

Effects of flooding stress in ‘Micro-Tom’ tomato plants transformed with different levels of mitochondrial sHSP23.6

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(With 5 figures)

Abstract

Soil flooding is an environmental stressor for crops that can affect physiological performance and reduce crop yields. Abiotic stressors cause changes in protein synthesis, modifying the levels of a series of proteins, especially the *heat shock proteins* (HSP), and these proteins can help protect the plants against abiotic stress. The objective of this study was to verify if tomato plants cv. Micro-Tom from different genotypes with varying expression levels of MT-sHSP23.6 (*mitochondrial small heat shock proteins*) have different responses physiological to flooding. Plants from three genotypes (untransformed, MT-sHSP23.6 sense expression levels and MT-sHSP23.6 antisense expression levels) were cultivated under controlled conditions. After 50 days, the plants were flooded for 14 days. After this period half of the plants from each genotype were allowed to recover. Chlorophyll fluorescence, gas exchange, chlorophyll index, leaf area and dry matter were evaluated. Flood stress affected the photosynthetic electron transport chain, which is related to inactivation of the oxygen-evolving complex, loss of connectivity among units in photosystem II, oxidation-reduction of the plastoquinone pool and activity of photosystem I. The genotype with MT-sHSP23.6 sense expression levels was less sensitive to stress from flooding.

Keywords: *Solanum lycopersicum* Mill, photosynthesis, chlorophyll fluorescence, stress.

Efeitos do estresse por alagamento em plantas de tomateiro ‘Micro-Tom’ transformada com diferentes níveis de sHSP23.6 mitocondrial

Resumo

O alagamento do solo é um estressor ambiental para as culturas e pode afetar o desempenho fisiológico e reduzir a produtividade das culturas. Estresses abióticos causam mudanças na síntese de proteínas, modificando os níveis de uma série de proteínas, em especial as proteínas de choque térmico (HSP) e essas proteínas são conhecidas por proteger as plantas contra estresses abióticos. O objetivo deste estudo foi verificar se as plantas do tomateiro cv. Micro-Tom de distintos genótipos com diferentes níveis de expressão da MT-sHSP23.6 (proteínas mitocondriais de choque térmico com pequena massa molecular), têm diferentes respostas fisiológicas ao alagamento. As plantas de três genótipos (não-transformado, transformado com orientação antisense e transformado com orientação sense para MT-sHSP23.6) foram cultivadas sob condições controladas. Após 50 dias as plantas foram alagadas durante 14 dias. Após esse período as plantas de cada genótipo foram recuperadas. Foram avaliados fluorescência da clorofila, trocas gasosas, índice de clorofila, área foliar e massa seca. O estresse por alagamento afetou a cadeia de transporte de elétrons da fotossíntese, que está relacionado à inativação do complexo de evolução do oxigênio, perda da conectividade entre as unidades do fotossistema II, de oxidação e redução do pool de plastoquinona e atividade do fotossistema I. O genótipo com orientação sense MT-sHSP23.6 foi menos sensível ao estresse por alagamento.

Palavras-chave: *Solanum lycopersicum* Mill, fotossíntese, fluorescência da clorofila, estresse.

1. Introduction

Soil flooding is a major limiting factor for the growth and development of most crop species (Horchani et al., 2008, 2009). Various easily detected physiological responses have been reported as a result of physical and chemical changes in soil (Kozłowski, 1997). Because flooding is a significant problem in agriculture, studies have been conducted on various crop species (Zou et al., 2013) and have yielded the following results: 1) there is a significant transcriptional expression of genes involved in virtually every major biological functions; 2) gene transcripts that were significantly up – or down-regulated in response; 3) similar expression of transcription factors among species, especially in the α gene (sub 1A, HIF, ERF) and 4) genes encoding sHSP are strongly up-regulated (Mustroph et al., 2010), however, the effects of flooding vary across species and within a single species (Ezin et al., 2010, Perboni et al., 2012).

Abiotic stressors cause changes in protein synthesis, modifying the levels of a series of proteins, especially the *heat shock proteins* (HSP). One type of HSP, *small heat shock proteins* (sHSP), are produced in response to a wide variety of environmental stressors and act with other HSP to control stress prevention and recovery (Waters, 2013; Hüther et al., 2013). HSP act on protein synthesis and repair and protect the endomembrane system, helping to restore cellular homeostasis (Pegoraro et al., 2011).

Prior studies (Rampino et al., 2009; Hüther et al., 2013) have found that the HSP23.6 gene is typically expressed in response to various pressures, and its expression levels are strongly up-regulated during some developmental stages (Sarkar et al., 2009). Slight increase in *HSP23.6* gene transcription was reported in tomato fruits under hypoxia (Pegoraro et al., 2012). The low transcription and protein amount required may be due to a low number of processes they are involved. Thus, although this protein is located in the mitochondria, it is believed to act within the organelle itself to promote homeostasis of the plastids, as reported by our group in previous studies with MT-sHSP23.6 sense tomato Micro-Tom genotype, lower photosynthetic damage under high temperature in comparison to MT-sHSP23.6 antisense genotype, suggest in a high tolerance degree to heat due to these protein function (Hüther et al., 2013). It is known that these two organelles interact during redox depletion, the interchange of metabolites, energy and sub-cellular protection (Waters, 2013).

