PCP
PLANT & CELL PHYSIOLOGY

Differential Expression Patterns of Non-Symbiotic Hemoglobins in Sugar Beet (*Beta vulgaris* ssp. *vulgaris*)

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(Received September 6, 2013; Accepted January 23, 2014)

Regular Paper

Biennial sugar beet (*Beta vulgaris* spp. *vulgaris*) is a Caryophyllidae that has adapted its growth cycle to the seasonal temperature and daylength variation of temperate regions. This is the first time a holistic study of the expression pattern of non-symbiotic hemoglobins (nsHbs) is being carried out in a member of this group and under two essential environmental conditions for flowering, namely vernalization and length of photoperiod. *BvHb* genes were identified by sequence homology searches against the latest draft of the sugar beet genome. Three nsHb genes (*BvHb1.1*, *BvHb1.2* and *BvHb2*) and one truncated Hb gene (*BvHb3*) were found in the genome of sugar beet. Gene expression profiling of the nsHb genes was carried out by quantitative PCR in different organs and developmental stages, as well as during vernalization and under different photoperiods. *BvHb1.1* and *BvHb2* showed differential expression during vernalization as well as during long and short days. The high expression of *BvHb2* indicates that it has an active role in the cell, maybe even taking over some *BvHb1.2* functions, except during germination where *BvHb1.2* together with *BvHb1.1*—both Class 1 nsHbs—are highly expressed. The unprecedented finding of a leader peptide at the N-terminus of BvHb1.1, for the first time in an nsHb from higher plants, together with its observed expression indicate that it may have a very specific role due to its suggested location in chloroplasts. Our findings open up new possibilities for research, breeding and engineering since Hbs could be more involved in plant development than previously was anticipated.

Keywords: Beta vulgaris (sugar beet) • carbon allocation • chloroplast transit peptide • non-symbiotic hemoglobins (nsHbs) • photoperiod • vernalization.

Abbreviations: BvHb, sugar beet Hb; cTP, chloroplast transit peptide; EST, expressed sequence tag; Hb, hemoglobin; LD, long days; NO, nitric oxide, nsHb, non-symbiotic Hb; SD, short days; sHb, symbiotic Hb; trHb, truncated Hb.

The nucleotide sequences reported in this paper have been submitted to GenBank with accession numbers KF549980, KF549981, KF549982 and KF549983.

Introduction

Hemoglobins (Hbs) are widespread proteins belonging to the globin super family. These proteins are composed of a heme *b* prosthetic group held within an α-helical secondary structure comprised of helices A–H, known as the myoglobin-fold (Mb-fold) (Vazquez-Limon et al. 2012). Hbs are mainly recognized as oxygen transporters; however, this function corresponds to a relatively recent adaptation in vertebrates. The early functions must have been enzymatic and oxygen sensing (Vinogradov and Moens 2008).

Hbs are present in almost all eukaryote groups, and nowadays it is obvious that they share a common origin with bacterial globins (Vinogradov et al. 2011b). In plants, three types of Hbs have been identified: symbiotic (sHbs), non-symbiotic (nsHbs) and truncated (trHbs) (Kubo 1939, Taylor et al. 1994, Watts et al. 2001). Unlike the pentacoordinated sHbs found in the nodules of legumes, nsHbs show different degrees of hexacoordination due to a histidine that reversibly binds to the sixth coordination site of the heme iron (Arredondo-Peter et al. 1997, Duff et al. 1997, Bruno et al. 2007) and are also well distributed in diverse organs of bryophytes and angiosperms during all their developmental stages (Hunt et al. 2001, Garrocho-Villegas et al. 2007, Vinogradov et al. 2011a). NsHbs are divided into Class 1 (nsHb1) and Class 2 (nsHb2) based on phylogenetic analysis, expression pattern and oxygen binding properties (Trevaskis et al. 1997, Hunt et al. 2001). However,

Plant Cell Physiol. 55(4): 834–844 (2014) doi:10.1093/pcp/pcu027, available online at www.pcp.oxfordjournals.org

both classes have not been found in all plants. It appears that monocots lack nsHb2, but generally have one or more nsHb1s. Dicots most often carry at least one nsHb1 and one nsHb2, unless (as in the case of legumes and some other nodulating dicots) the nsHb2 has evolved into an sHb (Hunt et al. 2001, Garrocho-Villegas et al. 2007, Smagghe et al. 2009).

In Arabidopsis it has been demonstrated that the nsHb1 from *A. thaliana* (AtHb1) can complement all nsHb2 (AtHb2) functions, but not vice versa, indicating that some overlap in AtHb2 and AtHb1 function exists and that the presence of at least one of them is essential for the survival of young non-stressed seedlings (Hebelstrup et al. 2006). Some potential roles have been assigned to nsHbs. NsHb1 is believed to modulate hypoxically generated nitric oxide (NO) levels in order to improve the energy status of plants (Igamberdiev and Hill 2009) while nsHb2, specifically AtHb2, seems to improve the oxygen supply for mitochondrial respiration (Anderson et al. 1996, Spyrakis et al. 2011, Vigeolas et al. 2011).

Plant nsHbs are differentially expressed under different stresses (Dordas 2009). Their gene and protein expression in response to hypoxia, cold, nutrient deprivation and osmotic stress has been evaluated (Taylor et al. 1994, Nie and Hill 1997, Trevaskis et al. 1997, Sowa et al. 1998, Hunt et al. 2001, Wang et al. 2003, Bustos-Sanmamed et al. 2011) as well as their response to signaling compounds such as NO and hormones (Hunt et al. 2001, Ross et al. 2004, Ohwaki et al. 2005, Shimoda et al. 2005, Qu et al. 2006, Sasakura et al. 2006, Bustos-Sanmamed et al. 2011). As a result, a biological role for plant nsHbs in hormone signal transduction (Hill 2012) through the regulation of the effects of NO produced by the plant under different stress conditions (Mur et al. 2013) has been suggested.

Under normal growth conditions, nsHbs have been found to be expressed both in different organs and in different developmental stages of plants, with some preferential organ distribution between nsHb1 and nsHb2 (Hunt et al. 2001). Studies on plant Hb evolution have demonstrated that the evolution of 3-on-3 plant Hbs (including the acquisition of new functions) paralleled the major transitions in land plant evolution (Vazquez-Limon et al. 2012). To date, nsHbs have mainly been studied in monocotyledons (rice, barley and maize) and in two members of the core eudicots: rosids (Arabidopsis, *Lotus japonicus* and *Trema tomentosa*) and asterids (tomato and chicory). From all those studies, it has been concluded that the assigned functions have species-specific and stress-related features that have to be established in each particular case (Gupta et al. 2011).

To date, only one nsHb from the Caryophyllids (the third member of the core eudicots) has been added to the study of Hb evolution in plants (Vazquez-Limon et al. 2012), and no nsHb has ever been studied as a gene or protein, except from the finding of an incomplete expressed sequence tag (EST) corresponding to BvHb2 (Hunt et al. 2001), an nsHb2 from sugar beet (*Beta vulgaris* spp. *vulgaris*). As a result of its adaptation to temperate regions, cultivated sugar beet is a biennial crop whose transition from vegetative to reproductive growth will primarily rely on environmental cues such as a period of vernalization, i.e. a period of winter cold, followed by long days (LDs) in order to flower (Bell and Bauer 1942). Another important factor controlling flowering time in sugar beets is the bolting gene B, a dominant promoter of flowering that over-rides the need for vernalization (Abegg 1936). Plants carrying the dominant B allele behave as annuals and proceed to bolting and flowering as a direct response to LDs (Bell and Bauer 1942). Given its economic importance and its peculiar life cycle, a vast number of publications describing the metabolism of sugar beet throughout its development have been published (Cooke and Scott 1993). It has been demonstrated that photosynthesis and carbohydrate metabolism will depend on the developmental stage of the plant (Oltmanns et al. 2006). The reproductive stage will be characterized for its high energy demand for bolting, flowering and seed production, as observed by the changes in carbon translocation (carbohydrates are mobilized from the tap root towards the leaves while the opposite happens during the vegetative stage) as well as the increase in photosynthetic rates (Fondy et al. 1989).

