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Overexpression of *BvHb2*, a Class 2 Non-Symbiotic Hemoglobin from Sugar Beet, Confers Drought-Induced Withering Resistance and Alters Iron Content in Tomato

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Abstract: Drought stress is one of the major threats to agriculture and concomitantly to food production. Tomato is one of the most important industrial crops, but its tolerance to water scarcity is very low. Traditional plant breeding has a limited margin to minimize this water requirement. In order to design novel biotechnological approaches to cope with this problem, we have screened a plant cDNA library from the halotolerant crop sugar beet (*Beta vulgaris* L.) for genes able to confer drought/osmotic stress tolerance to the yeast model system upon overexpression. We have identified the gene that encodes BvHb2, a class 2 non-symbiotic hemoglobin, which is present as a single copy in the sugar beet genome, expressed mainly in leaves and regulated by light and abiotic stress. We have evaluated its biotechnological potential in the model plant *Arabidopsis thaliana* and found that *BvHb2* is able to confer drought and osmotic stress tolerance. We also generated transgenic lines of tomato (*Solanum lycopersicum*) overexpressing *BvHb2* and found that the resulting plants are more resistant to drought-induce withering. In addition, transgenic lines overexpressing *BvHb2* exhibit increased levels of iron content in leaves. Here, we show that class 2 non-symbiotic plant hemoglobins are targets to generate novel biotechnological crops tolerant to abiotic stress. The fact that these proteins are conserved in plants opens the possibility for using Non-GMO approaches, such as classical breeding, molecular breeding, or novel breeding techniques to increase drought tolerance using this protein as a target.

Keywords: plant hemoglobin; non-symbiotic; sugar beet; tomato; drought stress; iron; overexpression; *Beta vulgaris*; *Solanum lycopersicum*; water deprivation

1. Introduction

The human population is currently about 7 billion and, according to the Food and Agriculture Organization FAO, it is expected to increase to up 9 billion in 2050. To provide a robust food supply for this growing population, agriculture productivity must increase concomitantly. Abiotic stress, and specifically drought is a major constraint for global food yield [1]. Drought affects millions of

people per year and is considered to be one of the main causes of famines. In the near future this problem is likely to increase due to anthropogenic global warming and to the fact that most of the population growth is occurring in arid and semiarid areas. Traditional plant breeding has improved the effectiveness of some crops under drought stress [2], but there is a consensus in the scientific community that traditional plant breeding is not going to be enough to secure food production to a growing population, and therefore, plant biotechnology, including genetic engineering and new breeding techniques, will be required to increase food production. The use of biotechnological strategies to develop crops with increased yield even under adverse environmental conditions is a means of addressing this objective [3]. The development of crops able to grow in arid lands will allow the extension of cultivable lands or increase the productivity of already established agricultural soils and thus, increase food production and diminish the water footprint, mainly in developing countries. It is known that drought is one of the main factors driving deforestation in developing countries [4].

There are reports in the literature of transgenic plants whose drought tolerance has been improved [5]. This improvement has been attained by the engineering of genes responsible for the synthesis of organic osmolytes, transcription factors, LEA proteins, and hormones [6]. In most cases, the election of the transgene is based on previous knowledge of plant physiology or data from systems biology experiments. These strategies may present several problems, such as late identification of growth limiting factors, which can result in very modest increases in tolerance. In other cases, the overexpression of the transgene can cause some side effects [7] or produce a yield penalty that affects production under non-stressed conditions [8,9]. One approach to override these problems and to identify the limiting factors for growth under stress conditions is to screen for genes able to confer tolerance. Heterologous expression of plant genes in yeast is a powerful technique to identify genes with potential to develop plants tolerant to abiotic stress [10,11]. Using this approach, we previously isolated and characterized a Serine O-Acetyl transferase [12] and a TIP aquaporin [13]. In the present report, we describe BvHb2, a class 2 non-symbiotic plant hemoglobin from the halotolerant crop *Beta vulgaris*, identified by expressing a plant cDNA library in an osmosensitive strain of the baker's yeast *Saccharomyces cerevisiae*.

Plant hemoglobins were originally identified in the rhizobium nodules of nitrogen fixing leguminoses. Its most characterized function is to transport oxygen in an extreme low oxygen environment [14]. Early evidence of hemoglobins in non-leguminous plants was first reported by Bogusz et al. in 1988 [15]. The first non-symbiotic hemoglobin was identified by Taylor in 1994 in barley [16]. Subsequent studies identified several families of non-symbiotic hemoglobin. Class 2 non-symbiotic hemoglobins (subdivided into class 2a and class 2b) have low oxygen affinity [17,18] and, unlike class 1 hemoglobins, are not induced by hypoxia [19]. There is an additional class of plant hemoglobins, class 3 or truncated hemoglobins, with unusual binding properties for O_2 and CO independent of the concentration of these molecules [20].

Several previous reports have described the effects of overexpressing plant hemoglobins. In Arabidopsis, overexpression of *AtGLB1* increases tolerance to hypoxia [21] and hydrogen peroxide [22]. Overexpression of *AtGLB1* or *AtGLB2* promotes early bolting by scavenging NO [23]. Overexpression of class 1,2, or 3 hemoglobins alters shoot organogenesis [24], and when *AtGLB2* is expressed in developing seeds of arabidopsis, it leads to an increase in polyunsaturated fatty acids, total oil content, and elevated energy state [25]. This diversity of effects and phenotypes suggest that plant hemoglobins may be targeted for improving resistance to multiple stresses [18]. A characterization of the five non-symbiotic hemoglobins from *Lotus japonicus* (Regel) K. Larsen unveiled their differential role in stress responses [26].

