



Expressing the sunflower transcription factor HaHB11 in maize improves waterlogging and defoliation tolerance

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Abstract

The sunflower (*Helianthus annuus*) transcription factor HaHB11 (*H. annuus* Homeobox 11) belongs to the homeodomain-leucine zipper family and confers improved yield to maize (*Zea mays*) hybrids (Hill × B73) and lines. Here we report that transgenic maize lines expressing *HaHB11* exhibited better performance under waterlogging, both in greenhouse and field trials carried out during three growth cycles. Transgenic plants had increased chlorophyll content, wider stems, more nodal roots, greater total aerial biomass, a higher harvest index, and increased plant grain yield. Under severe defoliation caused by a windstorm during flowering, transgenic genotypes were able to set more grains than controls. This response was confirmed in controlled defoliation assays. Hybrids generated by crossing B73 *HaHB11* lines with the contrasting Mo17 lines were also tested in the field and exhibited the same beneficial traits as the parental lines, compared with their respective controls. Moreover, they were less penalized by stress than commercial hybrids. Waterlogging tolerance increased via improvement of the root system, including more xylem vessels, reduced tissue damage, less superoxide accumulation, and altered carbohydrate metabolism. Multivariate analyses corroborated the robustness of the differential traits observed. Furthermore, canopy spectral reflectance data, computing 29 vegetation indices associated with biomass, chlorophyll, and abiotic stress, helped to distinguish genotypes as well as their growing conditions. Altogether the results reported here indicate that this sunflower gene constitutes a suitable tool to improve maize plants for environments prone to waterlogging and/or wind defoliation.

Introduction

Maize (*Zea mays*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) are the crops with the largest production worldwide, providing 60% of the global caloric human intake (UN Food and Agriculture Organization, 2021). Maize is used for human nutrition but also for ethanol synthesis and animal feeding. It is a C4 summer crop grown as single-cross (i.e. F1) hybrids presenting high heterosis expression conducive to high grain yield and large biomass production (Duvik, 2005). The improved yield observed in modern hybrids was mainly attributed to enhanced leaf area duration and post-silking crop growth (Rajcan and Tollenaar, 1999), as well as to improve radiation and water use efficiencies (Curin et al., 2020). Despite these positive traits, maize is remarkably vulnerable to stress conditions during the critical period of the kernel set (Cerrudo et al., 2013) due to the dominated condition within the plant of the grain-bearing organ (the ear) with respect to the pollen-producing organ (the tassel). Therefore, most breeding efforts have focused on enhancing abiotic stress tolerance (Tollenaar and Wu, 1999; Chen et al., 2016) and reducing apical dominance (Tollenaar and Wu, 1999; Duvik et al., 2004).

Although great efforts are devoted to maize breeding, the target production environments of this species are exposed continuously to abiotic stress that penalizes grain yields (Pedersen et al., 2017). Among abiotic stress factors and due to global warming, the incidence of floods that expose crops to waterlogging is rising every decade worldwide (Pedersen et al., 2017). Flooding events predominate in several areas of the main maize cropping regions. For instance, May 2018–April 2019 was the wettest 12-month period in 124 years of records in the United States (NASA Earth Observatory, 2019), producing a marked delay in maize sowing date due to soggy soils (U.S. Department of Agriculture, 2019) and a decline in grain yield (UN Food and Agriculture Organization, 2019). According to the projection based on multiple climate models, this scenario will not get better; excessive early season rainfall events will increase in most parts of the world during this century (Hirabayashi et al., 2021). Global climate change also impacts the severity of storms and hail incidence, indicating a significant increase in such phenomena. Strong winds and hail produce different degrees of damage to maize crops depending upon the defoliation intensity and the opportunity of the event (Battaglia et al., 2019).

The adaptation of rice to flooding has been deeply studied, since this species is resilient to anaerobic soil conditions. The investigation about this harmful stress was divided into that provoked by waterlogging (root system inundation) and the generated by submergence of the aerial system (Bailey-Serres et al., 2012a; Voeselek and Bailey-Serres, 2015). Waterlogging causes quick soil O₂ depletion because rhizosphere microbes rapidly consume it, provoking changes in the fixation of nitrogen and other nutrients. Another effect of waterlogging is a decrease in soil pH, that increases toxic metals and phosphorous solubility (Bailey-Serres and Voeselek, 2008; Setter et al., 2009). In these conditions,

plants become unable to cope with evaporative demand, reducing gas exchange and growth (Bramley et al., 2007). Gas diffusion is reduced 10⁴-fold, limiting not only oxygen for aerobic respiration but also the CO₂ needed for photosynthesis (Abiko et al., 2012).

When plants sense these environmental changes, they trigger molecular signaling pathways to cope with the stress, including specific modulation of gene expression and hormone homeostasis (Voeselek and Bailey-Serres, 2015). Among the hormones involved in plant response to flooding, ethylene plays a key role (Bailey-Serres and Voeselek, 2010; Bailey-Serres et al., 2012b; Voeselek and Sasidharan, 2013; Loreti et al., 2016; Sasidharan et al., 2018). Ethylene accumulates in the cells, eliciting the formation of reactive oxygen species (ROS), which play a dual role as signaling molecules and causing oxidative stress damage (Sasidharan et al., 2018; Yamauchi et al., 2018).

Adaptation to waterlogging stress also involves the fine-tuning of several genes, mostly associated with carbohydrate transport, anaerobic metabolism, cell wall remodeling, and detoxification. Among these genes, there are those encoding the enzymes invertase (INV); phosphohexose isomerase (PGI); glyceraldehyde-3-phosphate-dehydrogenase (GAPDH); phosphoglycerate kinase (PGK), and alcohol dehydrogenase (ADH) (Zou et al., 2010; Arora et al., 2017; Du et al., 2017). The root system is the most affected by waterlogging, and the adaptive changes include the generation of new adventitious roots and aerenchyma constituting a barrier to radial oxygen loss (ROL) (Abiko et al., 2012; Loreti et al., 2016; Yamauchi et al., 2018).

Defoliation stress is caused by varied factors such as storms, hail, leaf diseases, and herbivore attacks, causing a total or partial reduction in the leaf area of plants, leading frequently to reduce light interception, and consequently less biomass production through photosynthesis. In early defoliation events, the grain yield penalization is usually low provided the apical meristem is not injured causing plant death and stand reduction (Battaglia et al., 2019). By contrast, partial defoliation during the critical period for kernel set may decrease seed yield dramatically (Battaglia et al., 2019), depending upon the reduction caused in plant growth rate (Andrade et al., 1999). The extent of yield penalization due to defoliation during the active grain-filling period will depend upon the reduction caused to the source-sink ratio during this stage (Borrás et al., 2004).

Environmental factors are perceived by plants that display signal transduction pathways resulting in the degradation of superfluous biomolecules and the synthesis of others needed to deal with stress. In the first steps of such molecular responses, transcription factors (TFs) play a crucial role as master switches able to activate or repress entire metabolic pathways. In plants, there are numerous TFs (more than 1,500 in the model *Arabidopsis thaliana*) classified in families, mainly according to the conserved DNA binding domain. Among these families, the homeodomain-leucine zipper (HD-Zip) is unique to this kingdom and was

associated with abiotic stress responses (Perotti et al., 2017). Members of this family present high conservation of the HD-Zip domain, and the sequencing of whole genomes of different species revealed other uncharacterized functional motifs located in the N- and C-termini of these proteins (Arce et al., 2011). Notably, in sunflower (*Helianthus annuus*) and other Asteraceae species, there are HD-Zip I proteins exhibiting distinctive carboxy-termini. Among these divergent members, there is HaHB4, which confers tolerance to drought in wheat and soybean (*Glycine max*) plants (González et al., 2020), and HaHB11, which enhanced yield in B73 lines and Hill hybrids (Raineri et al., 2019). HaHB11 also conferred flooding tolerance to Arabidopsis plants, both to waterlogging and submergence (Cabello et al., 2016).

HaHB11 maize plants were previously assessed in greenhouse and field trials during three growing seasons. Phenotyping was carried out by measuring different traits conducive to characterize plant and crop growth, such as stem width and height, leaf area, total biomass, anthesis-silking interval (ASI), light interception, and grain yield (Raineri et al., 2019).

Based on this previous analysis, we had three main objectives: to evaluate the performance of *HaHB11* plants under waterlogged conditions, to obtain hybrids to test whether the beneficial characteristics observed in the lines were maintained, and to use remote sensing techniques aiming at differentiating transgenics from controls.

In this article, we describe greenhouse and field trials revealing that maize plants expressing the sunflower TF HaHB11 exhibit enhanced tolerance to waterlogging. Moreover, during one of these trials, a strong storm that provoked severe defoliation revealed that transgenic plants were able to withstand the negative effects of defoliation better than the nontransgenic ones. This response was corroborated in subsequent controlled assays. We obtained hybrids, using transgenic and nontransgenic B73 lines crossed to the contrasting MO17 parent. Transgenic hybrids had increased plant grain yield compared to the controls. Finally, nondestructive spectral analysis (remote-sensing) along the cycle of field-grown plants allowed distinguishing controls from transgenic genotypes.

Results

HaHB11 transgenic maize plants exhibit increased tolerance to waterlogging compared with controls in greenhouse and field assays

Although enormous differences exist between sunflower (the species from which *HaHB11* was isolated) and the model plant Arabidopsis, the evolutionary distance between Arabidopsis and maize is even greater. Hence, we wondered if the waterlogging tolerance, conferred by HaHB11 to Arabidopsis, was conserved in maize.

First, we carried out waterlogging assays with plants grown on pots filled with sand in the greenhouse. Several characteristics were assessed in two independent transgenic lines and null segregants, used as controls (B73). During the treatment

period, transgenic plants developed longer roots with increased biomass and achieved a larger leaf area than controls (Figure 1, A, B, D, and E). Moreover, compared with the null segregants, the leaves of *HaHB11* plants showed higher stomatal conductance (Figure 1C). Overall, these results suggest an increased waterlogging tolerance of *HaHB11* plants.