In this study, we report the finding of four Hb genes in the genome of sugar beet, three nsHbs and one trHb. Also, expression studies have been carried out to evaluate where and when the nsHbs are expressed. In the analysis, two essential environmental conditions for flowering, namely vernalization and different photoperiods, i.e. LDs and short days (SDs), were included. The results are discussed within the context of what is well known about sugar beet metabolism and physiology in order to open up new possibilities of research in the nsHb field. The analysis of the nsHb genes revealed the presence of a leader peptide at the N-terminal region in one of them; the relevance of this new finding is discussed.

Results

Structure and organization of nsHbs

Three different nsHb genes were found in sugar beet: *BvHb1.1*, *BvHb1.2* and *BvHb2*. Integration with its physical map localized *BvHb1.1* and *BvHb1.2* on chromosome 9 with roughly 5 kb distance between them; whereas *BvHb2* was located on chromosome 3. Their sizes, including introns, were 2,517, 2,233 and 3,446 bp, respectively. The alignment of the genomic sequences with their corresponding cDNA indicated that the coding sequence of *BvHb1.1* contains five introns, while *BvHb1.2* and *BvHb2* carry four and three introns, respectively. All gene models are fully backed up by mRNaseq reads, including reads which span splice junction (**Supplementary Fig. S1**). Their gene structure was compared with the corresponding sequences from Arabidopsis and rice (**Fig. 1**). Structural differences were found among the nsHb1 genes whereas nsHb2 genes showed a common structure with four exons and three introns. It is important to highlight that even though nsHb1 genes in sugar beet have a different number of introns, the position of the last three is quite conserved and similar to that of rice and Arabidopsis (**Fig. 1**). Overall, our results indicate that the evolution of nsHb genes has been complex and

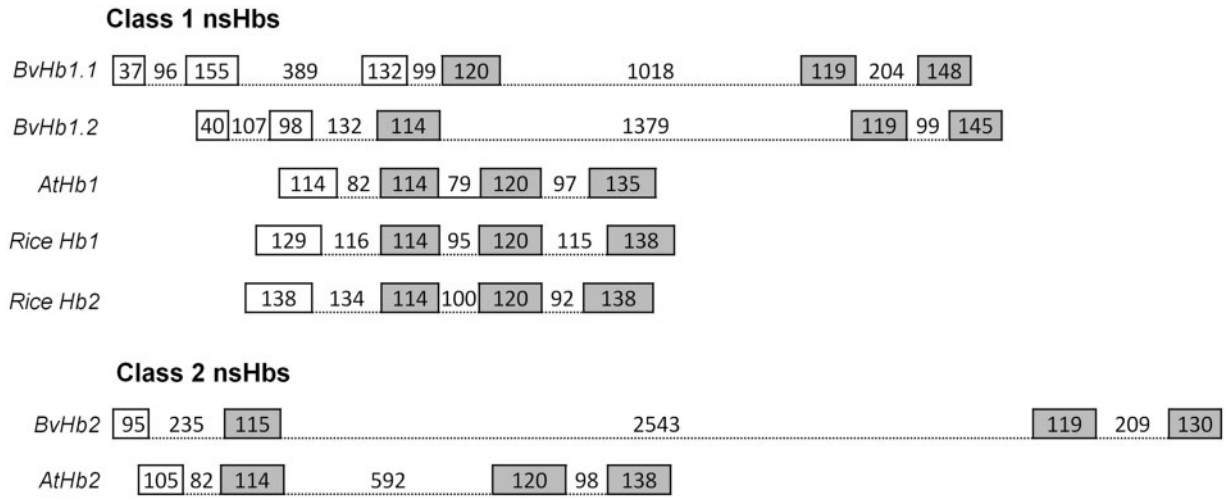

Fig. 1 Structure of sugar beet non-symbiotic hemoglobin (nsHb) genes. They are compared with other nsHbs from *Arabidopsis* (GenBank accession Nos. U94998 and U94999) and rice (GenBank accession Nos. AF335504 and U76028). Exons and introns are represented by boxes and dotted lines, respectively. Conserved exons are in gray. Lengths are shown in bp.

subject to significant duplication and deletion events where introns do not appear to be relevant to globin gene evolution (Lira-Ruan et al. 2002, Wang et al. 2003, Vinogradov et al. 2011a, Vazquez-Limon et al. 2012).

Subcellular localization of nsHbs

Analysis of the coding regions of the nsHb genes predicted three proteins of 236, 171 and 152 amino acids residues with molecular masses of 27.0, 19.8 and 17.9 kDa, respectively. Phylogenetic analysis of the protein sequences revealed that the first two belong to Class 1 (BvHb1.1 and BvHb1.2) and the last one to Class 2 (BvHb2) (**Supplementary Fig. S2**). As previously reported (Hunt et al. 2001), BvHbs were well separated into three groups: nsHbs (Class 1 and Class 2), sHbs and trHbs (Garrocho-Villegas et al. 2007). Interestingly, BvHb1.1 was placed in an independent branch paired with an nsHb1 from grape (GenBank accession No. CBI32538.3), suggesting a close similarity between them.

The amino acid sequences of BvHb1.1 and BvHb1.2 were 67% identical to each other and 58% and 57% identical to BvHb2. When compared with AtHb1, BvHb1.1 and BvHb1.2 were 68% and 74% identical. BvHb2 was 76% identical to AtHb2.

As expected, the three proteins contained the proximal HisF8, the distal HisE7, PheCD1, PheB10 and ProC2 (**Supplementary Fig. S3**). These amino acid residues are conserved among all plant Hbs, with the exception of trHbs (Watts et al. 2001, Garrocho-Villegas and Arredondo-Peter 2008). Protein alignment revealed that BvHb1.1 was significantly longer at the N-terminus by approximately 70–80 residues, and only comparable with the above-mentioned nsHb1 from grape, which also had a long N-terminal region (**Fig. 2**). The analysis of BvHb1.1 with the online server TargetP identified this long N-terminal region as a chloroplast transit peptide (cTP). This means that BvHb1.1 is synthesized as a precursor protein that will be translocated to the chloroplast where this extension will be cleaved by the general stromal processing peptidase (SPP) (Richter and Lamppa 1998). The cTPs do not have a very strong conserved sequence; however, they are characterized by having a three-domain structure: (i) an N-terminal domain usually beginning with MA, rich in S, L, A and T and potentially terminating with P; (ii) a central domain lacking acidic residues (D/E) with over-representation of S, L and P which slightly decrease towards the cleavage site; and (iii) a C-terminal cleavage cite domain enriched in R and with a loosely conserved motif VRA↓AA around the cleavage site (von Heijne et al. 1989, Zybailov et al. 2008). ChloroP predicted position 30 as the cTP cleavage site (**Fig. 3**) and, as this peptide did not completely fulfill the previous cTP description, we decided to analyze the nsHb1 from grape (GenBank accession No. CBI32538.3) that was very similar to BvHb1.1. This nsHb1 was also shown to have a cTP, but its cleavage site was predicted to be at position 78, just before the start of Helix A (**Fig. 2**; **Supplementary Fig. S3**). Previously it was reported that the only available cTP cleavage site predictor, which is the one from ChloroP, is not very precise and that its true positive (~86%) and false-positive rates (~35%) need to be further improved (Emanuelsson et al. 2007, Zybailov et al. 2008). Therefore, after a close observation of the detailed output and the alignment of both proteins, it is probable that the cleavage site in BvHb1.1 is at position 77 and not 30 as predicted. Evidently, experimental data are needed to confirm the real position of the cleavage site. On the other hand, the evidence is strong enough to affirm that sugar beet and grape both have an nsHb1 with a leader peptide carrying information to be translocated into their chloroplasts.

