specific since all other sugars, including the non-phosphorylated sugars (Glc, Tre) and Glc1P did not show a binding behaviour (Fig. 4.1.1-B).

<table>
<thead>
<tr>
<th>Dissociation constant ($K_D$) in mM</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tre6P</td>
<td>2.580 ± 0.189</td>
<td>6.790 ± 0.687</td>
<td>5.160 ± 0.368</td>
</tr>
<tr>
<td>Glc6P</td>
<td>35.900 ± 2.750</td>
<td>72.000 ± 7.670</td>
<td>39.900 ± 2.400</td>
</tr>
</tbody>
</table>

Table 4.1: Interaction of BAM8 with Tre6P/Glc6P. Dissociation constants were determined by MST in three independent experiments.

4.1.2 BZR1-BAMs stably bind to the preferred DNA motif

A complementary approach to study bimolecular interactions in vitro is Surface Plasmon Resonance (SPR). It is a label free, surface bound technique where one of the interaction partners is immobilized on a synthetic polycarboxylate surface of a sensor chip and the putative interaction partner is supplied in a continuous flow of solution. The synthetic polycarboxylate itself is attached to a thin gold film on a glass slide. Binding of an interaction partner to the surface leads to a change in refractive index on the glass side of the chip and alters the angle of minimal reflected intensity, which is proportional to the mass of material bound. The change in angle is visualised as an arbitrary unit called resonance unit (RU), which reflects the binding event and is plotted against time. In principle, different kinetic characteristics such as the association and dissociation rate as well as the dissociation constant ($K_D$) can be determined (for detail see also https://www.sprpages.nl). Using this system, I first looked at the previously characterized interaction of BZR1-BAMs with double stranded oligonucleotide containing the BZR1-BAM Responsive Element (BBRE, CACGTGTG) or a mutated version of it (mBBRE, CACTTGTG) (Soyk, Simkova et al. 2014). For that purpose, a 30 base-pair long double stranded oligonucleotide containing the respective binding motif was immobilized on the reaction cell (ds-BBRE: 510 RU, ds-mBBRE: 480RU), whereas the reference cell was covered with a single stranded poly-AT oligonucleotide (ss-oligoAT). Subsequently, the surface was exposed to a series of increasing (0 to 200 nM) concentration of recombinant His-tagged BAM7 or BAM8. To be confident about integrity of the surface during the measurements, the surface was subsequently exposed to decreasing concentrations (200 to 0 nM) of BAM7 or BAM8. Indeed, the signals of the increasing and decreasing concentration series were close
to identical. The program used was the ‘Association Experiment’ (see Materials and Methods, 3.2.3) with the difference that the association period was shortened to 60 s at a flow rate of 30 µl min⁻¹ and the regeneration by 1 M NaCl was carried out for 60 s with a stabilisation period of 60 s. For both BAM7 and BAM8 there was a concentration-dependent increase in signal strength (RU) on both oligonucleotides (BBRE and mBBRE) (Fig. 4.1.2), indicating the association of both proteins to the double stranded DNA (ds-BBRE and ds-mBBRE). However, in the case of ds-mBBRE the signal was lower on exposure to the same concentration of protein (compared to the ds-BBRE) and most of the signal was lost gradually after injection of the protein stopped, whereas for ds-BBRE it remained constant with only little dissociation at higher protein concentrations. The maximum signal at 200 nM BAM8 was 1600 RU on ds-BBRE vs. 800 RU on ds-mBBRE. For BAM7 the maximum signal at 200 nM protein was 700 RU on ds-BBRE vs. 120 RU on ds-mBBRE. This observation suggests that the association and binding of BAM7 and BAM8 to the BBRE is higher than to the ds-mBBRE and BAM8 generally binds with higher affinity than BAM7. Furthermore, binding to the BBRE seems to be very stable, and it was impossible to retrieve a dissociation constant. In contrast, the BZR1-BAMs readily dissociated from the ds-mBBRE, with a more pronounced effect for BAM8 than for BAM7. It seemed that the binding of BAM8 to the BBRE reached saturation because there was barely a difference in the final amount of protein immobilized between 100 and 200 nM protein injection. For BAM7 on ds-mBBRE/ds-BBRE and BAM8 on ds-mBBRE the association did not reach a plateau. Notably, BAM8 does not show a typical association curve. The sigmoidal shape of association mainly at higher concentrations (> 50 mM) indicates that there is most likely a second reaction occurring (e.g. dissociation of a component, e.g. due to decay of the dimer or higher order oligomer). Likewise, a slight loss in signal during the dissociation phase at high protein concentrations (> 50 mM) was observed and may support a decay of protein-protein interactions. Another observation was that BAM7 had a rather high unspecific association to the reference cell (120 RU) and the baseline increased by 380 RU in the course of the experiment, indicating that not all protein was washed off during the regeneration step with 1 M NaCl and a significant amount of BAM7 accumulated on the reaction surface. Therefore, the results obtained with the BAM7 protein have to be treated with caution. To reduce unspecific binding to the reference surface, BSA instead of the ss-oligoAT for blocking the reference cell was used but this did not help to overcome the problem. We therefore decided to focus the subsequent experiments primarily on BAM8. Nevertheless, this experiment allowed us to get qualitative information confirming the previously observed protein-DNA interaction for the two proteins. It showed us the stable nature of binding, particularly for BAM8. However, no quantitative information in respect to the kinetic constants behind
Figure 4.1.2: Reduced affinity and binding strength of BZR1-BAMs to ds-mBBRE compared to ds-BBRE, shown by SPR. (A) Binding of BAM7 to ds-BBRE and ds-mBBRE. The reaction cell surface was covered with 510 RU ds-BBRE (upper panel) or 480 RU ds-mBBRE (lower panel) and exposed to increasing concentrations of BAM7 protein (0 to 200 nM). Once the highest concentration (200 nM) was reached, decreasing (200 nM to 0) concentrations of BAM7, to confirm intactness of the surface in the course of the experiment, were applied. Sample injection was set to 60 s and the subsequent dissociation time was 120 s and 300 s on the ds-BBRE and ds-mBBRE surfaces, respectively. Note the differences in RU and time scale for upper and lower panels. (B) Same experiment as described in (A) but with BAM8 instead of BAM7. Binding of BAM8 to ds-BBRE (upper panel) and ds-mBBRE (lower panel).
4.1.3 Sugars impact BAM8’s affinity for the preferred DNA motif

Next, I tested how the association behaviour of BAM8 to ds-BBRE was affected by the presence of different sugars (subsequently called ‘Association Experiment’). As described for the previous experiment, ds-BBRE was immobilised in the reaction cell (500 RU). BAM8 (37.5 nM) was injected in the presence or absence of 5 mM sugar. In addition to the sugars tested for the MST, also 2-deoxy-D-glucose (2dGlc), 2-deoxy-D-glucose6-phosphate (2dGlc6P) and 6-deoxy-D-glucose (6dGlc) were included in this experiment. Out of this list of sugars, Tre6P had the strongest impact on BAM8; it consistently reduced the association of BAM8 to ds-BBRE by around half, whereas Tre did not trigger any changes in BAM8’s binding behaviour (Fig. 4.1.3-A). Suc6P also consistently reduced the association of BAM8 but to a much lesser extent than Tre6P. The other sugars showed either no (Glc, 2dGlc, 6dGlc, Fru, Suc, Mal, Glc1P, 2dGlc1P, Fru6P), small (Man6P and Mal1P) or inconsistent (Man) reductions in association. The presence of Glc6P introduced a very distinct association behaviour; according to the SPR signal it seemed as if association was reduced during the injection phase, but was compensated during the dissociation phase and ultimately led to a slightly lower binding level than in the absence of the sugar. A closer look at the signal outputs from the different flow cells revealed that Glc6P does not influence association in the reaction cell but renders BAM8 able to associate unspecifically to the reference cell (ss-oligoAT), from which it releases slower after injection stop than from the reaction cell, which was an effect unique to Glc6P. Double referencing (subtraction of the reference cell from the reaction cell, see also materials and methods section 3.2.3) therefore creates this artefact of ‘reduced’ association during injection, which slowly ‘recovers’ in the subsequent dissociation period. The reason for this altered affinity of BAM8 to ss-DNA in the presence of Glc6P is unclear. It might indicate a conformational change of BAM8, but further investigations would be needed to test this hypothesis.

To further investigate the effects of the most promising candidates Tre6P, Suc6P and Glc6P, we performed the above described ‘Association Experiment’ with concentration series of the sugars but constant BAM8 concentration (37.5 nM). This revealed that sugars which did not affect the association of BAM8 at 5 mM, did not do so in the range of 0-20 mM (Glc, Glc1P) or 0-10 mM (Tre, Suc). In contrast, Tre6P, Suc6P and Glc6P all showed a concentration-dependent response. At 10 mM, Tre6P almost completely abolished binding whereas increasing Suc6P concentrations had only a very small effect. Exposing BAM8 to 5mM Glc6P and 5 mM Tre6P simultaneously caused the same effect as 5 mM Tre6P alone. The effect of Tre6P seemed to override the effect of Glc6P (Fig. 4.1.3-B).
To verify that these results were truly due to the sugar’s effect on the protein and not due to a change in the SPR surface property (e.g. dissociation of immobilised DNA) we injected all sugars onto the same surface as in the experiment above, but without protein. All the tested sugars showed a weak (<30 RU) signal change during injection, which disappeared instantaneously after injection stop. This may be attributed to an unstable association of the sugars to the surface, or differences in refraction index changes on the reference and the reaction surface. Importantly, the surface was unchanged ($\Delta$ RU=0) after injection stop (Fig. 4.1.3-C).
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Figure 4.1.3: BAM8 association and binding to ds-BBRE is reduced in a concentration-dependent manner by Tre6P and Suc6P. (A) Impact of sugars on the association of BAM8 to ds-BBRE. The SPR reaction surface was covered with 500 RU ds-BBRE and exposed to 37.5 nM BAM8 in presence or absence of 5 mM sugars (Tre6P, Suc6P, Glc6P, Glc1P, Mal1P, Fru6P, Man6P, 2-deoxyGlc6P, UDP-Glc and the corresponding neutral sugars). Results for Fru6P, Fru, 2-deoxyGlc6P, 2-deoxyGlc and UDP-Glc show the same behaviour as Glc1P (not shown). Injection time was set to 200 s and dissociation time to 120 s. Representative results of three independent experiments are shown. Furthermore, the same experiment was performed once under higher flow rate (30 µl min⁻¹) and twice with BSA covering the reference surface instead of oligo-AT. The output of the latter two experiments was essentially the same as is shown here. (B) Tre6P, Suc6P and Glc6P reduce the amount of BAM8 associating to ds-BBRE in a concentration-dependent manner. The SPR reaction surface was covered with 325 RU ds-BBRE and exposed to 37.5 nM BAM8 in presence of varying concentrations of Tre6P, Suc6P, Glc6P, Glc1P or the corresponding non-phosphorylated neutral sugars. Samples were injected during 200 s and dissociation time was set to 120 s. Experiment was performed once. (C) SPR surface covered with ds-BBRE remains intact upon exposure to a range of sugars. The SPR reaction surface was covered by ds-BBRE and exposed to HBS buffer containing 5 mM sugar (Tre6P, Suc6P, Glc6P, Glc1P, Mal1P, Fru6P, Man6P and the corresponding neutral sugars). Injection lasted for 30 s and the following dissociation time was set to 60 s. Shown results are representative for the results of the other sugars. Experiment was performed once.
4.1.4 Sugars impact on BAM8’s binding stability to the preferred DNA motif in a concentration-dependent manner

Following the observation that sugars and sugar-phosphates were able to change BAM8’s affinity for the preferred DNA motif, we assessed also the response of already bound BZR1-BAMs to exposure to sugars. To address this, a ‘Binding Experiment’ was performed for which chip surface preparation and procedure was carried out as described in the Materials and Methods (section 3.2.3). In brief, coverage of the reaction cell by ds-BBRE was 300 RU. BAM8 (37.5 nM) was stably bound to the surface (400 RU) and subsequently the analyte (sugar, 5mM) was injected for 45 s. After a dissociation time of 60 s the surface was regenerated by NaCl (1M), which triggered the dissociation of the protein from DNA without causing the disintegration of the dimerised oligonucleotides.

Of all sugars tested, Tre6P and Suc6P led to a consistent dissociation of BAM8 from DNA (Fig 4.1.4-A). For all the other sugars (except for 2deoxy-Glc6P), the signal before and after injection did not deviate from the baseline. The results for 2deoxy-Glc6P were inconsistent between the two repetitions performed. Remarkably, Glc6P also did not cause dissociation of BAM8 even though it showed interaction with BAM8 in MST and caused a low reduction in association of BAM8 to ds-BBRE (Fig. 4.1.1-B, 4.1.3-A).

To further investigate the sugar-triggered dissociation of BAM8 from dsDNA, the two positive candidates Tre6P and Suc6P as well as Glc6P, Glc1P and the corresponding neutral sugars were tested.
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Figure 4.1.4: BAM8 binding stability to ds-BBRE in presence and absence of different sugar (-phosphates) (A) BAM8 stably bound to ds-BBRE dissociates in the presence of Tre6P and Suc6P. The SPR reaction surface was covered with 300 RU ds-BBRE and exposed to 37.5 nM BAM8 for 200 s leading to a coverage of 400 RU. A range of sugars (Tre6P, Suc6P, Glc6P, Glc1P, Man6P, Fru6P, 2-deoxyGlc and the corresponding non-phosphorylated neutral sugars) at 5 mM were injected for 45 s and dissociation was observed for 60 s. Representative results of two experiments are shown. (B) BAM8 bound to ds-BBRE dissociated in presence of Tre6P and Suc6P in a concentration-dependent manner. The SPR reaction surface was covered with 490 RU ds-BBRE and exposed to 37.5 nM BAM8 for 200 s leading to a coverage of 630 RU. A range of sugars (Tre6P, Suc6P, Glc6P, Glc1P and the corresponding neutral sugars) at different concentrations were injected for 45 s and dissociation was observed for 60 s. Experiment was performed once.

for their concentration-dependent response in a ‘Binding Experiment’. The reaction cell was covered with 490 RU ds-BBRE and BAM8 (37.5 nM) was immobilised to obtain a surface binding of 630 RU and was subjected to three different concentrations of each sugar. As observed previously, all neutral sugars (Glc, Suc, Tre) as well as Glc1P and Glc6P did not cause any significant dissociation. In contrast, Suc6P and Tre6P triggered a clear concentration-dependent dissociation (Fig.4.1.4-B). Of the initially immobilised BAM8 (630 RU), roughly 33% (210 RU) was caused to dissociate by 7.9 mM Tre6P. A considerably smaller fraction (14%, 90 RU) of protein were washed off by 10 mM Suc6P. This suggested that BAM8’s binding to ds-BBRE is susceptible to the presence of Tre6P and to a lesser extent to Suc6P.

4.1.5 Sugars impact BAM7’s affinity and binding stability to the preferred DNA motif

An ‘Association-’ and a ‘Binding-Experiment’ were also carried out with BAM7. In both cases, there was a relatively high baseline increase relative to the obtained association signal, as described previously (section 4.1.2). However, the same tendencies as for BAM8 (section 4.1.3, 4.1.4) could still be observed:
Association was significantly reduced only by Tre6P and, to a lesser extent, by Suc6P (data not shown). The ‘Binding Experiment’ showed, that Tre6P caused a dissociation of BAM7 from ds-BBRE, whereas all the other sugars did not show an effect (data not shown). Thus, while there was the same tendency observable for BAM7 and BAM8, the less reliable nature of the results meant that further experiments with BAM7 were not undertaken.

4.1.6 Intact DNA-binding domain and BAM-domain are required for stable binding to the preferred DNA motif

As described previously (section 1.2.5), BZR1-BAM proteins with amino-acid substitutions in residues known to be important for DNA binding, catalysis and proposed to be impaired in substrate binding were generated (Soyk, Simkova et al. 2014). Neither BZR1-BAM showed detectable β-amylase activity (Reinhold, Soyk et al. 2011) and an amino acid substitution previously shown to greatly reduced enzymatic activity of enzymatically active soybean β-amylase (GmBMY1) (Kang, Tanabe et al. 2005) did not impair transcription factor activity (Soyk, Simkova et al. 2014). However, in vitro and in planta experiments showed that BAM8 with amino acid substitutions in residues that either impair DNA-binding (BAM8-dna) or the proposed substrate (or putative ligand) binding (BAM8-lig), were no longer fully functional.

For the current study, BAM8-FL (full length), BAM8-dna and BAM8-lig were subjected to SPR to assess if their affinity for DNA was impacted by the presence of sugars. First, we intended to reproduce the DNA affinity results and carried out an ‘Association Experiment’. Proteins were all produced and purified as previously described (Fig. 4.1.1-A). The reaction cell was covered with 260 RU of ds-BBRE. All proteins were applied to the surface at a concentration of 75 nM. As expected from the previous study (Soyk, Simkova et al. 2014), the binding of BAM8-FL was highest compared to the two other forms (BAM8-dna, BAM8-lig) (Fig. 4.1.5-A). Surprisingly, BAM8-dna binding was not reduced to a similar extent as BAM8-lig, with the latter showing only marginal binding. In an independent ‘Association Experiment’ with BSA immobilised on the reference surface instead of the ss-oligoAT (see section 3.2.3), the reduced binding of BAM8-dna compared to BAM8-FL was confirmed and even more pronounced (data not shown).

The rather surprisingly modest reduction in binding efficiency to ds-BBRE of BAM8-dna compared to BAM8-FL made us further investigate this effect. BAM8-dna was subjected to an ‘Association Experiment’ in which a range of protein concentrations were used (0.39 – 200 nM). The experiment was essentially carried out as described earlier for BAM8-FL (see section 4.1.2, Fig. 4.1.2-B, upper panel). The difference was that there were two regeneration steps of NaCl (1M) included with a contact time of 30 s and stabilisations periods of 30 s and 60 s, respectively. The injection time of the protein was the same but the dissociation time was extended from 120 s to 600 s. The surface coverage by ds-
BBRE was 490 RU. After the initial association during protein injection, the signal decreased gradually following injection stop (Fig. 4.1.5-B). This loss in signal reflects the loss of BAM8-dna from the surface. At the end of the 600 s dissociation time only 13 % of the originally associated BAM8-dna (100 nM) was still present on the surface. This led to the conclusion that the amino acid substitution in BAM8-dna results in a reduced association rate and even more importantly a reduced binding stability (compare to BAM8 on Fig. 4.1.2-B, upper panel).