Decreased photosynthesis under flooded conditions may be associated with disturbances of photochemical and/or biochemical processes and PSII (photosystem II) is very sensitive to water stress, perhaps due to damage to the oxygen-evolving complex (Wright et al., 2009) or the PSII reaction centers (Slabbert and Krüger, 2011).

Chl (chlorophyll) *a* fluorescence kinetics can be used to calculate fluorescence parameters that characterize the functioning of the photosynthetic apparatus (Yusuf et al., 2010). Analyzing fluorescence transient may be useful for assessing the effects of stressors on plant performance.

The objective of this study was to verify if tomato plants cv. Micro-Tom from different genotypes with varying expression levels of MT-sHSP23.6 (*mitochondrial small heat shock proteins*) have different responses physiological to flooding.

2. Material and Methods

2.1. Plant materials and growth conditions

Tomato seeds (*Solanum lycopersicum* Mill. cv. Micro-Tom) were used in the experiment. The seeds were either untransformed or had been transformed to various expression levels of MT-sHSP23.6 mitochondrial sense and antisense, as described by Hüther et al. (2013).

Seeds from all three genotypes were sown in Gerbox containers and after 15 days transplanted to 500 mL plastic pots containing sand substrate. The seedlings were placed in growth chambers under controlled conditions (photosynthetic photon flux density of approximately 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ above the center of the pots, 10 hour photoperiod and temperature of $21 \pm 3^\circ\text{C}$). The plants were watered daily, and a nutrient solution described by Hoagland and Arnon (1950) was applied three times per week. Fifty days after sowing (DAS), 10 plants from each genotype were subjected to flooding (water level 2-3 cm above the substrate) for 14 days. To evaluate recovery from stress, at the 10 days of start the experiment, half of the flooded plants from each genotype were returned to normal water levels. Plants in the control treatment group remained under normal water levels.

2.2. Photosynthetic characteristics - fluorescence transient and gas exchange

Measurements for determining the fluorescence transient (curve OJIP) and gas exchange were taken on the first, third, seventh, tenth and fourteenth day of the experiment. During plant recovery, measurements were taken three and seven days after the excess water was drained.

The fluorescence transient was measured, using a portable fluorometer (Model Handy PEA, Hansatech Instruments, King's Lynn, Norfolk, UK), in the first completely expanded young leaves, which were first adapted to dark environment for 30 minutes. After the adaptation period, a flash of 3,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was applied to induce the fluorescence transient. The fluorescence intensity was measured from 50 μs (initial fluorescence F_0) to 1 s, and the parameters for the JIP-test were calculated as proposed by Strasser and Strasser (1995).

The same leaves used to measure fluorescence transient were used to measure gas exchange with a portable infrared CO_2 analyzer (model LI-6400XT LI-COR, Inc., Lincoln, NE, USA) using a photosynthetic photon flux density of 1,500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a CO_2 concentration of 380 $\mu\text{mol mol}^{-1}$ inside the chamber. The following variables were measured from 10:00 to 11:00 h net CO_2 assimilation rate (A) [$\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$] and intracellular CO_2 concentration (C_i) [Pa]. The values of A and C_i were used to calculate the apparent carboxylation efficiency

(A/C_v) [$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$]. The leaf area (LA) and dry matter of the stem, petiole, leaves and roots were measured at the end of the experimental period.

2.3. Statistical analyses

A completely randomized experimental design was used, with five replicates per treatment. The mean values for gas exchange and the growth parameters were compared using Tukey's tests ($p < 0.05$). Chl *a* fluorescence was measured in three leaves per plant for a total of 15 measurements per treatment.

3. Results

3.1. Analysis of OJIP fluorescence transients by the JIP-test: evaluation of photosynthetic performance

The investigation of Chl (chlorophylls) *a* fluorescence induction (Kautsky curve) and JIP-test parameters revealed that: a) the relative variable fluorescence in step J (V_j) increased in waterlogged plants from WT and MT-sHSP23.6 antisense genotypes; b) the normalized total complementary area above the OJIP transient curve (S_m - total complementary area normalised above the transient OJIP); c) the number of cycles of reduction, oxidation and re-reduction of Q_A from the time when the light was turned on until reaching F_M (N - turnover number as reduction, oxidation, re-reduction of Q_A in time span from light until reaching F_m) decreased in flooded plants from the genotypes WT and MT-sHSP23.6 antisense (Figure 1). There was no effect of flooding on plants from the genotype with MT-sHSP23.6 sense. During plant recovery (Figure 2), the OJIP fluorescence transient did not return to control levels in plants from the genotypes WT and MT-sHSP23.6 antisense, even after the full recovery period.

The absorption flux (of antenna Chl) per RC (Reaction centre) (Q_A^- - reducing PSII reaction center) (ABS/RC), trapping flux per RC (leading to Q_A^- reduction) (TR₀/RC) and electron transport flux (further than Q_A^-) per RC (ET₀/RC) were not significantly affected by flooding for any of the genotypes (Figure 1). However, electron flux reducing end electron acceptors at the PSI (photosystem I) acceptor side, per RC (RE₀/RC) in WT and MT-sHSP23.6 antisense plants began to decrease on the first day after the initiation of flooding, and this decrease continued as flooding continued. For plants with MT-sHSP23.6 sense, this pattern was more evident after the third day of flood stress. Seven days after the end of flooding, RE₀/RC had only recovered in plants with MT-sHSP23.6 sense (Figure 2).