For practical reasons and similar to previous reports where plant Hbs lack leader peptides (Ross et al. 2002, Hill, 2012, Vazquez-Limon et al. 2012), BvHb1.2 and BvHb2 are designated as cytoplasmic until their real location is determined, as other plant Hbs have been localized in the cytosol (Ross et al. 2001),

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CBI32538.3    --MLNTKTPATTATMLISNATQDAKNISKICATHGSGSTSLYRQSLELSWVRRDG-SVQG
BvHb1.1       MAFMSTKTPASNATCISNAVTLNGNFACIKKSNFKSGFT-------ELSWVKREGFQIQG
                ::.*****:.** : . .* :.:  .   :.. ** *       *****:*:* .:**

CBI32538.3    LFCRTPRLITSVEKCR---RLEVRAFTEEQEALVVKSWSSMKKNAGELSLKFFLRIFEIA
BvHb1.1       LFCKNPSFITSVRKRNDNGGLIVKAFSEEQEALVVKSWNAMKKNASELALKFFLRVFEIA
              ***:.* :****.* .    * *:**:***********.:*****.**:******:****

CBI32538.3    PSAKKLFSFLRDS--DVPPEQNPKLKPHALSVFVMTCESAIQLRKAGRVTVRESNLIDLG
BvHb1.1       PTAKKLFSFLRDSSDDVPLEKNPKLKAHALTVFSMTCESAVNLRKAGKVTVKESNLKDLG
              *:***********  *** *:*****.***:** ******::*****:***:**** ***

CBI32538.3    ATHFKYGVVDEHFEVTKYALLETIKEAVPDMWSPEMKSAWAEAYDQLVAAIKKEMKPPQT
BvHb1.1       TTHFKYGVADEHFEVVRFALLETIKEAVPEMWSAEMKEAWKEAYDQLVAAIKQEMKSPEQ
              :*******.******.::***********:***.***.** ***********:***.*:

CBI32538.3    S--
BvHb1.1       ALQ
              :
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Fig. 2 Sequence alignment of BvHb1.1 and one nsHb1 from grape (*Vitis vinifera*). The sequences share 69% similarity. The cleavage positions of the leader peptide predicted by ChloroP are underlined.

nucleus (Seregelyes et al. 2000, Sainz et al. 2013) and plastids (Smagghe et al. 2007, Kim et al. 2013).

Tissue localization of expression of nsHb genes

Information about the expression of nsHb genes under normal growth conditions is limited, as are the number of species studied (Arredondo-Peter et al. 1997, Trevaskis et al. 1997, Duff et al. 1998, Lira-Ruan et al. 2001, Wang et al. 2003, Bustos-Sanmamed et al. 2011). The expression profiling of the three nsHb genes was carried out under normal condition across different tissues of sugar beet plants (**Fig. 3**). In general, *BvHb2* was the most expressed gene in all the analyzed organs, except seeds, where *BvHb1.1* had a twice as high expression level. On the other hand, *BvHb1.2* was the least expressed in almost all organs, except seeds, where it reached its highest expression. These results differ from those presented by Bustos-Sanmamed et al. (2011) where an nsHb1 was the most expressed in all plant organs they examined. Interestingly, the expression of both nsHb1 genes is important in seeds, as *BvHb1.1* is the one with the highest expression and *BvHb1.2* is basically only expressed in this organ. As previously suggested, it is probable that the expression of nsHb1 genes during early seed germination and post-germination may reflect a participation in maintaining energy and redox status within the embryo (Hebelstrup et al. 2007). This can also be supported by the fact that *BvHb1.1* will increase and attain its maximum expression in the hypocotyl. *BvHb2* has the opposite tendency, with very low expression in seeds but high expression in the other organs; mainly during the vegetative stage. During the reproductive stage, both *BvHb1.1* and *BvHb2* will increase their expression again in flowers, an organ where the energy demand is high, as observed by the changes in carbon translocation and photosynthetic rates (Cooke and Scott 1993).

When our results on nsHb1 expression are compared with those of previous studies, they are similar to Arabidopsis and barley where nsHb1 have been detected in young germinating seedlings and hypocotyl (Trevaskis et al. 1997, Duff et al. 1998, Hunt et al. 2001), but different from *L. japonicus* and tomato where they have instead been found in roots of mature plants (Wang et al. 2003, Bustos-Sanmamed et al. 2011). On the other hand, the expression of nsHb2 in leaves and flowers of Arabidopsis, *L. japonicus* and tomato agrees with our results. In roots, nsHb2 expression is quite variable: high in *L. japonicus*; very low in sugar beet and Arabidopsis; and no at all in tomato (Trevaskis et al. 1997, Hunt et al. 2001, Bustos-Sanmamed et al. 2011).

Expression of nsHbs across different developmental stages

The expression of *BvHb1.1* and *BvHb2* in leaves was analyzed across different developmental stages. *BvHb1.2* was not included in this study because of its very low expression in leaves (**Fig. 4**). Both *BvHb1.1* and *BvHb2* are expressed throughout plant development and, as in our previous analysis, *BvHb2* was expressed more (up to 40 times) than *BvHb1.1*. Following the expression pattern during vernalization, *BvHb1.1* showed higher expression post-vernalization compared with before vernalization (**Supplementary Fig. S4**). This expression then returned to pre-vernalization levels to increase again just before the bolting stage, a process that is correlated to flowering initiation that leads to embryogenesis. On the other hand, *BvHb2* returned to its pre-vernalization expression levels after vernalization, increasing afterwards to reach its maximum levels just before bolting. Interestingly, they both were up-regulated shortly before and during the bolting stage, reflecting the effect of this developmental stage on their expression (**Supplementary Fig. S5**).

Similar to our results, a constant expression of nsHb1 genes was observed in leaves of rice collected at different

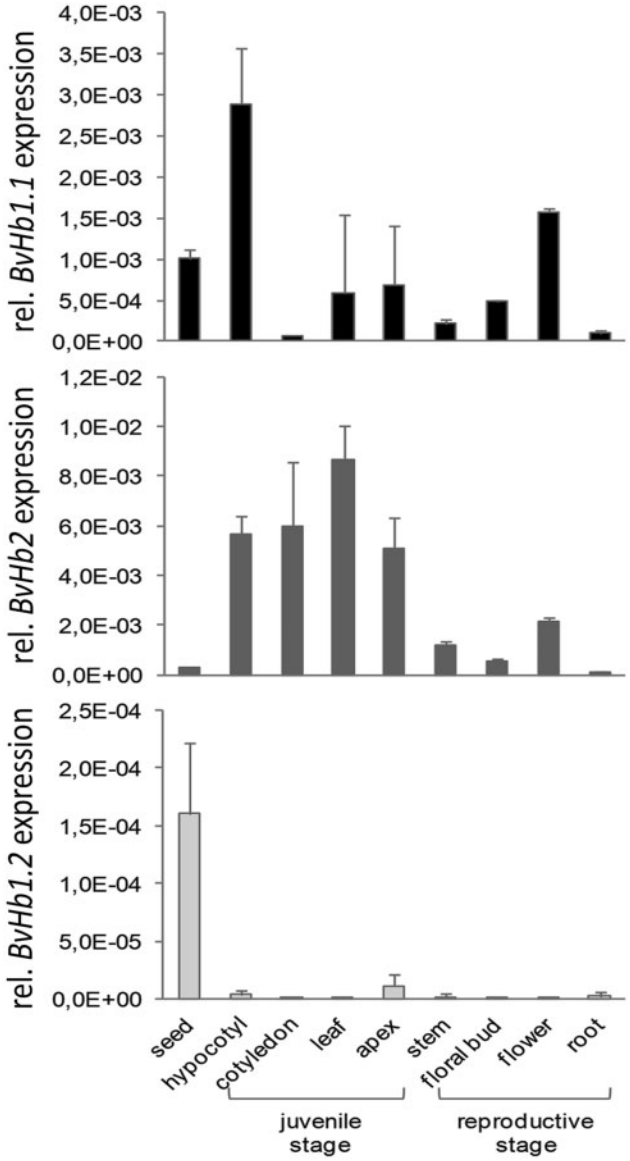

Fig. 3 Expression analysis of *BvHb1.1*, *BvHb1.2* and *BvHb2* in different tissues. Biennial sugar beet plants grown in LDs with a photoperiod regime of 18 h light/6 h dark at a temperature of 18°C. Samples were harvested at Zeitgeber time (ZT) 8 (i.e. 8 h after lights on). Data represent the mean ± SE ($n = 3$).