Next, association efficiency of BAM8-dna (37.5 nM) onto ds-BBRE (200 RU) upon the influence of sugars was tested twice in an ‘Association Experiment’ as described before with the only difference that the dissociation time was set to 300 s. Mannose and all phosphorylated sugars except Man6P and Glc1P reduced BAM8-dna association to different extents, whereas all other neutral sugars did not show an effect (Fig 4.1.5-C). An identical experiment with BAM8-dna (75 nM) binding to ds-BBRE but with BSA immobilised to the reference surface led to similar results (data not shown). Overall, it seems that besides the reduced association ability and faster dissociation from the DNA of the BAM8-dna compared to BAM8-FL, BAM8-dna is also more susceptible to disturbances in binding to ds-DNA by a range of phosphorylated sugars. This may cause an even more pronounced impact on binding events in vivo and would be in agreement with in planta data where BAM8-dna was suggested to be essentially non-functional (Soyk, Simkova et al. 2014).

Even though BAM8-lig binding to ds-BBRE was marginal compared to BAM8-FL and BAM8-dna (see Fig. 4.1.5-A), we tested if BAM8-lig showed detectable sensitivity in its association behaviour by the presence of sugars at a concentration of 5.9 mM. An ‘Association Experiment’ with BAM8-lig (37.5 nM) was carried out with a prolonged dissociation time of 300 s on a surface covered by ds-BBRE. The experiment showed that Tre6P and Glc6P led to a major reduction in association, whereas all other sugars caused a negligible small or no reduction at all (Fig. 4.1.5-D). The impact of Tre6P on BAM8-lig association to DNA was confirmed in a second experiment. Interestingly, Suc6P did not negatively impact association of BAM8-lig even though it impacts slightly on BAM8-FL association (see section 4.1.3, Fig.4.1.3-A). Overall, it seems surprising that BAM8-lig is not totally insensitive to Tre6P. If the amino acid substitution in the substrate binding cleft indeed blocked ligand binding, one would expect ligand insensitivity of BAM8-lig. However, one may consider that the glutamate (E) to arginine (R) amino acid exchange (E623R in BAM8) was proposed under the assumption the putative ligand would be a neutral sugar. In case of a sugar-phosphate as the ligand, the positively charged arginine could trigger a different effect, apparently still leading to non-functionality of the protein (Soyk, Simkova et al. 2014) but through a different mechanism than originally anticipated. Furthermore, the E to R amino acid exchange was modelled to take more than one different confirmations (Soyk, Simkova et al. 2014). Possibly one confirmation mimics the constitutively ligand bound state (no DNA binding), whereas
other confirmations still allow ligand binding and therefore also show susceptibility to Tre6P. If the latter configurations would be less likely (abundant) than the first one, this would explain the overall low binding of BAM8-lig and the susceptibility to Tre6P of the little protein that is bound. However, further experiments would need to confirm this speculation.
Figure 4.1.5 (A-C): Binding of BAM8-FL, BAM8-dna and BAM8-lig to BAM8’s preferred DNA motif (ds-BBRE) and their susceptibility to the presence of sugars. (A) BAM8-FL, BAM8-dna and BAM8-lig at a concentration of 75 nM were injected onto the reaction cell covered with 260 RU ds-BBRE for 200 s. The dissociation period was set to 120 s. (B) Double stranded BBRE (ds-BBRE, 490 RU) was immobilised on the reaction surface and increasing (0-200 nM) concentrations of BAM8-dna were applied. Once the highest concentration (200 nM) was reached, decreasing (200 nM to 0) concentrations of BAM8-dna were sequentially injected. The injection time was 60 s whereas the dissociation time was 600 s. Experiment was performed once. (C) BAM8-dna (37.5 nM) was applied to 200 RU ds-BBRE in presence or absence of 5 mM sugars (Tre6P, Suc6P, Glc6P, Glc1P, Man6P, Mal1P, Fru6P and the corresponding non-phosphorylated sugars). Injection time was 200 s and dissociation time 300 s. The experiment was performed twice; representative results are shown.
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Figure 4.1.5 (D): BAM8-lig (37.5 nM) was subjected to 490 RU ds-BBRE in presence or absence of 5.9 mM sugars (Tre6P, Suc6P, Glc6P, Glc1P, Man6P, Mal1P, Fru6P and the corresponding non-phosphorylated sugars). Injection time was 200 s and dissociation time 300 s. Response to Mal1P, Mal, Fru6P and Fru were similar to those of Suc and Suc6P (not shown). Experiment was performed once. Results for Tre6P and Tre were confirmed in a second, independent experiment.

4.1.7 Impact of Tre6P on transcription factor activity of BAM8 in vivo

In vitro data (MST and SPR) identified Tre6P as the most promising candidate as a putative ligand of BAM8. To investigate the biological significance of this compound in respect to transcriptional activity of BAM8 in vivo, transactivation assays in double mutant (bam7 bam8) protoplasts (Yoo, Cho et al. 2007) were performed. In addition to the usual reporter and effector and transfection control, enzymes modulating Tre6P levels were expressed simultaneously. Tre6P levels were increased by the E. coli trehalose 6-phosphate synthase gene (otsA) and decreased by the expression of the E. coli or rice trehalose 6-phosphate phosphatase genes (otsB and OsTPP1, respectively) (Kaasen, McDougall et al. 1994, Shima, Matsui et al. 2007). Previous studies showed that overexpression of 35S:otsB in Arabidopsis triggered detectable growth phenotypes (larger leaves, late flowering and reduced branching) but Tre6P levels were only decreased in seedlings (Zhang, Primavesi et al. 2009) and not adult plants (Wingler, Delatte et al. 2012, Yadav, Ivakov et al. 2014). This might be because Tre6P is much higher abundant in E. coli compared to Arabidopsis and therefore the bacterial enzyme has insufficient affinity to effectively act on the very low Tre6P levels in plants. The rice TPP1 was measured to have a much higher affinity (lower dissociation constant K_D) for Tre6P compared to otsB (Shima,
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Matsui et al. (2007). We therefore expected a more efficient reduction of Tre6P and a more pronounced effect of OsTPP1 than otsB co-expression on BAM8 activity. Strikingly, in protoplast transactivation assays, performed under moderately high light (150 μmol photons m⁻² s⁻¹) with Suc in the incubation medium, otsA caused no significant reduction in BAM8 activity, whereas otsB as well as OsTPP1 both led to significantly reduced activity of BAM8 (Fig. 4.1.6-A). These results were obtained twice in independent experiments. The expression of the Tre6P metabolising enzymes and BAM8 were confirmed by immunoblotting (Fig. 4.1.6-C). Furthermore, quantification of Tre6P (kindly measured by John Lunn, Max Planck Institute in Potsdam-Golm) in the same protoplasts samples confirmed significantly enhanced Tre6P concentrations in otsA expressing protoplasts (roughly 100-fold than the empty vector control). In contrast, expression of otsB and OsTPP1 only led to marginally reduced Tre6P levels (Fig. 4.1.6-B). Cytosolic concentration of Tre6P was calculated using previously reported estimates of the cytosol in volume (5.4 % of total cell volume) and assuming that 71.5 % of the Tre6P is in the cytosol (Winter, Robinson et al. 1994, Martins, Hejazi et al. 2013). Furthermore, it was assumed that protoplasts are spheres with a diameter of 30 μm. The measured cytosolic Tre6P concentrations were in the range of previous reports (Martins, Hejazi et al. 2013).

To test if the obtained results (opposite to what was expected from the in vitro data) were the response to a newly established homeostasis of Tre6P after a long incubation time (16h) and/or were potentially biased by the presence of sucrose in the incubation buffer, which might have led to a disturbed Suc:Tre6P nexus, an experiment with shorter incubation times, lower light intensity (50 μmol photons m⁻² s⁻¹) and without sucrose in the buffer was performed. Shorter incubation times (2h, 6h) resulted in very low Luciferase expression leading to low LUC/GUS ratios and no observable difference in treatments (not shown). The fact that LUC expression was more affected than GUS may be due to a lower GUS turnover rate than LUC (Koo, Kim et al. 2007) or the fact that LUC signal requires two steps for synthesis (BAM8 expression before BBRE-LUC can be transcribed) compared to one for GUS production. In three independent experiments, we observed that omitting sucrose from the incubation buffer resulted in an activation of BAM8 in presence of otsA and reduced activity in low Tre6P (otsB/OsTPP1) samples (Fig. 4.1.6-D) after 16h of incubation. Tre6P levels of these samples were not quantified but are assumed to be comparable as in the previous measurement (which included Suc in the buffer). The in vitro and in vivo results suggest that Tre6P reduces BAM8’s affinity and binding to the preferred DNA binding motif BBRE but at the same time enhances its transcription factor activity in vivo. In contrast, lower concentrations of Tre6P allows a more stable binding of BAM8 to the BBRE (Fig. 4.1.3-A) but reduced its activity significantly (Fig. 4.1.6-A, -D). Even though counterintuitive, these findings may propose a more sophisticated mode of action than the simple direct relationship between tight binding and higher transcription factor activity. It is possible that the BAM8 system needs the
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Figure 4.1.6: In vivo evidence for regulation of BAM8 activity by Tre6P. (A-C) BAM8 transcription factor activity under moderately high light conditions (150 μmol photons m⁻² s⁻¹) was measured by the protoplast based transactivation assay. The effector BAM8 was expressed in presence of Tre6P synthesising (otsA) and metabolising (otsB, OsTPP1) enzymes. The empty vector pEG201 was used as ‘no effector’ control DNA. The luciferase gene (LUC) under the control of the minimal 35S promoter in combination with twelve repetitions of the BBRE (CACGTGTG) was used as reporter. As a transfection control the β-glucuronidase (GUS) gene was co-expressed. Transcription factor activity is expressed as the LUC/GUS ratio normalised to the ‘no effector’ control (A). Error bars represent Standard Deviation (SD) of three technical replicates. Asterisks indicate a significant difference from BAM8 control transfection (t-test, p<0.05). The experiment was repeated twice independently with similar results. A representative example is shown. Tre6P levels were quantified in these protoplasts after 16 h of incubation by LC-MS/MS and expressed as concentration in the cytosol (B). Error bars represent Standard Error (SE) of the three replicates. Protein expression was confirmed by immunoblotting using anti-HA (BAM8 and...
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The dynamic of binding and dissociation to perform at its full capacity. Additionally, tight binding of BAM8 to the motif might block other co-regulating factors from binding which are required for transcription initiation. Or, as suggested by Soyk, Simkova et al. 2014, they (particularly BAM7) might compete with repressors for the binding site, which explains why most BBRE-driven genes are repressed in bam7 bam8 mutants.

4.1.8 Overlap of genome wide expression changes induced by elevated or reduced Tre6P and disturbed BZR1-BAMs levels (mutant and overexpressing plants).

Assuming that BAM8 activity as transcription factor is regulated by Tre6P there should be a considerable overlap between genes deregulated by BZR1-BAMs and by varying concentrations of Tre6P. To get an initial idea I mined publically available data. The gene set representing BZR1-BAM regulated genes was obtained from transcriptional analysis of seedlings (wild-type, bam8.1, bam7 bam8, BAM8-ox) grown on soil and harvested at the age of 14 days either at 4 h into the light or 4 h into the dark (Reinhold, Soyk et al. 2011). The set included all genes which were deregulated by at least 2-fold in any of the four genotypes relative to one of the other genotypes. This resulted in a list of 1628 and 734 genes for samples harvested during the day and night, respectively. The list of 5889 genes regulated by high Tre6P was obtained from seedlings constitutively overexpressing E. coli TPS (35S:otsA) and grown in liquid culture for 7 days under continuous light in 150 µmol photons m⁻² s⁻¹ (Zhang, Primavesi et al. 2009). The same study compiled a list of 257 genes deregulated by reduced Tre6P levels by overexpressing the E. coli TPP (35S:otsB). A cut off of a 2-fold change relative to the wild-type was used for the latter two lists. For all the following analyses, the term ‘deregulated by chance’ is defined as the ratio of the number of 35S:otsA or 35S:otsB deregulated genes relative to the number of genes present in the whole genome (33,323).

Of the gene set deregulated by BZR1-BAMs at night, 274 were commonly deregulated by 35S:otsA. These 274 genes represent 37 % of the total BZR1-BAM deregulated genes, which is clearly more than the 17 % that would be expected by chance. Out of the 274 commonly regulated genes, a subset of 111 genes were significantly deregulated in BAM8-ox vs. wild-type (wt). Within this subset the majority of genes up-regulated in BAM8-ox (67) was also up-regulated in 35S:otsA (71 %), whereas genes down-regulated in BAM8-ox (44) were predominantly also down-regulated in 35S:otsA (61 %) (Fig. 4.1.7). The same comparison of 35S:otsA with BZR1-BAMs regulated genes harvested during the day gave an
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Overlap of 531 genes, which corresponds to 33% compared to 17% expected by chance. Of these, the majority of BAM8-ox down-regulated (75) were also down regulated in 35S:otsA (68%) but fewer of the BAM8-ox up-regulated genes (136) were also up in 35S:otsA (47%).

Comparing the transcriptional de-regulation of BZR1-BAMs in the night with 35:otsB showed that 25 genes were commonly regulated, which corresponds to 3.4% compared to 0.8% expected by chance. Among those 25 genes, the subgroup of BAM8-ox regulated genes showed an inverted correlation between BAM8-ox and 35S:otsB. All of the BAM8-ox up-regulated genes (3) were 35S:otsB down-regulated (100%), whereas of the BAM8-ox down-regulated genes (12) the majority (75%) were 35S:otsB up-regulated. In contrast, in light there was a positive correlation between BAM8-ox and 35S:otsB regulated genes. 29 genes were commonly deregulated by BZR1-BAMs and 35S:otsB, which are 1.8% of the total BZR1-BAMs deregulated genes (0.8% expected by chance). The majority of BAM8-ox down-regulated genes (8) were also down-regulated by 35S:otsB (75%) and the majority of BAM8-ox up-regulated genes (9) were also up-regulated by 35S:otsB (89%).

In conclusion, it seems that in the dark, BAM8 and Tre6P concentration-dependent gene expression correlates in a positive manner; many gene transcripts induced by BAM8 can be induced by high Tre6P (35S:otsA) and gene transcripts reduced by BAM8 are reduced under low Tre6P (35S:otsB). This is what one would expect from the protoplast in vivo study. In the light, this correlation does not occur. Here, gene transcripts induced by BAM8 are equally up and down regulated by high Tre6P levels (35S:otsA). In contrast, low level of Tre6P induced genes tend to overlap with genes induced by BAM8. What needs to be noted is that the number of genes deregulated by 35S:otsB is rather small and therefore the results have to be treated with caution. Furthermore, the experimental settings (growth conditions) of the compared data sets are considerably different. Deeper transcript analysis of plant material grown and harvested in parallel are necessary to obtain more conclusive results.

It should be mentioned, that neither BAM7 nor BAM8 are among the Tre6P (35S:otsA, 35S:otsB) deregulated genes themselves. Results from consulting publicly available transcription data (from Genevestigator, https://genevestigator.com) also argue against transcriptional regulation of BAM7 and BAM8 by sugars (Glc, Suc) (for details of the search see material and methods, 3.5.1). Interestingly, both putative BZR1-BAM direct target genes (BUP1, BUP2) are deregulated by Glc. This supports modulation of the transcriptional response to sugar downstream of the BZR1-BAMs and strengthens the hypothesis of BZR1-BAMs acting as sugar sensors, which feed into the transcriptional network.
Figure 4.1.7: Transcriptional overlap between Tre6P and BZR1-BAM deregulated genes. Transcriptome data of *E. coli* TPS (35S:otsA) overexpressing or *E. coli* TPP (35S:otsB) overexpressing Arabidopsis seedlings grown in liquid culture (Zhang, Pramvesi et al. 2009) was compared to BZR1-BAM deregulated genes (Reinhold, Soyk et al. 2011). The BZR1-BAM regulated genes were identified from 14 days old seedlings harvested 4 h into the light or dark period, which showed a at least 2-fold change between one of the following investigated genotypes (*bam8.1, bam7 bam8, BAM8-ox*, wild-type). Of the BZR1-BAM and 35S:otsA or BZR1-BAM and 35S:otsB co-regulated genes (in dark grey ellipse) it is indicated what fraction is deregulated by BAM8-ox (in number of genes, light grey ellipse). Of those BAM8-ox up- or down-regulated genes, the fraction of 35S:otsA (first and second panel) or 35S:otsB (third and fourth panel) co-regulated genes are indicated.

4.1.9 Interaction with other proteins involved in sugar-signalling (TPS1 and GeBPL)

Apart from identifying a putative sugar ligand of the BZR1-BAMs, another approach to assess the involvement in sugar-signalling was through studying protein-protein interactions with known sugar-signalling components. A previous protein immunoprecipitation experiment performed on Arabidopsis liquid cell cultures identified a number of interaction partners of BAM7 and BAM8 (Soyk, PhD Thesis and unpublished). Among the BAM8 interacting proteins there was the GeBPL-gene (At4g25210), described as a DNA-binding, storekeeper-like transcription factor (STKL) that belongs to the GLABROUS1 enhancer binding protein (GeBP) family (Soyk, unpublished). GeBPL was seen to be
involved in transcriptional regulation by interacting with mediator and acting on the G-box cis-element (Shaikhali, Davoine et al. 2015). The GeBP family contains GeBP, GeBP-like, storekeeper and storekeeper-like (STKL) proteins (https://www.ebi.ac.uk/interpro/entry/IPR007592), of which AtSTKL1 and AtSTKL2 have recently been shown to be involved in Glc sensitivity response (Chung, Lee et al. 2016). Furthermore, the storekeeper homologue of potato (STK) was identified as the regulator of the Suc-responsive patatin promoter (Zourelidou, de Torres-Zabala et al. 2002). The interaction of BZR1-BAMs with GeBPL was assessed by bimolecular fluorescence complementation (BiFC) in tobacco epidermal cells. According to this method there was a clear interaction of GeBPL with BAM7 and BAM8 (Fig. 4.1.8-A).