The maximum quantum yield for primary photochemistry (TR₀/ABS = $F_v/F_m = \phi_{P_0}$) and Ψ_{E_0} (probability, at time 0, that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-) was slightly influenced. However, the quantum yield of electron transport from Q_A^- to the intersystem of electron acceptors (ϕ_{E_0} - quantum yield for electron transport -ET) decreased as a function of duration of flooding, especially after the seventh day of flooding. This pattern was only observed in MT-sHSP23.6 antisense plants. Of the yield parameters, ϕ_{R_0} (efficiency/probability

with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor side) was most sensitive to flooding in all genotypes (Figure 1). In WT and MT-sHSP23.6 antisense

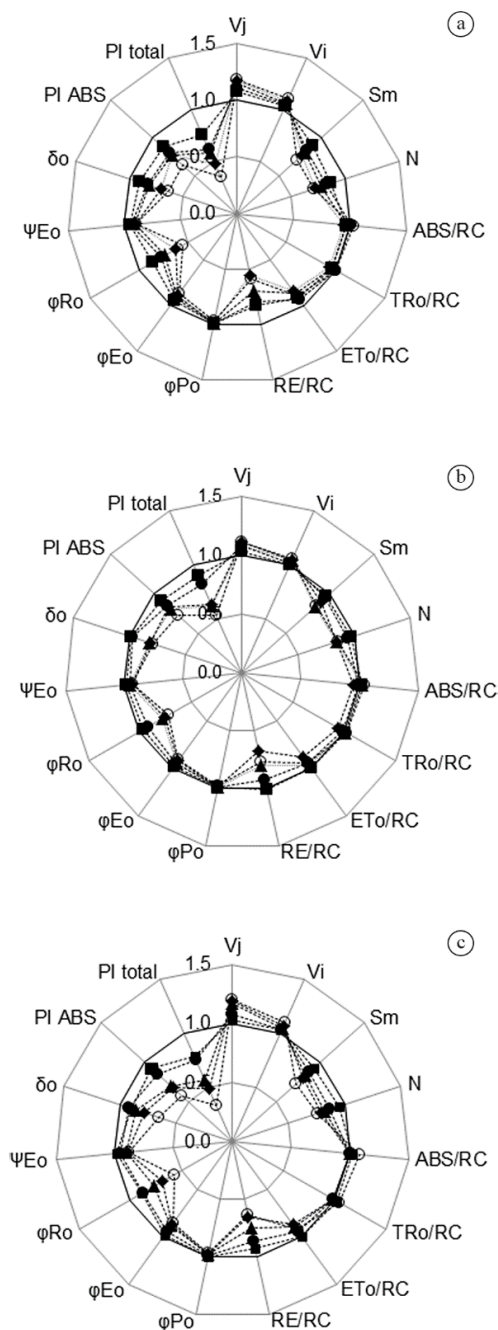


Figure 1. Radar plot of several parameters calculated by the JIP-test of the tomato plants 'Micro-Tom' with different expressions of HSP after flooding stress. (a) Wild type, (b) MT-sHSP23.6 sense, (c) MT-sHSP23.6 antisense. (—) control, (—■—) 1, (—●—) 3, (—▲—) 7, (—◆—) 10 and (—○—) 14 days of the flooding. The heavy black line represents the normalized values of the parameters of the control.