Here, we present evidence that BvHb2 confers tolerance to drought stress in the model plant *Arabidopsis thaliana* and in a horticultural crop, tomato *(Solanum lycopersicum* L.). Tomato is the most consumed horticultural crop worldwide. In 2018, the world production was about 182 million tones, with China and India as the main producers worldwide [27]. Tomato plants produce a great yield of fruit and total biomass during its life cycle, so its water demand is very high [28]. When exposed

to water deficit, tomato increases its root/aerial ratio, which causes a drastic reduction of plant size and fruit yield. Therefore, there is a considerable interest in the development of new tomato plant lines with lower water requirements. Traditional plant breeding has been used to screen the available commercial stocks to select for drought resistant genotypes [29] or introduce genes from drought tolerant wild relatives, such as *Solanum pennellii* (Correll) D'Arcy [30]. The transformation of tomato plants with foreign genes can broaden the strategies of tolerance improvement. The first protocol for transforming tomato plants with *Agrobacterium tumefaciens* (currently *Rhizobium radiobacter*) was published in 1986 [31], after that, there have been a lot of modifications and improvements. Nowadays, most protocols are based on explants and in vitro cultivation from cotyledons [32]. There are reports in the bibliography describing transgenic lines of tomato with enhanced fruit [33–35]; and resistance to pest and pathogen [36–38]; herbicides [39] and abiotic stress [32,40,41]. At the present moment, there are no transgenic tomato varieties on the market, even though the first commercialized transgenic crops were the Flav Savr tomatoes developed by Calgene [42] and shortly after the endless summer tomato by Monsanto [43,44]. In both cases, the desired trait was a delayed ripening, but they were withdrawn from the market. There are several descriptions in the literature of transgenic tomato plants that show tolerance to drought or salt stress obtained by overexpression of yeast genes [32,45–47]; plant genes, such as the aquaporin SlTIP2;2 [48]; an osmotin gene from tobacco [49]; the bacterial *codA* gene [50]; the transcription factor ATHB7 [51]; the CaRma1H1 gene, a hot pepper ring finger E3 ubiquitin ligase [52]; the dehydrin Tas14 [53]; and the microRNA 169 [54]. In most cases, the selection of the transgene was based on evidence provided by results in other plants or data base mining of results of gene expression during abiotic stress. Here, we propose a novel strategy based on using a crop resistant to abiotic stress as a source of genes to biotechnologically improve an abiotic stress sensitive crop.

In this work, we demonstrate how a halotolerant industrial crop (*Beta vulgaris*) can be used as a source of genes to increase abiotic stress resistance in crops with lower tolerance to abiotic stress (*Solanum lycopersicum*) using two different model systems (*Saccharomyces cerevisiae* and *Arabidopsis thaliana*) to screen and test for the interesting genes. Using this strategy, we found a class 2 non-symbiotic hemoglobin from *Beta vulgaris* (BvHb2) that is able to confer tolerance to drought-induced withering and alter iron content upon overexpression. We observed these phenotypes in the model system *A. thaliana* and in transgenic tomato lines derived from a commercial tomato variety. To our knowledge, this is the first evidence of a biotechnological improvement of abiotic stress in a crop plant using a class 2 non-symbiotic hemoglobin.

2. Materials and Methods

2.1. Screening of a cDNA Library from NaCl-Induced Sugar Beet Leaves and Isolation of BvHb2

The plant cDNA library from salt-stressed sugar beet leaves used in this study was previously described in the following references [55,56]. cDNA was ligated into the λPG15 phage and inserted into the pYPGE15 shuttle expression plasmid [57]. The library was used to transform the yeast mutant strain JM164 (*S. cerevisae* W303-1A, mat a/α, *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* GAL+, *gpd1::TRP1*) by the LiCl method [58] and selecting the colonies able to grow at 1.7M sorbitol, as described [12]. Yeast were grown as described in [59]. Standard methods for yeast culture were used. The plasmid DNA was isolated and retransformed to confirm that the phenotype was dependent on the plasmid and not a chromosomal mutation and sequenced. The pYPGE-*BvHb2* plasmid was named JM179.

2.2. Homology Modelling

To model the structure of BvHb2 we used the RAS MOL platform [60] using the published structure of rice Hb1 as a template [61].

2.3. Plant Material and Growth Conditions for Sugar Beet

Seeds of *B. vulgaris* cv Dita [55,56] were sterilized for 3 min in pure ethanol and washed three times with sterile water and placed on sterile vermiculite at 25 °C to germinate. Ten-day-old seedlings were transferred to plastic pots containing 1 L of sterilized peat moss/vermiculite (1:1, *v/v*). Plants were grown under greenhouse conditions (23 ± 2 °C, 70 ± 5% relative humidity and 16 hr light/8 hr dark. For the light experiments, 3-week-old plants were transferred for 24 h to a dark chamber and then transferred to a chamber with continuous white, red, far red, or blue light. At the indicated times, leaf samples were collected and frozen in liquid nitrogen prior to RNA extraction.

2.4. RNA Isolation and Methods

Total RNA was isolated from the indicated parts of sugar beet or Arabidopsis or tomato leaves by phenol/chloroform extraction followed by precipitation with LiCl [62] and stored at −80 °C. RNA was subjected to DNase treatment and reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen), following the manufacturer's instructions. To rule out the possibility of genomic DNA contamination, all the cDNA sets were checked by running control PCR reactions with aliquots of the same RNA that had been subjected to the DNase treatment, but not to the reverse transcription step. Northern blot using the *BvHb2* probe was carried out as described [13]. qRT-PCR analysis was performed using an iCycler iQ apparatus (BioRad, Hercules, CA, USA). The sequences of the primers used for PCR amplifications were as follows: actin8 (At1g49240 5'-AGTGGTCGTACAACCGGTATTGT-3' and 5'-GAGGATAGCATGTGGAAGTGAGAA-3' and for *BvHb2*, 5'-CAGAGAAAGATGAAGCTTTGG-3', and 5'-CCTTCATCTCAGCTTTGATGG-3'. Individual real-time RT-PCR reactions were assembled with oligonucleotide primers (0.15 μM each), 10.5 μL of 2× iQSYBR Green Supermix (Bio-Rad; containing 100-mM KCl, 40-mM Tris-HCl pH 8.4, 0.4-mM dNTPs, 50-U/μL iTaq DNA polymerase, 6-mM MgCl2, 20-nM SYBR Green I, 20-nM fluorescein) plus 1 μL of a 1:10 dilution of each corresponding cDNA in a final volume of 21 μL. We used 6 biological replicates in each case.

The PCR program was as follows: 3 min incubation at 95 °C to activate the hot-start recombinant Taq DNA polymerase, followed by 32 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C, where the fluorescence signal was measured. Standardization was carried out based on the expression of the *A. thaliana* actin 8 gene in each sample. The relative abundance of transcripts was calculated by using the $2^{-\Delta\Delta Ct}$ method [63]. Experiments were repeated three times, with the threshold cycle (Ct) determined in triplicate, using cDNAs that originated from six RNAs extracted from six different biological samples. Negative controls without cDNA were used in all PCR reactions.