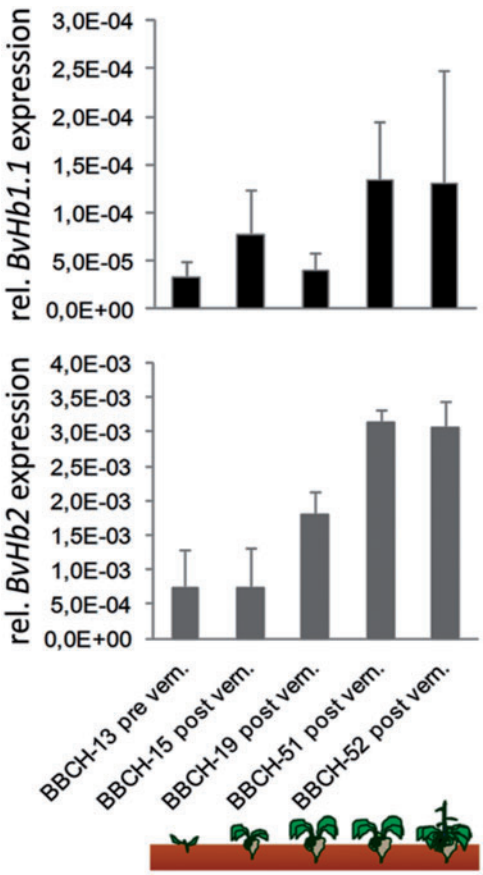

Fig. 4 Expression analysis of *BvHb1.1* and *BvHb2* during different developmental stages. Biennial sugar beet plants were grown in LDs with a photoperiod regime of 18 h light/6 h dark at a temperature of 18°C. Samples consisted of leaves harvested at ZT6. Data represent the mean ± SE ($n = 3$).

developmental stages (Lira-Ruan et al. 2001). Since these authors did not analyze the expression during flowering, it is not possible to know if they are also affected during that stage.

Expression of vsHbs during vernalization

Previous studies have examined the expression of nsHbs in response to low temperatures as a chilling stress where plants have been exposed to 4°C for 24 and 48 h (Trevaskis et al. 1997, Shimoda et al. 2005, Sasakura et al. 2006, Bustos-Sanmamed et al. 2011). However, for many plants, cold is a normal and even essential stage of their life cycle that is known as vernalization. These plants must go through a prolonged period of cold to overcome a block to flowering (Kim et al. 2009). We investigated the response of beet nsHbs in leaves during vernalization (**Fig. 4**) as this is the organ where vernalizing temperatures are perceived (Crosthwaite and Jenkins 1993). During this period, *BvHb1.2* was only expressed at very low levels, showing some increase at the beginning of vernalization, but afterwards its expression decreased and remained very low and invariant until the end, meaning that its gene expression is inactive during this period of cold. On the other hand, both *BvHb2* and *BvHb1.1* exhibited a significant response to vernalization. The expression of *BvHb2* was higher than that of *BvHb1.1*, reaching its maximum expression in the middle of vernalization and returning back to initial levels at the end of the period. In contrast, the expression of *BvHb1.1* increased gradually and reached its maximum at the end of vernalization. It is clear that even though both *BvHb1.1* and *BvHb2* are expressed during vernalization, it is the latter that has a high predominance over the entire period. The ratio between them is 10 times lower by the end of vernalization compared with the beginning.

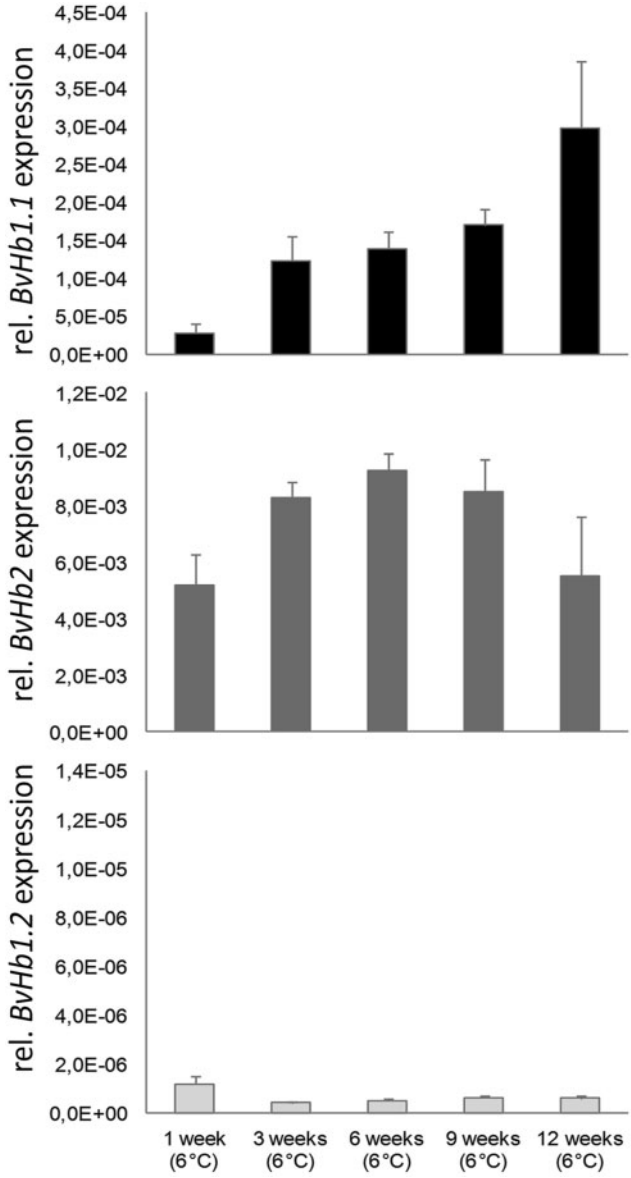

Fig. 5 Expression analysis of *BvHb1.1*, *BvHb1.2* and *BvHb2* during vernalization. Biennial sugar beet plants were grown in SDs with a photoperiod regime of 12 h light/12 h dark at a temperature of 6°C for 12 weeks. Samples consisted of leaves harvested at ZT4. Data represent the mean ± SE ($n = 3$).

Expression of nsHbs under different photoperiodic conditions

In sugar beet, carbohydrate metabolism and translocation are affected by the transition between the light and dark periods. Carbon that is exported from leaves at night or during times of slow photosynthesis comes mainly from diurnal starch reserves rather than from accumulated sucrose (Fondy et al. 1989). To investigate further the role of nsHbs in the metabolism of sugar beet, we examined their expression in leaves during diurnal cycles with different photoperiods before vernalization. Plants were grown under SD and LD conditions in two independent

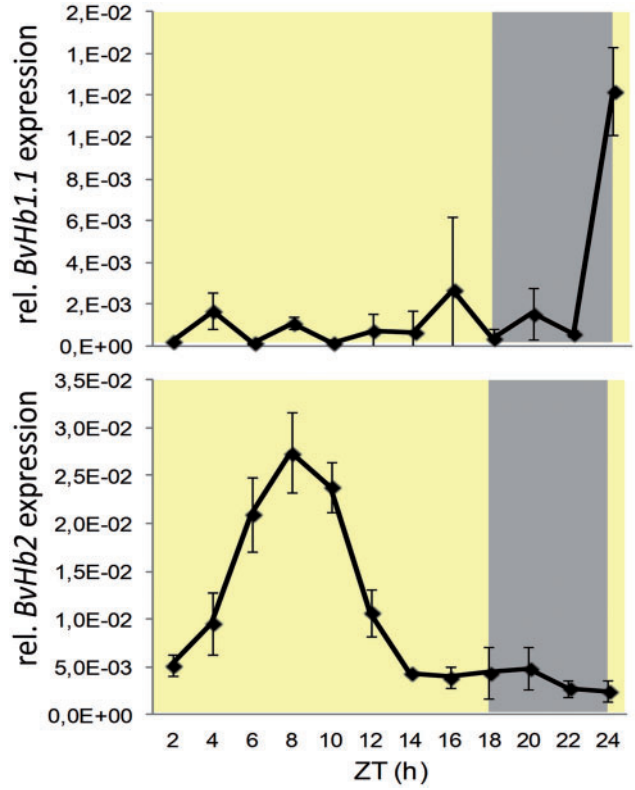

Fig. 6 Diurnal expression analysis of *BvHb1.1* and *BvHb2*. Biennial sugar beet plants were grown in LDs at a temperature of 18°C. Leaf samples were harvested every 2 h. Data represent the mean ± SE ($n = 5$).

experiments. Similar to the analysis at different developmental stages, *BvHb1.2* was not included due to its low expression in leaves. *BvHb1.1* and *BvHb2* showed a different and contrasting expression pattern under both LDs and SDs (**Fig. 6**; **Supplementary Fig. S6**). *BvHb2* was highly up-regulated during the light period in both LDs and SDs, with very little to no expression during the dark period. *BvHb1.1*, on the other hand, had very low expression with no large variation except for a sudden increase at the end of the dark period, just before the light period starts during LDs. The results were reinforced when annual plants were also analyzed (**Supplementary Fig. S7**). Such a rise indicates a transition in the leaf metabolism that is not triggered by an obvious change in the environment of the plant but by some other endogenous signal that prepares the leaves for the time when the light period will start. In the case of sugar beet, the metabolism in leaves is governed strongly by the light/dark transitions as well as the length of the day (Fondy and Geiger 1985, Fondy et al. 1989). The absence of such a rise in SDs indicates that the above endogenous signal did not need to be activated. Whether the activation of such a signal responds to a change in metabolic status or not is something that needs to be further explored. Overall, our results indicate that *BvHb1.1* is unaffected by daylight whereas *BvHb2* is very responsive, with a conserved expression amplitude regardless of the length of the day.