Trehalose 6-phosphate synthase 1 (TPS1, AT1G78580) was another direct BAM7 (but not BAM8) interacting candidate identified in the protein immunoprecipitation experiment mentioned above (Soyk, PhD Thesis). It cannot be excluded, that BAM8 indirectly interacts with TPS1 via BAM7, since BAM7 and BAM8 heterodimerise (Soyk, Simkova et al. 2014). TPS1 was a particularly interesting interaction candidate, because through its interaction BAM7 (BZR1-BAMs) might experience a different
microenvironment in terms of Tre6P concentration than the average cellular concentration. As previously described, the in vitro MST study determined a dissociation constant (K_D) of BAM8 with Tre6P in the low milli-molar range. Contrasting to that, Tre6P levels determined in 5 weeks old Arabidopsis seedlings were calculated to be in the low micro-molar range in the cytosol (Martins, Hejazi et al. 2013). Assuming that the reactivity of BAM8 in the in vitro study is representative for the in vivo situation, that
would question BAM8 as a potential Tre6P sensor. Nevertheless, there are reports that the local concentrations of Tre6P may vary considerably depending on tissue and cell type (Vandesteene, Lopez-Galvis et al. 2012, Wahl, Ponnu et al. 2013). Direct physical interaction of the BZR1-BAMs with TPS1 could establish a defined Tre6P micro-environment. The protein-protein interaction of BAM7 and BAM8 with TPS1 was assessed by BiFC in tobacco epidermal cells. However, there was no detectable interaction of TPS1 with neither BAM7 nor BAM8 (Fig. 4.1.8-B). The positive control (BAM8 with BAM8) showed a signal. Expression levels visualised by immunoblotting indicated well detectable levels of expression for TPS1 and BAM8, whereas BAM7 was weakly expressed. This raised the question whether there were sufficient amounts of BAM7 present to reveal an interaction with TPS1. However, in parallel BAM7-YFPc was co-expressed with GeBPL-YFPn and showed a nuclear signal, even though expression levels of BAM7 were equally low as for the BAM7 with TPS1 co-expression sample. In conclusion, protein-protein interaction of BAM7 and BAM8 with TPS1 could not be shown by BiFC under the experimental settings used.

4.1.10 Growth inhibition caused by external glucose and sucrose supply is BAM-domain dependent

The best described sugar sensor in plants is HEXOKINASE 1 (HXK1). HXK1 was identified as a glucose sensor in a mutant screen for plants with reduced glucose sensitivity (Rolland, Moore et al. 2002, Moore, Zhou et al. 2003). In analogy to that experiment, it was tested if plants with distorted BZR1-BAM levels (mutants or constitutive BAM8 over-expressers) also showed an altered sensitivity to Glc. Previous experiments showed that bam7bam8 mutants are hyper- and BAM8-ox plants hyposensitive to 6% Glc (Reinhold and Soyk, unpublished). To replicate and further investigate this issue, plants overexpressing a BAM8 protein with suggested impairment in ligand binding in the bam8.1 background (BAM8-lig) (Soyk, Simkova et al. 2014) were included in this study. Plants were grown for 11 days on media containing different concentrations of Glc in continuous light. On the 11th day the plants were rated in ‘green’ or ‘pale’. In general, the fraction of green relative to pale plants reduced drastically with increasing Glc concentration. This effect was most pronounced for bam7bam8; at 250 mM Glc half of the plants already showed a non-green phenotype, and at 305 mM Glc all were pale. In contrast, at 333 mM Glc more than half (60%) of BAM8-ox seedlings were still green. An intermediary phenotype was seen for BAM8-lig and the two controls bam8.1 and wild-type, indicating that the suggested inability of BAM8-lig to bind the putative ligand (Soyk, Simkova et al. 2014) led to Glc sensitivity (Fig. 4.1.9-A). Growth on plates containing same concentrations of mannitol (0 - 333 mM) led to a reduction in seedling size but greening was still 100% even at the highest concentration (333 mM mannitol). To further elucidate the mechanism behind the abovementioned effect, bam7bam8 and BAM8-ox combinations with hxx1 were created and the homozygous population (F3 generation) was exposed to
increasing Glc concentrations, as mentioned above. Results of this experiment were inconclusive: Both bam7 bam8 hxx1 and BAM8-ox hxx1 plants showed a hxx1-like resistance pattern and were distinguishable from bam7bam8 and BAM8-ox, respectively (Fig. 4.1.9-B). Growth on same concentrations of mannitol did not lead to an impairment of greening. From these results we cannot draw a clear conclusion regarding the mechanism. It seems that the two pathways (BZR1-BAM and HXX1) do not act completely independently since the BAM8-ox hxx1 clearly did not show an additive resistant effect. Further, under our experimental setup the BAM8-ox showed are more pronounced Glc insensitivity than the hxx1 mutant.

Due to the close relationship between Tre6P and Suc (Yadav, Ivakov et al. 2014) we extended our investigations on the response of plants lacking or overexpressing BZR1-BAMs to exposure of increased Suc concentrations. Experiments with seedlings of bam8.1, bam7 bam8, BAM8-ox, BAM8-lig and wild-type genotype grown under the same conditions as mentioned before (11 days continuous light), on medium containing increasing concentrations of Suc (0-278 mM) or Man were carried out. Seedlings were rated into ‘pale/very small’ or ‘green/regular size’. The results showed that with increasing Suc concentrations, the fraction of pale or very small seedlings was considerably higher in the bam7 bam8 double mutant compared to the wild-type and BAM8-ox (Fig. 4.1.9-C). The single mutant bam8.1 and the BAM8-lig also showed a reduced fraction of regular green seedlings compared to wild-type and BAM8-ox. Interestingly, at these concentrations of Suc, the wild-type did not behave differently from BAM8-ox with both showing only little impairment by the presence of 278 mM Suc. Previous reports showed that seedlings grown on 300 mM Suc in solid minimal Arabidopsis media failed to develop true leaves and have pale and purple cotyledons (Laby, Kincaid et al. 2000). However, no quantitative information was given in that study. The fact that BAM8-lig behaved like bam8.1 is most likely due to its bam8.1 background and confirms the inability of BAM8-lig to fulfil BAM8’s native function. Another observation was that in all these experiments there was the tendency of lower germination rates with increasing sugar (Suc and Man) concentrations in the medium, attributed to a general response to osmotic stress and not to sugar-signalling. In general, bam7 bam8 had lower, and BAM8-ox higher germination rates than the average, but this was not consistent in all experiments.
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A)

% green seedling

305 mM Mannitol

bam7 bam8 BAM8-ox wild type

bam8.1 BAM8-ig

305 mM Glucose

bam7 bam8 BAM8-ox wild type

bam8.1 BAM8-ig
Results and Discussion

B)

305 mM Mannitol

305 mM Glucose

BAM8-ox hxl1  bam7 bam8  hxl1  BAM8-ox hxl1  bam7 bam8  hxl1

BAM8-ox  bam7  bam8  wild type

BAM8-ox  bam7  bam8  wild type
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4.1.11 Miss-expression of BAM8 in sugar mutants does not restore their growth defect

Plants defective in primary carbon metabolism often show reduced or impaired growth (Caspar, Huber et al. 1985, Niittyla, Messerli et al. 2004, Seung, Lu et al. 2016). One reason for this is the disturbed or reduced availability of resources, another reason might be aberrant sugar signals, which transduce a wrong information regarding the energy status and ultimately lead to an inhibition of growth (Lastdrager, Hanson et al. 2014). To tackle the question if BZR1-BAMs are involved in sugar-signalling and trigger a growth-related response, the BZR1-BAM double mutant (bam7 bam8) and BAM8-ox were crossed to mutants with known abnormal sugar levels or known impaired sugar sensing. The chosen sugar mutant plants were defective in the chloroplastic phosphoglucomutase (pgm1), the cytosolic disproportionating enzyme (dpe2), the maltose transporter (mex1), isoamylase1 (isa1) or hexokinase1 (hxk1). Pgm1 leads to an increase in various soluble sugars during the day and equal or lower amounts
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of these during the night. Suc presumably accumulates mainly in the cytosol, whereas Glc and Fru are stored in the vacuole (Caspar, Huber et al. 1985, Winter, Robinson et al. 1993). Mutation in DPE2 (dpe2) triggers a Glc, Mal, maltotriose (G3) and maltotetraose (G4) increase during day and night (Chia, Thornecroft et al. 2004). Mal was determined to localise to the cytosol and chloroplast in roughly equal amounts, whereas G3 and G4 more likely accumulate in the chloroplast (Lu, Steichen et al. 2006). Mutation in DPE2 (dpe2) triggers a Glc, Mal, maltotriose (G3) and maltotetraose (G4) increase during day and night (Chia, Thornecroft et al. 2004). In isa1, Mal levels peak at the end of the day and decrease to wild-type levels during the night. Mex1 shows a significant Mal increase specifically in the chloroplast (Niittyla, Messerli et al. 2004, Lu, Steichen et al. 2006) during the day and the night. As mentioned above, hxxk1 is impaired in sugar-signalling but does not show a consistent growth defect under standard conditions.

To quantify growth defects, leaf area (LA), fresh weight (FW) and dry weight (DW) were measured. Mutant combination of bam7bam8 or BAM8-ox with the sugar mutants (pgm1, dpe2, mex1, isa1, hxxk1) grown under 12/12 light/dark conditions for up to 41 days showed that for all three criteria (FW, DW, LA) the sugar mutant background determined the growth capacity. In none of the cases did the absence or exaggerated presence of BAM7 or BAM8 led to a reversion or enhancement of the phenotype in terms of growth (Fig. 4.1.10-A). It seems that the growth defect is more likely due to unsuitable carbon-availability rather than aberrant (BZR1-dependent) sugar signals.

Looking at other characteristics such as target gene expression, chlorophyll accumulation or leaf bending in these mutant combinations might answer if BZR1-BAMs dependent sugar-signalling plays a role in processes other than growth control. Therefore, expression of BUP1, which is a good candidate as a direct target since it is strongly upregulated in BAM8-ox and downregulated in bam7bam8 (relative to wild-type) and contains one BBRE motif in the 1,000 bp-promoter, was quantified. Expression levels were measured from a pool of seedlings harvested at midday, grown for 14 days under 12h/12h light/dark cycle (120 µmol photons m⁻² s⁻¹) in all above mentioned mutant combinations of bam7bam8 or BAM8-ox with isa1, mex1, pgm1, dpe2 and hxxk1 and the respective single mutants. Expression was normalised to the house keeping gene PP2A and represented relative to the wild-type expression level. BUP1 was not markedly deregulated in any of the single sugar mutants (isa1, mex1, pgm1, dpe2) but was upregulated two-folds in hxxk1 (Fig. 4.1.10-B). This upregulation was consistent in the hxxk1 bam7bam8 compared to bam7bam8, which indicates that there must be an BZR1-BAM independent contribution leading to the increased expression. In bam7bam8 mex1, bam7bam8pgm1, bam7bam8dpe2 BUP1 was downregulated and in bam7bam8 isa1 upregulated compared to bam7bam8, which overall seems like an amplification of the tendency seen for the single mutants. There are possibly other sugar-responsive factors regulating BUP1, which have a larger impact in the absence of the BZR1-BAMS. The quantification of BUP1 transcript in BAM8-ox isa1, BAM8-ox mex1, BAM8-ox pgm1, BAM8-ox dpe2,
BAM8-ox hxx1 showed a general upregulation compared to the wild-type (as expected) and some variation compared to the BAM8-ox. However, this variation correlates to BAM8 expression (which also varied in these samples, see Fig. 4.1.10-C) except for BAM8-ox mex1 and BAM8-ox dpe2, where BUP1 expression was reduced even though BAM8 expression was enhanced compared to BAM8-ox. Overall these results confirm the previously observed activating effect of BAM8 on BUP1 expression. Furthermore, it seems that BUP1 is slightly deregulated by the sugar context in the sugar mutants. However, this effect cannot be solely assigned to BAM8’s action. There must be one or more other factors involved that have a negative impact on BUP1 expression in the presence of one or several sugars. Furthermore, from these results it is also not possible to assign a particular sugar as the cause of the observed changes.

**A)**

- Left top: leaf area (cm²) vs DAG for wild type, bam7 bam8, mex1, and mex1 bam7 bam8.
- Right top: leaf area (cm²) vs DAG for wild type, BAM8-ox, mex1, mex1 BAM8-ox.
- Left bottom: leaf area (cm²) vs DAG for wild type, bam7 bam8, isal, and isal bam7 bam8.
- Right bottom: leaf area (cm²) vs DAG for wild type, mex1, mex1 bam7 bam8, and mex1 BAM8-ox.
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4.1.10: Growth defect in ‘sugar mutants’ (mex1, dpe2, pgm1) is most likely not triggered by a misregulation of BZR1-BAM target genes. (A) Combinatorial mutants of bam7 bam8 or BAM8-ox with mex1, dpe2, pgm1, isa1 and hxx1 were generated. The homozygous (F3) generation was compared to the parental lines in terms of leaf area (LA), fresh weight (FW) and dry weight (DW) over a time period of 20-42 day after germination (DAG). Mutant combinations with pgm1, mex1 and dpe2 followed the same pattern, whereas hxx1 and isa1 mutant combinations followed another pattern. The experiment was repeated twice. Representative results of mex1 and isa1 are shown for the LA (four top panels). Representative results for the resemblance of FW and DW mutant combinations of mex1 are shown (lower two panels). Error bars represent SD for LA and SE for FW and DW. (B) Relative expression of BUP1 was measured in pools of seedlings grown for 14 days at a 12/12 h light/dark cycle and harvested at midday (6h into the light). Chosen genotypes were the same as mentioned in (A). The expression level of BUP1 was measured by qPCR, normalised to PP2A and expressed relative to the expression in wild-type. Error bars represent the SD of two (parental lines and BAM8-ox combinatorial mutants) and three (bam7 bam8 combinatorial mutants) technical replicates. (C) BAM8 expression in wild-type, BAM8-ox and BAM8-ox combinatorial mutants, quantified and plotted as described for (B). Error bars represent SD of three technical replicates.

4.1.12 Discussion

4.1.12.1 Identification of a sugar ligand of BZR1-BAMs

BZR1-BAMs have arisen with the emergence of vascular plants. Intriguingly they have retained their β-amylase domain even though it has very low or no measurable enzymatic activity (Reinhold, Soyk et al. 2011, Soyk, Simkova et al. 2014). The most plausible explanation, also considering the combination with the transcription factor domain, is a sensing function. To date there has been no direct evidence of a ligand-protein (BZR1-BAM) interaction. In this study, the first experimental evidence was generated which supports the physical interaction of the BZR1-BAMs with a sugar ligand. Out of 13 tested phosphorylated and non-phosphorylated mono- and di-saccharides, Glc6P and Tre6P showed direct
interaction with BAM8 *in vitro* (MST). Complementary experiments using SPR generated further indirect evidence for a sugar-protein interaction by testing the impact of sugars on BZR1-BAM’s binding behaviour to the preferred double stranded DNA motif (BBRE). Whereas Tre6P and Suc6P consistently reduced DNA affinity and binding stability, Glc6P triggered a more obscure effect; it increased the affinity for single stranded DNA (reference cell), whereas affinity for the ds-BBRE was only slightly reduced and binding stability to ds-DNA was not impacted. The observed impact of Suc6P on BAM8 in the SPR but not the MST experiment might have a sensitivity reason; if the Suc6P-BAM8 interaction is relatively weak, it might not be detectable by MST but cause an observable difference on SPR, where a relatively small loss of BAM8 from the chip surface is readily detectable. Alternatively, Suc6P could be a false negative in the MST, meaning that the parameters (mass, charge, hydration shell) that lead to changes in migration behaviour level each other out. Structurally Glc6P and Tre6P are very similar, the difference is that Tre6P is composed of two Glc units, which are linked through a \( \alpha,\alpha-1,1 \) glucoside bond. They both carry the phosphate group at the C6 position. Suc6P, which is composed of a glucose and a fructose unit, carries the phosphate group on the Fru moiety. While still a disaccharide, it is therefore less similar to Tre6P, especially considering that the phosphate group seems to play a crucial role for the protein-ligand interaction. Tre6P and Suc6P have in common that they only occur in the ring-conformation, whereas Glc6P alternates between two conformations (ring- and linear-form). The effect of all three sugars on BAM8 may therefore be seen as the sensing of three different ‘criteria’. However, from the *in vitro* data it seems more plausible that Tre6P is the actual ligand and Glc6P and Suc6P cause in some of the experimental conditions a similar yet much more moderate response due to their structural similarity to Tre6P. It is doubtful whether the higher affinity of BAM8 in presence of Glc6P for random single stranded DNA is of any biological significance.