2.5. Construction of Arabidopsis thaliana Transgenic Plants and Growth Conditions

Arabidopsis thaliana plants (ecotype Columbia 0) were grown under greenhouse conditions (16 h light/8 h dark, at 23 ± 2 °C and 70 ± 5% relative humidity) in pots containing a 1:2 vermiculite:soil mixture. Plants were irrigated twice a week with nutrient solution for 3 weeks, as described [64]. The JM881 (pXCS-*35S::BvHb2*) plasmid and the empty control plasmid pXCS-HA.STREPII [13,65] were introduced into the C58C1 *A. tumefaciens* strain by electroporation. Transformed bacteria were grown in standard media. Plants were transformed by flower infiltration [66]. Transgenic plants transformed with the *35S:BvHb2* and the *bar* gene or with the *bar* gene were selected in pots by adding Basta 0.76 mM, and the expression of the transgene was further confirmed by quantitative reverse transcription (qRT)-PCR from six biological replicates, as described in Section 2.4. We generated about 10 transgenic lines for each construction and selected 3 lines presenting mendelian segregation and a similar expression of the transgene. Experiments were performed with T2 homozygous lines. We also confirmed that none of the lines transformed with the control plasmid presented a distinctive phenotype (except the Basta tolerance), when compared with the non-transgenic parental line (Col. 0). Experiments were performed with 10 to 30 plants. A detailed description of the in vitro and hydroponic growth conditions can be found in [64,67]. For the drought stress experiments, seeds of the indicated lines were planted in pots

(10 plants per pot). Pots containing control plants were irrigated as indicated above, whereas drought conditions were applied by withholding water or nutrient solution in 3-week-old plants. Pictures were taken after 4 days. The height of plants was determined after completing the development under normal watering conditions or three weeks after stopping the irrigation.

2.6. Plant Material, Tomato Transformation and Evaluation of Transgenic Plants and Progenies

Tomato seeds from the UC-82 variety were provided by INTERSEMILLAS S.A. (Valencia, Spain). Seeds were immersed for 10 min in a solution of 25% commercial bleach (40 g/L active chlorine), washed twice with sterile deionized water for 5 min each and then sown in Petri dishes containing nutrient medium MB (MS (Murashige and Skoog) salts including vitamins, 2% sucrose, 0.6% plant agar (DUCHEFA, the Netherlands)). Cotyledons were obtained from 13-day-old plantlets and transferred to Petri dishes (90 × 15 mm) with shoot induction medium (SIM): Murashige and Skoog salts, 3% (*w/v*) Sucrose, 0.7% Plant agar and 0.2 mg/L zeatine riboside [68]. Plates were kept in the dark for 1 day. Then, cotyledon explants were infected with *A. tumefaciens* GV3101: pMP90RK [69] either transformed with JM881 or with the empty control vector pXCS-HAStrep [13,65]. After co-cultivation for 1 day, explants were washed with liquid MB medium supplemented with 200 mg/L cefotaxime and 100 mg/L vancomycine and transferred to SIM medium containing both antibiotics plus 1 mg/L Basta. After 20 days, explants were subcultivated in the same medium, but with 1.5 mg/L of Basta to ensure a proper selection of transformants. The selected transformants were transferred to tubes with MB medium supplemented with 100 mg/L of Ticarcillin-clavulanate to eliminate any remaining Agrobacterium. In all cases, the pH of the media was adjusted to 5.8 and sterilized at 121 °C for 20 min. Growth regulators and antibiotics were purified by filtration and added to sterilized media. Cultures were incubated in a growth chamber at 26 °C ± 2 °C under a 16 h photoperiod with cool white light provided by Sylvania cool white F37T8/CW fluorescent lamps (90 μmol m^{-2} s^{-1}). Regenerated plantlets were acclimatized in pots (80 × 80 × 95 mm) with Hortimix® substrate and transferred to a growth incubator at 25–27 °C with 16 h of light at 71 mmol m^{-2} s^{-1} photon flux density and 62% relative humidity and then transferred to the greenhouse. Transformations were confirmed by using PCR with primers checkGLB2D: 5′-CAGAGAAAGATGAAGCTTTGG-3′ and checkGLB2R: 5′-CCTTCATCTCAGCTTTGATGG-3′ with 51 °C annealing temperature and 2 min of extension. We generated about 10 different transgenic lines and selected 3 different transformant lines presenting mendelian segregation and similar expression of the transgene. Experiments were performed with homozygous T2 plants. Experiments were performed with 10 to 30 plants.

For the drought assays transgenic stable homozygotic T2 progenies transformed either with the JM881 or with the empty vector were cultivated in 35X44 mm plant pots until the plants had 3–4 true leaves. Plants were arranged in a complete random block design with six blocks where the different seed sources were randomized within the block. Seedlings were grown in a growth chamber under controlled conditions set at a 23 °C, relative humidity at 70%, and a photoperiod of 16 h (130 μmol m^{-2} s^{-1}). Seedlings were watered to full capacity every 2 days, alternatively twice with water and once with complete Hoagland's nutrient solution containing all essential macro- and micro-nutrients. After 8 weeks of growth, healthy plants of similar size from each seed source, accounting for 6–8 replicates per seed source, were randomly assigned to control and drought treatment; control seedlings were irrigated every 2 days whereas drought conditions were applied by withholding water. Withering was visually monitored after 5 days. We repeated the experiment three times.

For iron content determination, plant tissue from 2-month-old plants was dried at 70 °C for 4 days. Dry weight was determined, and ions were extracted by a 30 min incubation in 1 mL of 0.1M HNO$_3$ at room temperature. Then samples were centrifuged, and supernatant was diluted with 4 mL of milliQ water and filtered (22 μM). Iron was determined by atomic absorption spectroscopy using a SensAA (GBC Scientific Equipment), as described [70].

2.7. Statistical Analysis

The data analysis for this paper was generated using the Real Statistics Resource Pack software (Release 6.8). Copyright (2013–2020) Charles Zaiontz [71]. The means were considered to be significantly different at $p < 0.05$ after Tukey's honestly significant difference (HSD) test [72].