In the field, waterlogging usually persists for several days but not along all the life cycle. Hence, after the stress treatment, plants were placed into larger pots and were allowed to recover and grow in normal conditions. At the end of the life cycle, *HaHB11* transgenic plants developed larger leaf and stem areas and an extended period of leaf greenness and concurrent delayed senescence (Figure 1, F–I). These trends were accompanied by increased biomass (Figure 1H). Moreover, such plants had more nodal roots (Figure 1J) and enhanced kernel number (Figure 1L), whereas individual kernel weight (Figure 1K) did not differ between genotypes. Described traits explained the increased plant grain yield (Figure 1M) and healthy aspect of the produced kernels (Figure 1N). Notably, most differential traits between controls and transgenics, observed in normal conditions trials (Raineri et al., 2019) were maintained after this stress treatment.

Even though greenhouse assays gave us a preliminary idea about the performance of *HaHB11* transgenic plants after a waterlogging episode, field-grown maize is exposed to a combination of environmental conditions (irradiance level, wind, evaporative demand, etc.) that may modify results obtained in the greenhouse. However, the generation of waterlogging conditions in the field is rather difficult. Hence, we designed a mixed test to evaluate the performance of waterlogged maize (see Methods). Waterlogging was applied for 14 d to V4 plants grown in a large pot in the field (Figure 2A). After that, plants were transferred to soil and grown in standard conditions until harvest (Figure 2A). To further understand the distinctive root phenotype observed in the greenhouse (Figure 1, A, D, and E), we performed and analyzed transversal cuts. This study indicated that the transgenic genotype developed more xylem vessels than controls (Figure 2, B and C). Similar to the greenhouse scenario, *HaHB11* plants exhibited delayed senescence and increased chlorophyll content and light interception than controls (Figure 2, D–F), and developed more nodal roots, wider stems, and greater total aerial biomass (Figure 2, G–I), indicating a better recovery from waterlogging than their control counterparts. Regarding grain yield determination, transgenic plants partitioned more biomass into grains (Figure 2J), showing increased plant grain yield, explained by an improved grain setting (Figure 2, L and K).

HaHB11 transgenic maize plants withstood the hardship of an unexpected severe windstorm and exhibited improved performance than controls under defoliation

During the third waterlogging assay in the field, a severe windstorm with gusts of 107 km h⁻¹ hit the crop 11 d after

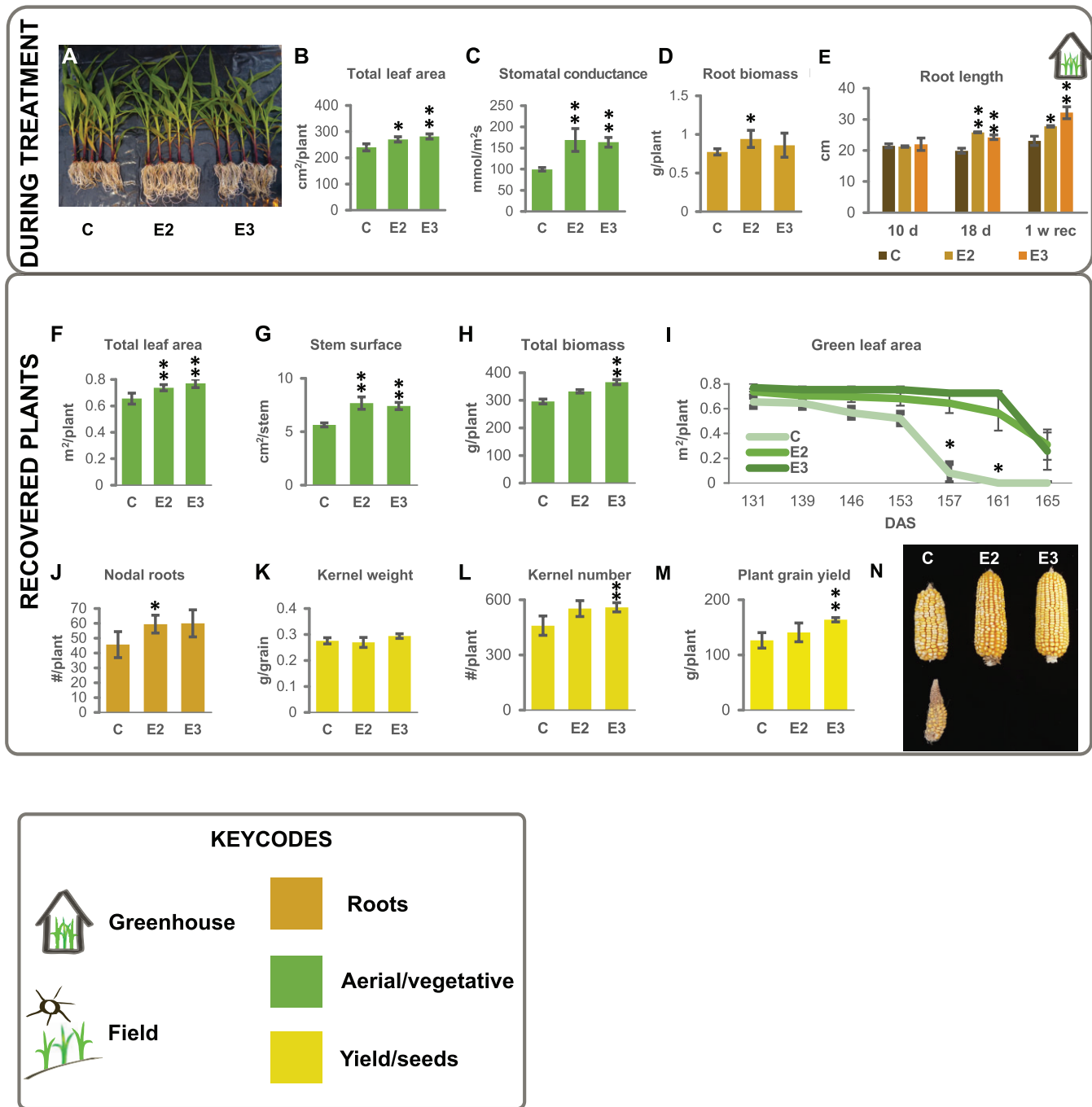


Figure 1 Transgenic plants expressing *HaHB11* exhibit enhanced waterlogging tolerance compared with their B73 controls in the greenhouse. Illustrative picture of maize plants subjected to waterlogging for 18 d (A). Total leaf area (B) and root biomass (D) after 18 d of waterlogging. Stomatal conductance after 7 d of treatment (C). Root length after 10 and 18 d of waterlogging treatment plus 1 week of recovery (E). Total leaf area at silking (F), stem surface area (G), total biomass (H), green leaf area (I), number of nodal roots (J), kernel weight (K), kernel number (L), and plant grain yield (M). Illustrative pictures of ears at harvest of control (C), and transgenic events (E2 and E3) in B73 background (N). Data represent means \pm standard error of the mean (SEM) of at least four biological replicates. Differences across means were analyzed by a Student's *t* test; asterisks indicate significant differences with respect to the control genotype (* for $P < 0.05$ and ** for $P < 0.01$). Bottom: codes used in all the illustrations.

silking, when still in the critical period for grain setting. Plants that were not killed and remained standing were completely defoliated with leaves preserving only their mid-ribs (Supplemental Figure S1). Surprisingly, transgenic *HaHB11* plants accumulated more plant biomass and

doubled plant grain yield of controls at maturity (Supplemental Figure S1). To confirm this serendipitous finding, we performed a greenhouse assay. We manually defoliated the plants 11 d after silking with similar results to those of the field (Supplemental Figure S2). These results