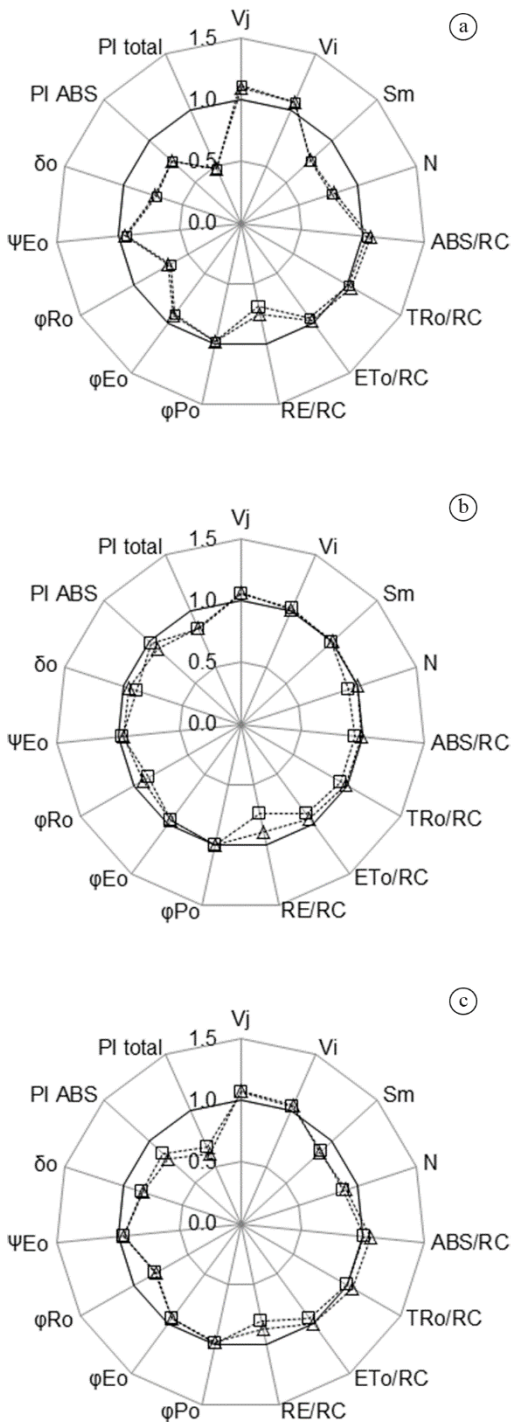


Figure 2. Radar plot of several parameters calculated by the JIP-test of the tomato plants ‘Micro-Tom’ with different expressions of HSP after three (□) and seven (Δ) days of the recovery after flooding stress. (a) Wild type, (b) MT-sHSP23.6 sense, (c) MT-sHSP23.6 antisense. The heavy black line represents the normalized values of the parameters of the control (—).

plants, the effects of flood stress on ϕ_{Ro} were observed immediately after flooding began, and ϕ_{Ro} did not return to control levels after recovery in these plants (Figure 2).

Both performance index (PI_{ABS} - performance index for energy conservation from exciton to the reduction of intersystem electron acceptors e PI_{total} - performance index for energy conservation from exciton to the reduction of PSI end acceptors) decreased during the waterlogging treatment, whereas MT-sHSP23.6 sense genotype responded more effectively. In addition, MT-sHSP23.6 sense recovery its performance index similar to control upon return to normoxic conditions (Figure 2).

3.2. Analysis of the OJIP fluorescence transient: normalizations and subtractions

Normalizations and subtractions among steps of the OJIP fluorescence transient were used to thoroughly assess the effects of flooding on the O, J, I and P steps of the fluorescence transient. This approach allowed differences in kinetics to be identified and revealed hidden bands among the O, J, I and P steps. We present only measurements taken on the 14th day of flooding and the 7th day of recovery relative to the control.

The fluorescence transient data were normalized as follows:

- a) between steps O (50 μ s) and K (300 μ s) as relative variable fluorescence [$W_{OK} = (F_t - F_0)/(F_k - F_0)$] and as kinetic difference [$\Delta W_{OK} = (W_{OK}^{(treatment)} - W_{OK}^{(control)})$] (Figure 3a-c). It was obtained and identified positive L-band (at approximately 150 μ s) in flooded plants from all genotypes. The positive L-band was not present in MT-sHSP23.6 sense plants after seven days of recovery from flooding.
- b) between steps O (50 μ s) and J (2 ms) as relative variable fluorescence [$W_{OJ} = (F_t - F_0)/(F_j - F_0)$] and as kinetic difference [$\Delta W_{OJ} = (W_{OJ}^{(treatment)} - W_{OJ}^{(control)})$] (Figure 3 d-f). This procedure revealed the K-band (at approximately 300 μ s). Flooding caused the positive K-band to appear in MT-sHSP23.6 antisense plants, and the K-band remained even after recovery.
- c) between steps O (50 μ s) and I (30 ms) as relative variable fluorescence [$W_{OI} = (F_t - F_0)/(F_i - F_0)$] ($W_{OI} \geq 1$) (Figure 4 a-c). This procedure enabled the evaluation of I-P phase events. Maximum amplitude of $W_{OI} \geq 1$ reflects the size of the electron acceptor pool on the PSI electron acceptor side. In this study, amplitude decreased with flooding for all genotypes, and this effect was smaller in MT-sHSP23.6 sense plants. This effect was only reversed in MT-sHSP23.6 sense plants during recovery from flooding (Figure 4b).
- d) between steps I and P as [$W_{IP} = (F_t - F_i)/(F_p - F_i)$] (Figure 4 d-f). The inverse of the time to reach $W_{IP} = 0.5$ is an estimate of the global rate of reduction of electron acceptors of PSI. In this study, there were no clear differences in the global rate of reduction of final electron acceptors in flooded plants relative to control plants.