Preliminary data regarding BAM7’s interaction with a sugar ligand and the subsequent impact on ds-BBRE binding was also acquired. The robustness of these data is questionable due to experimental difficulties; heterologously expressed BAM7 is of lower purity and shows significantly higher unspecific association to the reference surface and relatively high irreversible association to the SPR surface in the course of the experiment. To obtain more reliable results one would have to invest into optimising experimental conditions. Nevertheless, the observed tendencies indicated that BAM7 shows essentially the same behaviour as BAM8 regarding the impact of sugars onto association and binding to the ds-BBRE. The affinity to ds-BBRE of BAM7 compared to BAM8 seems lower altogether. At this point it may also be mentioned that in general, comparison between binding levels of different proteins to the same reaction surface is only semi-quantitative. Best results are obtained if measurements are carried out on the very same surface. Further inaccuracies depend on the precision of the determined protein concentration. Variation in the affinity to the reference surface also bias the result.
Comparisons of different BAM8-versions (BAM8-dna, BAM8-lig, BAM8-FL) exposed to the same SPR surface covered with ds-BBRE confirmed results previously obtained in an DPI-ELISA experiment performed by Soyk, Simkova et al. 2014. In both studies, DNA-affinity of the putative ligand-binding (BAM8-lig) and the DNA-binding mutant (BAM8-dna) were reduced compared to the full length BAM8 (BAM8-FL). However, the reduction for BAM8-dna in our experiment was less pronounced than expected (compared to the study of Soyk, Simkova et al. 2014), possibly due to the semi-quantitative nature of these SPR measurements in this respect. As mentioned above, it is not possible to quantitatively compare two different proteins regarding the amount binding to the SPR surface. Another reason, of biological nature, was observed in the follow-up experiment; BAM8-dna was considerably more susceptible to a wider range of mainly phosphorylated sugars. In planta, this would lead to an even more pronounced inability to bind DNA and allow gene expression than under in vitro conditions. The increased dissociation rate of BAM8-dna further contributes to overall lower binding (as observed in Soyk, Simkova et al. 2014). In contrast BAM8-lig showed a massive reduction in DNA-binding affinity as was shown before (Soyk, Simkova et al. 2014). Surprisingly, BAM8-lig was still sensitive to Tre6P even though ligand binding was predicted to be prevented or greatly reduced by introducing the E623R amino acid substitution. However, modelling was not performed under the assumption that the ligand would be a negatively charged disaccharide. In the case of Tre6P as the ligand, the substitution of the negatively charged glutamate by a larger and positively charged amino acid might prevent the ligand from binding due to steric limitations (as proposed by Soyk, Simkova et al. 2014). The introduced positive charge may also increase the affinity of the protein to the ligand and make the ligand-protein interaction more stable. If that was the case, BAM8-lig would actually be more sensitive to Tre6P but should show a binding affinity comparable to BAM8 in the absence of Tre6P, which according to our experiments is not the case. Alternatively, BAM8-lig may already behave though in the ‘bound’ confirmation even in the absence of the ligand, which would explain the marginal binding even in the absence of Tre6P but not the residual sensitivity to the ligand. Possibly a combination of the two scenarios occurs: as mentioned in section 4.1.6, the E623R amino acid exchange was modelled to take on different confirmations (Soyk, Simkova et al. 2014). The predominant form may be stuck in the ‘bound’ confirmation, whereas others (less abundant confirmations) still allow ligand binding, potentially with an even higher affinity. According to that scenario, the largest fraction of BAM8-lig would not bind ds-BBRE and a minor fraction would bind but be susceptible to Tre6P. Being more critical, there is undoubtedly a concern about BAM8-lig integrity. Even though it was possible to express and purify the protein, BAM8-lig was always lower abundant than the two other isoforms (see Fig.4.1.1-A), which might indicate a lower stability and/or miss-folding. To clarify if the protein is correctly folded and in the native state one could employ techniques such as circular dichroism (CD) or
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a partial protease digestion. The latter technique would result (under optimised conditions) in a wide diversity of degradation products in case of mis-folding and distinct degradation products (e.g. according to different protein domains) in case the protein is correctly folded. Showing BAM8-lig's integrity is important to substantiate the above-mentioned hypothesis regarding its activity.

4.1.12.2 Role of trehalose 6-phosphate on BZR1-BAM activity in vivo

Another concern raised upon the in vitro data are the relatively low binding affinity of BAM8 to Tre6P (high \( K_D \) values), especially considering the very low abundance of Tre6P reported in planta. However, it may be noted that there is a range of Tre6P concentrations in Arabidopsis reported today (1-10 nmol g\(^{-1}\) FW), which is usually extracted from whole plant parts without separating into specific cell-/tissue types (Schluepmann, Pellny et al. 2003, Martins, Hejazi et al. 2013). Particular parts of the plants, e.g. meristematic tissue (Wahl, Ponnu et al. 2013) may contain considerably higher concentrations. Furthermore, since it is also known that there are different Tre6P:Suc ratios present in Arabidopsis depending on tissue type and developmental stage (Figueroa and Lunn 2016), one can imagine that there is actually a wider diversity in Tre6P concentrations found in plants than determined to date. Additional to variance in different cell-/tissue types, there may also be higher concentrations of Tre6P in close proximity of Tre6P synthesising enzymes. However, the hypothesised interaction of BZR1-BAMs with TPS1 could not be confirmed. Nevertheless, because we do not yet fully understand BZR1-BAMs localisation in terms of tissue and cell type and its precise time of action, it cannot yet be ruled out that it may be exposed and acting in locations of high Tre6P concentrations. Another plausible explanation for the discrepancy between the in planta situation and the in vitro data is that standard buffers used for MST and SPR do not sufficiently reflect the in planta conditions. Furthermore, as-yet unknown co-factors might be necessary that influence binding affinities of the protein for the ligand.

Testing the response of BAM8 activity to modulated Tre6P levels in a protoplast based reporter system confirmed Tre6P as a good candidate as metabolic signal sensed by BAM8. There were two conditions tested; firstly, protoplasts were incubated in medium supplemented with Suc and under moderately high light. Secondly, an incubation medium without Suc was used together with low light. The two conditions led to slightly different results: In both cases, the presence of otsB (low Tre6P, quantified only for high light) reduced BAM8 activity. In contrast, presence of otsA (high Tre6P, quantified only for high light) had no influence in high light, but exerted an activating effect on BAM8’s transcriptional output in low light. The fact that high Tre6P (if anything) activates BAM8 was opposite to what we expected from the SPR analysis. It seemed that binding stability to the preferred DNA binding motif did not correlate with higher gene expression activation. As mentioned earlier, this might indicate that a dynamic system of association and dissociation of BAM8 to/from DNA is required for it to be fully functional. Since we do not know if any or which other components collaborate with BAM8 to activate
gene expression, it is impossible to conclude with one action model. Encouragingly, comparison with genome wide expression changes was broadly congruent with the transactivation assay.

When comparing expression changes upon overexpression of otsA (35S:otsA) or otsB (35S:otsB) to BZR1-BAM regulated genes it became evident that the correlation was different depending on the light conditions of sampling. Most of the genes activated by BAM8 at night were activated under high Tre6P (35S:otsA) and repressed by low Tre6P (35S:otsB). This correlated well with the finding of the transactivation assay carried out under low light, which showed higher transcriptional activation of BAM8 in the presence of otsA and lower activity when co-expressed with otsB. In contrast, genes activated by BAM8 during the day were up- and down-regulated by high Tre6P in equal proportion and most were induced by low Tre6P. This fits the transactivation assay results of unaffected BAM8 activity by otsA in high light but contradicts the observed reduced BAM8 activity in presence of otsB. However, I consider the comparisons of BAM8-ox with 35S:otsA as more meaningful, since the number of deregulated genes are roughly 10-times higher than in 35S:otsB (15-17 for 35S:otsB, 111-211 for 35S:otsA). Overall, the outcome of the genome wide expression change comparisons and the transactivation assay look promising. Particularly during night/low light conditions, there seems to be a good correlation between BAM8 and high-Tre6P upregulated genes. From these findings, several new questions arise: Are BZR1-BAMs acting in different mechanisms or modes during day and night? Does BZR1-BAM sensitivity to Tre6P vary from day to night? Are there other signalling pathways interfering with the BZR1-BAMs response during the day but not the night (‘diluting’ Tre6P responses during the day)?

Interestingly, diverging functions during day and night have been proposed for Tre6P. Whereas during the day Tre6P appears mainly to influences carbon partitioning between Suc and organic acids and amino acids (via PEP carboxylase and nitrate reductase), at night Tre6P seems to regulate starch remobilisation by linking sink demand with starch mobilisation in source organs (Martins, Hejazi et al. 2013, Figueroa, Feil et al. 2016). Ethanol-induced otsA expression (iTPS) led to reduced starch degradation and increased starch levels at the end of the night (Martins, Hejazi et al. 2013). From that, one would conclude that BZR1-BAM deregulated plants might also have an impact on starch metabolism. However, neither the double mutant (bam7bam8) nor BAM8-ox showed significantly changed starch content at the middle of the night (Reinhold, Soyk et al. 2011).

A genetic approach to investigate the interaction of gene regulation by Tre6P and the BZR1-BAMs may be addressed by crossing 35S:otsA with BAM8-ox and 35S:otsA with bam7bam8 double mutants. If BAM8 is indeed a Tre6P sensor at least some of 35S:otsA upregulated genes should no longer respond in 35S:otsA bam7 bam8 or show an even stronger response in 35S:otsA BAM8-ox. The smaller overlap between BAM8-ox upregulated genes with Tre6P deregulated genes during the day may be due to
changed BZR1-BAM activity upon post-translational modification (such as phosphorylation or redox-regulation) or the result of interference with the BR-signalling pathways. Arguments for the second hypothesis are the previous observation that BZR1-BAMs most efficiently influence brassinosteroid-signalling pathways under conditions of low BR saturation such as during the day or treatment with brassinozole (BRZ) (S. Soyk, unpublished). BR-mediated signalling is weakened in the light due to its missing interaction partner (PIF4), which is sent for degradation upon light perception and the repression by the DELLAs (Jaillais and Vert 2012, Oh, Zhu et al. 2012). This would explain the higher number of BR and BZR1-BAM co-regulated genes during the day and the reduced correlation with 35S:otsA up-regulated genes.

4.1.12.3 Physiological output of BZR1-BAMs’ response to elevated sugar concentrations

The growth defect of the single sugar mutants (isa1, mex1, dpe2, pgm1) could not be assigned to BZR1-BAM dependent sugar sensing and signalling. Combinatorial mutants of the sugar mutants with bam7 bam8 or BAM8-ox showed the same growth phenotype as the parental single mutant. If elevated sugar levels perceived by BZR1-BAMs would have been the major cause for the stunted phenotype of the sugar mutants, one would have expected at least a partial reversion of the growth defect in the combinatorial mutants. Furthermore, the abundance of BUP1 transcript at midday (identified as a direct transcript of BAM8, described in section 4.3.5) did not markedly deviate in any of the single sugar mutants compared to the wild-type, indicating that none of the overrepresented soluble sugars present in isa1, mex1, dpe2 or pgm1 modulated BAM8’s activity on the BUP1 promoter. Hence, the use of sugar mutants and the characteristics investigated (BUP1 expression, growth phenotype) did not allow us to observe activity changes of the BZR1-BAMs upon exposure to elevate sugar levels. The only interesting observation from these experiments was that in mex1 BAM8-ox and dpe2 BAM8-ox, BUP1 expression seemed reduced relative to BAM8 abundance. The mex1 mutants have increased Glc, Suc, Fru during the day but lower Glc and Fru at night, whereas Suc does not significantly change (Niittyla, Messerli et al. 2004). The dpe2 mutants have higher Glc during day and night and lower Suc during the night (Chia, Thornycroft et al. 2004). Both mutants most likely experience a status of carbon starvation at night, which was shown to lead to low Tre6P concentrations in wild-type plants (Yadav, Ivakov et al. 2014). Assuming that mex1 and dpe2 are low in Tre6P during the night would fit our model of Tre6P activating BAM8 activity. What weakens the argument is that these differences are only seen in the BAM8-ox mutant combinations but not the single sugar mutants. Furthermore, pgm1 was shown to have reduced Tre6P at the end of the night (Lunn, Feil et al. 2006) and is well known to experience carbon starvation at night but does not follow the same trend in our experiment. To substantiate this hypothesis, Tre6P
would have to be measured in the sugar mutants (most importantly *mex1, dpe2* and *pgm1*) and correlated to transcriptional analysis and BAM8 abundance in the very same biological tissue.

Sensitivity to external sugars is rather complex and, in addition to sugar signals, also hormone signalling pathways and nitrate abundance are known to play a role in sugar sensitivity (Lastdrager, Hanson et al. 2014). ABA biosynthesis or signalling mutants were seen to be Glc insensitive, whereas ethylene insensitive mutants are Glc hypersensitive and low nitrate concentrations also render plants more susceptible to elevated sugars (Yanagisawa, Yoo et al. 2003, Dekkers, Bezstarosti et al. 2008, Cho, Sheen et al. 2010). Both, HXK1 and Tre6P, were shown to be involved in sugar sensitivity and they are at least partially interconnected, likely via ABA-signalling (Avonce, Leyman et al. 2004) (see section 1.3.6.2). It was shown that HXK1-mediated signalling increased ABA abundance (Ramon, Rolland et al. 2008). Furthermore, increased Tre6P levels such as in *TPS1*-ox led to reduced Glc sensitivity, reduced ABA-responsiveness as well as reduced ABA accumulation (Avonce, Leyman et al. 2004). In contrast, reduced Tre6P levels as in *35S:otsB* plants show hypersensitivity to glucose (Schluepmann, Pellny et al. 2003). Our results also suggest that BZR1-BAMs are involved in Glc and Suc sensitivity. *BAM8*-ox plants are less susceptible to elevated Glc levels, whereas *bam7 bam8* plants are more susceptible to elevated Glc and Suc. Intriguingly, overexpression of BAM8 only caused a *hxk1*-like Glc insensitivity if the protein contained a functional BAM-domain. BAM8-lig overexpressing plants had a wild-type-like susceptibility to elevated external Glc. This supports the hypothesis that BAM8 acts as sugar sensor which occurs via a functional BAM-domain. Mutant combinations with *hxk1* showed that the *hxk1* and *BAM8*-ox glucose insensitivity trait was not additive. *BAM8*-ox *hxk1* plants showed an intermediary sensitivity between *hxk1* and *BAM8*-ox, *BAM8*-ox essentially being slightly more resistant than *hxk1*. Nevertheless, an overlap or convergence between BZR1-BAMs and *hxk1*-signalling is supported by the observed upregulation of BUP1 in *hxk1*. However, the precise molecular mechanism cannot be determined from these data.

Neither can it be determined whether the altered Glc/Suc sensitivity in *BAM8*-ox and *bam7 bam8* is Tre6P dependent or an independent mechanism. Considering previous data of Tre6P’s impact on BAM8 activity support the hypothesis that BAM8 acts as (one of the) Tre6P sensors. The direct link between BAM8 and Tre6P could be clarified in a genetic approach: One would expect that *TPS1*-ox *bam7 bam8* at least partially lose its resistance to elevated Glc compared to the parental *TPS1*-ox and the *TPS1*-ox *BAM8*-ox has increased or retained resistance, in case that BZR1-BAMs transduce the Tre6P signal. Of course, this would only apply if *BAM8*-ox and *bam7 bam8* have not yet reached the extremes in terms of Glc sensitivity/resistance phenotypes. Furthermore, ABA concentrations in *BAM8*-ox and *bam7bam8* could be measured to assess if they also show a reduced and increased ABA accumulation, respectively.
This would reveal if the Glc insensitivity of BAM8-ox and hypersensitivity of bam7 bam8 is achieved through the hormone ABA.

Publicly available expression data show that the BZR1-BAMs are neither deregulated by Glc nor Suc themselves but the putative direct target BUP1 is responsive to Glc (but not Suc). Hence, the level of regulation must be down-stream of the BZR1-BAMs, further supporting a sensing/signalling role for them. If the putative BZR1-BAM related sugar-signalling is dependent or facilitated by the GeBPL protein has to be further investigated. The previous identified interaction of GeBPL with mediator, which is a facilitator of regulatory signals from promoter-bound transcription factors to be transmitted to the RNA polymerase II machinery, might be important for the BAM8 triggered gene activation.
4.2 Spatial appearance of BZR1-BAMs

To understand a protein’s function *in planta* it is important to know its subcellular localization and expression pattern within the plant. Previous studies have shown that BZR1-BAMs localize to the nucleus. This was revealed by transient expression of the fluorescently labelled protein under the control of the constitutive CaMV 35S promoter in protoplasts and tobacco epidermal cells as well as in stably transformed Arabidopsis (Reinhold, Soyk et al. 2011, Soyk, Simkova et al. 2014). Furthermore, their expression pattern was assessed by using a promoter BZR1-BAM:GUS fusion construct. The promoter was defined as the 2 kb region upstream of the predicted start of transcription. According to that study BAM7 was mainly expressed in young leaves and young flowers. In contrast, BAM8 was only weakly expressed and showed inconsistent results between different lines. The low expression of BAM8 raised some doubts because BAM8 is usually well detectable by immunoblotting whereas native BAM7 is not. It was therefore speculated that cis-regulatory elements in the 5’UTR of the gene or the first intron (which were omitted in these constructs) may influence the proper expression pattern.

4.2.1 Plant wide BZR1-BAM expression pattern

To overcome the doubts of omitted and potentially important cis-regulatory elements in the 5’UTR or the first intron within the gene, new constructs were generated by Klara Simkova (unpublished data) and stably transformed into wild-type *Arabidopsis thaliana*. Roughly 2,000 bp upstream of the start of the second exon (genomic sequence) were cloned in front of the GusA gene (Fig. 4.2.1-A, green construct). The chosen genomic region included the promoter sequence, the 5’ UTR, the first exon, the first intron and a few base pairs of the second exon. This construct was stably transformed into wild-type Arabidopsis plants. Three independent, homozygous transgenic lines were selected. The expression pattern was assessed in the T3-generation of young seedlings (3 weeks) grown on half-strength MS medium and floral tissue of plants grown on soil.