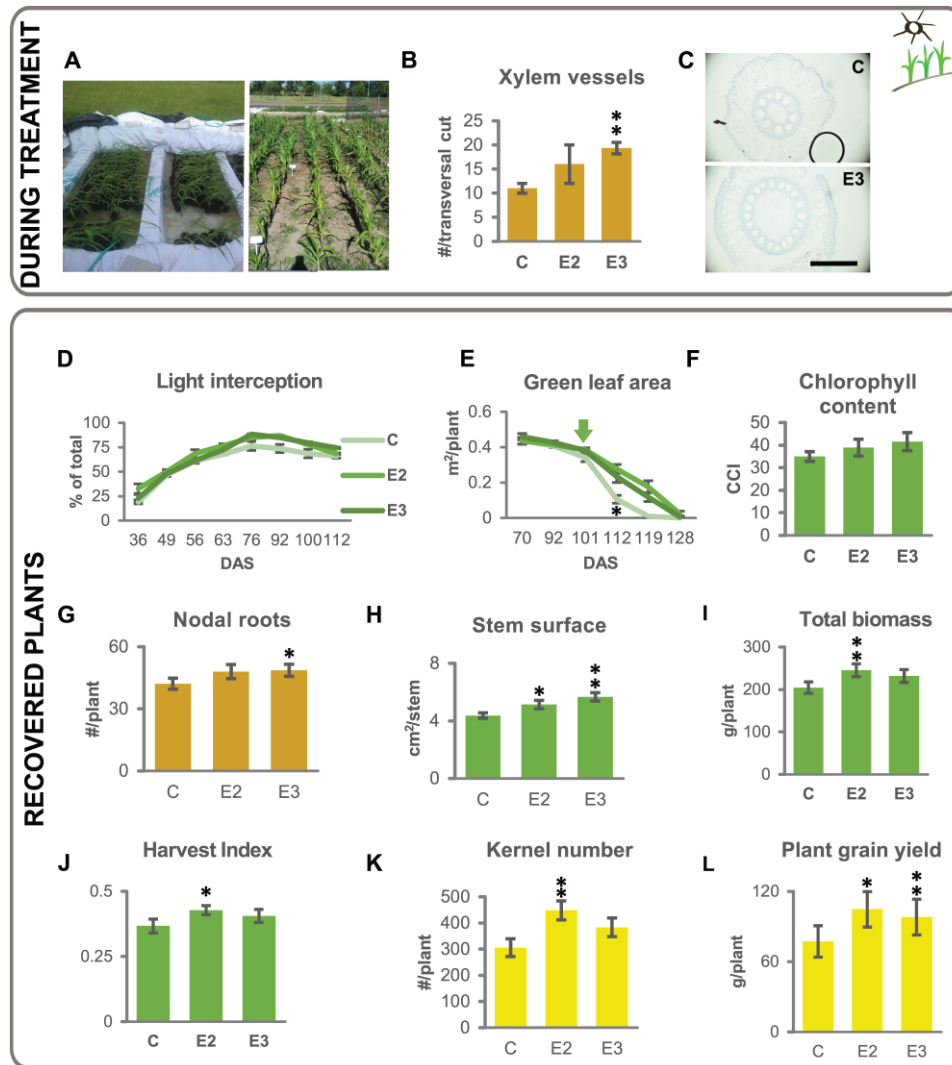


Figure 2 Field-grown transgenic plants expressing *HaHB11* tolerate waterlogging better than their B73 controls. Illustrative picture of waterlogging treatment in the field (left, A) and plants placed on soil after treatment (right, A). Xylem vessels per transversal root cuts (B) and image of the stained cross sections of control and E3 transgenic plants (C), after 2 weeks of waterlogging. Scale bars represent 1.5 mm. Light interception and green leaf area during the life cycle (D, E). Ear green leaf index, 100 d after sowing (DAS) measured as chlorophyll/carotenoid index (F). Nodal roots number (G), stem surface area (H), total biomass (I), HI (J), kernel number, and plant grain yield (K, L) of plants at harvest. Data represent means \pm SEM of at least three biological replicates. Differences across means were analyzed by a Student's *t* test; asterisks indicate significant differences respect to the control genotype (\dagger for $P < 0.05$ and $**$ for $P < 0.01$). C: control non-transgenic line; E2 and E3: maize independent transgenic lines carrying the gene *HaHB11*.

strongly suggested that transgenic plants can withstand defoliation during seed filling better than the controls, and, therefore, reduced the associated penalization to grain yield.

Is *HaHB11* able to improve the already enhanced growth promoted by heterosis in F1 maize hybrids?

Original transgenic maize plants were obtained in the Hill hybrid (a cross between the A188 and B73 inbreds) of poor performance compared with commercial hybrids. Hence, the progeny of Hill was backcrossed to B73 to recover the phenotype of this inbred line and reduce phenotypic segregation (Raineri et al., 2019). The beneficial effect of *HaHB11* on

several agronomic traits was detected, albeit at different extents, dependent on the heterozygosity levels.

Heterosis in maize usually increases yields around 72%–254% under no-stress conditions (Duvik, 2005; Munaro et al., 2011). Thus, we wondered if *HaHB11* would be able to maintain the previously described beneficial traits when expressed in an improved hybrid background, or the benefits conferred by the transgene may be masked due to the enhanced heterosis conferred by the cross of inbreds representative of contrasting heterotic groups. We performed crosses between B73 (transgenic and control plants) and the Mo17 public lines. The former belongs to the Reid Yellow Dent Group and the latter to the Lancaster Sure Crop

Group, and crosses between them have been widely studied (Troyer, 1999). In greenhouse assays, carried out in normal growth conditions, transgenic F1 hybrids, from the B73 \times Mo17 cross, accumulated more biomass, and exhibited delayed senescence (Figure 3, A–C). Moreover, similar to the results obtained with E2 and E3 lines (Figure 1), transgenic hybrids achieved significantly higher kernel number and plant grain yield than controls (Figure 3, D and E). These results strongly suggested that *HaHB11* expression could still improve hybrid plants, at least in standard conditions.

HaHB11 transgenic hybrids exhibited enhanced tolerance to waterlogging and defoliation in greenhouse and field assays

To determine *HaHB11* hybrids performance under abiotic stress conditions, we carried out defoliation and waterlogging assays first in the greenhouse. Defoliation was performed manually 11 d after silking on transgenic and control

hybrids. The leaf area removed from *HaHB11* plants was slightly larger than from controls because individual leaf area was larger among plants of the former (Figure 3F). Despite defoliation, at the end of the cycle, transgenics had increased biomass, seed weight, and kernel number than control plants (Figure 3, G–I). These results were similar to those obtained with the parental line B73, both in the field and the greenhouse. Notably, the F1 hybrid DK72-10 included as a reference, for comparison with a commercial product currently used by farmers, showed similar results as *HaHB11* lines.

Regarding waterlogging, root development was assessed by measuring different traits related to flooding tolerance. Similar to *HaHB11* lines, transgenic hybrids exhibited increased root volume and biomass, as well as a higher number of xylem vessels/pith area than controls (Figure 4, A–C, E). To understand whether these differential traits lead to differences in ROL, we treated the roots with methylene-blue. Figure 4D confirmed that control roots lost more

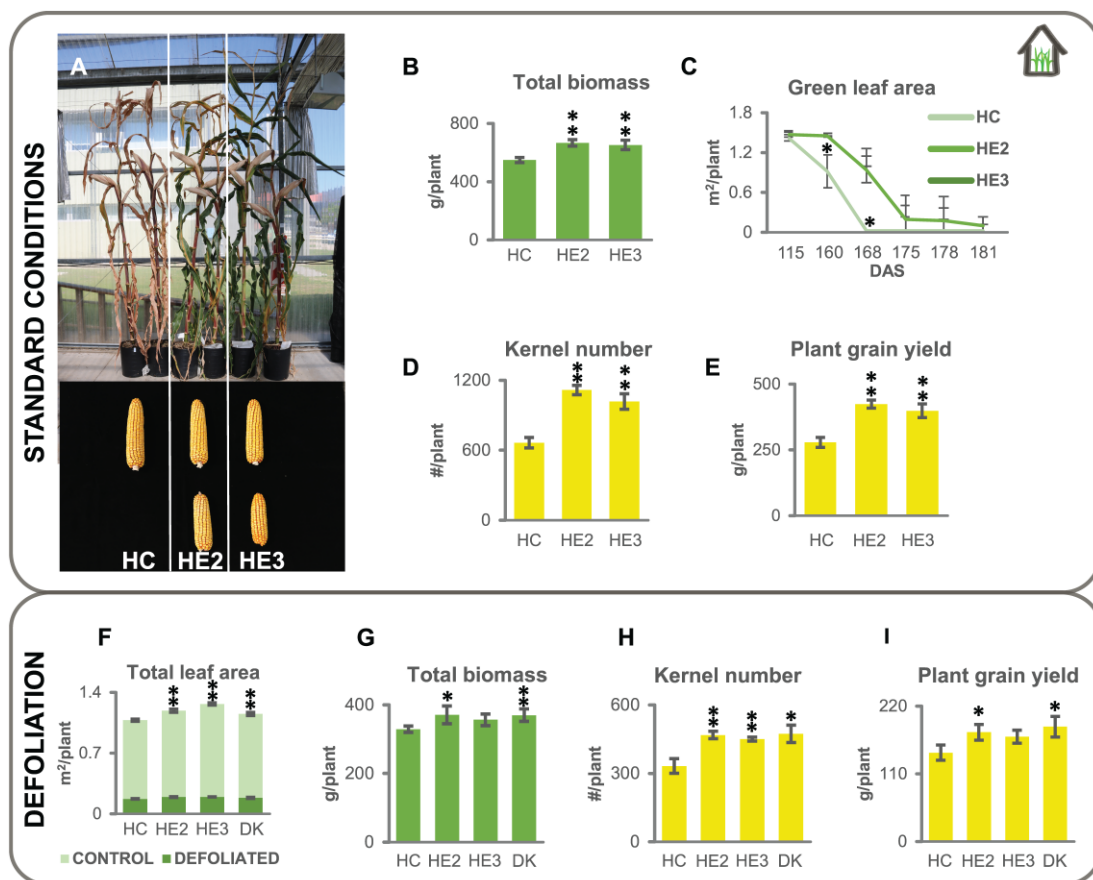


Figure 3 *HaHB11* transgenic hybrids tested in the greenhouse exhibited delayed leaf senescence and greater plant grain yield than controls in standard growth conditions and after defoliation. Upper panel: Illustrative photograph of B73 \times Mo17 hybrids plants and their ears (A). Total biomass (B). Senescence as indicated by green leaf area during grain filling (C). Kernel number (D) and plant grain yield (E) at the end of life cycle. Plants were grown in normal conditions. Lower panel: Total leaf area of plants before (F, light green), and after defoliation (F, dark green). Total biomass, kernel number, and plant grain yield of defoliated plants at harvest (G, H, I, respectively). Data represent means \pm SEM of at least four biological replicates. Differences across means were analyzed by a Student's *t* test; asterisks indicate significant differences respect to the control genotype (* for $P < 0.05$ and ** for $P < 0.01$). HC: control non-transgenic hybrid; HE2 and HE3: maize independent transgenic hybrids carrying the gene *HaHB11*; DK: commercial hybrid DK72-10.