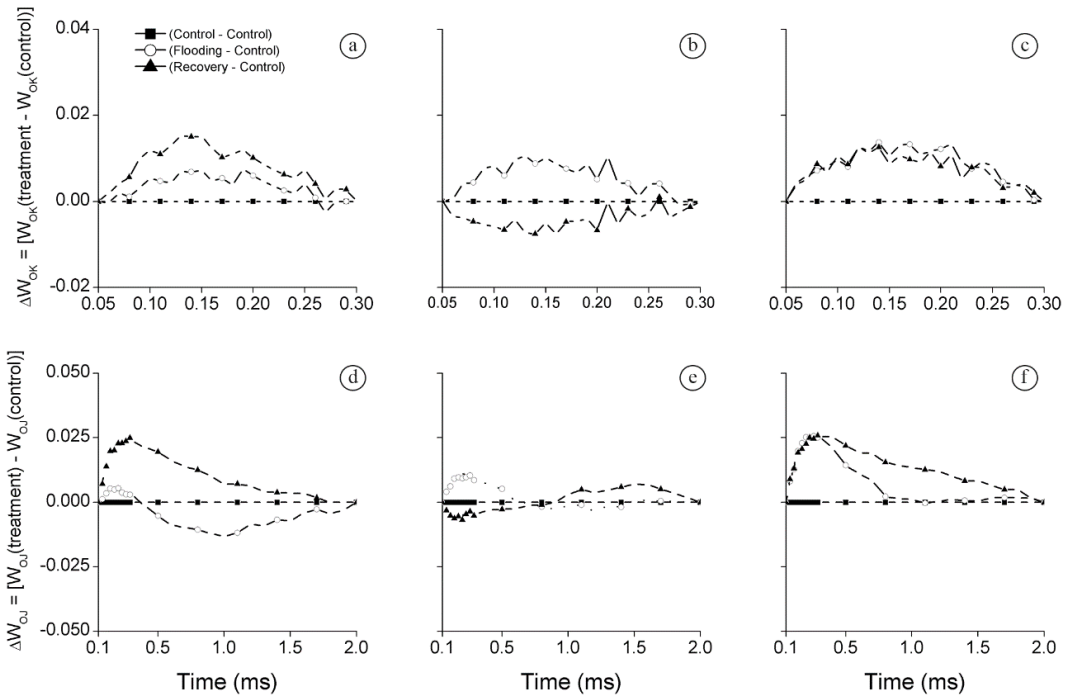


Figure 3. The difference in the kinetics of double normalization at F_0 and F_K [$\Delta W_{OK} = W_{OK[\text{treatment}]} - W_{OK[\text{control}]}$] (a-c) and the difference in the kinetics of double normalization at F_0 and F_j [$\Delta W_{OJ} = W_{OJ[\text{treatment}]} - W_{OJ[\text{control}]}$] (d-f) to the tomato plants 'Micro-Tom' with different expressions of HSP after flooding stress by 14 days (flooding) and flooding by seven days and seven days of the recovery (recovery). (a) and (d) - Wild type; (b) and (e) - MT-sHSP23.6 sense; (c) and (f) - MT-sHSP23.6 antisense.

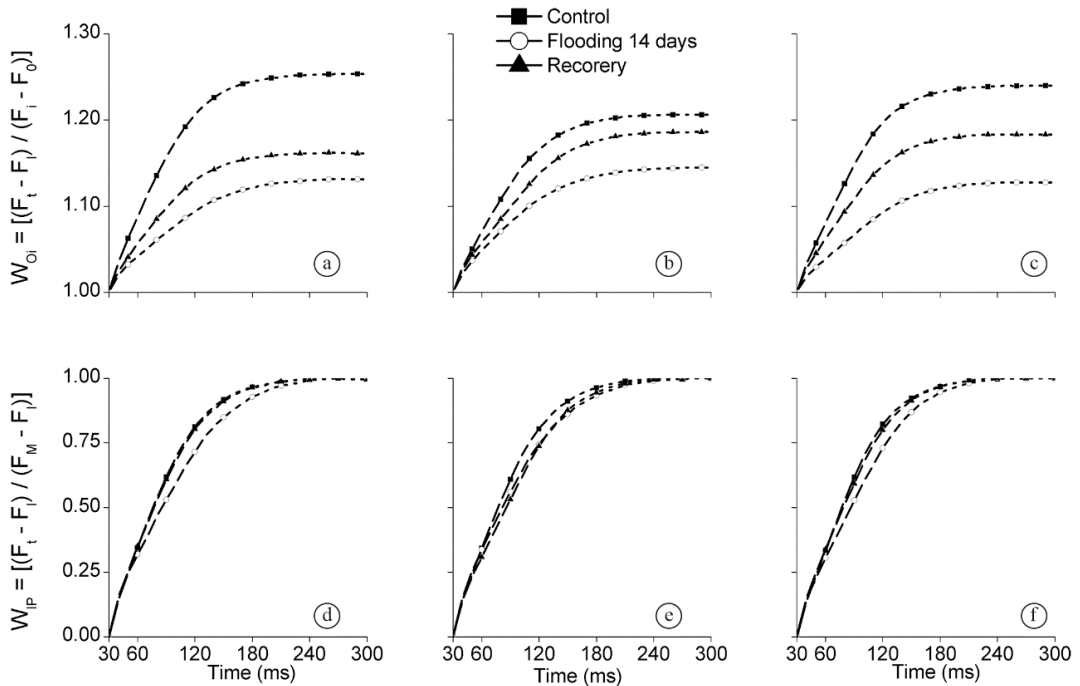


Figure 4. Double normalization at the F_0 to F_1 [$W_{O1} = (F_t - F_0) / (F_1 - F_0)$] (a-c) and double normalization at the F_1 to F_p [$W_{IP} = (F_t - F_1) / (F_m - F_1)$] (d-f) to the tomato plants 'Micro-Tom' with different expression levels of HSP after flooding stress by 14 days (flooding for 14 days) and flooding by seven days and seven days of the recovery (recovery). (a) and (d) - Wild type; (b) and (e) - MT-sHSP23.6 sense; (c) and (f) - MT-sHSP23.6 antisense. Events for $W_{O1} \geq 1.0$ illustrate the differences in the pool size of the end electron acceptors.