For BAM7 and BAM8 there was a considerable diversity in signal strength between the three independent, homozygous lines (#8.4, #6.1, #4.3 for BAM7, #6.6, #4.2, #2.1 for BAM8). In seedlings, BAM7 was expressed in the vasculature of cotyledons and true leaves (old and young), in the hydathodes and the root vasculature. The weaker the expression was, the less evident became the signal in cotyledon and leaf vasculature. The most consistent signal, even in low expressing lines, was in the hydathodes (Fig. 4.2.1-B). BAM8 was expressed in the same tissues as mentioned for BAM7 and was overall stronger. Additionally, it was found throughout the leaf (not restricted to the vasculature) particularly in young leaves and the root tip. In floral tissue, BAM7’s expression was strongest in in the flower stem and weaker in young and old stamens. BAM8 was predominantly expressed in the stem and the stamens of young flower buds (Fig. 4.2.1-C). In the stronger expressing lines, GUS staining was also seen in the petals, pistils and weakly in the siliques (but not seeds).
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A)

BAM7
-1564 -1531

pB7:BAM7 Δ
GUSA
pBAM7
BAM7 (gene without STOP)
HA/GFP
Tnos

BAM8
-1467 -1164

pB8:BAM8 Δ
GUSA
pBAM8
BAM8 (gene without STOP)
HA/GFP
Tnos

B)

pB7:GusA #8.4
pB7:GusA #6.1
pB7:GusA #4.3

pB8:GusA #6.6
pB8:GusA #4.2
pB8:GusA #2.1

pB7:GusA
#8.4
#6.1
#4.3

pB8:GusA
#6.6
#4.2
#2.1
4.2.2 Localization of the ‘native’ Arabidopsis BZR1-BAM proteins

I generated a new GFP-tagged construct, which included all elements of the native genes, with exception of the 3’ UTR which was excluded and replaced by the Nos-terminator (Tnos). The intention was to get an as close to native expression and localization pattern as possible (Fig. 4.2.1-A, purple construct).
Transient expression in tobacco epidermal cells indicated a very clear nuclear localization for both pBAM7:BAM7-GFP (pB7:B7-GFP) and pBAM8:BAM8-GFP (pB8:B8-GFP) products (Fig. 4.2.2-A). Subsequently, stable transformation in the respective Arabidopsis single mutants (bam7.1, bam8.1) and the double mutant (bam7 bam8) were generated. For both proteins three independent, single-insertion homozygous lines were identified. Confocal fluorescence microscopy confirmed the nuclear localization of BAM7 and BAM8 also in these lines (Fig. 4.2.2-B). Roots and young leaves of 2-week-old seedlings grown on half-strength MS medium were investigated. Root cells were additionally stained with propidium iodide (PI) to reveal the cell walls. BAM7 showed overall a lower GFP signal than BAM8, indicating lower protein abundance. Furthermore, for BAM7 only two out of three transgenic lines expressed the protein in amounts detectable by fluorescence microscopy, even though in all three lines BAM7 was detectable by immunoblotting (in bam7.1 and bam7 bam8 background). BAM7 and BAM8 were both present in nuclei throughout the root. In the above ground tissue BAM7 was seen in nuclei of guard cells and frequently as an additional bright spot inside the guard cell, but outside the nucleus. BAM8 was found in nuclei of both guard cells and epidermal cells (Fig. 4.2.2-B). In contrast to the uniform distribution of BAM8 within the nucleus, the signal from BAM7 showed speckling. To clarify if the bright dots seen in pB7:B7-GFP expressing plants were indeed outside the nucleus, crosses with plants expressing the nuclear marker proH1.1:H1.1-RFP (generated by Marek Whitehead and kindly provided by C. Baroux lab, University of Zürich) or pUBQ10:H2B-RFP pUBQ10:YFP (von Wangenheim, Fangerau et al. 2016) were generated. The F1 generation confirmed the bright spots in pB7:B7-GFP were located outside of the nucleus (Fig. 4.2.2-C). However, out of all screened T1 transformant-lines (>10), only these two lines (#5, #2) showed a detectable fluorescence and BAM7 expression in these lines is considerably higher than in the wild-type (native BAM7 not detectable) (see Fig. 4.2.2-C). The occurrence of this extra-nuclear signal was more frequent in the higher expressing pB7:B7-GFP line (#5.1 see Fig. 4.2.2-E) but could not be assigned to a known cellular structure. It is possible that the origin of this extra-nuclear signal is an artefact caused by the unnaturally high protein abundance.

To test if the functional integrity of GFP-tagged BAM7 and BAM8 was retained, the phenotypes of the double mutant plants expressing pB7:B7-GFP and pB8:B8-GFP were analysed. After 19 days of growth in long day conditions (16/8h light/dark) the double mutant showed elongated petioles compared to the wild-type, as described before (Reinhold, Soyk et al. 2011). We expected this phenotype to be reverted in the complemented lines. The plant line pB7:B7-GFP #8.8 looked wild-type-like whereas #3.1 and #11.1 only partially rescued the phenotype. In the lines pB8:B8-GFP #23.2 and #6.2 the long-petiole phenotype was also complemented, whereas for #10.1 it was less clear. The results for BAM8 reflect the abundance of the BAM8-GFP protein (Fig. 4.2.2-D,-E).
Results and Discussion
C) $pB7.B7\cdot GFP/bam7.1 \times H2B\cdot RFP + PM\cdot GFP$

- **GFP + Brightfield + RFP**
- **GFP**
- **RFP**

- **pB7.B7\cdot GFP/bam7.1 \times pH1.1\cdot RFP**

- **GFP + Brightfield + RFP**
- **GFP**
- **RFP**
### Results and Discussion

#### pB8:B8-GFP/bam8.1 x H2B-RFP + PM-GFP

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#### pB8:B8-GFP/bam8.1 x pH1.1-RFP

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Figure 4.2.2: Subcellular- and tissue-specific localisation of BZR1-BAM proteins. Fluorescent signal of the translational reporter construct (see 4.2.1A) was monitored by confocal laser scanning microscopy. (A) Epidermal cells of *N. benthamiana* were transiently transformed by *A. tumefaciens* carrying the construct (pB7:B7-GFP and pB8:B8-GFP) described in 4.2.1A. Localisation was monitored 3 days after infiltration. Right panel is a close up of the section indicated on the middle panel. Green: GFP fluorescence. Scale bar 20 µm. (B) The GFP-tagged BZR1-BAMs driven by the respective native promoter, were stably transformed into bam7.1, bam8.1 or bam7 bam8 Arabidopsis plants (pB7:B7:GFP/bam7.1, pB7:B7:GFP/bam7 bam8, pB8:B8:GFP/bam8.1, pB8:B8:GFP/bam7 bam8). Localisation was assessed in young true leaves (two left panels) and roots (right panel). Green: GFP fluorescence; Red: Chlorophyll (leaves) and cell walls stained by propidium iode (roots). Scale bars as indicated. (C) Co-localisation of BZR1-BAMs with nuclear markers was assessed in true leaves of young seedlings (growth conditions as in (B)). Heterozygous F1 generation seedlings of the crosses between two independent pB7:B7-GFP or pB8:B8-GFP lines with either proH1.1:H1.1-RFP or pUBQ10:H2B-RFP pUBQ10:YFP were imaged. Red: RFP fluorescence; Green: GFP fluorescence; Scale bar as indicated. (D) Phenotype of 19 days old seedlings grown in 16/8 hs light/dark conditions to test complementation of the bam7 bam8 double mutant phenotype by pB7:B7-GFP and pB8:B8-GFP. Three independent homozygous transformant lines were investigated. (E) Protein
4.2.3 Characteristics of the basic helix-loop-helix (bHLH)-like domain impact distribution of BAM7 and BAM8 within the nucleus

The N-terminal transcription factor domain of the BZR1-BAMs contains a basic helix-loop-helix (bHLH)-like domain. In classical bHLH proteins, the basic domain is responsible for its DNA binding property. The largest proportion of bHLH proteins in Arabidopsis (74%) have a preference for the E-box (CANNTG). The HLH domain enables dimerization (Ma, Rould et al. 1994) (for more details see section 1.2.7). The first amino acid (leucine 27, L27) identified as predominantly important for dimerization (Toledo-Ortiz, Huq et al. 2003), is conserved in BZR1-BAMs. Mutation in L27 to glutamate (E) was shown to prevent dimerization and thereby impaired functionality of PAR1 (Carretero-Paulet, Galstyan et al. 2010, Galstyan, Cifuentes-Esquível et al. 2011). In contrast, the corresponding mutation in the BZR1-BAMs (L97E in BAM7, L114E for BAM8) did not lead to a complete abolishment of BZR1-BAM heterodimerisation (L. Luginbühl, Master Thesis). In another study about HFR1 (long hypocotyl in far red light), two of the conserved hydrophobic amino acid residues within the helix domain, which were previously reported to be required for dimerization (Voronova and Baltimore 1990), were substituted (V172L173 to D172E173) and indeed led to the inability of HFR1 to heterodimerisation (Hornitschek, Lorrain et al. 2009). To date it is clear that BZR1-BAMs can homo- and heterodimerise but it is unclear, what role homo- or heterodimerisation plays in their function. Dimerization mutants would help to understand their mode of action. Another attempt to create a dimerization mutant according to the HFR1 example was therefore initiated. The corresponding residues in BZR1-BAMs were identified as V114 and I115 in BAM7 and V131 and I132 in BAM8. Homo- and heterodimerisation was assessed by Bimolecular Fluorescence Complementation (BiFC) experiments with BAM7 V114D I115E and BAM8 V131D I132E in tobacco epidermal cells. Homo-dimerisation localisation and behaviour of BAM7 V114D I115E and BAM8 V131D I132E was indistinguishable from that of the wild-type BAM7 and BAM8 proteins, respectively (Fig. 4.2.3). Heterodimerisation of BAM7 V114D I115E-YFPn with BAM8 V131D I132E-YFPC (and vice versa) led to a highly speckled signal in the nucleus. Speckles were numerous and small (>10 speckles/nucleus). Interestingly, heterodimerisation of wild-type BAM7 and BAM8 proteins also showed a speckled distribution within the nucleus, but it was usually limited to 1-4 speckles. According to immunoblotting most of the putative dimerization mutants were expressed slightly less than the wild-type proteins (not shown). From this we concluded that the introduced amino acid substitutions in BAM7 and BAM8 do not abolish dimerization and they do not impact the homodimerisation behaviour. However, BAM7 V114D I115E and BAM8 V131D I132E heterodimerisation may
differ slightly compared to the wild-type BAM7 and BAM8. The biological meaning or significance of this alteration cannot be concluded at this point but it seems unlikely that the different behaviour was

Figure 4.2.3: Amino acid exchange of two residues in the bHLH-like domain of BZR1-BAMs previously shown to be important for protein dimerisation does not prevent homo- and heterodimerisation of BZR1-BAMs nor changes their cellular localisation, but leads to speckle formation in the nucleus. BAM7 or BAM8 were transiently co-expressed with BAM7 V114D I115E or BAM8 V131 I132E in leaves of N. benthamiana by infiltration of A. tumefaciens. Each protein was C-terminally fused to either the C-terminal half of YFP (YFPc) or the N-terminal half of YFP (YFPn). A binary p35S:p19 construct was co-infiltrated to suppress gene silencing (Lindbo 2007). Green: BiFC fluorescence; Red: chlorophyll. Scale bar: 20 μm. Protein abundance was assessed by immunoblotting using the anti-Myc and the anti-Ha antibody for the YFPn and YFPc, respectively. Equal loading was tested by Penceau staining.
attributable to differences in expression levels. It is possible that the isoleucine identified in BZR1-BAMs does not correspond to the conserved leucine in the other bHLH proteins. It has to be kept in mind that the BZR1-BAMs are not classical bHLH proteins but only bHLH-like. Another possibility is that the enzyme (BAM) domain of these proteins play a role in dimerization. Introducing these amino acid substitutions into a truncated BZR1-BAM, which is reduced to its N-terminal (BZR1-) domain, might answer the question to which extent the BZR1-domain of the BZR1-BAMs behaves as a classical bHLH domain.

4.2.4 Discussion

The transcriptional fusion construct used to analyse tissue specific localisation of the BZR1-BAMs contains the gene specific promoter, the 5’UTR, the first exon and the intron and should (theoretically) reflect the endogenous expression pattern. BAM7 was predominantly seen in the vasculature of seedlings and the anthers. BAM8 was localised to the vasculature, the mesophyll and endodermis of young leaves and most floral tissues, overall showing a slightly stronger expression than BAM7. These findings are only partially in agreement with publicly available expressional profiles (eFP Browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), which indicate slightly enhanced expression in the shoot apex and young flowers if looking at very small scales of expressional differences (data not shown) but do not show any enrichment of BAM7 nor BAM8 in the vasculature. According to this database, both transcripts are about equally low abundant in different developmental stages and tissue types compared to the active β-amylases (BAM1, BAM3) (Fig. 4.2.4 lower panel). This is in agreement with the transcription levels analysed by quantitative PCR in different tissues (seedlings, rosettes, stem and inflorescence), which showed low variation in relative abundance in the different tissue types (Reinhold, PhD Thesis). Furthermore, BAM8 appears slightly more abundant than BAM7, particularly in leaves (eFP Browser, data not shown), which correlates with my experimental data (as mentioned above). A higher expression of BAM8 than BAM7 is supported by the structure of the 5’UTR where no intron is present in BAM8 compared to one for BAM7. Analysis of intron length and abundance in 5’UTRs showed that more highly expressed genes tend to have shorter or no intron (Barrett, Fletcher et al. 2012). To address the question if the observed higher BAM7 expression in vascular tissue is real or rather an artefact, the predicted expression levels of BZR1-BAMs were compared to a known vascular protein, SUCROSE-PROTON SYMPORTER 2 (SUC2). Whereas SUC2 is clearly higher abundant in vascular tissue (phloem) compared to surrounding tissues, there was no enrichment seen in the central root cylinder for neither BAM7 nor BAM8 (Fig. 4.2.4 upper panel). Hence, the apparent enrichment of BAM7 in the vasculature might be due to the fact that the very low abundant BAM7 transcript is invisible, except in tissue types with lower cell size (vasculature, hydathodes) and therefore higher transcript concentration.
This means that the publicly available expression data supports the overall low abundance of BZR1-BAM transcript, the higher abundance of BAM8 than BAM7 and the expression of both BZR1-BAMs in roughly all tissue types. Latter being in agreement with previous results performed by Reinhold (PhD Thesis) but not fully supported by my results. In contrast, the enrichment of BAM7 transcript in the vasculature was not confirmed and supported the speculation that it is due to an artefact caused by the low BAM7 transcript, which is only visible in more condensed cell-types. This interpretation would also match the results obtained from the translational fusion construct, where BAM7-GFP and BAM8-GFP were observed throughout the leaves with higher abundance in young leaves than cotyledons but without an obvious enrichment in the vasculature (not quantified). Expression in the vasculature and later migration to the mesophyll seems rather unlikely. A relative enrichment of BAM7 in guard cells compared to mesophyll cells as seen in this study was not observed by a study which quantified guard cell enriched transcripts (Bates, Rosenthal et al. 2012). My observation of BAM7 mainly appearing in guard cells might therefore again be attributed to a ‘concentration effect’, similar as described for BAM7’s apparent expression in the vasculature. However, further investigations would be needed to clarify this issue.

Further insights gained from the new translational reporter was the nuclear localisation of BAM7 and BAM8 in vegetative tissue (epidermis, mesophyll and trichomes), which appears to be independent of the presence or absence of the other BZR1-BAM. The same subcellular localisation was obtained for the expression of pb7:B7-GFP and pb8:B8-GFP in the single mutant or double mutant background.

The complementation experiment of bam7 bam8 double mutants with pb7:B7-GFP and pb8:B8-GFP to test the functionality of the construct was not fully conclusive, mainly due to the subtle phenotype of bam7 bam8 as such. Another way of testing the complementation capacity of the inserted construct (pb7:B7-GFP and pb8:B8-GFP) would be by looking at the recovered expression of BZR1-BAM target genes (such as BUP1 or BUP2), which are downregulated in the double mutant compared to the wild-type. The loss of a single BZR1-BAM does not cause a phenotype and a transcription profile significantly different from the wild-type whereas the loss of both (bam7 bam8) does.

Altogether, the data acquired suggests BAM7 and BAM8 to localise to the identical tissues and cellular structures but probably differing in their abundance. Partial redundancy and/or cooperation of the two proteins seems plausible. Furthermore, there are several lines of evidence supporting the idea that they interact and form heterodimers: BAM7 and BAM8 were identified to interact by a co-immunoprecipitation experiment from Arabidopsis cell culture followed by tandem mass spectrometry analysis. The recombinantly expressed proteins were also shown to interact in vitro by Microscale Thermophoresis (MST) (Soyk, PhD Thesis). The microscopic method BiFC confirmed the heteromeric interaction as well. To further disentangle their function, it would therefore be
very useful to have isoforms incapable of dimerising. Attempts to generate such a modified protein were not successful so far. Also, the amino acid substitutions used here (BAM7 V114D I115E and BAM8 V131D I132E) did not prevent BAM7 and BAM8 from interacting with itself or the other counterpart. It may be that they differ from the classical bHLH proteins too strongly in order to lead to the same effect as in previously characterised examples (PAR1, HFR1) (Hornitschek, Lorrain et al. 2009, Carretero-Paulet, Galstyan et al. 2010, Galstyan, Cifuentes-Esquivel et al. 2011). It has to be kept in mind that the BZR1-BAMs have, additionally to the comparably small bHLH-like containing BZR1-domain (20 kDa), a much larger BAM-domain (50 kDa). However, up to date it has not been investigated if the BAM-domain on its own also interacts. Maybe the bHLH-like domain in BZR1-BAMs renders them DNA binding but is not the only domain responsible for dimerization. The substitution of the conserved glutamic acid 13 (position number according to Toledo-Ortiz, Huq et al. 200) in the basic domain by an aspartic acid in BAM7-dna disabled the protein form binding to

Figure 4.2.4: Visualisation of expression levels according to the eFP Browser. Absolute expression levels of SUC2 (At1g22710), BAM7 (At2g45880), BAM8 (At5g45300), and BAM3 (At4g17090) are shown. Note, the colour code is the same for the same type of image (roots, upper panel; seedling and mature plant, lower panel).
DNA and created a transcriptional profile similar to the double mutant \((\text{bam7 bam8})\) (Soyk, Simkova et al. 2014). This led to a recruitment model (Soyk, PhD Thesis) that postulates BAM7’s function to be facilitating BAM8’s binding to DNA through direct protein-protein interaction and a dominant negative effect of BAM7-dna by sequestering BAM8 off the DNA.
4.3 Crosstalk with light-signalling

BZR1-BAMs act as transcription factors and have a considerable overlap with brassinosteroid-signalling pathways in respect to their genome-wide gene expression profile as well as their preferred DNA binding motifs (BBRE and BRRE, respectively). Apart from the brassinosteroid-responsive BRRE, the eight letter BBRE motif also contains a particular type of E-box, a G-box. The G-box is known to be targeted by different types of bHLH proteins among which there are many light-regulated proteins (e.g. MYC2, PIF4, PIF3, PIL1, PIL2) (Toledo-Ortiz, Huq et al. 2003, Sessa, Carabelli et al. 2005, Kazan and Manners 2013). The G-box in the BBRE-target motif together with the observed phenotype of adult plants, which have shorter petioles in BAM8-ox and longer petioles in bam7bam8 double mutants, led to the suggestion that the BZR1-BAMs might crosstalk with light-signalling, potentially acting as integrators of metabolic cues into the light-signalling network. To date there is no such component known, yet it seems beneficial for plants to integrate and coordinate its nutritional status (represented by metabolic cues) with the present or anticipated potentially available energy source (light). A comparison (performed in this study) between published light regulated transcriptional profiles with BZR1-BAM deregulated genes showed that there is indeed a substantial overlap between the two pathways at the transcriptional level. Publicly available expression data showed that the BZR1-BAMs themselves are not (less than two-fold) deregulated by light treatments but their putative direct targets BUP1 and BUP2 show changes in expression upon light treatment (Genevestigator). Mainly the transfer from low light (or darkness) to higher light intensity triggered an upregulation of BUP2 and a downregulation of BUP1. These findings indicate that the suspected integration of a metabolic cue into light-signalling more likely occurs downstream of the BZR1-BAMs, at the level of their target genes. However, the transfer from low to high light also introduces a change in metabolic status and the regulation of BZR1-BAM target genes may only indirectly be modulated by light. To further assess the potential role of BZR1-BAMs in the light-signalling framework, I investigated the response of BZR1-BAM mutant and overexpressing plant to different light regimes at the physiological as well as at the transcriptional level. Furthermore, an interaction study with the bHLH transcription factor MYC2 was carried out and a Chromatin Immunoprecipitation (ChIP) experiment confirmed the binding of BAM8 to the promoter of BUP1 and BUP2.