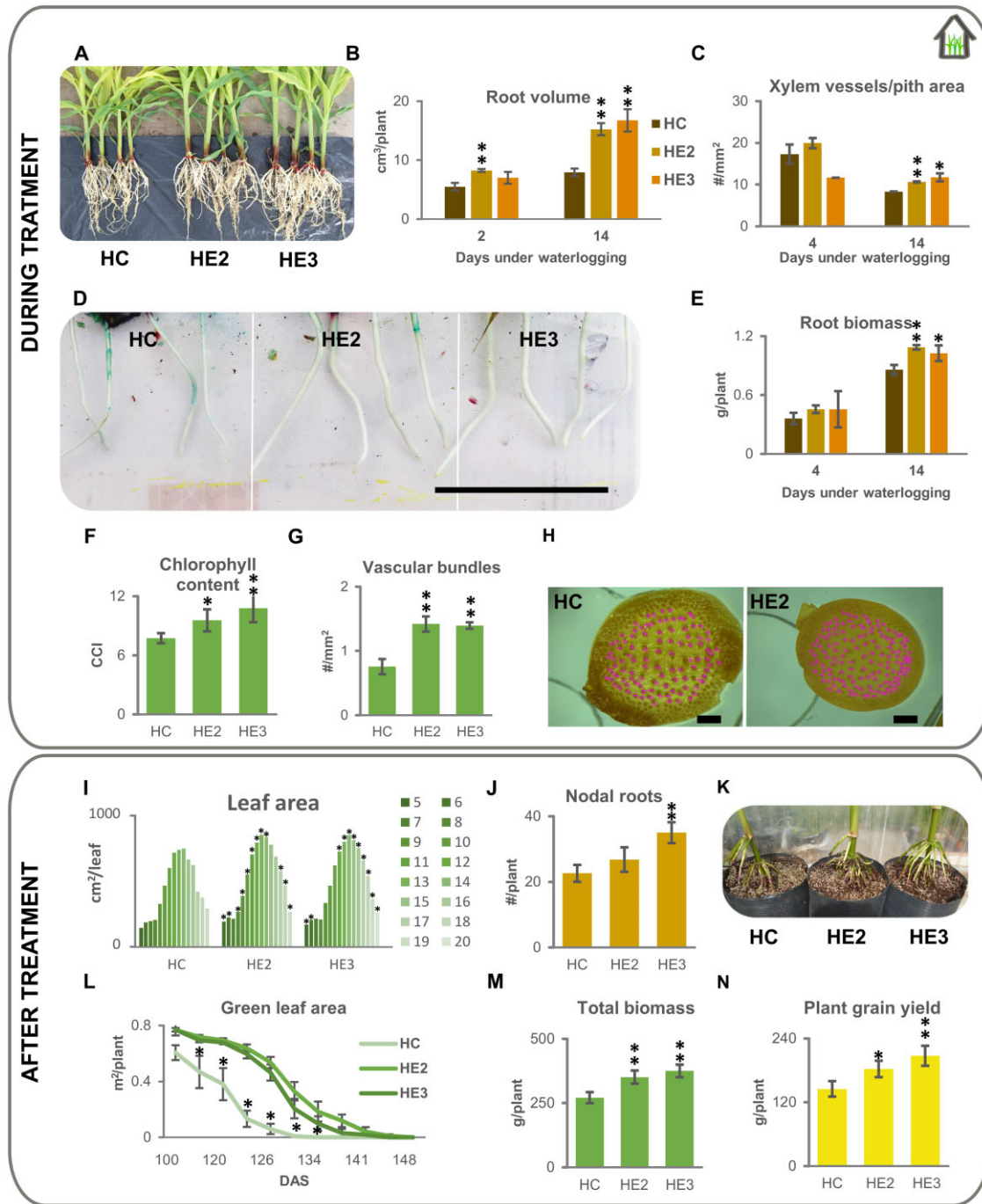


Figure 4 Transgenic hybrids expressing *HaHB11* showed improved performance during waterlogging stress and after recovery than controls in the greenhouse. Upper panel: Illustrative image of hybrid controls (HC) and transgenic events (HE2 and HE3) after 2 weeks of waterlogging treatment (A). Root volume (B), xylem vessels per pith area (C), and root biomass (E). Illustrative photograph of roots stained for 1 h with methylene blue, taken 2 weeks after initiating waterlogging treatment (D). Bar scale represents 10 cm. Chlorophyll content of the fifth leaf after 14 d of waterlogging treatment. F, Number and illustrative picture of vascular bundles in transversal sections of stems (G, H). Vascular bundles are marked with a pink cross. Bar scale in (H) represents 2 mm. Lower panel: Phenotype of maize plants subjected to 14 d of waterlogging, and then grown in normal conditions until harvest. Individual leaf area from leaf 5–20 (I). Total nodal roots and representative photograph of the plants (J, K). Green leaf area of plants at the end of grain filling (L). Total biomass and plant grain yield at harvest (M, N). Data represent means \pm SEM of at least four biological replicates. Differences across means were analyzed by a Student's *t* test; asterisks indicate significant differences respect to the control genotype (* for $P < 0.05$ and ** for $P < 0.01$).

oxygen (blue-stained roots) than *HaHB11* roots, indicating that this mechanism could be contributing to the hypoxia tolerance showed by the transgenic plants. This result may explain the enhanced chlorophyll content (Figure 4F) and the number of vascular bundles (Figure 4, G and H) of transgenic plants with respect to controls detected on 14 d after waterlogging when plants were already growing in standard conditions. At flowering, leaves were larger in the transgenics than in the control plants (Figure 4I), and both independent events exhibited delayed senescence (Figure 4L). Moreover, the transgenics showed a higher number of nodal roots, compared with controls on the hybrid background (Figure 4, J and K). All these characteristics explained, at least in part, the increased total biomass and plant grain yield of *HaHB11* plants (Figure 4, M and N).

In the field, the results were similar to those observed in the greenhouse when roots were evaluated after 12 d of waterlogging treatment. In transversal cuts of adventitious roots, transgenic plants developed a higher number of xylem vessels/pith area (Figure 5, A and D). Tissue damage was detected on the pith of control roots, whereas the medulla was intact in the transgenic ones (Figure 5A). Such damage could be generating an impaired function in control roots. Moreover, transgenic roots accumulated more lignin than the control ones (Figure 5B), suggesting that controls lost more radial oxygen than transgenics. Stating that transgenic plants deal better with the oxidative stress triggered by waterlogging, NBT staining was carried out 7 d after the treatment, resulting in reduced superoxide accumulation in *HaHB11* plants (Figure 5C).

Once the plants were growing in standard field conditions, the phenotype was assessed until the end of the life cycle. Individual leaf area was larger in *HaHB11* recovered plants compared with controls, and as in other mentioned assays, they had delayed senescence and developed more biomass (Figure 5, E–G). Kernel number and grain yield were higher for *HaHB11* plants in control conditions as well as after defoliation and waterlogging (Figure 5, H–J). The results suggest that plant grain yield increase is mainly due to the enhanced kernel number (Figure 5H). As expected, the DK72-10 hybrids had the highest plant grain yield compared with the rest of the evaluated genotypes in standard growth conditions (Figure 5J). However, penalization after defoliation was the highest for this hybrid, and its seed yield was similar to that of transgenic *HaHB11* plants (Figure 5J).

***HaHB11* modulates the expression of genes involved in carbohydrate metabolism, detoxification, and waterlogging response**

To unravel the molecular basis of waterlogging tolerance exhibited by *HaHB11* plants, we selected genes described as differentially regulated in tolerant accessions or after waterlogging treatments.

Transcriptional regulation is a dynamic and fine-tuned process that changes depending on various factors such as stress conditions. We were particularly interested in gene expression kinetics in *HaHB11* plants modulated by waterlogging. Samples were harvested from roots of plants grown in the greenhouse 1 d after treatment initiation, and from roots and leaves of field-grown plants on 4, 6, and 12 d after treatment initiation.

In roots, expression levels of *GAPDH*, *ADH1*, *PHOSPHOHEXOSE ISOMERASE 3 (PGI3/PHI3)*, *β-EXPANSIN (EXPBE7)*, *POLYGALACTURONASE (PG8)*, *SUCROSE TRANSPORTER 1*, *APETALA2-EREBP-TRANSCRIPTION FACTOR 83 (AP2/EREBP83)*, *GOLDEN2-LIKE 1*, *HEAVY METAL TRANSPORT/DETOXIFICATION SUPERFAMILY PROTEIN*, *HEXOKINASE 8*, *MINIATURE SEED1 (MN1)*, *PGK5*, and *INV2*, mainly involved in carbohydrate metabolism and transport, were assessed. In the greenhouse, *PGI3*, *GAPDH*, *INV2*, and *AP2/EREBP83* were differentially induced in *HaHB11* plants, whereas *ADH1*, *PGK5*, *MN1*, and *HMT* showed the opposite regulation (Figure 6 and Supplemental Figure S3). The scenario changed in the field. In these conditions, the more remarkable results were the earlier induction of *EXPBE7* in the transgenics (4 d) compared with controls (6 d), and the faster repression of *GADPH* 4 d after treatment (Figure 6). Among the selected genes, several did not show differential regulation between genotypes and others were not detectable in roots or leaves harvested at these developmental stages (Supplemental Figure S3).

In leaves, the evaluated genes were *GAPDH*, *ADH1*, *PGI3*, *SUGAR WILL EVENTUALLY BE EXPORTED TRANSPORTER 13a*, and *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 3*. Such genes are involved in carbohydrate metabolism and transport. Among them, those showing substantial differences between genotypes were *GAPDH*, *ADH*, and *PGI3*. *GAPDH* was only induced in *HaHB11* plants after 12 d of treatment (Supplemental Figure S3), whereas *PGI3* showed higher expression levels in the transgenic leaves than in the control ones, both after 4 or 12 d of treatment (Supplemental Figure S3). Other evaluated genes did not show differential regulation between genotypes or treatments (Supplemental Figure S3).