3.3. Gas exchange

Net CO₂ assimilation rate (*A*) (Figure 5 a-c) decreased under waterlogging conditions in all genotypes, though the reduction was high in the beginning of the treatment. On the other hand, WT and MT-sHSP23.6 sense genotypes, after seven days of waterlogging, increased the net photosynthetic rate close to the control, whereas MT-sHSP23.6 antisense genotype showed decrease time-dependent under waterlogging. When plants returned to normoxic conditions an increase in *A* parameter was shown by all genotypes. However, in WT plants, *A* remained below control levels even after

seven days of recovery. Regarding carboxylation efficiency (Figure 5 d-f), a similar behavior to liquid photosynthetic net was observed, and the reduction in *A/Ci* ratio is probably due to damage in the biochemistry reactions of photosynthesis.

3.4. Growth parameters

Plants from the genotypes WT and MT-sHSP23.6 antisense showed significant differences in terms of leaf area, mainly in waterlogged genotypes MT-sHSP23.6 antisense (Table 1). Leaf area was not affected by flooding

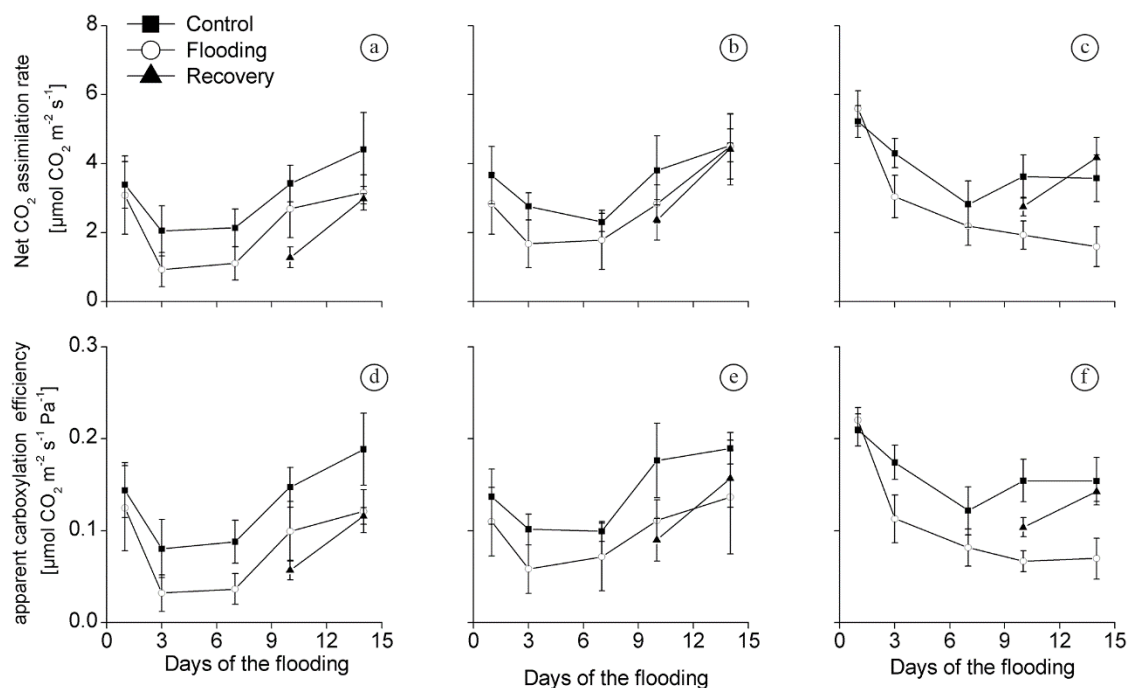


Figure 5. Net CO₂ assimilation rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (a-c) and apparent carboxylation efficiency ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$) (d-f) to the tomato plants ‘Micro-Tom’ with different expression levels of HSP after flooding stress by 14 days (flooding for 14 days) and flooding by seven days and seven days of the recovery (recovery). (a) and (d) - Wild type; (b) and (e) - MT-sHSP23.6 sense; (c) and (f) - MT-sHSP23.6 antisense.

Table 1. Growth parameters in tomato plants cv. Micro Tom measured at the end of the experimental period (14 days of flooding more seven days of recovery).

Genotype	Treatment	Leaf area [cm ² plant ⁻¹]	Dry shoot weight [g plant ⁻¹]	Dry root weight [g plant ⁻¹]	Dry matter total [g plant ⁻¹]	Shoot/root dry matter ratio
Wild type	Control	256 ± 16 a*	1.12 ± 0.29 a	0.17 ± 0.02 a	1.30 ± 0.15 a	6.4 ± 0.9 b
	Flooding	147 ± 15 b	1.00 ± 0.16 a	0.08 ± 0.16 b	1.02 ± 0.10 a	9.6 ± 1.3 a
	Recovery	157 ± 13 b	0.87 ± 0.24 a	0.13 ± 0.02 a	1.06 ± 0.05 a	6.1 ± 0.3 b
MT-sHSP23.6 sense	Control	218 ± 17 a	1.10 ± 0.13 a	0.19 ± 0.04 a	1.29 ± 0.08 a	6.0 ± 0.7 b
	Flooding	198 ± 14 a	1.16 ± 0.16 a	0.11 ± 0.02 a	1.28 ± 0.05 a	10.0 ± 1.0 a
	Recovery	233 ± 21 a	1.09 ± 0.21 a	0.18 ± 0.05 a	1.28 ± 0.10 a	6.4 ± 1.1 b
MT-sHSP23.6 antisense	Control	250 ± 24 a	1.01 ± 0.23 a	0.23 ± 0.09 a	1.07 ± 0.09 a	5.3 ± 0.8 a
	Flooding	103 ± 27 c	0.85 ± 0.22 a	0.07 ± 0.04 b	0.64 ± 0.07 b	5.6 ± 1.2 a
	Recovery	164 ± 15 b	0.66 ± 0.14 a	0.23 ± 0.03 a	1.08 ± 0.07 a	4.0 ± 0.9 a