4.3.1 Overexpression of BAM8 prevents normal hypocotyl elongation in response to true shade and neighbour detection

Plants closely monitor their light environment in terms of light quantity and quality. Neighbouring or overgrowing plants trigger a so-called shade avoidance syndrome (SAS), which leads to a variety of physiological adaptations, such as induced elongation growth, hyponastic leaves, reduced lamina size and early flowering (de Wit, Galvao et al. 2016). Due to the phenotypes of BAM8-ox and bam7bam8
(reduced and increased petiole length in adult plants, respectively), we aimed to investigate the impact of distorted BZR1-BAMs abundance under conditions of shade. Induced hypocotyl elongation at the seedling stage upon shading is one of the best characterized responses in terms of SAS and was used as a readout for our purpose. Shade can be perceived by plants as ‘true shade’, or ‘neighbour detection’. ‘True shade’ describes a situation where the plant is below the canopy of other plants and therefore involves a reduction in blue and red light intensity, the latter simultaneously leading to a reduction of the R/FR ratio. ‘Neighbour detection’ is indicative for an enrichment of FR light due to
reflection by neighbouring plants, thereby reducing the red to far red ratio (low R/FR) without altering the overall light intensity (for further details also see section 1.4.3.1). The ‘true shade’ as well as the ‘neighbour detection’ response were compared in the single mutants (bam7.1, bam8.1) the double mutant (bam7 bam8), the overexpressors (BAM7-ox, BAM8-ox), and the wild-type. The pif4 pif5 double mutant has a well described impaired hypocotyl elongation response to shade (Lorrain, Allen et al. 2008) and was used as a control. In three independent neighbour detection experiments, we consistently observed that BAM8-ox and pif4 pif5 had a significantly reduced elongation response upon a low R/FR treatment as well as in high R/FR light control treatment (Fig. 4.3.1-A, right panel). In two out of the three experiments bam8.1 also showed a reduced elongation response in low R/FR, but this was attributed to delayed germination. Two independent true shade experiments essentially led to the same result as the neighbour detection experiment (Fig. 4.3.1-A, right panel). These results indicated

Figure 4.3.1: Reduced shade avoidance response in BAM8-ox cannot be compensated by adding a carbon signal or source. (A) Plants lacking or overexpressing BZR1-BAMs were first grown in white light (high R/FR) conditions for 4 d and subsequently exposed for 3 d to a neighbour detection (high PAR, low R/FR) or true shade (low PAR, low R/FR) treatment (see right and left panel, respectively). In the control treatments, light intensity was in each case the same as in the shade mimicking treatment but at high R/FR. Data represent means ± SE. Different letters indicate significant difference (p<0.01). (B) Plants lacking or overexpressing BZR1-BAMs were exposed for 5 d to different fluence rates of pure blue, red and far red light. As controls the photoreceptor mutants cryptochrome 1 (cry1), phytochrome B (phyB) and phytochrome A (phyA) were included. Hypocotyl length of plant grown for 5d in darkness (lower right panel). (C) Same experiment as described in (A), except that the control treatment was always in ‘high PAR, high R/FR’ and the initial growth in white light was for 4.5 d, followed by 5 d of low R/FR or control treatment. Furthermore, plants were grown on 1/2 MS media or 1/4 MS media supplemented by 2 % Glc or Suc. Low PAR indicates light intensities of 17 μmol m⁻² s⁻¹ (upper left and right panel) and high PAR light intensities of 195 μmol m⁻² s⁻¹ (lower panel). Data represent means ± SE.
that BAM8-ox has a defect in elongation growth comparable to the one previously described for pif4 pif5 (Lorrain, Allen et al. 2008) in low and high R/FR and irrespective of the overall light intensity (neighbour detection and true shade). Further investigations are needed to clarify if this can actually be called a shade-specific response for BAM8. Since reduced hypocotyl elongation is only observed in BAM8-ox and no opposite response is seen in the bam7 bam8 double mutant, it may also be a result of ectopic expression.

To investigate if this reduced elongation of BAM8-ox was an inability to respond to particular light qualities, fluence rate experiments in pure red (R), blue (B) or far red (FR) were performed. As non-responsive controls, mutants in the main photoreceptor in the respective light spectra were used (cry1 for B, phyB for R, phyA for FR, for details see section 1.4.1). Testing the response to pure blue light was particularly interesting since a shade response can also be triggered by low blue light intensities (Keuskamp, Pollmann et al. 2010, Keller, Jaillais et al. 2011, Pedmale, Huang et al. 2016). Upon 5 d growth in the corresponding light conditions, no substantial genotype dependent elongation response was seen in any of the treatments (B, R, FR) (Fig. 4.3.1-B). In R and B both BAM7-ox and BAM8-ox had the tendency of being shorter but the effect was small. The corresponding dark control indicated that BAM8-ox does not have a fundamental elongation defect; after 5 d of growth in the dark it showed hypocotyl lengths indistinguishable from all the other genotypes (Fig. 4.3.1-B, lower right panel).

With the proposed function of BZR1-BAMs as metabolic sensors in mind, shade avoidance experiments in the presence of sugars (Glc or Suc) were performed. Plants were grown for 4.5 d in high R/FR and another 5 d in low R/FR or the control treatment (see methods). If a compensatory effect in BAM8-ox relative to the wild-type in presence of sugars occurred this would indicate a negative impact of sugars on BAM8 activity, thereby releasing the repressive action of BAM8 on hypocotyl elongation. In case that the sugar reinforced BAM8 activity, one would expect an unchanged or further reduced hypocotyl elongation response in BAM8-ox compared to the wild-type. Results showed that Glc as well as Suc induced elongation growth specifically in low R/FR at low light intensities (low photosynthetically active radiation, PAR) (Fig. 4.3.1-C, upper panels). In low R/FR and high R/FR at high PAR, no major promotion in hypocotyl growth by sugars was seen in any of the genotypes (Fig. 4.3.1-C, lower panel). Probably this indicates, that the induction of hypocotyl growth by Glc and Suc under ‘low PAR, low R/FR’ was mainly caused by addition of a carbon source, rather than a sugar signal. Alternatively, the putative sugar signal might have already been saturated at high PAR and only became apparent at low PAR. Furthermore, the pattern of BAM8-ox responding less to low R/FR compared to the other genotypes was consistent in the presence or absence of 2 % Glc or Suc (Fig. 4.3.1-B). Since these results did not look promising to help us confirm the existence or the identity of the putative sugar ligand, I performed the experiment only once for each case. It therefore indicates a trend but is of limited reliability.
4.3.2 Gene expression changes upon neighbour detection treatment

Another approach to assess if the BZR1-BAMs interfere transcriptionally with the light-signalling pathway is to compare transcriptional profiles of BZR1-BAMs and a known light regulated transcription factor, PHOTOTYPHOE INTERACTING FACTOR 4 (PIF4) (Casal 2012). To eliminate secondary effects which appear in transcription data, I focused on gene sets of direct target genes, if available. For this purpose a set of 1537 PIF regulated PIF4 target genes (Oh, Zhu et al. 2012) were compared to a group of 394 putative direct target genes of BAM8. The latter was defined as genes deregulated by at least two-fold (up-regulated in BAM8-ox vs. wild-type and down-regulated in bam7bam8 vs. wild-type) in a data set of a previous study (Reinhold, Soyk et al. 2011). A considerable number (55 genes; 14.0 %) were commonly regulated (4.6 % to be expected by chance). Amongst those, there were some well known proteins such as ATHB4 (ARABIDOPSIS THALIANA HOMEBOX-LEUCINE ZIPPER PROTEIN 4), ATHB2, PAR1 (PHY RAPIDLY REGULATED 1), LSH6 (LIGHT SENSITIVE HYPOCOTYL 6) and LHS10 (Fig. 4.3.2-A). Further analysis showed that out of the 55 BZR1-BAM/PIF4 co-regulated genes, approximately half (25) were also BR regulated BZR1 direct targets (Sun, Fan et al. 2010). All genes of the above-mentioned group (ATHB4, ATHB2, PAR1, LSH6, LHS10) were among those. This indicates the strong link between the BZR1-BAM and BR regulated transcriptional outputs.

To complement this literature-based data and to investigate at what time point of the shade response the BZR1-BAMs play a role, expression of a number of genes were quantified by reverse transcription followed by quantitative PCR (qPCR) at an early time point of SAS. Gene to be analysed were chose according to their known involvement in the shade avoidance response. These were namely: PAR1, HFR1, ATHB4, ATHB2, IAA19 and PRE1. Furthermore, the putative direct target of BAM8, BUP1 (At5g22580) and a brassinosteroid synthesis gene CPD (Szekeres, Nemeth et al. 1996) were included. HFR1, PRE1, IAA19 and CPD are most likely not direct targets of BAM8 since they are all downregulated in BAM8-ox, whereas ATHB4, ATHB2 and PAR1 might be direct targets (Reinhold, Soyk et al. 2011). Including putative direct and indirect targets of BAM8 should help to distinguish between the level of deregulation. Plants were grown as described for the shade avoidance response in neighbour detection. Instead of 3 d of shade treatment, seedlings were harvested 2 h after treatment-start in pools of 30
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seedlings (4 replicates each, except for bam7 bam8, where 3 replicates were taken). The analysed gene expression changes were therefore the short-term response to shade. All the known shade-responsive genes (PAR1, HFR1, ATHB2, IAA19 and PRE1) were upregulated after 2 h of shade, as expected, but not deregulated in a BAM8 dependent manner (same response in all genotypes). The only exception, ATHB4, was only marginally upregulated by shade but massively induced by BAM8-ox and repressed in bam7bam8, as previously reported (Reinhold, Soyk et al. 2011). The low responsiveness of ATHB4 to shade was surprising because previous studies have shown a fast (1 h) response to shade (Carabelli, Sessa et al. 1993, Sorin, Salla-Martret et al. 2009). Possibly the expression already declines in our conditions (2 h of exposure to shade). BUP1 was deregulated in white light as expected and did not respond to shade. The brassinosteroid synthesis gene CPD was not substantially impacted by shade and marginally (less than two-fold) downregulated in BAM8-ox, bam8.1 and bam7 bam8 (Fig. 4.3.2-B). From these results, it seems that the BZR1-BAMs do not significantly impact the short term transcriptional response to shade.

To test if the long-term shade response, which showed a physiological change in BAM8-ox was also reflected at the transcriptional level, plants (BAM8-ox, bam7 bam8, wild-type) were grown on soil for 12 d in high R/FR light (115 µmol photons m⁻² s⁻¹) and then either kept in high R/FR or transferred to low R/FR (16 µmol photons m⁻² s⁻¹) for another 4 d. Plants were harvested in pools on the fourth day at midday (6 h into the light). Expression levels of ATHB2, ATHB4 and BUP1 were assessed. ATHB4 was significantly up-regulated by BAM8 but did not show a big difference between shade or non-shade treatment. In contrast, ATHB2 was greatly upregulated in all genotypes in response to shade but to a much lower extent in BAM8-ox. These findings correlate with a previous study where ATHB2 was shown to promote cell expansion in hypocotyls (Steindler, Matteucci et al. 1999). BUP1 behaved as expected but was not responsive to shade as observed earlier.
Figure 4.3.2: Light and brassinosteroid regulated, BZR1-BAM responsive genes. (A) The overlap between PIF regulated PIF4 targets (Oh, Zhu et al. 2012) and BZR1-BAM regulated putative BZR1-BAM direct targets (downregulated in bam7 bam8 or upregulated in BAM8-ox by at least two-fold) (Reinhold, Soyk et al. 2011) are 55 genes. Of these light/BZR1-BAM commonly regulated genes, 25 are also found within the BR regulated BZR1-direct targets (Sun, Fan et al. 2010). (B) Transcriptional response upon 2 h of neighbour detection treatment (low R/FR) in comparison to the white light control treatment (high R/FR). Expression levels of six known shade-responsive genes (ATHB2, ATHB4, PAR1, HFR1, PRE1 and IAA19) were quantified. BUP1 is a putative direct target of BZR1-BAMS and CPD is involved in BR biosynthesis. The first four above mentioned genes were among the 25 BR/light/BZR1-BAMS targeted genes (see A). Expression was normalised against the house keeping gene PP2A and expressed relative to the white light grown wild-type. Relative expression is plotted in logarithmic scale (log2). Each bar represents the average relative expression of 4 pools of 30 seedlings each (except bam7 bam8; 3 pools) ± SE of the replicates. (C) Transcriptional response of ATHB4, ATHB2 and BUP1 after extended shade (low R/FR) or white light (high R/FR) treatment (4 d). RNA was extracted from a pool of seedlings grown on soil. Data represents average ± SD of two technical replicates.
4.3.3 Localisation of BAM8 upon shade treatment

A change in the activity as a transcription factor upon shading might be related to a redistribution within the cell, e.g. through interaction with PHYB as shown for the PIFs (Duek and Fankhauser 2005). To test this, seedlings stably expressing GFP-tagged BAM8 (pB8:B8-GFP) driven by its native promoter were grown in white light (PAR=137 µmol photons m⁻² s⁻¹, R/FR =7.2 for light range definition see section 3.1.5) for 14 d at 12/12 h light/dark cycle. On the 15th day at 3 h into the light, seedlings were exposed to 4.5 h of low R/FR (PAR=127 µmol photons m⁻² s⁻¹ R/FR=0.6) or high R/FR (control) light and immediately mounted onto the confocal microscope for imaging. Localisation of BAM8-GFP was found predominantly in the nucleus, irrespective of the light treatment (Fig. 4.3.3). These findings indicate, that the altered physiological and transcriptional response to shade in BAM8-ox most likely is not dependent on cellular re-location of BAM8 within this 4.5 h time period.

4.3.4 Interaction with light regulated protein MYC2

From the list of previously-identified putative protein interactors of BZR1-BAMs by protein IP (Soyk, unpublished) one candidate, MYC2, was seen as a BAM8 (but not BAM7) interactor. The bHLH protein MYC2 has been characterised as a major player in jasmonate-signalling with further roles in the interaction with other phytohormone- and light- (phytochromes) signalling, including shade avoidance, and the circadian clock (Kazan and Manners 2013). Furthermore, MYC2 was shown to be localised to the nucleus (Lorenzo, Chico et al. 2004, Chini, Fonseca et al. 2009) and bind to the G-box and G-box variants (Dombrecht, Xue et al. 2007). MYC2 has many confirmed bHLH interaction partners and a wide
number of predicted interactors (e.g. PIF4, BES1-INTERACTING MYC-LIKE 1 (BIM1) a regulator of BR-signalling) (Chandler, Cole et al. 2009, Chen, Wen et al. 2012, Kazan and Manners 2013). In terms of phenotypic shade avoidance response, the myc2 mutant was shown to behave opposite to BAM8-ox; upon low R/FR treatment myc2 showed an increased hypocotyl elongation (Robson, Okamoto et al. 2010). Altogether, this led me to speculate that MYC2 and BAM8 might have an overlapping range of action. This made MYC2 an interesting candidate to test for direct protein-protein interaction. For this, BiFC experiments of MYC2 with BAM7 and BAM8 were carried out. In a series of three experiments, there was consistent interaction observed between MYC2-YFPn and BAM8-YFPc but not BAM8-YFPn and MYC2-YFPc. No or very weak interaction was seen for BAM7 with MYC2. The positive control of MYC2 homodimerisation was positive (Fig. 4.3.4). The reasons for this construct-dependent interaction of BAM8 and MYC2 were unclear. It could be due to unequal protein expression levels or the conformational nature of the interaction, which enabled the N- and C-terminal part of YFP to interact in one case (MYC2-YFPn and BAM8-YFPc) but not the other (BAM8-YFPn and MYC2-YFPc). The original data set which suggested a MYC2-BAM8 interaction (IP-data) showed a consistent interaction over four

![BiFC images](image)

**Figure 4.3.4: Protein-protein interaction of BZR1-BAMs with MYC2.** BAM7 or BAM8 together with MYC2 were transiently co-expressed in leaves of *N. benthamiana* by infiltration of *A. tumefaciens*. Each protein was C-terminally fused to either the C-terminal half of YFP (YFPc) + Ha-tag or the N-terminal half of YFP (YFPn) + Myc-tag. A binary p35S:p19 construct was co-infiltrated to suppress gene silencing (Lindbo 2007). Protein expression was visualised by immunoblotting (BAM8-YFPn/c:104 kDa, BAM7-YFPn/c: 104 kDa, MYC2-YFPn/c: 94 kDa). Green: BiFC fluorescence; scale bar: 20 μm.
experiments, but the peptide count was rather low in each case. This may indicate that the interaction is weak or temporarily limited, which could explain why we could not convincingly confirm the interaction of BAM8 and MYC2 by BiFC.