To assess whether the observed transcriptional changes affected carbohydrate contents, sucrose and starch concentrations were evaluated in roots and leaves of waterlogged plants. In leaves, the hybrids HE2 and HE3 had more sucrose than controls before the waterlogging treatment (Figure 6B). During the treatment, sucrose, glucose, protein, chlorophyll, and carotenoid contents did not significantly change between genotypes (Supplemental Figure S4). After 4 and 7 d of waterlogging, starch content was similar in all the genotypes; however, 12 d after initiating the treatment, controls accumulated more starch in the leaves than transgenic hybrids, suggesting that *HaHB11* plants were more efficient to deliver carbohydrates to other active growing sinks. In agreement, roots of *HaHB11* hybrids exhibited more starch

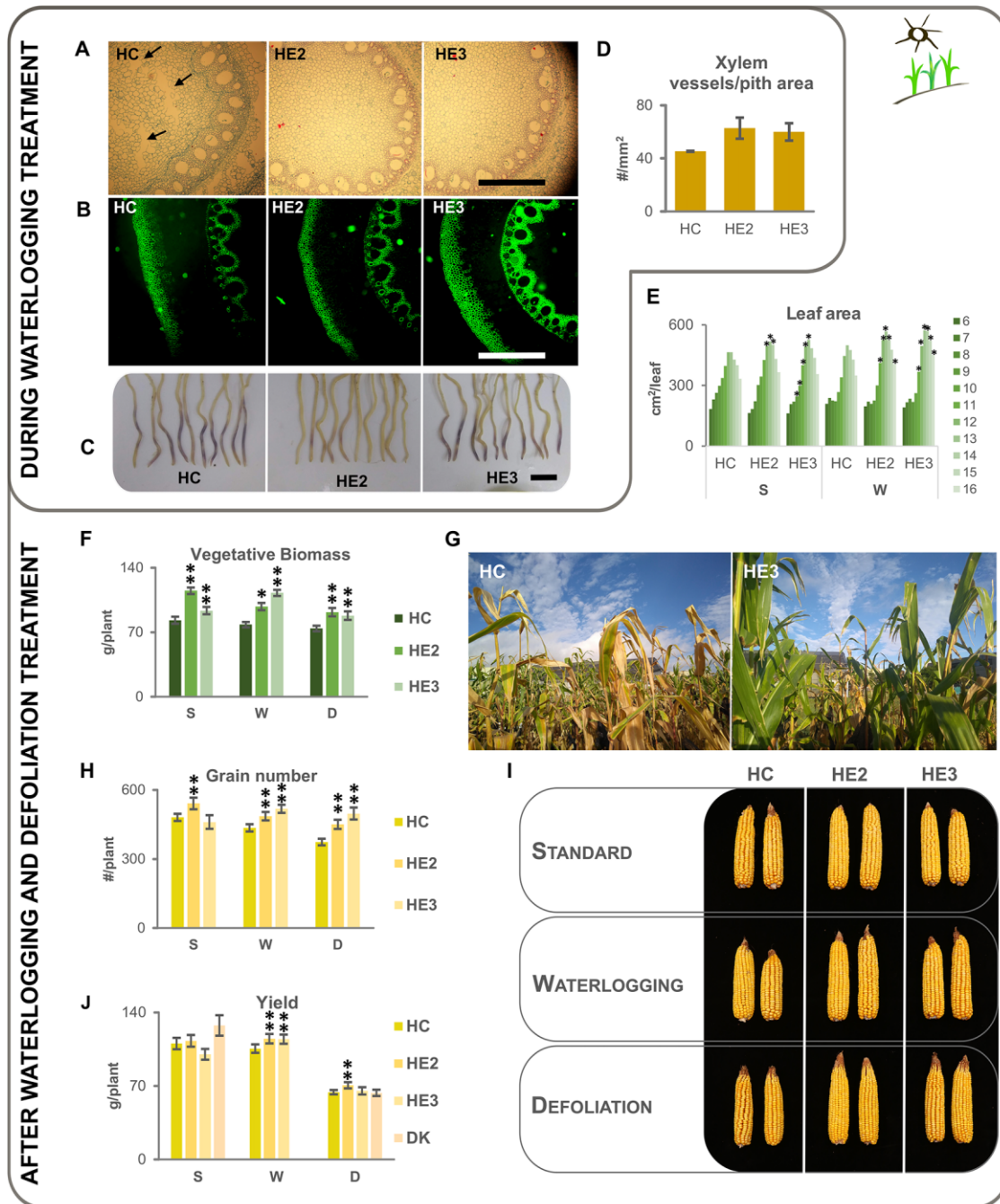


Figure 5 Field-grown transgenic hybrids carrying *HaHB11* exhibited a better performance than their controls grown in standard conditions and after waterlogging or defoliation treatments. Upper panel: Transversal cuts of adventitious roots, 12 d after initiating waterlogging treatment; samples were taken 4.5 cm from the root tip (from 6 to 8 cm length roots), stained with safranin-fast green and captured with white light (A), or epifluorescence (B). Illustrative picture of roots 7 d after the waterlogging treatment, stained with NBT (C). Number of xylem vessels per pith area in stems of plants waterlogged for 14 d (D). Scale bars in (A) and (B) represent 1 mm; in (C) the bar represents 1 cm. Lower panel: Phenotype of plants grown in normal conditions (S), defoliated 15 d after silking (D), or subjected to 14 d of waterlogging (W). Individual leaf area (from leaf 6 to 16, E). Vegetative biomass, kernel number, and plant grain yield of plants at harvest (F, H, and J, respectively). Illustrative photograph of control hybrids (HC) and the transgenic event HE3 100 DAS (G). Representative picture of ears (HC, HE2, and HE3) after different treatments (I). Arrows in A point at tissue damage on the pith. Data represents means \pm SEM of at least three biological replicates. Differences across means were analyzed by a Student's *t* test; asterisks indicate significant differences respect to the control genotype (* for $P < 0.05$ and ** for $P < 0.01$).

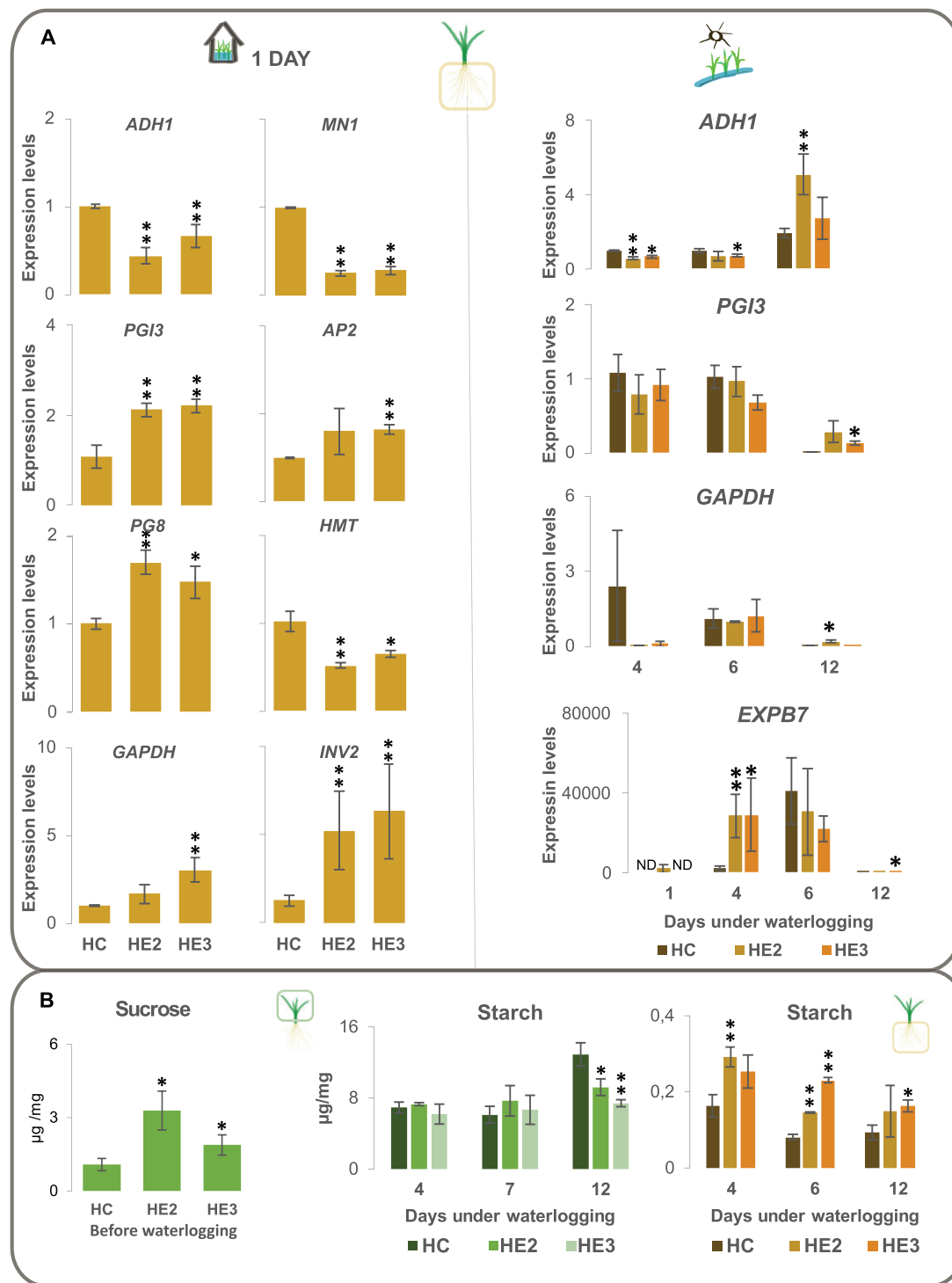


Figure 6 Transgenic HaHB11 hybrids showed differential expression of genes related with waterlogging tolerance and carbohydrate contents. A, Transcript levels of *ADH*, *PGI3*, *GAPDH*, *INV2*, *PG8*, *MN1*, *AP2*, *EXPB7*, and *HMT* genes involved in waterlogging response in *HaHB11* transgenic roots of the control (HC) and transgenic hybrids (HE2 and HE3), evaluated one day after initiating waterlogging treatment (left panel), or after 4, 6, and 12 d of treatment (right panel). B, Sucrose and starch contents before or after waterlogging treatment in leaves and roots of the control (HC) and transgenic hybrids (HE2 and HE3). Quantification of mRNA levels was performed by RT-qPCR. Each value was normalized with its endogenous *ACTIN* according to the ΔCt method and then with the value obtained in the control (HC), arbitrary assigned a value of one (with the $\Delta\Delta\text{Ct}$ method). The ID codes for the tested genes are listed in [Supplementary Table S3](#). Each point is the average of four plants and error bars represent $\text{SEM} \times 2$. Differences across means were analyzed by a Student's *t* test; asterisks indicate significant differences respect to the control genotype (* for $P < 0.05$ and ** for $P < 0.01$).

than controls after 4, 7, and 12 d of treatment (Figure 6B), whereas glucose and protein contents did not differ between control and transgenic hybrids (Supplemental Figure S4).