Discussion

The presence of a leader peptide in an nsHb1

Three nsHbs were found in sugar beet. A significant finding is no doubt the fact that *BvHb1.1* carries the information to be translocated into chloroplasts. To date, no transit sequences have ever been found at the N-termini of these proteins in higher plants. Previously, sequence alignments of moss Hbs and a number of sHbs and nsHbs (Arredondo-Peter et al. 2000) indicated that the size of the polypeptide at the N-terminus (pre-helix A) decreased over time (Ross et al. 2002) and that it functioned as a leader peptide, similarly to *Chlamydomonas* T1 trHb, which is translocated to chloroplasts (Couture et al. 1994). Our findings suggest that, at least in some species, this leader peptide is still conserved. Thus, some nsHbs can still be translocated to chloroplasts, and not all nsHbs became cytoplasmic during evolution as has been suggested previously (Ross et al. 2002, Vazquez-Limon et al. 2012). This is supported by the fact that two other nsHb1s also have a cTP in their N-terminal region, one from grape and other from spinach (*Spinacia oleracea*), the latter being part of the Amaranthaceae family like sugar beet. The analysis of an early draft of the spinach genome revealed the presence of three nsHbs, with one nsHb1 carrying a cTP (data not shown). Similarly to sugar beet, spinach has two nsHb1 genes located on the same chromosome, approximately 2 kb from each other (data not shown). These nsHb1 genes have obviously arisen through a duplication event that took place in an ancestor of spinach and sugar beet. It is probable that the ancestral nsHb1 gene had that long N-terminal region, but after duplication one of the new copies lost it over time through deletion events (Ross et al. 2002). Thus, one of them continued to be translocated to the chloroplast while the other stayed in the cytoplasm. To our knowledge, this is the first time that an nsHb1 from higher plants has been shown to carry information to be translocated into chloroplasts. NsHbs have previously been associated with this organelle when staining, and linear aggregates were observed co-localizing with the chloroplast during immunolocalization studies of somatic embryogenesis in chicory (*Cichorium intybus*) (Smagghe et al. 2007). Moreover, chloroplasts are the major site of heme biosynthesis in higher plant cells (Cornah et al. 2002), and studies on chloroplasts of Arabidopsis (Arnaud et al. 2006) and soybean (Jasid et al. 2006) have determined that this organelle contributes to NO synthesis in vivo. Therefore, a relationship between BvHb1.1 and NO might be plausible based on present knowledge where nsHb1 proteins are recognized as NO scavengers (Gupta et al. 2011, Spyrakis et al. 2013). However, more research needs to be done in order to confirm this possibility or to unveil the role of these proteins.

Differential expression of cytoplasmic nsHbs

NsHbs are differentially expressed (Hunt et al. 2001). In the case of sugar beet, we could also observe differential expression in some organs and developmental stages; but also some coincidences were found. Both *BvHb1.1* and *BvHb1.2* are mainly expressed in organs related to seed germination and early plant growth, such as seeds and hypocotyl, as previously reported (Duff et al. 1998, Hunt et al. 2002). *BvHb2* is expressed at a very low level in seeds but instead it is highly expressed in leaves during the juvenile stage when the accumulation of sucrose in the storage root is at its maximum (Oltmanns et al. 2006). When the reproductive stage is reached, before and during bolting, and in flowers, both *BvHb1.1* and *BvHb2* are expressed. Our results indicate that both cytoplasmic nsHbs indeed have a very differential expression (nsHb1 in young germinating seedlings and hypocotyl, and nsHb2 in leaves and flowers), while some coincidences can be found between *BvHb1.1* and *BvHb2*.

Interestingly, cytoplasmic nsHbs had opposite expression, with *BvHb2* being the most highly expressed and *BvHb1.2* the least. This indicates that *BvHb2* is more active in cell metabolism, with probably some overlapping functions as previously reported in Arabidopsis (Hebelstrup et al. 2006); on the other hand, *BvHb1.2* is only active in seeds where it should have a more specific role that cannot be completely assumed by *BvHb2*. *BvHb1.1* is basically expressed in all organs during development of the plant, except in roots of mature plants where no nsHb was expressed. Based on our results, we believe that *BvHb2* has an active role in the cytoplasm, maybe even taking over *BvHb1.2* function, while *BvHb1.1* has a much more specific role mainly due to its specific and confined location in the chloroplast.

Expression during vernalization and photoperiod

Previous studies suggested that both nsHb1 and nsHb2 were expressed in response to cold as they were analyzed during the first 24–48 h of cold exposure (Trevaskis et al. 1997, Shimoda et al. 2005, Sasakura et al. 2006, Bustos-Sanmamed et al. 2011). However, plants acclimate to cold within a 24 h period, and after this time the cold-sensing mechanism becomes desensitized (Zarka et al. 2003). Our results indicate that the expression of *BvHb1.1* is not related to cold acclimation but instead is related to changes in plant metabolism due to long-term exposure to low temperature. The chloroplast is the central switch of the plant's response to cold (Crosatti et al. 2013), and photosynthesis is one of the most temperature-sensitive processes in plants (Stitt and Hurry 2002). The adaptation to low temperatures is achieved by shifting protein expression, by chloroplast–nucleus cross-talk, to compensate their decreased activities (Crosatti et al. 2013, Yamori et al. 2014) which would explain the gradual increase of *BvHb1.1* expression. Low temperature promotes photoinhibition that increases with light intensity (Hetherington et al. 1989), initiating a signal transduction pathway which coordinates photosynthesis-related gene expression (Pfannschmidt et al. 2009). Therefore, a way to determine if the translocation of BvHb1.1 to chloroplasts is related to photosynthesis would be to assess its photoperiodic regulation at low temperatures given that at warm temperatures it did not show variation. As previously reported for nsHb1 from

barley (Igamberdiev and Hill 2009), the presence of *BvHb1.1* could also be due to the presence of NO. It has been demonstrated that NO production increases during cold (Zhao et al. 2009) and, even though the actual origin has not been demonstrated, chloroplasts could be an important source (Jasid et al. 2006). In the hypothetical scenario where *BvHb1.1* interacts with NO, *BvHb1.1* could help to adjust the NO levels which, if high, may lead to impairment of the photosynthetic machinery (Jasid et al. 2006).

Similarly to *BvHb1.1*, the expression of *BvHb2* does not correspond to a cold-sensing mechanism but it is not part of the adaptation to long-term exposure to low temperature either. Low temperature induces modifications of carbohydrate metabolism (Stitt and Hurry 2002). In this study, we observe that *BvHb2* is almost constitutively expressed in leaves during developmental stages where sugar beet plants are subject to changes in temperature and photoperiod that affect both photosynthesis and carbohydrate metabolism (ap Rees et al. 1988, Hetherington et al. 1989, Strand et al. 1997). During the juvenile stage before vernalization, the metabolism of sugar beet is directed towards sucrose accumulation in the tap root (Oltmanns et al. 2006); during vernalization, carbohydrates are depolymerized, resulting in an increase in soluble sugars (Chouard 1960); and, after vernalization, the stored carbohydrates are mobilized from the tap root to the leaves to be utilized for bolting, flowering and seed production (Oltmanns et al. 2006). The photoperiodic behavior of carbon allocation in sugar beet leaves (Li et al. 1992) is strikingly similar to the photoperiodic behavior of *BvHb2* expression. Both starch accumulation and *BvHb2* expression decrease before the end of the day period and are independent of light and daylength. *BvHb2* expression is also under endogenous circadian control, as is carbon allocation (Li et al. 1992). During vernalization, the presence of oxygen is indispensable (Chouard 1960) probably as a way to alleviate photosynthesis inhibition which otherwise would reduce sucrose translocation (Servaites and Geiger 1974). A high expression of *BvHb2* in photosynthesizing young leaves would be important for the fast translocation of sucrose (Fondy et al. 1989) as this process is inhibited by low oxygen (Servaites and Geiger 1974). Therefore, a possible increment of oxygen availability by BvHb2 as suggested for AtHb2 (Spyrakis et al. 2011, Vigeolas et al. 2011) cannot be disregarded. Our results indicate that, in the case of sugar beet, a possible role for BvHb2 related to carbon metabolism/allocation needs to be further investigated.