3. Results

3.1. Isolation and Characterization of BvHb2

The first objective of this work was to find genes from the halotolerant crop *Beta vulgaris* useful for developing biotechnological applications. For this purpose, we screened a plant cDNA library from *Beta vulgaris* leaves treated with NaCl, [56,73]. This library harbors the plant cDNAs in a yeast episomal plasmid. The expression of the plant genes is controlled by the strong constitutive promoter from the phosphoglycerokinase (*PGK1*) gene and the terminator of the *CYC1* gene [57]. We transformed this library in an osmosensitive yeast strain, which is a diploid strain homozygous for the *gpd1* mutant allele [12], and we isolated plasmids able to confer the ability to grow in 1.7 M sorbitol. After obtaining 241.000 independent transformants we plated them onto the high osmolarity media, where the JM164 strain cannot grow. We isolated seven cDNAs. Six contained a Serine O-Acetyl-transferase (BvSAT1), which has already been described [12], and one a plasmid contained a 872 bp cDNA encoding an open reading frame (ORF) of 456 bp (Figure 1a). Standard BLAST analysis showed that our cDNA was identical to the cDNA clone ys016149 (accession number Be590299; GenBank) that encodes a putative class 2 non-symbiotic hemoglobin [74,75]. A BLASTp analysis against the *Beta vulgaris* genome data base [76] confirmed that the sequence we have identified was a bona fide sugar beet gene. The sequence was identical to the hypothetical protein KMT16735, encoded by the *BVRB_3g050180* gene, which is located in chromosome 3, between positions 2,300,796 and 2,304,597. The genomic structure of this gene presents four exons and three introns (Figure 1b). We did not find any paralogues, pointing to a single copy of this gene in the *Beta vulgaris* genome, similar to what has been observed in most plants [77].

The translation of the 456bp ORF predicted a protein of 152 amino acids (Figure 1c). The WoLF pSORT algorithm predicted a cytoplasmic localization for this protein. We used the RASMOL software to model the predicted 152 amino acids of BvHb2 using the structure of RiceHb1 as a template [78] (Figure 1c). As expected, we found a 3-on-3 alpha-helical sandwich embracing a hexacoordinated heme group (Figure 1d). We found that the amino acids relevant for the heme binding pocket in BvHb2 are His 63 (distal histidine) and His 98 (proximal histidine). The other residues nearest to the ligand binding site in BvHb2 are Phe30, Phe44 and Val67 (Figure 1c,d). According to our predicted model the distance between the distal and proximal histidine is 6.84 Å (Figure 1d).

We further investigated whether the plasmid containing *BvHb2* could confer tolerance to different stresses in yeast. We found that overexpression of *BvHb2* enhanced yeast growth under heat stress (37 °C) and in the presence of $CuSO_4$ and sodium nitroprusside (Figure 1e). We did not find any phenotype when sodium or lithium chloride was added to the medium or under continuous growth at 10 °C suggesting that the tolerance was not due to a change in the monovalent cation concentration or in membrane fluidity (data not shown).

3.2. Regulation of BvHb2 Expression

Once the identity of BvHb2 as a class 2 non-symbiotic plant hemoglobin was confirmed, we investigated its expression pattern in *Beta vulgaris*. First, we studied the expression of *BvHb2* in different plant organs. For this purpose, we extracted RNA from root, hypocotyl, petiole, cotyledon, and leaves, and examined the *BvHb2* gene expression by Northern blot. In 3-week-old plants grown in soil under no stress conditions, *BvHb2* is mainly expressed in leaves and weakly in hypocotyl. We detected only minor expression in other organs (Figure 2a). This observation is in agreement with

previous reports [79] and is consistent with the fact that the cDNA library used in our initial screening was derived from *B. vulgaris* leaves.
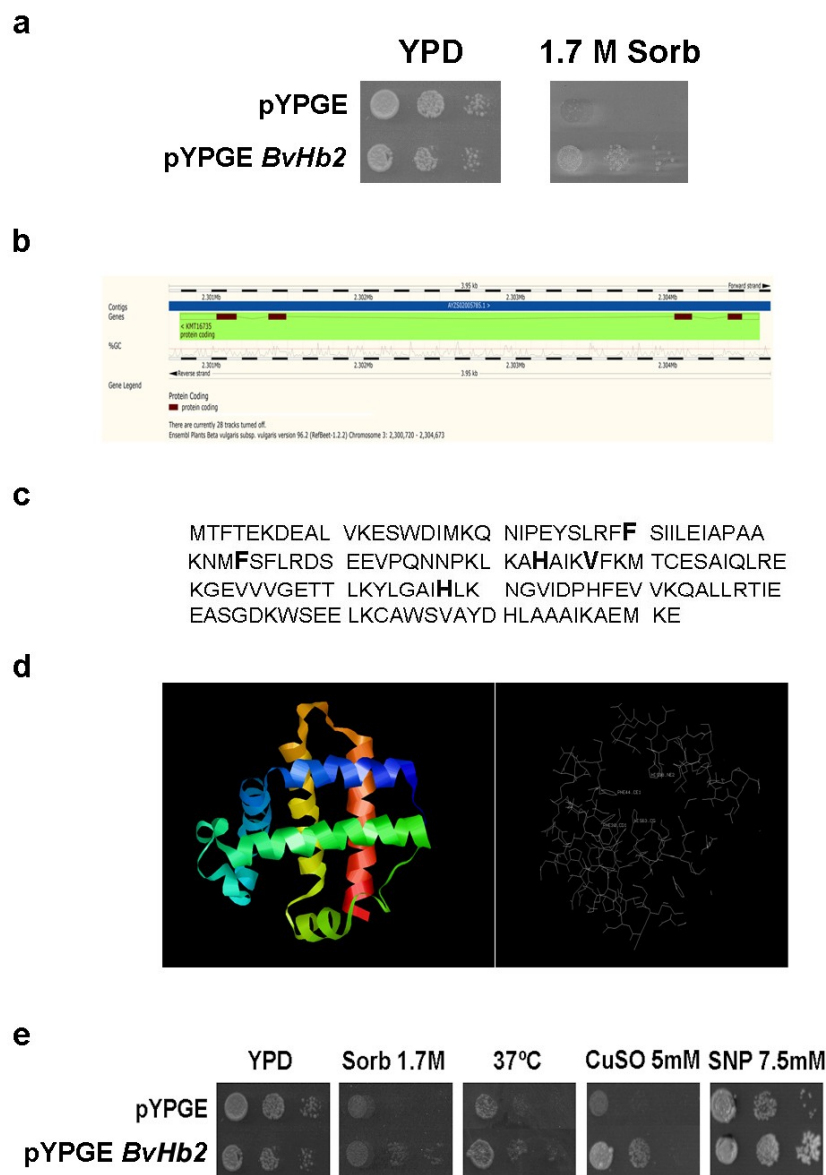
a