In situ canopy spectral reflectance helped discriminate maize genotypes nondestructively across growing conditions

Traditional ground-based crop phenotyping of secondary, physiological traits aimed at breeding is usually limited by the number of plants that can be evaluated and is being replaced by nondestructive methods such as spectral images (Reynolds et al., 2021), which produce a large number of data and consequently the need of adequate computing tools for their analysis. Evaluation of canopy spectral reflectance in crops is done predominantly by studying a collection of vegetation indices (VIs) and comparing their performance to select a single one or a few of them that better represent a trait of interest (Reynolds et al., 2021). It is less customary to explore the potential of several VIs jointly for capturing differences among genotypes within a single environment (Arias et al., 2021), as we did in the current study using a set of 29 selected VIs to discriminate maize genotypes grown under different conditions.

Statistical significances for VI values were analyzed for the factor genotype with a one-way analysis of variance (ANOVA) and a post hoc Tukey test. We chose a *P*-value of 0.05 for statistical significance. Each ANOVA was performed on a set of 162 VI values, comprising 18 measurements per plot on the nine plots per treatment (three repetitions for each of the three genotypes). One ANOVA per date and treatment was performed, giving a total of 261 (three dates, three treatments, and 29 VIs) ANOVAs for VI and their corresponding post hoc tests. When the spectral behavior of the evaluated genotypes was analyzed, we detected that the set of VIs that allowed their discrimination varied across treatments. For plants grown in control conditions, 21 VIs successfully discriminated genotypes carrying *HaHB11* from controls during the grain-filling stage, whereas, for the defoliation assay, only 14 VIs did the same (Supplemental Figure S5). For the waterlogging condition, the measurement carried out on the first date revealed differences in VI values, E3 being always significantly different from its control while E2 was only clearly discriminated from the non-transgenic line in the control condition (Supplemental Figure S5). The VIs considering biomass, chlorophyll, and abiotic stress clearly differentiated transgenics from controls (Figure 7). Furthermore, principal component analysis (PCA) for all treatments and all genotypes, showed the weights of the selected VIs, with vectors along PC1 and PC2 components far from 0, meaning that all of them are relevant to discriminate between genotypes. Besides, when a PCA was done per treatment, the VIs showed significant loadings,

but they did not maintain the same clustering pattern (Supplemental Figure S5).

The enhanced biomass, seed number, and plant grain yield exhibited by *HaHB11* plants were robust and consistent traits across all the genetic backgrounds and tested conditions

Given the variety of growing conditions, environments, and genetic backgrounds in which the performance of the transgene *HaHB11* was tested, we performed a statistical analysis to determine the robustness of the differences in the evaluated traits as well as the relationship among them. Variance and multivariate analysis, including a PCA, were carried out using all the data, considering inbreds, hybrids, greenhouse, and field assays in control and stress conditions (Supplemental Table S1 and Figure 8A).

Considering waterlogging effects, the PCA explained 89.5% of the variation produced by this condition early in development (Figure 8A). Within each water regime, hybrids had larger plant grain yield than inbreds and in the control condition, larger plant grain yield than under waterlogging stress. In general, both transgenic events, but particularly E3, had a larger plant grain yield than the control. Plant grain yield was associated with harvest index (HI) (acute angles between vectors) and did not respond to the variation in stem section, days to flowering, and ASI (vectors in right angle). Inbreds were located towards positive values of the PC2, with a higher number of roots and leaves and a longer vegetative period. Hybrids had enhanced HI (independently of the soil water condition) and longer ASI (particularly under waterlogging).

Aiming at knowing which traits are more related to the performance of *HaHB11* plants in different conditions, an additional PCA was carried out considering only hybrids grown in the field subjected to stress caused by waterlogging or defoliation. The PCA explained 76.9% of the total variability. Hybrids carrying *HaHB11* always had a larger grain plant yield than the control under stressful conditions. Collectively, plant grain yield was tightly related to leaf area, total biomass, grain yield components, and HI; it had no relationship with leaf number and was negatively related to the extension of the vegetative period and the ASI. Within each growing condition, transformed genotypes tended to have larger kernel number than the control, whereas the opposite trend was verified for individual kernel weight (Figure 8B).

Discussion

The expected second generation of transgenic plants is still absent in the market. The reasons for this are diverse, including the negative public perception of genetically modified crops and the fact that abiotic stress-tolerant crops do not represent a universal business since they are limited to target environments (Chan et al., 2020). However, the need for stress-tolerant crops remains actual and current due to global climate change and the increase of natural disasters.

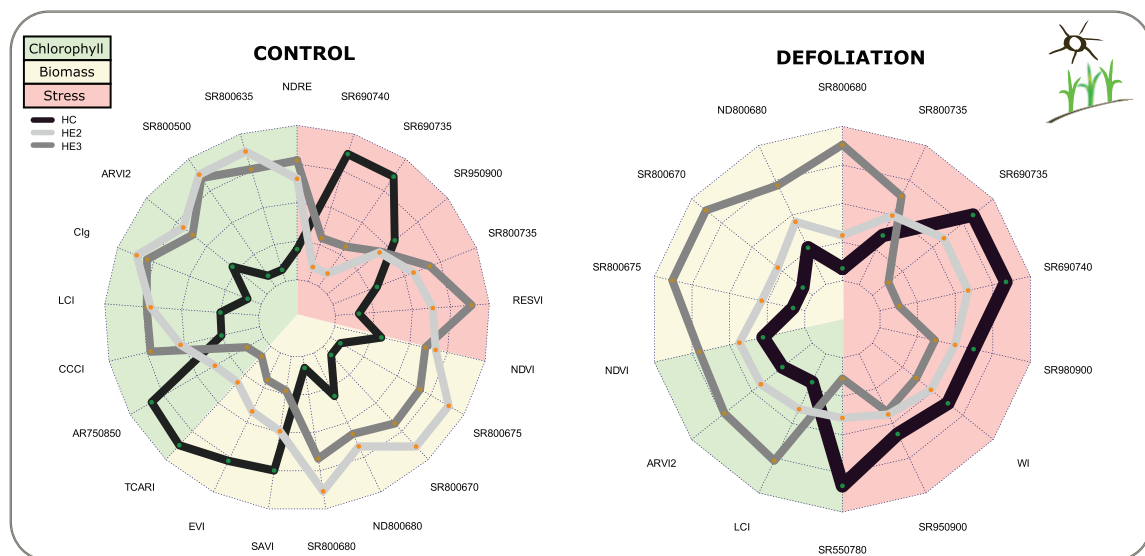


Figure 7 VIs computing spectral data are able to discriminate genotypes and treatments. Radar plots showing significantly different values ($P < 0.01$) of VIs between genotypes for standard and defoliation treatments. Black line: control genotype (HC). Light gray and gray lines: transgenic E2 and E3 genotypes, respectively. Background colors indicate the applicability of the VIs. Green: chlorophyll, pink: water stress, light pink: biomass.

Although drought is still the major constraint for crop yield worldwide, flooding and severe storm episodes augment their frequency with direct consequences for food and fuel production. Particularly, between 2006 and 2016, 65% of economic damage registered in crops was caused by abiotic stresses linked to excess water (UN Food and Agriculture Organization, 2017). To diminish the impact of such problems, breeders and biotechnologists work hard to obtain crops with improved behavior when exposed to abiotic and biotic stress factors. Usually, these efforts are not cooperative and there is abundant scientific literature describing the research of stress-tolerant plants not tested in the field but only under controlled conditions or presenting very slight improvements (Sadras et al., 2020). Another important aspect of this research area is the slow but constant replacement of manual measurements by high-throughput phenotyping with modern, automated equipment that produces large databases, and demands big data analysis. In this work, we presented the results of interdisciplinary research work, starting from molecular biology in the laboratory to spectral phenotyping in the field, performed to test the sunflower gene *HaHB11* as a potential tool to improve stress tolerance in maize.

The sunflower TF *HaHB11* has already been described as a transgene in the model *Arabidopsis* (Cabello et al., 2016) and maize plants (Raineri et al., 2019). In maize, transgenic plants were obtained in the ancient hybrid Hill (AxB) background and then backcrossed several times with the B73 line. When evaluated in greenhouse and field conditions under irrigation, these plants showed improved plant grain yield, mainly supported by a higher kernel number than controls.