In summary, this study shows that the expression of nsHbs in sugar beet is tissue and stage specific. This, together with the fact that a class 1 BvHb can probably be located in chloroplasts, has important implications concerning the physiological role of Hbs. Based on their levels of expression, the possibility that BvHb2 would deliver oxygen and that Class 1 BvHbs would have a signaling role cannot be dismissed. We believe that our findings open up new possibilities for research in the field of Hbs in order to enhance our understanding of their role in plant metabolism. We therefore believe that the characterization and analysis of plant Hbs from divergent species would provide us with more opportunities for breeding and engineering of plants, since Hbs are more involved in plant development than was previously anticipated.

Materials and Methods

Plant material and growth conditions

Biennial sugar beet plants (G018B0 line) were used in the study. They were grown in climate chambers at 18°C under LD conditions with a photoperiod of 18 h light/6 h dark, or SD conditions with 18 h dark/6 h light. Light intensity was set to a photon flux of 200 μmol m^{-2} s^{-1}. The plants used for vernalization were grown for 2 weeks at 18°C in SDs (12 h light/12 h dark). Vernalization treatment consisted of 15 weeks of cold exposure at a temperature of 6°C where plants were grown in SD conditions (12 h light/12 h dark). Afterwards the plants were slowly acclimatized for 2 weeks by stepwise temperature increases from 6 to 18°C and then transferred to LD conditions (18 h light/6 h dark).

Isolation of BvHb1-1 and BvHb2 transcripts

Two incomplete sugar beet ESTs, BI543289 and BE590299 (Hunt et al. 2001) from the NCBI database, showing strong homology with plant Hb proteins were identified. The EST sequences were used to isolate the transcripts for two different Hb genes, here named *BvHb1.1* and *BvHb2*, respectively. The transcripts were isolated from leaf samples using the following primers: BI543289F, BI543289R, BE590299F and BE590299F (**Supplementary Table S1**). The amplicons were subsequently sequenced.

In silico gene structure characterization and identification of a leader peptide in an nsHb

The gene structure of *BvHb1.1*, and *BvHb2* was determined based on homology searches in the latest version of the sugar beet genomic sequence using the transcript sequences of both genes. Gene models were derived from automated predictions calculated using the Augustus software (Stanke et al. 2008), incorporating transcriptional evidence. In total, 580 million reads from Illumina sequencing, together with 36,000 public sugar beet ESTs and 282,000 cDNA reads from 454 sequencing were used for evidence-based gene prediction (Dohm et al. 2014). For each gene model, Augustus assigns a score ranging from 0% (no evidence) to 100% (all gene features supported by evidence). A third nsHb gene and a trHb gene were additionally found in the latest version of the sugar beet genome (RefBeet1.1: http://bvseq.molgen.mpg.de/index.shtml) which has been corrected for homopolymer errors and misassemblies. We have named them *BvHb1.2* and *BvHb3*, respectively (**Supplementary Table S2**; **Supplementary Fig. S1**). Each of the four genes is supported by 100% evidence. Their accession

numbers in GenBank are: KF549980 (*BvHb1.1*), KF549981 (*BvHb1.2*), KF549982 (*BvHb2*) and KF549983 (*BvHb3*).

The identification of the leader peptide as a cTP in BvHb1.1 was done with the online server TargetP (Emanuelsson et al. 2007). For the identification of the cleavage site position, the online ChloroP server was used (Emanuelsson et al. 1999). Both servers are hosted at the Center for Biological Sequence analysis (CBS) at the Technical University of Denmark (DTU).

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA v. 5.2 (Tamura et al. 2011) The full protein sequences of BvHb1.1, BvHb1.2, BvHb2 and BvHb3 were aligned against various plant Hbs from Arabidopsis (*A. thaliana*), rapeseed (*Brassica napus*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), barley (*Hordeum vulgare*), apple (*Malus domestica*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), poplar (*Populus trichocarpa*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), grape (*Vitis vinifera*) and maize (*Zea mays*). In this analysis, symbiotic, non-symbiotic and truncated Hbs were used. The evolutionary history was inferred using the Neighbor–Joining method (Saitou and Nei 1987). The analysis involved 39 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 111 positions in the final data set.

Expression analysis

Total RNAs from various tissues and developmental stages of biennial sugar beet plant were isolated using the RNAqueous®-4PCR or RNAqueous®-96 kits (Ambion, Inc.) and DNase treated using the DNA-free™ Kit (Ambion, Inc.). A 1 μg aliquot of total RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.). The expression of *BvHb1.1*, *BvHb1.2* and *BvHb2* was quantified by means of quantitative real-time PCR (RT-qPCR) using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Inc.) on an ABI7500 Real-Time PCR System (Applied Biosystems Inc.) in a 20 μl final reaction volume. The PCR conditions were as follows: primary denaturation at 95°C for 10 min followed by 40 amplification cycles of 15 s at 95°C and 1 min at 60°C. At least three biological replicates were used and each sample was run in triplicate. The comparative C_T ($\Delta\Delta C_T$) method (Schmittgen and Livak 2008) was applied. Relative expression levels were calculated and normalized to the geometric mean of *BvGAPDH* (Reeves et al. 2007) and *BvICDH* (Pin et al. 2010) expression. Primers used for detection of *BvHb1.1*, *BvHb1.2* and *BvHb2* transcripts were BvHb1.1-F, BvHb1.1-R, BvHb1.2-F, BvHb1.2-R, BvHb2-F and BvHb2-R (**Supplementary Table S1**).

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the European Union [Alβan Programme Doctoral Scholarship to N.L-E.]; the Federal Ministry of Education and Research, BMBF ['AnnoBeet' FKZ 0315962B to H.H.]; Plant Link [http://www.plantlink.se/].

Disclosures

The authors have no conflicts of interest to declare.

References

Abegg, F.A. (1936) A genetic factor for the annual habit in beets and linkage relationship. *J. Agric. Res.* 53: 493–511.

Anderson, C.R., Jensen, E.O., LLewellyn, D.J., Dennis, E.S. and Peacock, W.J. (1996) A new hemoglobin gene from soybean: a role for hemoglobin in all plants. *Proc. Natl Acad. Sci. USA* 93: 5682–5687.

ap Rees, T., Burrell, M.M., Entwistle, T.G., Hammond, J.B., Kirk, D. and Kruger, N.J. (1988) Effects of low temperature on the respiratory metabolism of carbohydrates by plants. *Symp. Soc. Exp. Biol.* 42: 377–393.

Arnaud, N., Murgia, I., Boucherez, J., Briat, J.-F., Cellier, F. and Gaymard, F. (2006) An iron-induced nitric oxide burst precedes ubiquitin-dependent protein degradation for *Arabidopsis* AtFer1 ferritin gene expression. *J. Biol. Chem.* 281: 23579–23588.

Arredondo-Peter, R., Hargrove, M.S., Sarath, G., Moran, J.F., Lohrman, J., Olson, J.S. et al. (1997) Rice hemoglobins (gene cloning, analysis, and O2-binding kinetics of a recombinant protein synthesized in *Escherichia coli*). *Plant Physiol.* 115: 1259–1266.

Arredondo-Peter, R., Ramírez, M., Sarath, G. and Klucas, R.V. (2000) Sequence analysis of an ancient hemoglobin cDNA isolated from the moss *Physcomitrella patens* (Accession No. AF218049) (PGR00-040). *Plant Physiol.* 122: 1457.

Bell, G.D.H. and Bauer, A.B. (1942) Experiments on growing sugar beet under continuous illumination. *J. Agric. Sci.* 32: 112–141.

Bruno, S., Faggiano, S., Spyrakis, F., Mozzarelli, A., Abbruzzetti, S., Grandi, E. et al. (2007) The reactivity with CO of AHb1 and AHb2 from *Arabidopsis thaliana* is controlled by the distal HisE7 and internal hydrophobic cavities. *J. Amer. Chem. Soc.* 129: 2880–2889.