YPD   1.7 M Sorb

pYPGE

pYPGE *BvHb2*

b

c

MTFTEKDEAL   VKESWDIMKQ   NIPEYSLRF**F**   SIILEIAPAA
KNM**F**SFLRDS   EEVPQNNPKL   KA**H**AIK**V**FKM   TCESAIQLRE
KGEVVVGETT   LKYLGAI**H**LK   NGVIDPHFEV   VKQALLRTIE
EASGDKWSEE   LKCAWSVAYD   HLAAAIKAEM   KE

d

e

YPD   Sorb 1.7M   37°C   CuSO 5mM   SNP 7.5mM

pYPGE

pYPGE *BvHb2*

Figure 1. *BvHb2* encodes a class 2 non-symbiotic plant hemoglobin, which confers osmotic stress tolerance upon overexpression in yeast. (**a**) *BvHb2* confers osmotic stress tolerance in yeast upon overexpression. Serial dilutions (1/10, 1/100, and 1/1000) of control JM164 yeast cells transformed with the empty plasmid (pYPGE) and yeast cells transformed with the pYPGE plasmid containing the *BvHb2* cDNA (pYPGE *BvHb2*) were spotted onto YPD medium and grown at the indicated sorbitol concentration. Pictures were taken after 5 days. (**b**) Genomic structure of *BvHb2*. The gene is on chromosome 3 and has four exons and three introns. (**c**) Analysis of *BvHb2* translated sequence. In bold are the two coordination histidines and the amino acids in close contact with the ligand. (**d**) BvHb2 model using the Rasmol software. Left: protein ribbon diagram. Right diagram indicating the coordination histidines. (**e**) *BvHb2* confers pleiotropic stress tolerance in yeast upon overexpression. Serial dilutions (1/10, 1/100, and 1/1000) of control JM165 yeast cells transformed with the empty plasmid (pYPGE) and yeast cells transformed with the pYPGE plasmid containing the *BvHb2* cDNA (pYPGE*BvHb2*) were spotted onto YPD medium at the indicated temperature or containing the indicated additive. Pictures were taken after 5 days.
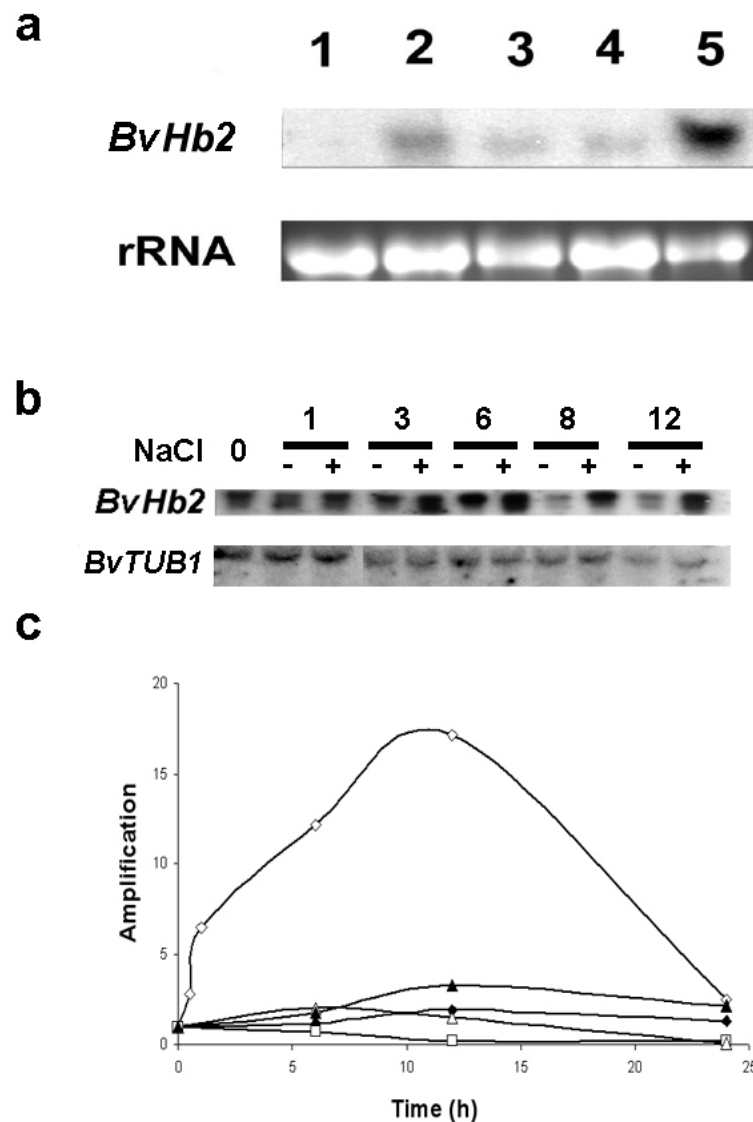
Figure 2. *BvHb2* is mainly expressed in leaves and upregulated by stress and white light. (**a**) Northern blot analysis of total RNA (15 μg) from root (1), hypocotyl (2), petiole (3), cotyledon (4), and leaf (5) from sugar beet. The lower panels show the amount of 26S rRNA loaded for each treatment (ethidium bromide staining). (**b**) Northern blot analysis of total RNA (15 μg) from sugar beet leaves. RNA was isolated from 3-week-old sugar beet leaves at 0, 3, 6, 8, and 12 h after growing with (+) or without (−) 250 mM NaCl. The same RNA blot was hybridized with an α3 tubulin probe (*BvTUB1*) from *B. vulgaris* used as control of filter transfer. (**c**) Amplification of *BvHb2* (fold change) determined by real time quantitative PCR in 3-week-old plants kept in the dark (filled diamonds) or exposed to white light (empty diamonds), blue light (filled triangles), red light (empty triangles), or far-red light (empty squares). For clarity errors bars were not included, but errors were less of 10% of the value (*n* = 6).

The sugar beet cDNA library used to identify *BvHb2* was constructed from plants under abiotic stress induced by sodium chloride, so we checked whether NaCl could regulate *BvHb2* expression. We found that *BvHb2* expression was high when the first samples were taken but decreased with time under control conditions. The NaCl treatment could counteract this decrease and maintained *BvHb2* expression high over time (Figure 2b). Samples were cultivated in a greenhouse with natural illumination and first samples were taken in the morning, after several hours exposed to natural light. There is evidence in the literature that *BvHb2* expression is regulated by light [79]. We further

investigated this regulation trying to sort out which type of light could be driving *BvHb2* expression. For this, we maintained plants for 24 h in dark, and then transferred them to growth chambers with no light (dark), or illuminated with white, blue, red, or far-red light. Samples were taken at the indicated time point and RNA levels monitored by quantitative RT-PCR. Only white light was able to induce expression of *BvHb2*, but after 12 h even though the illumination was maintained, expression decreased (Figure 2c), therefore *BvHb2* expression depends on the presence of white light and we cannot discard an effect of the circadian rhythm.