4.3.5 BAM8 preferably binds the BBRE containing chromatin region of the BUP1 and BUP2 promoter

Direct interaction of BAM7 and BAM8 with DNA has been shown in different in vitro experiments (DPI-ELISA, Reinhold et al. 2011; SPR, this study) and their action as transcription factors in vivo was confirmed by transactivation assays as well as micro-array experiments (Reinhold, Soyk et al. 2011, Soyk, Simkova et al. 2014). However, the direct binding of BZR1-BAMs to chromatin in planta has not been shown yet. A Chromatin Immunoprecipitation (ChiP) experiment was performed to confirm the BAM8-DNA interaction. Arabidopsis plants (bam8.1) were stably transformed with a HA-tagged BAM8, which was driven under its endogenous promoter (pB8:B8-HA/bam8.1). Three independent

**Figure 4.3.5:** Chromatin Immunoprecipitation with BAM8-HA. (A) Of three independent Arabidopsis lines, which are stably expressing HA-tagged BAM8 driven by its endogenous promoter (pB8:B8-HA), protein abundance (BAM8-HA) was visualised by immunoblotting using the anti-BAM8 antibody and compared to wild-type and bam8.1 plants. For ChiP, line #2.8 was chosen. (B) Shearing efficiency was assessed by separating sheared and non-sheared chromatin according to their size on a 1.5 % agarose gel. DNA was visualised by ethidium bromide staining. Clearly, un-sheared chromatin is of large size (>20'000 bp), whereas the sheared chromatin peaks around 400 base pairs (bp). Optimal fragment size ranges between 200 and 600 bp. (C) After Chromatin Immunoprecipitation, the abundance of two different regions of the BUP1 and BUP2 promoter was quantified by qPCR. One amplified region spanned the BZR1-BAM Responsive Element (BBRE spanning), whereas the other region was roughly 750 bp upstream of the predicted BAM8 binding site (upstream BBRE). The promoter region abundance was expressed as relative enrichment in the pB8:B8-HA expressing line #2.8 compared to the wild-type.
transformed lines were chosen and plants homozygous for the pB8:B8-HA insert selected. Immunoblotting confirmed the presence of BAM8 (Fig. 4.3.5-A). All lines had higher BAM8-HA abundance than native BAM8 abundance in the wild-type. To avoid artefacts created by overexpression, I chose the line with the BAM8 levels closest to that of the wild-type (pB8:B8-HA/bam8.1 #2.8) for the subsequent ChIP experiments. Shearing efficiency was confirmed (Fig. 4.3.5-B) and the immunoprecipitation (IP) was carried out using a Ha-specific antibody. Abundance of the BBRE spanning promoter sequence, as well as a promoter region 790 bp (BUP1) and 743 bp (BUP2) upstream of the BBRE were quantified and expressed as relative enrichment to the abundance of the respective fragment in the wild-type IP. In both cases the BBRE spanning fragment was enriched (approximately three- to four-fold), whereas the upstream region was enriched to a lower extent (1.5-2-fold) (Fig. 4.3.5-C). These results confirmed the direct binding of BAM8 to the promoter of BUP1 and BUP2 and support the binding preference for the BBRE motif. Our intention was to scale this experiment up and sequence the immunoprecipitated chromatin fragments to get an idea of the genome wide BAM8 binding sites. Unfortunately, due to technical problems I never succeeded in precipitating a chromatin quantity required for sequencing.

4.3.6 Discussion

Investigations of neighbour detection and true shade experiments at the seedling stage showed that BAM8-ox has an impaired response in both growth conditions (low R/FR) and the control treatment (high R/FR) which is comparable to the pif4 pif5 mutant. This reduced responsiveness occurred irrespective of the overall light intensity (PAR). We can exclude that BAM8-ox suffers from a general elongation growth defect, since they elongate normally in etiolated growth (in darkness) and do not respond markedly different from wild-type to intensities of pure red, blue and far red light regimes. The latter results would need further confirmation because the experiment was performed once only. Overall, there is resemblance to the pif4 pif5 double mutant phenotype, which are indistinguishable from wild-type when grown in the dark but have reduced elongation growth in low and high R/FR light (Lorrain, Allen et al. 2008, Lorrain, Trevisan et al. 2009). The SAS at the adult stage (e.g. petiole length) was not tested, but the phenotype of BAM8-ox (stunted) and the bam7bam8 double mutant (elongated petioles) (Reinhold, Soyk et al. 2011) seem to be in agreement with the phenotype observed at the seedling stage. Measuring transcript levels of two shade-responsive PIF4-target genes (ATHB2 and ATHB4) after 5 d of low R/FR treatment in seedlings further supported our hypothesis of the BZR1-BAMs being involved in an interplay with light-signalling pathways. ATHB4 was greatly upregulated in BAM8-ox, which correlates with short hypocotyls upon shading in ATHB4 overexpressing plants (Sorin, Salla-Martret et al. 2009), whereas ATHB2 was down-regulated compared to the wild-type in response to low R/FR. Previous reports showed that overexpression of ATHB2 led to long hypocotyls in response
to shade (Steindler, Matteucci et al. 1999, Casal 2012). Therefore, a relative reduction in \( \text{ATHB2} \) expression indirectly infers a reduced hypocotyl elongation. Since BZR1-BAMs are known to be closely linked to BR-signalling and tend to act antagonistically to BR signals (Soyk, unpublished), the observed transcriptional changes may be BR dependent or independent. Indeed, BR is known to play a role in low R/FR response (described in detail later) and \( \text{ATHB2} \) and \( \text{ATHB4} \) are BR regulated direct BZR1 targets (Sun, Fan et al. 2010). Furthermore, \( \text{ATHB4} \) expression is oppositely regulated in BR mutants that have reduced BR perception (\( \text{bri-1} \), BR receptor) as compared to those that have a hyper stimulation of BR-signalling (\( \text{bzz1-D} \), positive dominant BZR1); \( \text{ATHB4} \) is induced in \( \text{bri-1} \) and repressed in \( \text{bzz1-D} \) (Sun, Fan et al. 2010). On the other hand, upregulation of \( \text{ATHB4} \) confers the plant more BR sensitive, which implies that \( \text{ATHB4} \) is not solely a downstream target of BR but also has some kind of a feed-back effect onto BR-signalling, which therefore complicates the network further (Sorin, Salla-Martret et al. 2009).

\( \text{ATHB2} \) expression was also shown to be downregulated by brassinolide (Vert, Nemhauser et al. 2005). It therefore seems plausible that counteraction or interference of the BZR1-BAMs with the BR-signalling pathway happens downstream of the actual cross section with light signals and is ultimately responsible for the defect in the shade avoidance response of \( \text{BAM8-ox} \). The missing influence of BZR1-BAMs on the very short term response to shade at the transcriptional level suggests an involvement not at the immediate output upon light perception, but rather at a later, growth modulating response, which seems reasonable if claiming that BZR1-BAMs role is mainly the contribution of a metabolic status signal.

The hypothesis that BZR1-BAMs integrate a metabolic signal into the light-signalling pathway could not be fully substantiated in this study. Supplementing the growth medium with either 2 % Suc or Glc showed that sugars promote the response to shade under resource limiting conditions (low PAR) but not under saturating conditions (high PAR). However, supplementing sugars did not revert the \( \text{BAM8-ox} \) phenotype in shade (neither at low nor high PAR), which means that either the BAM8 inhibitory effect on elongation growth is reinforced by sugars (and not released) or the method employed (hypocotyl elongation on sugar plates) was not appropriate to reveal BZR1-BAMs function in this context. What needs to be considered is that using the overexpressing plants creates an artificial situation and it is difficult to predict how the system would respond to the supplied ligand; overexpression of the sensor could make it more or less sensitive. Possibly another light regime instead of the 12/12 h night/dark cycle (e.g. long day, short day) or a nutritionally more limiting situation (lower sugar concentration in the media) would lead to different results. Considering previous results, which determined Tre6P as the most promising putative ligand of BAM8 and the fact that transcriptional changes upon increased Tre6P (in \text{otsA-ox}) had a higher overlap with BZR1-BAM regulated genes in the night than the day, short day conditions seem the most promising. Another explanation may be that
BAM8 plays two different roles; sugar dependent actions on the one hand and ligand independent actions on the other hand. The crosstalk with light signals might be ligand independent and rather based on the BR moderating (antagonising) function. Further experiments which include the ligand binding mutant (BAM8-lig) would possibly clarify this issue. Treatments of BAM8-ox plants with brassinolide to test if hypocotyl elongation upon sugar supply in shade is restored, could clarify if BAM8 integrates into the TOR-BR-dependent mechanism recently proposed (Zhang, Zhu et al. 2016).

To assess the mechanism through which the BZR1-BAMs might integrate into the light-signalling pathways, we had a closer look at available genome-wide transcriptional profiles and also tested the interaction with MYC2, a putative interaction partner of BAM8. For the former, a considerable overlap between PIF4-based light-signalling and BZR1-BAMs-signalling was observed. Roughly half of the light/BZR1-BAM overlapping genes were also identified as target of BR-signalling. This may not be surprising because PIF4 and BZR1 were shown to physically interact in vitro and in vivo and have a large (50 %) overlap in common target genes (Oh, Zhu et al. 2012). Keeping in mind the preferred DNA binding motif of BZR1-BAMs (BBRE), which comprises the BZR1 (BRRE) and the PIF4 (G-box) target motif, the overlap of the three transcription factors may be seen as a logic consequence. Besides the overlap in (putative) direct target genes, the BZR1-BAMs might exert an impact onto light-signalling through secondary effects such as modulating auxin related genes. Auxin has been characterised as one of the key hormones in the elongation response in hypocotyl and petiole upon shading (de Wit, Galvao et al. 2016). BZR1-BAMs deregulate several INDOLE-3-ACETIC ACID INDUCIBLE (IAA) genes (IAA19, IAA17, IAA5, IAA 14, IAA2, IAA1, IAA3, IAA29, IAA6) and AUXIN RESPONSE FACTOR (ARF) genes (ARF2, ARF5, ARF11) in 12/12 s light/dark grown seedlings (Reinhold, Soyk et al. 2011), IAA5s being the repressors of latter (ARFs). ARFs regulate auxin responsive genes (Li, Xie et al. 2016). The deregulation of these auxin related genes is mostly through repression in BAM8-ox, which makes it to be more likely an indirect regulation (considering that BAM8 is so far assumed to be a transcriptional activator). Overall it seems that auxin biosynthesis is not impacted by BZR1-BAMs (no deregulation of YUCCA genes) but the transcriptional read out is influenced. In respect to shade avoidance, BR and auxin responses are known to significantly interrelate, too (Kozuka, Kobayashi et al. 2010). It is therefore not surprising that some of the above mentioned BZR1-BAM/Auxin regulated genes are also targeted by BZR1 (IAA19 and ARF2) or BZR1 and PIF4 (IAA29).

In conclusion, it has to be admitted that through the experiments performed in this study it was not possible to show convincingly that the observed defect in elongation response of BAM8-ox is truly shade specific. Even though the phenotype resembles the widely-accepted shade specific pif4 pif5 double mutant, one has to keep in mind that we deal with ectopically, overexpressed BAM8, which might cause artefacts. Nevertheless, if assuming it is a shade specific response, I would claim that the
impact of BZR1-BAMs on the SAS is mainly through common target genes with light regulated transcription factors (such as PIF4) of which a large proportion is also BR targeted (through BZR1 and BES1) (Fig. 4.3.6). Integration of a metabolic signal can also be envisioned at this level. The modulation of BR biosynthesis or sensitivity by BZR1-BAMs I assume to be of minor importance in SAS since the BR synthesis gene CPD is not deregulated upon shading. Impacting auxin-signalling may occur at a secondary level of regulation and play role in fine-tuning the shade adaptation response. However, this is still very speculative and knowing the direct targets of BZR1-BAMs would greatly help to disentangle the phytohormone-light-BZR1-BAMs signalling network. The observed weak and partially inconsistent interaction of BAM7 and BAM8 with MYC2 was not convincing and does not allow to draw conclusions regarding the mechanism of BZR1-BAMs integration into light-signalling. One speculation is that light regulated transcription factors and the BZR1-BAMs are targeted to the same chromatin regions and coordinately bind the BBRE (containing the G-box) to regulate gene expression. The BiFC signal might rather be a result of the very close proximity and not an actual protein-protein interaction as such. The

Figure 4.3.6: Model of hypothesised BZR1-BAMs’ integration into light and hormone signalling pathways to modulate the Shade Avoidance Syndrome (SAS). Shade conditions are mainly perceived by phytochromes and cryptochromes (for details see section 1.4.3). Here we focus on shade perceived as low R/FR by phytochromes, of which the transcription factors PHYTOCHROME INTERACTING FACTORS (PIF) (particularly PIF4 and PIF5) are the major downstream players translating the light input into a transcriptional response. Downstream of the PIFs multiple signalling steps may ultimately trigger elongation growth. The two hormones auxin and brassinosteroid (BR) were identified as positive regulators of the shade avoidance syndrome (SAS). BR biosynthesis and sensitivity were shown to be enhanced upon shade (details see section 1.4.4.1). Auxin abundance and sensitivity are increased, whereas its spacial distribution is altered upon shading (details see section 1.4.4.2). One point of convergence of light, brassinosteroid and auxin signals is through direct interaction and common target genes of their downstream transcription factors (PIF4 for light, BZR1 for BR, ARF6 for auxin). For simplicity reasons the interdependency of brassinosteroid, auxin and gibberellic acid is not represented here (details see section 1.4.4.4). From our experimental data, BZR1-BAMs (particularly BAM8 and possibly BAM7) integrate most likely into the shade response through competing with BR signals. It remains unclear if sugars impact BAM8’s activity in respect to SAS. However, according to findings in this study sugars, if anything, enhance BAM8 activity, which indirectly represses elongation growth. Besides acting as a signal (possibly through BZR1-BAMs), sugars also have a growth promoting effect under light limited conditions, probably by providing the fuel in form of a carbon source. The model does not claim to be complete but depicts only the major players relevant for this aspect of shade avoidance. Segmented and full lines represent speculative and established links, respectively.
loss of MYC2 (in myc2), which leads to the opposite phenotype of the BAM8-ox, suggests a synergistic interaction rather than an antagonistic. The fact that bam8.1 does not show the myc2 phenotype in shade implies that MYC2 action is most likely not strictly BAM8 dependent.
5 Conclusion

5.1 Spatio-temporal occurrence of BZR1-BAMs’ in planta

Localisation of a given protein gives essential hints about its physiological function. The BZR1-BAMs transcriptional and translational fusion constructs (-GFP and -GUS constructs, respectively) roughly confirmed publicly available expression profiles (Genevestigator, eFPBrowser). BAM7 and BAM8 were seen to be low abundant throughout the plant (in seedlings) and predominantly expressed in floral and growing tissues (young leaves, shoot apex). Nevertheless, I encountered some inconsistencies between the observed location of expression and protein occurrence; the stronger expression of BAM7 and BAM8 in hydathodes (especially in the low expressing transgenic lines) and the predominant expression of BAM7 in the vasculature, was both not confirmed by increased protein abundance in those tissues (translational fusion construct). In contrast, increased BAM7 abundance in guard cells was not reflected by the expression fusion construct. These differences can most likely be attributed to misleading results caused by the visual nature of detection of these constructs; hydathodes, vasculature and guard cells are all more condensed tissues, which have smaller cell sizes compared to their surrounding tissues (e.g. mesophyll) and might therefore allow visualisation of signals otherwise indistinguishable from the background noise. Such an abundance-limiting effect was also reported for the Arabidopsis TPS1, for which the guard cells were the only tissue where it was detectable by an untargeted proteomic analysis (Zhao, Zhang et al. 2008). Another possible explanation for the observed inconsistencies are artefactual results generated by one or the other construct, due to its close to, but not identical to endogenous sequence.

Interestingly, metabolic sensing seems to play a role in the tissues where the BZR1-BAMs are expressed. The two subunits of the carbon starvation sensor SnRK1 (SnRK1α1 and SnRK1βγ) are reported to be most abundant in meristematic, elongating and differentiating zones of primary and lateral roots as well as in young leaf primorida (Bitrian, Roodbarkelari et al. 2011). Interestingly, the abundance of the SnRK1 and putatively BZR1-BAM regulating metabolite, Tre6P, is under spatiotemporal control due to cell- and tissue-specific expression of its metabolisable enzymes (TPPs) (Vandesteene, Lopez-Galvis et al. 2012). However, a correlation between Tre6P responsive SnRK1- and BZR1-BAM- modulated responses have not yet been investigated.

Besides tissue-specific localisation, subcellular localisation also greatly determines a protein’s functionality. Stable expression of fluorescently tagged BAM7 and BAM8 in Arabidopsis confirmed previous results (Reinhold, Soyk et al. 2011) regarding their localisation to the nucleus. BAM8 was evenly distributed, whereas BAM7 showed specking. Interestingly, a similar distribution pattern as BAM7 was also seen for the chimeric proteins composed of the BAM7 BAM-domain and the BAM8 N-
terminal part, which renders the protein to localise to discrete foci (Soyk, Simkova et al. 2014). The reciprocal construct showed uniform nuclear localisation (Soyk, Simkova et al. 2014). Hence, the (sensory) BAM-domain appears to influence BZR1-BAM’s distribution within the nucleus. Furthermore, the BAM-domain might also impact homo-/heterodimerisation. In contrast to bHLH proteins, it is not sufficient to mutate conserved residues of the bHLH-like BZR1-BAMs within the basic or HLH-like domain to prevent dimerization. This suggests that the BAM-domain impacts the protein’s localisation, dimerization and essentially its biological function, further supporting the idea that interaction of the BAM-domain with a metabolic signal (sugar) might have a wide impact on the protein’s functionality.

5.2 BZR1-BAMs interact with sugar-phosphates

An important role for the BAM-domain was also suggested in the sensing an as-yet undetermined putative ligand. This study generated the first direct evidence for a protein-carbohydrate interaction. BAM8 was determined to interact with Tre6P and Glc6P by MST, with a dissociation constant (K_D) in the low millimolar range. Further analysis showed that Tre6P consistently and effectively reduces BAM8’s association and binding stability to the ds-BBRE in a concentration-dependent manner. Related sugar phosphates, Glc6P and Suc6P, had a smaller impact on BAM8 and only reduced either association or binding stability, respectively. Binding of the sugar-phosphates to BAM8 may induce conformational changes in order to affecting DNA binding. Interestingly, upon binding to Glc6P, the migration behaviour of BAM8 within a temperature gradient (measured by MST) was opposite to that observed upon binding to Tre6P (slower and faster migration compared to the unbound state upon Glc6P and Tre6P interaction, respectively). Mass and charge changes upon binding Glc6P or Tre6P are presumably only marginally, or not different, if assuming that an equal number of ligand (Tre6P or Glc6P) molecules are bound. However, even if there was a difference in the number of bound ligands and therefore a difference in charge of the ligand-protein complex, one would probably not expect an opposite but rather a more accentuated migration behaviour of one of the two complexes. Alternatively, a significant difference in the hydration shell, another major determinant of the migration property, could be caused by a conformational change. To assess a ligand-dependent conformational change, a deuterium exchange experiment with detection by mass spectrometry could be performed. Why the two ligands (Glc6P, Tre6P) would trigger different protein confirmations can only be speculated about. They might bind to different binding sites within the protein thereby inducing different confirmations or the different physical-chemical properties of Glc6P and Tre6P may also lead to different impacts onto the protein confirmation. Further experiments, e.g. by running a native gel in presence and/or absence of Glc6P or Tre6P, followed by immunoblotting, could reveal different conformational changes triggered upon Glc6P or Suc6P binding. Ultimately it would be nice to resolve the protein’s structure by crystallography or NMR in presence or absence of one or the other ligand. To determine if interactions
with Glc6P and Suc6P are of biological relevance or solely a side-reactivity of Tre6P need further investigations. A possible approach is to mix Glc6P and Tre6P in cytosolic ratios and test the effect on DNA-affinity by SPR. Alternatively, a competition-MST experiment with constant protein and Tre6P but increasing Suc6P/Glc6P concentrations could give an indication about the substrate preference of BAM8.