Bustos-Sanmamed, P., Tovar-Mendez, A., Crespi, M., Sato, S., Tabata, S. and Becana, M. (2011) Regulation of nonsymbiotic and truncated hemoglobin genes of *Lotus japonicus* in plant organs and in response to nitric oxide and hormones. *New Phytol.* 189: 765–776.

Chouard, P. (1960) Vernalization and its relations to dormancy. *Annu. Rev. Plant Physiol.* 11: 191–238.

Cooke, D.A. and Scott, R.K. (1993) The Sugar Beet Crop. Science into Practice. Chapman & Hall.

Cornah, J.E., Roper, J.M., Pal Singh, D. and Smith, A.G. (2002) Measurement of ferrochelatase activity using a novel assay suggests that plastids are the major site of haem biosynthesis in both photosynthetic and non-photosynthetic cells of pea (*Pisum sativum L.*). *Biochem. J.* 362: 423–432.

Couture, M., Chamberland, H., St-Pierre, B., Lafontaine, J. and Guertin, M. (1994) Nuclear genes encoding chloroplast

hemoglobins in the unicellular green alga *Chlamydomonas eugametos*. *Mol. Gen. Genet.* 243: 185–197.
Crosatti, C., Rizza, F., Badeck, F.W., Mazzucotelli, E. and Cattivelli, L. (2013) Harden the chloroplast to protect the plant. *Physiol. Plant.* 147: 55–63.
Crosthwaite, S.K. and Jenkins, G.I. (1993) The role of leaves in the perception of vernalizing temperatures in sugar beet. *J. Exp. Bot.* 44: 801–806.
Dohm, J.C., Minoche, A.E., Holtgrawe, D., Capella-Gutierrez, S., Zakrzewski, F., Tafer, H. et al. (2014) The genome of the recently domesticated crop plant sugar beet (Beta vulgaris). *Nature* 505: 546–549.
Dordas, C. (2009) Nonsymbiotic hemoglobins and stress tolerance in plants. *Plant Sci.* 176: 433–440.
Duff, S.M.G., Guy, P.A., Nie, X., Durnin, D.C. and Hill, R.D. (1998) Haemoglobin expression in germinating barley. *Seed Sci. Res.* 8: 431–436.
Duff, S.M.G., Wittenberg, J.B. and Hill, R.D. (1997) Expression, purification, and properties of recombinant barley (*Hordeum sp.*) hemoglobin. Optical spectra and reactions with gaseous ligands. *J. Biol. Chem.* 272: 16746–16752.
Emanuelsson, O., Brunak, S., von Heijne, G. and Nielsen, H. (2007) Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* 2: 953–971.
Emanuelsson, O., Nielsen, H. and von Heijne, G. (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci.* 8: 978–984.
Fondy, B.R. and Geiger, D.R. (1985) Diurnal changes in allocation of newly fixed carbon in exporting sugar beet leaves. *Plant Physiol.* 78: 753–757.
Fondy, B.R., Geiger, D.R. and Servaites, J.C. (1989) Photosynthesis, carbohydrate metabolism, and export in *Beta vulgaris* L. and *Phaseolus vulgaris* L. during square and sinusoidal light regimes. *Plant Physiol.* 89: 396–402.
Garrocho-Villegas, V. and Arredondo-Peter, R. (2008) Molecular cloning and characterization of a moss (*Ceratodon purpureus*) nonsymbiotic hemoglobin provides insight into the early evolution of plant nonsymbiotic hemoglobins. *Mol. Biol. Evol.* 25: 1482–1487.
Garrocho-Villegas, V., Gopalasubramaniam, S.K. and Arredondo-Peter, R. (2007) Plant hemoglobins: what we know six decades after their discovery. *Gene* 398: 78–85.
Gupta, K.J., Hebelstrup, K.H., Mur, L.A.J. and Igamberdiev, A.U. (2011) Plant hemoglobins: important players at the crossroads between oxygen and nitric oxide. *FEBS Lett.* 585: 3843–3849.
Hebelstrup, K.H., Hunt, P., Dennis, E., Jensen, S.B. and Jensen, E.O. (2006) Hemoglobin is essential for normal growth of *Arabidopsis* organs. *Physiol. Plant.* 127: 157–166.
Hebelstrup, K.H., Igamberdiev, A.U. and Hill, R.D. (2007) Metabolic effects of hemoglobin gene expression in plants. *Gene* 398: 86–93.
Hetherington, S.E., He, J. and Smillie, R.M. (1989) Photoinhibition at low temperature in chilling-sensitive and -resistant plants. *Plant Physiol.* 90: 1609–1615.
Hill, R.D. (2012) Non-symbiotic haemoglobins—what's happening beyond nitric oxide scavenging?. *AoB Plants* 2012: pls004.
Hunt, P.W., Klok, E.J., Trevaskis, B., Watts, R.A., Ellis, M.H., Peacock, W.J. et al. (2002) Increased level of hemoglobin 1 enhances survival of hypoxic stress and promotes early growth in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* 99: 17197–17202.
Hunt, P.W., Watts, R.A., Trevaskis, B., Llewelyn, D.J., Burnell, J., Dennis, E.S. et al. (2001) Expression and evolution of functionally distinct haemoglobin genes in plants. *Plant Mol. Biol.* 47: 677–692.
Igamberdiev, A.U. and Hill, R.D. (2009) Plant mitochondrial function during anaerobiosis. *Ann. Bot.* 103: 259–268.
Jasid, S., Simontacchi, M., Bartoli, C.G. and Puntarulo, S. (2006) Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. *Plant Physiol.* 142: 1246–1255.
Kim, D.-H., Doyle, M.R., Sung, S. and Amasino, R.M. (2009) Vernalization: winter and the timing of flowering in plants. *Annu. Rev. Cell Dev. Biol.* 25: 277–299.
Kim, D.Y., Hong, M.J., Lee, Y.J., Lee, M.B. and Seo, Y.W. (2013) Wheat truncated hemoglobin interacts with photosystem I PSK-I subunit and photosystem II subunit PsbS1. *Biol. Plant.* 57: 281–290.
Kubo, H. (1939) Über hämoprotein aus den wurzelknöllchen von leguminosen. *Acta Phytochim. (Tokyo)* 11: 195–200.
Li, B., Geiger, D.R. and Shieh, W.J. (1992) Evidence for circadian regulation of starch and sucrose synthesis in sugar beet leaves. *Plant Physiol.* 99: 1393–1399.
Lira-Ruan, V., Ross, E.J.H., Sarath, G., Klucas, R.V. and Arredondo-Peter, R. (2002) Mapping and analysis of a hemoglobin gene family from *Oryza sativa*. *Plant Physiol. Biochem.* 40: 199–202.
Lira-Ruan, V., Sarath, G., Klucas, R.V. and Arredondo-Peter, R. (2001) Synthesis of hemoglobins in rice (*Oryza sativa* var. *Jackson*) plants growing in normal and stress conditions. *Plant Sci.* 161: 279–287.
Mur, L.A., Mandon, J., Persijn, S., Cristescu, S.M., Moshkov, I.E., Novikova, G.V. et al. (2013) Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants* 5: pls052.
Nie, X. and Hill, R.D. (1997) Mitochondrial respiration and hemoglobin gene expression in barley aleurone tissue. *Plant Physiol.* 114: 835–840.
Ohwaki, Y., Kawagishi-Kobayashi, M., Wakasa, K., Fujihara, S. and Yoneyama, T. (2005) Induction of class-1 non-symbiotic hemoglobin genes by nitrate, nitrite and nitric oxide in cultured rice cells. *Plant Cell. Physiol.* 46: 324–331.
Oltmanns, H., Kloos, D.U., Briess, W., Pflugmacher, M., Stahl, D.J. and Hehl, R. (2006) Taproot promoters cause tissue specific gene expression within the storage root of sugar beet. *Planta* 224: 485–495.
Pfannschmidt, T., Brautigam, K., Wagner, R., Dietzel, L., Schroter, Y., Steiner, S. et al. (2009) Potential regulation of gene expression in photosynthetic cells by redox and energy state: approaches towards better understanding. *Ann. Bot.* 103: 599–607.
Pin, P.A., Benlloch, R., Bonnet, D., Wremerth-Weich, E., Kraft, T., Gielen, J.J.L. et al. (2010) An antagonistic pair of FT homologs mediates the control of flowering time in sugar beet. *Science* 330: 1397–1400.
Qu, Z.L., Zhong, N.Q., Wang, H.Y., Chen, A.P., Jian, G.L. and Xia, G.X. (2006) Ectopic expression of the cotton non-symbiotic hemoglobin gene GhHbd1 triggers defense responses and increases disease tolerance in *Arabidopsis*. *Plant. Cell. Physiol.* 47: 1058–1068.
Reeves, P.A., He, Y., Schmitz, R.J., Amasino, R.M., Panella, L.W. and Richards, C.M. (2007) Evolutionary conservation of the FLOWERING LOCUS C-mediated vernalization response: evidence from the sugar beet (*Beta vulgaris*). *Genetics* 176: 295–307.
Richter, S. and Lamppa, G.K. (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. *Proc. Natl Acad. Sci. USA* 95: 7463–7468.