*Values followed by different letters are significantly different ($p < 0.05$; Tukey test) for each parameters and genotype. Means ± Standard Error (SE) (n = 5).

in MT-sHSP23.6 sense plants. Total dry matter was only affected by flooding in MT-sHSP23.6 antisense plants. However, for this genotype, the shoot/root dry matter ratio did not differ from its control. Although the shoot dry matter decreased, so did the root dry matter. Therefore, there were no significant differences between treatments. Flooded WT and MT-sHSP23.6 sense plants exhibited a greater increase in shoots than in roots, resulting in greater shoot/root dry matter ratios. The control of WT and MT-sHSP23.6 (sense ou antisense) genotypes exhibited a more pronounced drop in this ratio following the recovery period, with a relatively high increase in root dry matter and a relatively small increase in shoot dry matter.

4. Discussion

Photosynthetic responses genotypes to flooding were detected through changes in chlorophyll fluorescence parameters, suggesting that flooding caused changes in absorption and in the utilization of light energy by the electron transport chain for photosynthesis. The changes in JIP-test parameters were more pronounced in the WT and MT-sHSP23.6 antisense genotypes. Relative to the WT plants, the effects of flooding were more evident in the parameters related to oxidation and reduction of the electron acceptors of PSII (S_m and N). Flood stress resulted in fewer total electron transporters per reaction center, leading to a deficit in the reduction of Q_A .

For the parameters describing specific energy fluxes per reaction center, the electron flux reducing end electron acceptors at the PSI acceptor side, per RC (RE_0/RC) all genotypes were affected, with slight decrease or increase in MT-sHSP23.6 sense genotype, may be due to a higher stability of the membranes and its protection capacity to the cellular damage induced by the stress. Changes in this parameter resulted in a decrease in the parameters ϕ_{Ro} and δ_{Ro} (probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor side) relative to the control plants, demonstrating that there was a significant effect of flooding on the activity of PSI.

The performance indices (PI_{ABS} and PI_{total}) used in this study have been suggested to be the most efficient parameters for quantifying stress us genotypes because they are products of terms that express partial potentials for the conservation of energy at sequential bifurcations of photons absorbed by PSII for the reduction of intersystem electron acceptors (PI_{ABS}) and for the reduction of the final receptors of PSI (PI_{total}) (Strasser et al., 2010; Yusuf et al., 2010).

The parameter PI_{ABS} decreased with duration of flooding. According to Strasser et al. (2010), a large drop in PI_{ABS} can be explained by changes in one, two, or three parameters and shows a rapid decrease in the efficiency of the redox reaction in the electron transport chain. Slabbert and Krüger (2011) describe PI_{ABS} as a marker of plant vitality. In this study, the effect of flooding was most evident in the parameter PI_{total} , which showed the greatest decrease with flooding and reflected little energy conservation over time.

Yusuf et al. (2010) showed that decreases in this parameter indicates a negative "strain" on the system photosynthetic, and PI_{total} parameter is assumed to be the most sensitive of the JIP test, once allows to evaluate several paramaters from transient fluorescence OJIP.

It should be noted that the MT-sHSP23.6 sense genotype best maintained the flow of energy to PSI throughout the period of flood stress, suggesting that the MT-sHSP23.6 sense and the function of this gene in stress response mechanisms may have led to greater flood tolerance (Siddique et al., 2008). Dramatic decreases in PI_{total} lead to reduced PSII activity and counteract damage to the structure and function of PSI. The IP phase of the OJIP curve is related to electron transfer through PSI and the reduction of electron congestion caused by a transient block on the acceptor side of PSI due to inactivation of the ferredoxin NADP⁺ reductase (Strasser et al., 2010).

Regarding the recovery period (Figure 2) all parameters from JIP test returned to values similar to those observed in the control in MT-sHSP23.6 sense genotype. However, the parameters associated with the activity and functionality of the PSI were still having a residual flooding effect, particularly on the third day of recovery. It demonstrate that the recovery after a flooding stress allows the photosynthetic machinery return back to its full functionality, regarding its structure and functionality as determined by the parameters of efficiency, yield and performance. However, it was not observed in WT and MT-sHSP23.6 antisense genotypes, indicating that the structural and functional damage was not repaired during the recovery period evaluated, thus, linking these data with the functional capacity of HSP23.6 protein. Further more, the reestablishment of the homeostasis after a stress, once the transition from hypoxia to return to the oxygenation, promotes the formation of reactive oxygen species (Mustroph et al., 2010), and these proteins play an important role in activating the antioxidant system (Sule et al., 2004).