Maize is a species affected by flooding, mostly early in development (usually up to V2) and to a lesser extent in

subsequent stages (Zaidi et al., 2004). Several works were dedicated to analyzing the effects of waterlogging, also called excess soil moisture (ESM) on contrasting genetic backgrounds (hybrids and inbreds). Such studies, applying varied waterlogging treatments, focused on physiological traits or/and molecular mechanisms. The more robust results indicated that the genotypes exhibiting early adventitious rooting, aerenchyma formation, a barrier to ROL in roots, partial stomatal closure in leaves, and increase of NAD-ADH activity and starch accumulation in stem tissues were more tolerant to ESM than those that did not exhibit these traits (Zaidi et al., 2003). Notably, these attributes were common in induced hypoxia tolerance and allowed identifying associated Quantitative Trait Loci (Zaidi et al., 2003; Abiko et al., 2012). Increased ADH activity was a characteristic observed in adapted subtropical and tropical inbreds. Although the content of ethanol (the product of this enzyme) was higher in the susceptible genotypes, the ability to extrude it seemed to be increased in the tolerant ones (Zaidi et al., 2007). Tolerant and susceptible genotypes in advanced developmental stages differed in their ability to accumulate carbohydrates in stem tissues, the extension of the ASI, root porosity, and stomatal conductance (Zaidi et al., 2004). In agreement with these previous reports using different genetic backgrounds, *HaHB11* plants showed increased leaf area together with higher stomatal conductance, root length, and biomass than control plants after waterlogging treatments (Figures 1–4). Moreover, histological cuts of waterlogged roots evidenced tissue damage in the pith of control plants and an increased number of xylem vessels in the transgenics that could be associated with this stress response (Figures 1–5). Moreover, transgenic roots seemed to have a “tight” barrier to oxygen loss, compared to those of

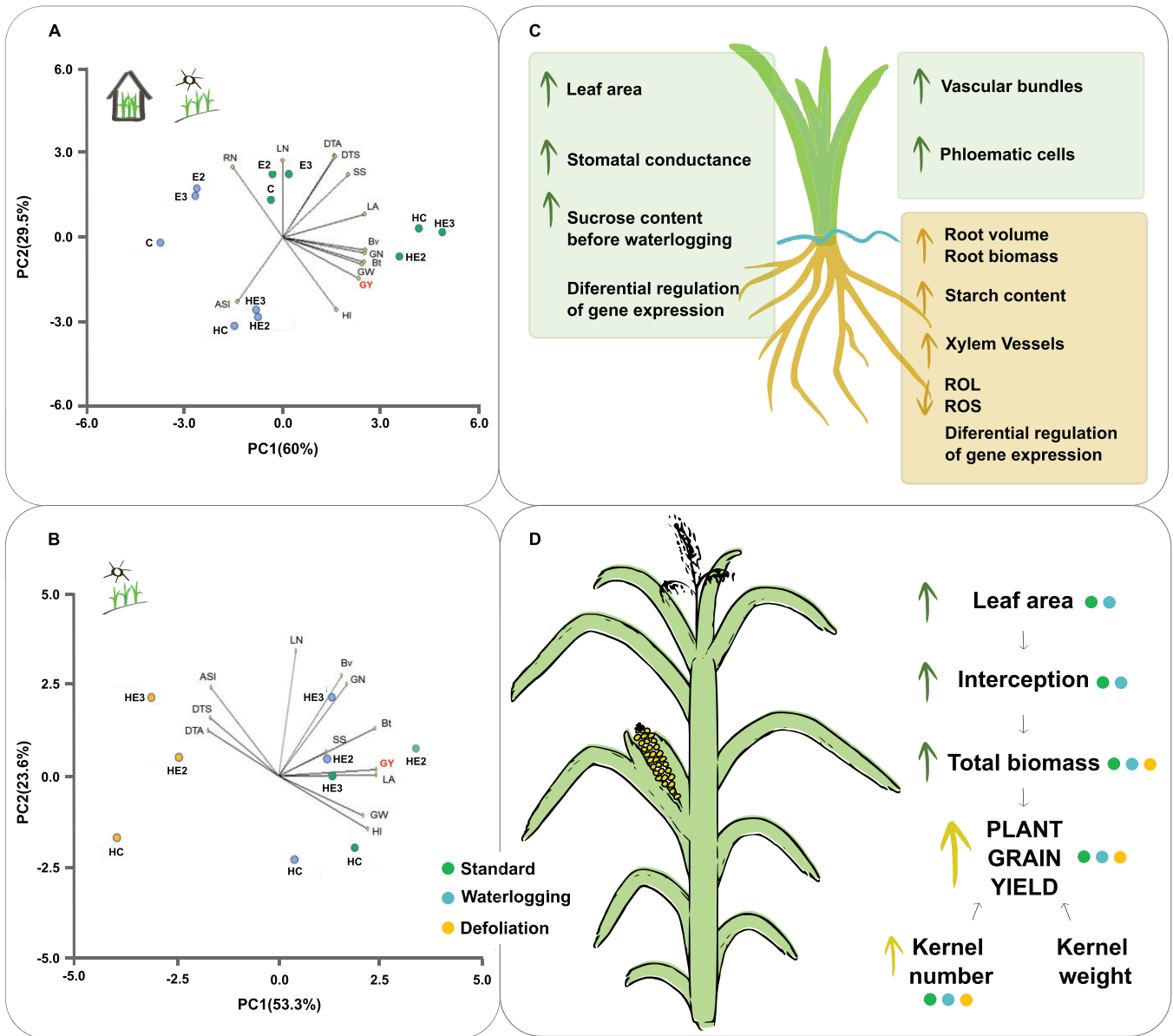


Figure 8 The beneficial effects conferred by *HaHB11* to maize plants are statistically robust. A and B, PCA performed using all the data presented in this manuscript. C, Summary of the differential traits assessed between transgenic and control maize plants at the vegetative stage (V3) subjected to waterlogging. D, Summary of the differential traits assessed between transgenic and control maize plants at harvest after different treatments. Circles indicate (i) plants grown in control (blue) or waterlogging (green) conditions, and (ii) plants exposed to defoliation (orange).

the wild type (Figure 5). After recovery, the transgenics exhibited an increase in nodal roots, stem surface, biomass, light interception, and chlorophyll, which resulted in an improved plant grain yield (Figures 1–5).

Regarding the mechanisms playing a role in waterlogging adaptation, inbreds showing susceptibility had a reduced dry matter translocation from source to sink tissues, which resulted in an inadequate grain filling (Kaur et al., 2021). Transgenic *HaHB11* plants, described here, accumulated more biomass and partitioned a larger part of it to kernels, setting a higher kernel number, resulting in increased plant grain yield compared to controls (Figure 5).

An interesting question is if the waterlogging tolerance observed in several inbred genotypes was maintained in hybrids. It was reported that morpho-physiological traits differed between normal conditions and waterlogging and that hybrids were superior to parental lines under stress. Most of the characteristics associated with ESM tolerance in hybrids corresponded positively with those of parental lines, but in normal moisture conditions, the effect of heterosis was more important than the contribution of the parental line (Zaidi et al., 2007).

In a more recent trial, different hybrids were tested in the V2 stage for their tolerance to waterlogging, evaluating

similar parameters as in inbreds, after 7, 14, and 21 d of ESM. Although all the assessed hybrids showed a decrease in the evaluated traits, differences were detected between the tolerant and the susceptible ones (Kaur et al., 2021).

Regarding the molecular level, tolerant genotypes exhibited adaptive mechanisms enabling hypoxia tolerance. These plants had upregulated genes encoding enzymes participating in carbon metabolism and signal transduction, such as *ADH*, sucrose synthase, aspartate aminotransferase, and NADP-dependent malic enzyme (Kaur et al., 2021). Notably, some of these genes were also differentially regulated in *HaHB11* plants, albeit not always in the same sense (up or down, Figure 6). This contrasting result can be explained by the high turnover of genes observed after different waterlogging treatments and conditions. In transcriptome analyses performed with waterlogged maize plants, a huge variation in gene regulation was reported (Rajhi et al., 2011; Arora et al., 2017; Du et al., 2017). The only gene robustly regulated across all assays encoded a polygalacturonase (GRMZM2G037431, Rajhi et al., 2011; Arora et al., 2017; Li et al., 2019), and it was also upregulated in *HaHB11* transgenic plants.

Crop defoliation may have different origins: biotic, like insect attack, or abiotic as heavy rain, wind, or hail storms. In the trials described here, a summer storm produced severe defoliation (Figure 5). Although the serendipitous nature of the event, it allowed us to learn that *HaHB11* plants performed better than controls in response to such harmful conditions. The crop yield depends on the quality (i.e. size and activity) of the photosynthates source and the ability to transport assimilates to sink tissues. In maize, a consistent trend in seed dry weight was observed when assimilates during active grain filling were dramatically diminished by defoliation (Borrás et al., 2004). We hypothesized that because the storm occurred when the number of grains was almost already established, and transgenic plants set more grains than controls, the source-sink relationship may have been more affected among the former than among the latter. To corroborate this hypothesis, we developed controlled defoliation assays (Figure 5). As expected, the commercial hybrid, used as control, produced more grain biomass than all other hybrids in potential growing conditions; however, after a defoliation treatment, it was more penalized than the transgenic hybrids, indicating that a tolerant parental line in *HaHB11* plants contributed to a better performance in such condition. It is important to note that the influence of defoliation on crop yield depends on its timing and severity. During vegetative stages, it may have no or little effect, whereas even a mild hail can produce a reduction on grain yield of 30% or more when it occurs from the start of the critical period onwards (Battaglia et al., 2019), depending upon the relative impact on light interception efficiency (Borrás et al., 2004; Cerrudo et al., 2013).

Described differences among genotypes across treatments and environments were confirmed by multivariate analysis and assessed through VIs indicative of variations in canopy

spectral reflectance. Rather than selecting the best VI for describing each evaluated trait (García-Martínez et al., 2020), we opted for a joint analysis of 29 VIs to capture differences among genotypes in a single environment (Arias et al., 2021). Although genotypes could be differentiated, it was not the same set of VIs that allowed their discrimination across treatments. On the one hand, this response is indicative of the capacity of VIs to track the fast changes in plant metabolism in response to the environment (Reynolds et al., 2021). On the other hand, described shifts in the way VIs ranked genotypes along the cycle alert on the need for further research aimed to understand the interrelation between spectral data and differential gene expression among genotypes.