Ross, E.J., Shearman, L., Mathiesen, M., Zhou, Y.J., Arredondo-Peter, R., Sarath, G. et al. (2001) Nonsymbiotic hemoglobins in rice are synthesized during germination and in differentiating cell types. *Protoplasma* 218: 125–133.

Ross, E.J., Lira-Ruan, V., Arredondo-Peter, R., Klucas, R.V. and Sarath, G. (2002) Recent insights into plant hemoglobins. *Rev. Plant Biochem. Biotech.* 1: 173–189.

Ross, E.J., Stone, J.M., Elowsky, C.G., Arredondo-Peter, R., Klucas, R.V. and Sarath, G. (2004) Activation of the *Oryza sativa* non-symbiotic haemoglobin-2 promoter by the cytokinin-regulated transcription factor, ARR1. *J. Exp. Bot.* 55: 1721–1731.

Sainz, M., Perez-Rontome, C., Ramos, J., Mulet, J.M., James, E.K., Bhattacharjee, U. et al. (2013) Plant hemoglobins may be maintained in functional form by reduced flavins in the nuclei, and confer differential tolerance to nitro-oxidative stress. *Plant J.* 76: 875–887.

Saitou, N. and Nei, M. (1987) The neighbor–joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406–425.

Sasakura, F., Uchiumi, T., Shimoda, Y., Suzuki, A., Takenouchi, K., Higashi, S. et al. (2006) A class 1 hemoglobin gene from Alnus firma functions in symbiotic and nonsymbiotic tissues to detoxify nitric oxide. *Mol. Plant Microbe Interact.* 19: 441–450.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3: 1101–1108.

Seregelyes, C., Mustardy, L., Ayaydin, F., Sass, L., Kovacs, L., Endre, G. et al. (2000) Nuclear localization of a hypoxia-inducible novel non-symbiotic hemoglobin in cultured alfalfa cells. *FEBS Lett.* 482: 125–130.

Servaites, J.C. and Geiger, D.R. (1974) Effects of light intensity and oxygen on photosynthesis and translocation in sugar beet. *Plant Physiol.* 54: 575–578.

Shimoda, Y., Nagata, M., Suzuki, A., Abe, M., Sato, S., Kato, T. et al. (2005) Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in *Lotus japonicus*. *Plant Cell Physiol.* 46: 99–107.

Smagghe, B.J., Blervacq, A.S., Blassiau, C., Decottignies, J.P., Jacquot, J.P., Hargrove, M.S. et al. (2007) Immunolocalization of non-symbiotic hemoglobins during somatic embryogenesis in chicory. *Plant Signal. Behav.* 2: 43–49.

Smagghe, B.J., Hoy, J.A., Percifield, R., Kundu, S., Hargrove, M.S., Sarath, G. et al. (2009) Review: correlations between oxygen affinity and sequence classifications of plant hemoglobins. *Biopolymers* 91: 1083–1096.

Sowa, A.W., Duff, S.M.G., Guy, P.A. and Hill, R.D. (1998) Altering hemoglobin levels changes energy status in maize cells under hypoxia. *Proc. Natl Acad. Sci. USA* 95: 10317–10321.

Spyrakis, F., Bruno, S., Bidon-Chanal, A., Luque, F.J., Abbruzzetti, S., Viappiani, C. et al. (2011) Oxygen binding to *Arabidopsis thaliana* AHb2 nonsymbiotic hemoglobin: evidence for a role in oxygen transport. *IUBMB Life* 63: 355–362.

Spyrakis, F., Lucas, F., Bidon-Chanal, A., Viappiani, C., Guallar, V. and Luque, F.J. (2013) Comparative analysis of inner cavities and ligand migration in non-symbiotic AHb1 and AHb2. *Biochim. Biophys. Acta* 1834: 1957–1967.

Stanke, M., Diekhans, M., Baertsch, R. and Haussler, D. (2008) Using native and syntenically mapped cDNA alignments to improve de novo gene finding. *Bioinformatics* 24: 637–644.

Stitt, M. and Hurry, V. (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. *Curr. Opin. Plant. Biol.* 5: 199–206.

Strand, A., Hurry, V., Gustafsson, P. and Gardestrom, P. (1997) Development of *Arabidopsis thaliana* leaves at low temperatures releases the suppression of photosynthesis and photosynthetic gene expression despite the accumulation of soluble carbohydrates. *Plant J.* 12: 605–614.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739.

Taylor, E.R., Nie, X.Z., MacGregor, A.W. and Hill, R.D. (1994) A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. *Plant Mol. Biol.* 24: 853–862.

Trevaskis, B., Watts, R.A., Andersson, C.R., Llewellyn, D.J., Hargrove, M.S., Olson, J.S. et al. (1997) Two hemoglobin genes in *Arabidopsis thaliana*: the evolutionary origins of leghemoglobins. *Proc. Natl Acad. Sci. USA* 94: 12230–12234.

Vazquez-Limon, C., Hoogewijs, D., Vinogradov, S.N. and Arredondo-Peter, R. (2012) The evolution of land plant hemoglobins. *Plant Sci.* 191–192: 71–81.

Vigeolas, H., Huhn, D. and Geigenberger, P. (2011) Nonsymbiotic hemoglobin-2 leads to an elevated energy state and to a combined increase in polyunsaturated fatty acids and total oil content when overexpressed in developing seeds of transgenic *Arabidopsis* plants. *Plant Physiol.* 155: 1435–1444.

Vinogradov, S.N., Fernandez, I., Hoogewijs, D. and Arredondo-Peter, R. (2011a) Phylogenetic relationships of 3/3 and 2/2 hemoglobins in Archaeplastida genomes to bacterial and other eukaryote hemoglobins. *Mol. Plant* 4: 42–58.

Vinogradov, S.N., Hoogewijs, D. and Arredondo-Peter, R. (2011b) What are the origins and phylogeny of plant hemoglobins?. *Commun. Integr. Biol.* 4: 443–445.

Vinogradov, S.N. and Moens, L. (2008) Diversity of globin function: enzymatic, transport, storage, and sensing. *J. Biol. Chem.* 283: 8773–8777.

von Heijne, G., Steppuhn, J. and Herrmann, R.G. (1989) Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.* 180: 535–545.

Wang, Y.H., Kochian, L.V., Doyle, J.J. and Garvin, D.F. (2003) Two tomato non-symbiotic haemoglobin genes are differentially expressed in response to diverse changes in mineral nutrient status. *Plant, Cell Environ.* 26: 673–680.

Watts, R.A., Hunt, P.W., Hvitved, A.N., Hargrove, M.S., Peacock, W.J. and Dennis, E.S. (2001) A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. *Proc. Natl Acad. Sci. USA* 98: 10119–10124.

Yamori, W., Hikosaka, K. and Way, D.A. (2014) Temperature response of photosynthesis in C3, C4, and CAM plants: temperature acclimation and temperature adaptation. *Photosynth. Res.* 119: 109–117.

Zarka, D.G., Vogel, J.T., Cook, D. and Thomashow, M.F. (2003) Cold induction of *Arabidopsis* CBF genes involves multiple ICE (inducer of CBF expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiol.* 133: 910–918.

Zhao, M.G., Chen, L., Zhang, L.L. and Zhang, W.H. (2009) Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in *Arabidopsis*. *Plant Physiol.* 151: 755–767.

Zybailov, B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q. et al. (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS One* 3: e1994.