Normalizations and subtractions were applied to analyze the OJIP fluorescence transient, and these procedures permitted the detection of bands between the O and P steps. Using these normalizations, it was obtained and identified the positive L-band in all genotypes studied. The L-band (Stirbet et al., 1998) obtained by subtraction between K and O from variable fluorescence relative, is a good indicator of the connectivity of the PSII complex, as well as its connectivity is closely related to its size, big L-bands have weak connectivity (Yusuf et al., 2010). Thus, was observed that 14 days of waterlogging imposed a loss of connectivity between the units of the PSII in all genotypes, whereas MT-sHSP23.6 sense genotype was effective in maintaining the connectivity between units of PSII upon recovery (negative L-band) once, high connectivity results in better utilization of excitation energy and better stability of the (Strasser et al., 2004).

The analysis of fluorescence normalized between O (50 μ s) and J (2 ms) enabled the identification of the K-band at approximately 300 μ s. According to Yusuf et al. (2010), the presence of the positive K-band reflects inactivation of

the oxygen-evolving complex (especially the Mn complex) and/or an increase in the size of the functional PSII antenna. The K-band is normally observed in plants under heat stress. However, in this study, it was also identified in plants under flooding stress, especially MT-sHSP23.6 antisense plants, that the transition from recovery to normal conditions did not impose damages. However, in MT-sHSP23.6 sense plants were not identified a K-band either upon waterlogging and recovery in comparison to control plants, indicating an effective stability of the liberty oxygen complex in response to the flooding.

The analysis of the O-I phase helped clarify the sequence of events involved in exciton capture by PSII up to the reduction of plastoquinone (PQ) (Yusuf et al., 2010). The MT-sHSP23.6 antisense and WT genotypes showed the greatest changes in this phase during flood stress, suggesting that higher expressions of HSP may be correlated with increased flood tolerance, given that there was no limitation on PQ reduction and electrons were largely transferred to the cytochrome complex *b₆f*.

The kinetic difference between points I and P (30 ms to nearly 300 ms) reflects changes in the flow of electrons from plastoquinone (PQ) to the final electron acceptor of PSI (Yusuf et al., 2010). According to the results of our study, induced flood stress reduced the size of the PSI electron acceptor pool for all genotypes, suggesting that there was a reduction in the photochemical yield, but the effect was smallest in the genotype with a high expression of MT-sHSP23.6.

In our study, decreases in the rate of photosynthesis were mostly related to carboxylation efficiency, indicating that non-stomatal factors had the greatest effect on photosynthesis. Some published reports have suggested that the emergence of adventitious roots, which act as a new drain for photoassimilates, explains the maintenance of stomatal openings and transpiration rates under stress (Sauter, 2013) in these genotypes. However, at the end of the experiment the liquid assimilation rate of all genotypes recovered, indicating that a flooding stress can cause a metabolic disorder and these plants are able to recovery of CO₂ fixation, and there are reports that these proteins are important during the recovery after a flooding stress, by playing a synergistic action against cell damage (Sule et al., 2004).

In our experiment, WT plants had a smaller leaf area with flood stress, but total dry matter was not affected. A plant recovery period did not reverse this effect, and leaf area remained low. A similar pattern was observed in plants with MT-sHSP23.6 antisense. In contrast, plants with MT-sHSP23.6 sense did not show a decrease in leaf area with flood stress, but they did exhibit a higher ratio of shoot to root dry matter.

The most significant difference among genotypes in this study was the difference in the shoot:root dry matter ratio between the genotypes MT-sHSP23.6 sense and MT-sHSP23.6 antisense. Plants from the MT-sHSP23.6 sense genotype had a ratio of 10:1 under flood stress, while MT-sHSP23.6 antisense plants had a ratio of 5.6:1.

In addition, was observed necrosis and degradation in root tissues in MT-sHSP23.6 antisense genotype (Data not shown).

Thus, the limited plant growth cannot be explained by a single physiological process, even if photosynthesis is the dominant physiological process involved. Such alterations lead to stunted growth and gradual deterioration of the main roots, as observed in the roots of genotype MT-sHSP23.6 antisense in this study. Adventitious roots appeared on MT-sHSP23.6 sense plants after the 10th day of flooding, which may explain the greater tolerance of this genotype to stress from flooding. Plants from the other genotypes did not develop these roots.

The importance of *sHSP23.6* gene in response to hypoxia stress was reported by Waters et al. (2008) and Pegoraro et al. (2012), as being co-regulated under low oxygen levels, by encoding a protein within the mitochondria. As this organelle in plants is responsible for important metabolic processes such as photorespiration, oxidative phosphorylation, and process of the tricarboxylic acid cycle (Taylor et al., 2009) is believed that changes in the expression of specific genes during hypoxic stress (Pandey and Kim, 2012) plus a high expression of the *sHSP23.6* gene have played an important role in the energy redox state of the cells, compensating the hypoxic effects, mainly in MT-sHSP23.6 sense genotype.

Thus, among the genotypes analyzed, the MT-sHSP23.6 sense was more tolerant to the flooding and recovery treatment imposed and the results suggest that mitochondrial HSP play an important role in determining plant response to stress (hypoxia and recovery) and that the responses of plants can vary widely by genotype.

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Abbreviations: MT-sHSP23.6 antisense - Plants transformed in the anti-sense orientation; MT-sHSP23.6 sense - Plants transformed in the sense orientation; WT - Non-transformed plants.