3.3. Overexpression of BvHb2 in Arabidopsis thaliana Confers Drought Tolerance

BvHb2 was isolated for its ability to confer tolerance to osmotic stress upon overexpression in yeast. We wanted to investigate whether the tolerance conferred by *BvHb2* could be reproducible in a plant system. For this purpose, we used the model plant *A. thaliana* to investigate the effect of the overexpression of this protein and its biotechnological potential in plants. We inserted the *BvHb2* ORF into the pXCS2HA::STREP2 binary plasmid [65], which enables the transformation in plants and the expression of the transgene under the control of the 35S promoter and the NOS terminator, obtaining strong and constitutive expression. The resulting plasmid (named JM881) contains the *bar* gene, for transformant selection with Basta. We generated different transgenic lines and chose three different lines that presented strong expression and mendelian segregation of the Basta resistance gene (data not shown). We also generated a control transgenic line by transforming with the empty plasmid and selecting a line resistant to Basta, but with no other phenotype.

In order to check the phenotypes of the generated transgenic plants, we designed an experiment in hydroponic culture where the conditions can be finely controlled and a large number of plants can be monitored in parallel. We germinated plants and transferred them to liquid MS media or to liquid MS media containing mannitol to induce osmotic stress and measured the fresh and dry weight of the plants. Overexpression of *BvHb2* enhanced biomass accumulation under stress conditions but had no effect under control conditions (Figure 3a). Then we wanted to check whether this improvement under water deficit could also be observed in soil conditions. We designed an experiment in which we planted the different lines in pots. When plants developed rosette leaves, half of the plants of each line were maintained with normal watering, whereas watering was stopped for the other half. Transgenic lines maintained green leaves for longer (Figure 3b upper panel), and under water deficit epicotyls of transgenic lines were longer than control plants (Figure 3a lower panel).

The BvHb2 protein contains a heme group that binds iron. In order to determine whether the overexpression of *BvHb2* alters iron homeostasis in the transgenic lines, we measured the iron levels in leaves. We found that transgenic plants presented a higher iron content than control plants (Figure 3c).