Conclusions

In current research, we demonstrated the advantage of maize genotypes transformed with *HaHB11* to withstand transient episodes of ESM that take place early in the cycle. This response is probably linked to their improved root system because the negative effects of ESM in mentioned stages usually affect roots more than shoots (de San Celedonio et al., 2017), and we observed that starch content accumulated in leaves and decreased in roots of the control genotypes whereas the opposite trend occurred in genotypes transformed with *HaHB11*. The latter is indicative of an active metabolism despite the stressful anaerobic condition. Being the recovery of roots lower than that of shoots, plants bearing *HaHB11* may be in better conditions than the controls to withstand subsequent stressful scenarios along the cycle (e.g. defoliation, enhanced evaporative demand), which are not uncommon among field-grown plants.

Materials and methods

Plant material and growth conditions

Greenhouse assays

Maize (*Z. mays*) plants of different genotypes (B73 lines and B73 × Mo17 hybrids) were grown during 2017 and 2021 at the Institute of Agrobiotechnology, located at Santa Fe (31°38'17.1"S, 60°40'01.8"W). Plants were cultivated in 45 L pots (one plant/pot) in a randomized arrangement under long-day photoperiod (16/8 h light/dark cycles), with daily temperatures fluctuating between a mean minimum of 10°C and a mean maximum of 40°C. Pots were placed at a 30-cm distance between them. Artificial lights were turned on by a sensor when the intensity was under 600 $\mu\text{E}/\text{m}^2$ near the ear, and to complete the photoperiod regime. Sowing dates, as well as evaluated parameters, are described in Supplemental Table S2.

Field trials

Experiments were performed during 2017 and 2020 at the IAL, on a sandy soil of 2 m depth with low water-holding capacity and an organic "A" horizon of 15 cm. Evaluated germplasm included the following genotypes: controls (null segregants) and transgenic lines (E2 and E3) of B73 lines

(transformed Hill backcrossed three to four times to B73, Experiments 1 and 2) as well as B73 × Mo17 hybrids (Experiment 3) and commercial F1 hybrid DK 72-10 (all experiments). The sowing date took place at the beginning of November using a single stand density of 9 plants m⁻². Genotypes were distributed in a completely randomized design with three replicated plots for each genotype and treatment. Each plot had three rows of 1.25 and 0.5 m between rows. Plots were drip-irrigated along the whole cycle to keep the uppermost 1 m layer at field capacity and were fertilized with N (180 kg ha⁻¹ at sowing and 180 kg ha⁻¹ at tasseling) and P (100 kg ha⁻¹ at sowing). Plots were kept free of weeds, insects, and diseases. Daily mean temperature (in °C) and incident solar radiation (in MJ m⁻² day⁻¹) were obtained from a nearby meteorological station. All experiments were carried out after obtaining the corresponding authorization from the CONABIA (National Committee of Biotechnology) and Seeds National Institute (INASE).

Waterlogging treatments

In greenhouse experiments, plants (all the tested genotypes) with three expanded leaves (V3) were placed in a plastic pool. At the beginning of the day, enough water was added to cover half of the pot height. At midday, the water level raised until 1 cm above the ground continuously for 2 weeks.

In field assays, the pots were placed in pools at a density of 9 plants/m². At the V3 stage, plants were waterlogged during 14 d, keeping the water level 1 cm above the ground. After the treatment plants from all genotypes were removed from the pots and placed immediately in the experimental field and grown under irrigation until the end of the life cycle.

Partial defoliation assays

In the field trial, the first defoliation episode occurred 11 d after silking in the field trial carried out in spring–summer 2019. The second and third experiments were carried out in the field and greenhouse during 2020, respectively, by manually defoliating plants (75%–80% of the leaf surface defoliated), 11 d after silking leaving only the ear leaf and the leaf immediately above it. All ribs were kept, emulating the field defoliation.

Plant phenotyping in greenhouse and field conditions

Measurements were performed on four–seven plants per genotype (greenhouse). In field assays, eight plants from the central row of each plot which were tagged at V3, as previously described (Raineri et al., 2019). Three plots were sowed with each genotype resulting in 24 plants/genotype for leaf area assessment. To evaluate grain weight, 15–20 plants per plot were harvested, resulting in 45–60 per genotype and treatment. No significant differences were detected between plots of each genotype and treatment. The ASIs were registered for all plants. At silking, the total number of fully expanded leaves and total plant leaf area were measured.

Individual leaf area was computed as in Equation (1) (Montgomery, 1911).

$$\text{Leaf area} = \text{Maximum leaf length} \times \text{maximum leaf width} \times 0.75. \quad (1)$$

All tagged plants were oven-dried for estimation of total aerial plant biomass. Grain yield was expressed on a 14.5% wet basis. HI was estimated as the quotient between plant grain yield and total plant biomass (on a dry basis). The evolution of light interception efficiency (e_i) was assessed on the central row of each plot as previously described (Raineri et al., 2019).

Stomatal conductance

Stomatal conductance was measured with a porometer (Decagon SC-1). All measures were taken at midday.

Carbohydrate and chlorophyll contents

Starch, sucrose, glucose, and protein contents from roots and leaves of at least four plants were assessed as previously described (Cabello et al., 2016). Chlorophyll content was determined either by acetone extraction (Raineri et al., 2019) or by using a specific chlorophyll meter device (Cavadevices, <https://cavadevices.com/archivos/FOLLETOS/Clorofilio.pdf>).

Allometric measurements during waterlogging stress in roots

At least four plants were harvested and the roots were washed. Total adventitious roots, root length, and root were quantified. Root volume was assessed by a volumetric method: 80 mL of deionized water (V_W) was placed in a tube; then, the root system was completely submerged and the volume of water plus the root (V_{W+R}) was measured. The root volume (V_R) was calculated as follows: $V_R = V_{W+R} - V_W$.

ROL

The ROL in roots was measured as described (Watanabe et al., 2017). Four plants per genotype were selected after the waterlogging treatment. All the adventitious roots were removed, except one of 10–14 cm length. Plants having a single nodal root were placed in a pot with methylene-blue solution and photographed after 1 h of incubation.

NBT staining

Roots were collected and placed in a solution containing NBT 0.1 mg/mL in 25 mM Hepes pH 7.6 and 0.05% v/v Triton X-100. The samples were vacuum-infiltrated for 15 min and incubated for an additional hour at 37°C.

RNA isolation and expression analyses by real-time RT-PCR

Total RNA for reverse transcription quantitative PCR (RT-qPCR) was isolated from maize leaves or stems using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RT-qPCR was performed using an Mx3000P Multiplex qPCR system (Stratagene, La Jolla, CA, USA) as described before

(Raineri et al., 2019). Primers used are listed in [Supplemental Table S3](#).

Histology

Histology of the cross-sections was carried out as previously described (Cabello et al., 2016) and stained with safranin fast-green. The xylem and pith area were assessed using the free software ImageJ (Schneider et al., 2012). For lignin content, the cross-sections were evaluated using an epifluorescence microscope (Eclipse E200 microscope; Nikon, Tokyo, Japan, equipped with a Nikon Coolpix L810 camera); excitation at 365/10 and emission at 460/50.

Remote sensing analyses

Canopy spectral reflectance was measured using a compact shortwave NIR spectrometer (Ocean Insight). The instrument is sensitive to 1,024 wavelengths in the range from 632 to 1,125 nm with an optical resolution of 3 nm at full-width half-maximum. All measurements were performed between 10:00 and 14:00 h ART time (UTC 03:00), with the instrument positioned at a nadir view 50 cm above the canopy surface, with a diameter of the measured footprint of ~26 cm. Ten measurements were taken per plot. The upwelling light reflected from a 50 cm × 50 cm white reference material with 99% reflectance, was recorded before each canopy measurement allowing data acquisition during variable sky conditions. The integration time was adjusted to avoid saturation of the white signal and each measurement was the average of five successive scans. The measurements were homogeneously distributed over the plot to reduce border effects. Measurements were collected on 17 January 2020 (vegetative stage), 28 January 2020 (flowering), and 18 February 2020 (grain-filling stage). A typical outlier control based on standard deviation was implemented on each canopy spectral reflectance raw data.

Twenty-nine VIs were selected based on the range of available wavelengths and their applications. The selected indices, their formulas, and type of applications are shown in [Supplemental Figure S1](#).

Each VI was evaluated for each treatment per genotype combination. Only those that differed significantly ($P < 0.05$) between genotypes are discussed. Data analysis was conducted in R using the aov function and the post-hoc test was performed using the agricolae-package (Mendiburu, 2010).

Statistical analyses

A *t* test was used for the comparison of genotypes evaluated in the greenhouse and field experiments. ANOVA was used to assess the effect of treatments (control, waterlogging, and defoliation) and genotypes (line or hybrid, control or transgenic) in greenhouse and field experiments. Differences across means were analyzed by a Tukey test ([Supplemental Table S1](#)). PCA were used to evaluate the correlation among traits for the different genotypes and experiments, as well as for the VIs ([Figure 8](#)).

Accession numbers

Accession numbers of the genes evaluated in this work are available in [Supplemental Table S3](#).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. *HaHB11* plants doubled plant grain yield of controls after natural defoliation in the field.

Supplemental Figure S2. *HaHB11* plants set more kernels than control plants after the defoliation treatment in the greenhouse.

Supplemental Figure S3. Expression levels of genes related to waterlogging response.

Supplemental Figure S4. Carbohydrate, protein, and pigment contents in waterlogged plants during the treatment in control and transgenic hybrids.

Supplemental Figure S5. VIs can discriminate genotypes and treatments in field trials.

Supplemental Table S1. Statistical analysis of greenhouse and field assays.

Supplemental Table S2. Sowing dates and evaluated characteristics of each assay.

Supplemental Table S3. Oligonucleotides used in qPCR assays and genes IDs.

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Conflict of interest statement. None declared.

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