In this study, only preliminary data was obtained by MST and SPR experiments for BAM7. Extending the analysis to BAM7 would possibly help to understand the mechanism behind the differing behaviour between BAM8 and BAM7 in planta. It could be envisioned that BAM7 has a lower affinity for Tre6P than BAM8, which would explain its lower/not detectable transcription factor activity. This would require that they follow the same mechanism of activation upon Tre6P binding.

5.3 BZR1-BAMs integrate Tre6P abundance into transcriptional network

Previous studies performed by Soyk, Simkova et al. 2014 showed that DNA-binding is a prerequisite for transcription factor activity of BAM8. Results of the present study indicate that DNA-binding alone is not yet sufficient to render BAM8 maximally active. Counterintuitively to the in vitro results, in vivo experiments showed that increased Tre6P levels can, under certain conditions, enhance BAM8 activity even though reducing DNA-binding/-affinity. Interestingly, Tre6P’s activating effect was only observed in low light and in absence of Suc in the medium, whereas under high light and in presence of Suc, BAM8 activity was not markedly changed upon increased Tre6P. In contrast, irrespective of the light conditions and Suc presence, lowered Tre6P always reduced BAM8 activity. Hence, low Tre6P apparently reduced BAM8 activity even though allowing more stable binding to the ds-BBRE than under high Tre6P conditions. This suggested, that DNA-binding stability of BAM8 does not necessarily positively correlate with gene expression activation. A plausible explanation is that higher Tre6P abundance allows a more dynamic system with higher association and dissociation rates, possibly also involving a recruitment of other transcription factors, which further boost transcriptional activation (see Fig. 5.1). BAM7’s role might predominantly be to protect the BZR1-BAM binding site from BBRE-binding repressors (Soyk, PhD thesis) or alternatively, it may only play an activating role in other, as-yet unstudied conditions such as during ABA responses. BAM7 has an ABA-responsive-like (ABRE-like) motif in its promoter but regulation by ABA has never been assessed. Performing SPR and MST experiments under reducing or oxidative conditions or of phosphorylated or dephosphorylated BAM8 might indicate if post-translational modifications of BAM8 cause different behaviours in high or low light conditions. Since there is no experimental evidence supporting this hypothesis yet, it seems more likely that different metabolite profiles or the absence/presence of different interaction partners are responsible for the difference of BAM activity in the two conditions tested.
The observation of differences in function of BAM8 depending on the light conditions are supported by comparisons with genome wide expression profile changes. Genes up-regulated by BAM8-ox at night are mostly also upregulated by elevated Tre6P (35S:otsA), whereas genes up-regulated by BAM8-ox during the day are equally up- or downregulated by elevated Tre6P. Overall there are more genes up-regulated by BAM8-ox during the day (422 of which 136 are co-regulated by 35S:otsA) compared to the night (166 of which 67 are co-regulated by 35S:otsA)(Reinhold, Soyk et al. 2011), which suggests a direct metabolic signalling response at night and a signalling response more integrated with other signalling pathways (e.g. BR, light) during the day. The crosstalk of BZR1-BAM with other signalling pathways consequently lowers the direct correlation between BAM8 activation and Tre6P abundance, such as during the day. Experiments looking at morphological changes (hypocotyl length) indirectly support this hypothesis of more integration with other signalling pathways during the day than during the night. For example, BZR1-BAMs modulate BR-regulated developmental changes during photomorphogenesis but not skotomorphogenesis (Soyk, unpublished), which indicates a lower overlap of BZR1-BAM and BR regulated genes at night but a higher overlap during the day. Furthermore, a more direct metabolic signal at night is supported by the observation that the BBRE motif was enriched in bam8.1 repressed genes in the dark but not in the light (Reinhold, PhD thesis). One can imagine that the involvement of more signalling pathways triggers a wider secondary response, which introduces other regulatory elements and reduces the enrichment of BBRE. However, instead of this ‘dilution-hypothesis’, BAM8 may simply be less sensitive to Tre6P during the day, possibly due to post-translational modification or interaction with other proteins. Alternatively, according to the above-mentioned recruitment model, putative BAM8 interactors could predominantly be activators at night and a mix of activators and repressors during the day.

Irrespective of the mechanism leading to different downstream responses of BAM8 during the day or at night, the fact that we find differences at the two time-points is in agreement with results from Tre6P-signalling studies. The Suc/Tre6P nexus model proposes different effects of Tre6P under illuminated or dark conditions (Figueroa and Lunn 2016). In darkness, increased Tre6P promotes energy-consuming processes and reduces starch degradation, whereas during the day it predominantly promotes investment into organic and amino acids and repression of sucrose synthesis. The observed day/night difference of BAM8-activity therefore seems to be in line with different responses triggered by Tre6P during the day or night. No experimental evidence so far exists that would propose an involvement of BZR1-BAMs in the Suc/Tre6P nexus model. The BRZ1-BAM triggered response may act independently. A time course experiments as performed in Yadav, Ivakov et al. 2014 may test the involvement of BZR1-BAMs in the Suc-nexus model. Genetic combinatorial lines of TPS1-inducible, bam7 bam8 and BAM8-ox would need to be included.
5.4 BAM8 modulates light response indirectly via interference with brassinosteroid-signalling

The data acquired to date, supports a role of BZR1-BAMs in light-response processes. Indications were obtained from hypocotyl elongation upon shading, expressional response after long term shading (5 days) (this study) and petiole elongation at adult stage (Reinhold, Soyk et al. 2011). Among all tested light signals, a crosstalk was most convincing with shade perception. However, an interplay with other light-signalling inputs cannot be totally excluded. Nevertheless, I suspect an indirect rather than a direct involvement of BZR1-BAMs in light-signalling for several reasons: Firstly, no BAM8-dependent, short-term transcriptional changes of known PAR genes were observed upon exposure to neighbour detection conditions (low R/FR). Secondly, direct target genes of BAM8 (BUP1 and BUP2) do not respond to shade but were identified as responsive to low to high light transitions (Genevestigator). Besides the changes introduced regarding light quality and quantity, the transfer from low to high light also alters photosynthate (sugar) abundance, hence the metabolic status. It therefore seems possible that the BUPs only indirectly respond to light. Thirdly, BAM8 does not change its localisation upon shading, which might be expect if they were under direct control by a photoreceptor (e.g. PHYB). In brief, there is no compelling evidence for a direct involvement of the BZR1-BAMs in light-signalling. Also, the presence of the known light regulated motif (G-box) within the BBRE motif does not insist on a direct, but allows an indirect involvement. The G-box was seen to be enriched within promoters of PIF4 and BZR1 common target genes (Oh, Zhu et al. 2012) (also see section 1.2.6). Therefore, it seems plausible that BZR1-BAMs modulate the light triggered induction of BR-signalling, or compete for common (light/BR/BZR1-BAMs) targets. Either way, the convergence may occur through counteraction of BZR1-BAMs with BZR1-/BES1-signalling as shown previously (Soyk, unpublished). A downstream involvement rather than a direct involvement of BZR1-BAMs in light-signalling would also give more flexibility to integrate nutritional conditions and still is consistent with our principle hypothesis that BZR1-BAMs are metabolic sensors. So far, one experiment supports an integration of metabolic signals into shade response via BAM8: Under true shade conditions (low PAR) moderate concentrations of sugars (Glc, Suc) enhance hypocotyl elongation, which was repressed in \textit{BAM8-ox} (see Fig. 4.3.1-C). The fact that sugars induce longitudinal growth in shade/darkness, but not light, was previously shown to be BR dependent. In that study, an involvement of HXK1 was proposed (Zhang and He 2015). Another study identified PIF-dependent Suc-promoted hypocotyl elongation in the dark (Liu, Zhang et al. 2011, Stewart, Maloof et al. 2011), also pointing towards an integration of light- and nutritional-parameters. In our experiments, the suspected sugar signal seems to repress elongation growth via BAM8 (far weaker seen for \textit{BAM7-ox}). Even though we now have indications for Tre6P as putative ligand of BZR1-BAMs, the identity of the sugar leading to impaired shade responsiveness of \textit{BAM8-ox} is unclear. Tre6P
levels rise upon feeding of plants with Glc and Suc (Yadav, Ivakov et al. 2014), making Tre6P a possible candidate limiting the elongation response of plants grown in true shade. At the same time a wide range of other metabolites also change upon Suc/ Glc feeding and they cannot be excluded as potential signal for BAM8 in this context. To test the impact of Tre6P on hypocotyl elongation, genetic experiments of BAM8-ox combinations with lines overexpression Tre6P-metabolisable enzymes (e.g. BAM8-ox 35S:otsB, 35S:otsA, bam7 bam8 35S:otsA) could be performed. From a broader perspective, the acquired results lead to the speculation that shade triggered elongation responses are moderated under conditions of plentiful sugar availability to avoid an exaggerated investment into unnecessary longitudinal growth. Open questions remain regarding BAM7’s role in this light-sugar interplay. Furthermore, it has to be considered that most experimental evidence is based on the phenotype of BAM8-ox plants and only few opposite effects are also observed in the double mutant (bam7 bam8). Ectopic overexpression of BAM8 could generate misleading results.

Mechanistically, the BZR1-BAM-, light- and BR-signalling interplay is not resolved. They may converge at many commonly regulated (downstream) genes. The two HD-ZIP class II transcription factors ATHB2 and ATHB4 could be among these, since both are direct targets of BZR1 and PIF4. ATHB4 has been proposed as an integrator of shade perception and hormone-mediated growth (Sorin, Salla-Martret et al. 2009).

To substantiate a wider involvement of BR-signalling in BZR1-BAM-signalling during the day compared to the dark, reporter assays co-expressing BAM8 and BZR1/BES1 could be performed in the light and the dark to see if the inhibition of BAM8 by BZR1/BES1 seen during the day (Soyk, unpublished) is persistent during the night. The direct impact of Tre6P on BAM8 activity in planta can be assessed by direct comparison of genome wide expressional changes in genetic combinations of BAM8-ox and TPS1-inducible lines when grown under identical experimental conditions. Further treatment with the brassinosteroid biosynthesis inhibitor BRZ could address the question if the impact of Tre6P on BAM8 occurs with or without the involvement of BR-signalling.

5.5 BAM8-dependent high Glc insensitivity occurs via interference with abscisic acid-signalling

Previous reports showed that exposure to high Glc leads to enhanced ABA biosynthesis and ABA sensitivity via both HXK1-dependent and HXK1-independent pathways (Cheng, Endo et al. 2002, Rolland, Baena-Gonzalez et al. 2006). In contrast, increased Tre6P (in 35S:otsA) led to a moderation of ABA accumulation and reduced ABA and Glc sensitivity (Avonce, Leyman et al. 2004). Results presented in this study indicate that overexpression of BAM8 also confers insensitivity to high sugar (Glc, Suc) exposure in respect to seedling-greening. Sugar supplementation studies determined that all sugars, which can be converted to Suc (including Glc, Fru) lead to an increase in Tre6P concentration (Yadav,
Ivakov et al. 2014). Considering the responsiveness of BAM8 to Tre6P this leads to the hypothesis that high Glc results in increased Tre6P, hence a higher activity of BAM8, which in turn helps to achieve a balance in ABA abundance and sensitivity. This could explain why BAM8-ox is Glc hyposensitive, whereas bam7 bam8 is Glc hypersensitive. HXK1 and BAM8 may act independently but are linked via the ABA synthesis and responsiveness and therefore cannot be completely disentangled at the phenotypic level. Glc susceptibility of BAM8-lig overexpressing plants indicates the importance of an intact BAM-domain. The proposed model could be substantiated by some future genetic experiments: crosses of 35S:otsB (reduced Tre6P) with BAM8-ox plants would lead to a reversion of the BAM8-ox Glc hyposensitivity phenotype. Overexpression of otsA in the bam7 bam8 background should also no longer be Glc insensitive. Measuring ABA levels and sensitivity in BAM8-ox and bam7 bam8 could further help to support or reject this hypothesis.

5.6 BZR1-BAMs’ mode of action – a model

Compiling the data of this study, I propose the following simplified model: under conditions of low or no Tre6P in the wild-type, BAM7 and BAM8 stably bind the BBRE motif in target gene promoters and outcompete repressors (Y). BAM8 itself activates transcription, triggering moderate expression (Fig. 5.1-A). Comparable conditions one would expect in plant overexpressing BAM7-lig, BAM7-cat or BAM7-FL (Soyk, Simkova et al. 2014). When Tre6P concentrations are high – BAM8 (and BAM7) by themselves associate, activate but also readily dissociate from the BBRE. During association, they may simultaneously recruit an unknown protein interaction partner(s) (X), which promotes BBRE-driven transcription activation and potentially also prevents dissociation of BAM8 (and BAM7). The absence of factor X in the in vitro experiments would explain the unstable binding of BAM8 to the ds-BBRE in presence of Tre6P. Under these conditions, the target gene promoters would be occupied by the BZR1-BAMs or/and the factor X and would outcompete repressors (Y) (Fig. 5.1-B). The absence of functional BAM7 and BAM8, as in the double mutant (bam7 bam8) (Fig.5.1-C) or the BAM7-dna overexpressor, which sequesters BAM8 off the DNA, would allow repressing factor (Y) to bind to the BBRE and lead to an overall repression of BBRE-driven genes (Reinhold, Soyk et al. 2011, Soyk, Simkova et al. 2014). In the absence of functional BAM8 (as in bam8.1, BAM8-lig, BAM8-dna), BAM7 would bind the BBRE-motif and prevent total repression by repressor (Y) but in contrast to BAM8, could not activate gene expression on its own. In case of overexpression of functional BAM8 (BAM8-ox, BAM8-cat), an intermediary situation between Fig. 5.1-A and Fig. 5.1-B would apply (Soyk, Simkova et al. 2014).

5.7 BZR1-BAMs in a wider perspective

Despite being neglected for a long time, trehalose 6-phosphate /trehalose metabolism has recently attracted a lot of attention. Nowadays it has been acknowledged to impact wide domains of a plant’s
life, ranging from controlling growth rates and growth patterns, influencing developmental decisions, impacting photosynthetic capacity or channelling investment into particular metabolic pathways in favour of others (Schlümpmann, Pellny et al. 2003, Kolbe, Tiessen et al. 2005, Ponnu, Wahl et al. 2011, Figueroa, Feil et al. 2016). Furthermore, it was shown that modulation of the trehalose 6-phosphate /trehalose metabolism through overexpression of exogenous or endogenous TPS/TPP genes confer resistance to abiotic stress, particularly drought stress. These studies were carried out in model organisms such as Arabidopsis or tobacco (Romero, Belles et al. 1997, Miranda, Avonce et al. 2007) but also important staple crops including potato, rice and maize (Yeo, Kwon et al. 2000, Garg, Kim et al. 2002, Nuccio, Wu et al. 2015). The expression patterns TPP genes were shown to be distinct in terms of cell- and tissue types (Vandesteene, Lopez-Galvis et al. 2012). The importance of such a spatiotemporal regulation of expression became particularly evident in a large scale, field trial study with genetically modified maize plants, expressing the riche TPP-gene specifically in maize ears. Using one floral MADS-box gene expression regulator (OsMads6) but not another (OsMads13) to control TPP expression led to consistently improved yield upon drought stress (Nuccio, Wu et al. 2015). If claiming that the BZR1-BAMs act as Tre6P sensors, it therefore seems surprising that the absence or overexpression of the BZR1-BAMs (bam7 bam8 and BAM8-ox) do not show a wider impact on plant performance. Furthermore, one might expect more restricted expression patterns in respect to developmental stages and tissue types. Nevertheless, all investigated vascular plants encode BZR1-BAMs genes and most of them contain two distinguishable isoforms (clustering into two clades, corresponding to BAM7 and BAM8). In planta studies in Arabidopsis showed that both isoforms are required for full functionality (Soyk, Simkova et al. 2014). These findings imply an important function of BZR1-BAMs in plant and suggest that we potentially still underestimate their importance. Possibly through the techniques employed so far (ectopic expression and total knock out) we miss subtler but more important traits of them. Testing more specifically characteristics, which were seen to be altered upon modulated Tre6P levels/ organic (e.g. abiotic stress, reduced partitioning into Suc in favour of amino-, organic- acids, starch degradation at night) may answer some questions. Spatiotemporal more targeted knock-down or overexpression could lead to more specific phenotypes than the generic phenotypes observed so far. Furthermore, extending studies to staple crops might uncover yet unidentified effects (e.g. stronger impact on reproductive organs and consequently seed set). However, the limited impact of BZR1-BAMs on plant performance can also simply be explained by the fact that BZR1-BAMs cover only a certain spectrum of Tre6P-signalling but not the whole. The effect of Tre6P on plant performance would therefore be much wider than BZR1-BAM’s impact. For example, allosteric regulation of SnRK1 activity by Tre6P already covers a wide range of responses and is likely BZR1-BAM independent. However, further experiments are needed to clarify these questions.
The present and previous studies place BZR1-BAM-signalling into signalling networks undoubtedly of crucial importance to plant performance (Tre6P, BR, light-signalling). What the BZR1-BAMs precise role within this network is, remains still elusive and needs further experimental investigations. Nevertheless, already the fact that these proteins bind a sugar ligand and modulate transcriptional responses is spectacular as such and worth pursuing.

**Figure 5.1: Model of BZR1-BAMs proposed mode of action.** (A) Under conditions of low trehalose 6-phosphate (Tre6), BAM7 and BAM8 stably bind to their preferred DNA-binding motif (BBRE). BAM8 moderately activates gene expression. Repressors (Y) are outcompeted. (B) Under conditions of high Tre6P the system is more dynamic: BZR1-BAMs readily dissociate and associate from and to the BBRE. Either this state as such renders BAM8 more active, or potentially it allows the BZR1-BAMs to recruit other activator proteins. The Repressor (Y) is again outcompeted. (C) In the absence of BZR1-BAMs the BBRE is not occupied and allows repressors (Y) to act.
6 Literature