3.4. Obtention of Solanum lycopersicum Transgenic Lines Overxpressing BvHb2 and Evaluation for Drought Tolerance

After confirming the biotechnological potential of *BvHb2* overexpression in the model plant *A. thaliana*, we decided to test it in major industrial crop, such as tomato. As starting material, we used a standard commercial cultivar (UC82). We transformed cotyledon explants with *A. tumefaciens* containing the JM881 binary plasmid to generate the *A. thaliana* transgenic plants in previous experiments, (*35S:BvHb2* and *bar* gene for selection). Regenerated shoots were selected with Basta, and transgene insertion and expression were confirmed by PCR and quantitative RT-PCR. We selected three different T2 homozygotic transgenic lines for the subsequent experiments, and as a control, we used a T2 homozygous transgenic line transformed with the empty plasmid, in which we could not detect any phenotype. We investigated the tolerance of these lines to water stress by interrupting irrigation 3 weeks after germination. Transgenic plants expressing the *BvHb2* gene tolerated the water withdrawal longer than transgenic control plants transformed with the empty plasmid. The number of plants without withering symptoms after five days was significantly higher in three different transgenic lines (Figure 4a).
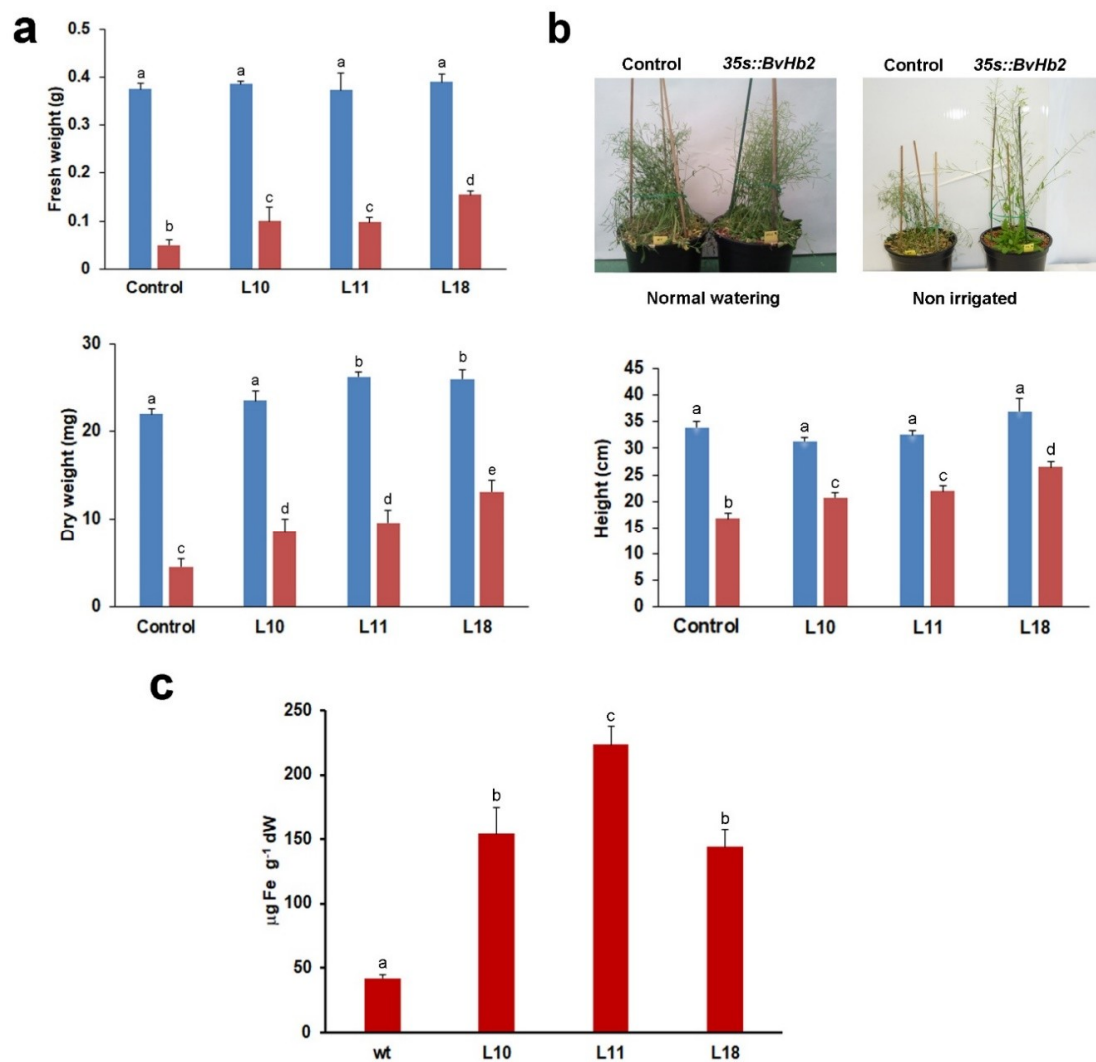
Figure 3. Arabidopsis transgenic lines overexpressing *BvHb2* present drought tolerance and increased iron content in leaves. (**a**) Lines overexpressing *BvHb2* accumulate more biomass upon osmotic stress. Plants were grown for six weeks in hydroponic MS medium (blue bars) or in hydroponic MS medium containing 120 mM mannitol (red bars). Bars represent means ± standard error. *X* axis represents the control line (transformed with the empty vector) and the different transgenic lines (transformed with 35S::*BvHb2*). Data with different letters differ significantly ($p < 0.05$), as determined by Tukey's HSD test ($n = 30$). (**b**) Upper panel: Overexpression of *BvHb2* conferred resistance to water deprivation. Irrigation was stopped in 3-week-old plants and pictures were taken after 4 days. Lower panel: The height of plants was determined after completing the development (cm) under normal watering conditions (blue bars) or three weeks after stopping the irrigation (red bars). Bars represent means ± standard error. *X* axis represents the control line (transformed with the empty vector) and the different transgenic lines (transformed with 35S::*BvHb2*). Data with different letters differ significantly ($p < 0.05$), as determined by Tukey's HSD test ($n = 30$). (**c**) Overexpression of *BvHb2* increases iron content in leaves. Leaves from 6-week-old plants were obtained. The dry weight was determined, and ions were extracted with HNO_3. Iron content was determined by atomic absorption spectroscopy. Bars represent means ± standard error. *X* axis represents the control line (transformed with the empty vector) and the different transgenic lines (transformed with 35S:*BvHb2*). Data with different letters differ significantly ($p < 0.05$), as determined by Tukey's HSD test ($n = 10$).
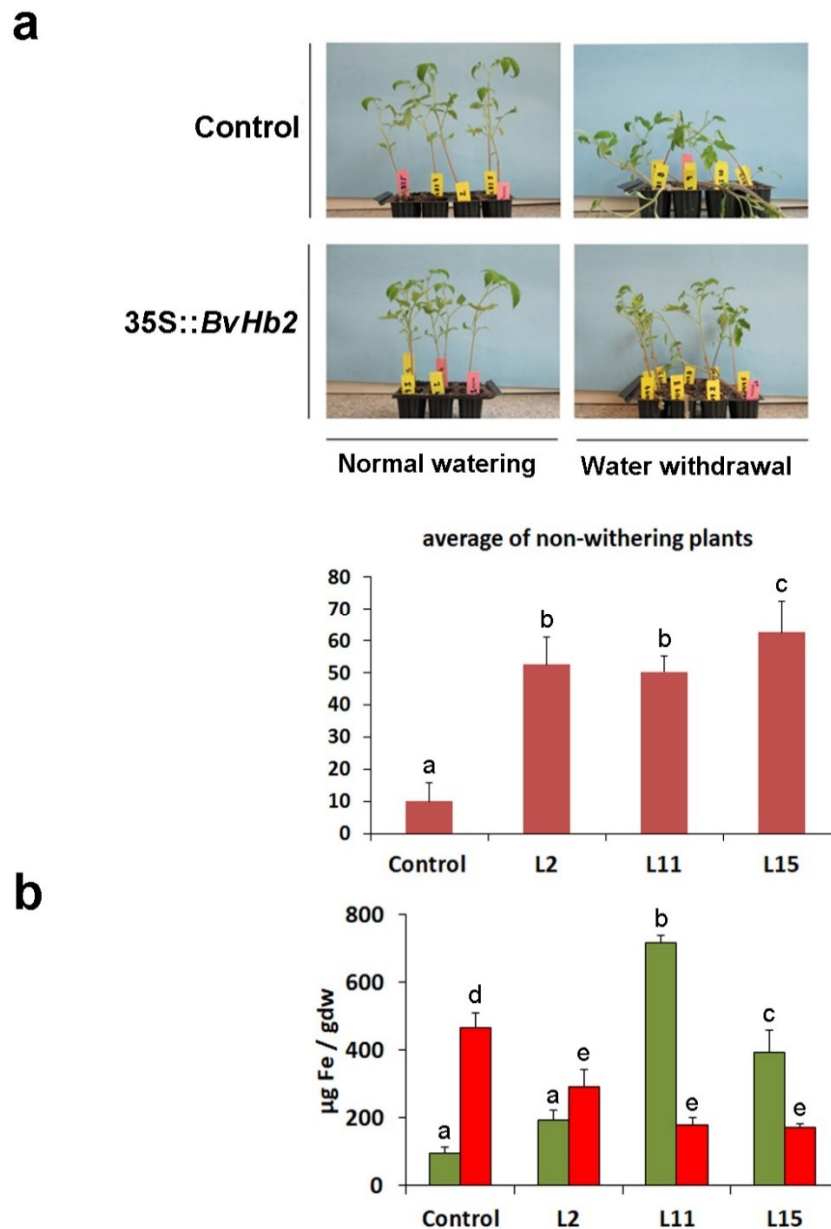
Figure 4. Transgenic tomato lines overexpressing *BvHb2* present higher drought-induced withering resistance and altered iron distribution. (**a**) Overexpression of *BvHb2* conferred resistance to water deprivation. Upper panel: irrigation was stopped for 3-week-old plants, and pictures were taken after 5 days. Lower panel: Quantification of withering plants. Bars represent means ± standard error. *X* axis represents the control line (transformed with the empty vector) and the different transgenic lines (transformed with 35S::*BvHb2*). Data with different letters differ significantly ($p < 0.05$), as determined by Tukey's HSD test ($n = 20$). (**b**) Overexpression of *BvHb2* increases iron content in leaves and decreases iron content in fruit. Plants were grown until mature fruits were developed (approximately 4 months). Leaves (green bars) and fruits (red bars) were obtained. Dry weight was determined, and ions were extracted with HNO_3. Iron content was determined by atomic absorption spectroscopy. *X* axis represents the control line (transformed with the empty vector) and the different transgenic lines (transformed with 35S::*BvHb2*). Bars represent means ± standard error. Data with different letters differ significantly ($p < 0.05$), as determined by the Tukey HSD test using biological replicates ($n = 10$).

We also checked whether the overexpression of *BvHb2* could have any effect on iron homeostasis, as we observed in Arabidopsis (Figure 3c). We cultivated control and *BvHb2* expressing lines under

standard conditions and measured the iron content in leaves and fruits. We found that transgenic tomato lines also accumulated more iron in leaves, but the iron content diminished in fruits (Figure 4b).

4. Discussion

The yeast overexpression approach has proven to be a powerful technique to identify genes with high biotechnological potential [10]. This technique has the limitation that yeast is a unicellular organism and many signal transduction pathways related to stress responses are not conserved. Genes encoding integral membrane proteins are also usually underrepresented in cDNA libraries (our unpublished observations), and not all plant cDNAs are suitable for heterologous expression in yeast due to differential codon usage [11]. Even with these limitations, the internal medium of yeast is very similar to a plant cell, and many biochemical mechanisms of ion homeostasis, drought or oxidative stress response are conserved. This has allowed for the identification of genes involved in mRNA metabolism [56], lipases [80], or translation initiation [81] that are able to confer abiotic stress tolerance when overexpressed in plants. Moreover, some yeast genes are described to confer abiotic stress tolerance upon overexpression in plants [32] or even increased yield [82].

We have found that overexpression of *BvHb2* is able to confer abiotic stress tolerance in three different organisms. This gene is not conserved in the yeast genome, as yeast only contains two flavohemoglobins [83]. It is known that plant non-symbiotic hemoglobins have an enzymatic action against reactive nitrogen species preventing nitrosamination of proteins and lipids. Nevertheless, evidence indicates that this role would be predominantly performed by class 1 non-symbiotic hemoglobins, while class 2 non-symbiotic hemoglobins would act as oxygen donors in developing tissues [18]. Our results confirm the differential roles and physiological effects of both hemogobins, as the overexpression of a class 1 non-symbiotic hemoglobin from spinach (*Spinacia oleracea* L.), a crop phylogenetically very close to *Beta vulgaris*, in *Arabidopsis thaliana* lowered nitrate and other abiotic stresses tolerance [84]. We showed that under physiological conditions in sugar beet, *BvHb2* is mainly expressed in leaves and the expression is regulated by white light and to a lesser extent by abiotic stress. This suggests a photosynthesis-related function. In addition, in the same screening in which we found *BvHb2*, we also found a serine acetyl transferase gene [12] and, using a different yeast growth media, an antioxidant enzyme (unpublished). Taken together, these results suggest that *BvHb2* may have some kind of antioxidant activity that is preventing the deleterious effect of stress, as it is known that oxidation is produced under abiotic stress conditions. BvHb2 is also able to alter iron distribution in tomato by promoting accumulation of iron in leaves and decreasing levels in fruit. Even though we have used the constitutive 35S promoter from cauliflower mosaic virus, *BvHb2* in its native conditions in sugar beet *BvHb2* is mainly expressed in leaves, so we cannot discard that the stability of the protein could be different in different tissues and this would explain the changes in the iron distribution.

In this report, we describe the complete process from screening for useful genes from a halotolerant crop (*B. vulgaris*), testing the effectivity in two different model systems (*S. cerevisiae* and *A. thaliana*) and finally developing a GMO crop (*S. lycopersicum*) with increased resistance to drought-induced withering (under greenhouse conditions). Tomato cultivation has a very variable water footprint, depending on crop yield and local agro-climatic and soil conditions [85]. Therefore, developing new varieties with reduced water requirements is a major objective for agronomist, and therefore, the strategy herein described has tremendous potential in order to develop crops with increased market potential. These two varieties were a commercial failure, and nowadays there are no transgenic tomatoes on the market. This commercial failure has several causes, the reticence of some consumers against GMO being one of them [86]. Setting aside the GMO debate, there are useful outcomes which can be developed from heterologous screening strategies that circumvent the generation of new GMO crops. The complete tomato genome was published [87]. Blast analysis showed that there is one locus which encodes a putative class 2 non-symbiotic hemoglobin gene (*Solyc03g071690.2*). We proved that class 2 non-symbiotic hemoglobins are a biotechnological target for engineering abiotic stress tolerance in tomato and, probably, increased organoleptic quality. Strategies such as TILLING microarrays or

molecular breeding can be used to increase the expression of the *Solyc03g071690.2* gene in tomato, or the native *Hb2* (also known as *GLB2*) in other crops, and gain the demonstrated tolerance without generating novel GMO lines, facilitating the commercial potential of these new lines. Iron deficiency is a worldwide agricultural problem that is especially serious in soils with high pH, such as calcareous soils, which comprise approximately 30% of cultivated soils worldwide [88]. There is another opportunity to develop the results presented in this study using new breeding techniques, such as using CRISPR/Cas9 technology to modify the promoter of *Hb2*, introducing the GMO trait in grafting rootstock, and then produce non-transgenic crops from the scion or stacking several traits to increase tolerance or to combine agronomical improvement with enhanced nutritional content. A final outcome of the present report is that we showed that expression of *BvHb2* alters the iron distribution in the plant. We used a constitutive 35S promoter and observed more accumulation in leaves and less in fruit (Figure 4b). Although further studies are required, we hypothesize that generating transgenics lines which overexpress *BvHb2* under the control of a fruit- or a seed-specific promoter, such as ACC-oxidase, E8, or USP [89], could result in plants producing biofortified fruits or cereals with higher iron content.

5. Conclusions

Here, we present a strategy to identify genes from an industrial halotolerant crop and transfer them to a major crop such as tomato, increasing resistance to drought-induced withering and an altered iron distribution. This strategy is based on the use of two different model systems and a final validation in an industrial crop. We found that yeast can be used to identify a plant gene (*BvHb2*) able to confer to abiotic stress tolerance (drought), and when this gene is overexpressed in a model plant (*Arabidopsis thaliana*) or in a horticultural crop (*Solanum lycopersicum*), it also induces phenotypes related to abiotic stress tolerance and alters iron distribution. These findings open the possibility for developing drought resistant crops with increased iron content using either biotechnological strategies or new breeding techniques to increase the expression of the Hb2 gene.

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