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Evaluation of Tomato Genetic Resources for Response to Water Deficit

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ABSTRACT

Water deficit strongly affects plant yield and quality. However, plants can minimize drought injury by adaptation mechanisms that have evolved to escape harmful conditions. The response to water deprivation is a complex trait controlled by several genes. In order to gain a deeper understanding of drought response mechanisms in tomato, a collection of 27 genotypes was studied under different water deficit conditions. Since developmental stages might be differently influenced by drought, analyses were carried out on young plantlets during fruit setting. The only genotype that showed good performances both as water retention and fruit production was the ecotype Siccagno. All the genotypes were analyzed at molecular level with the aim of detecting structural polymorphisms in selected stress-responsive genes. In addition, the expression level of a number of these genes was measured in the genotypes more tolerant to water deficit. Many polymorphisms were detected in six stress-responsive genes, and some could imply significant modifications in the protein structure. Furthermore, the expression analysis by RT-qPCR of three stress-responsive genes allowed arguing that a higher level of expression of the gene *erd*15 might be related to the better response to water deficit exhibited by Siccagno. Similarly, the lower expression of eight genes in the same genotype analysed through a microarray experiment confirmed the involvement of these stress-related genes in the tomato response to drought. Further investigations are required for a better comprehension of the mechanisms underlying response to water deficit in tomato by exploiting the genetic resource identified as more tolerant. The use of new technologies able to globally analyze structural polymorphism and expression level of genes will succeed to identify crucial genes involved in stress response in the ecotype Siccagno grown under different water regimes.

Keywords: Drought-Tolerance; Water Loss Rate; Relative Water Content; Yield Evaluation; Gene Polymorphisms

1. Introduction

Water deficiency is one of major environmental constraints that strongly affect cultivated plants, reducing growth and yield. However, plants can minimize drought injury by adaptation mechanisms that have evolved to escape harmful conditions [1]. The drought tolerance mechanism is a polygenic trait controlled by several small effect genes or QTLs and leads to physiological, biochemical and molecular changes, such as the synthesis of abscisic acid (ABA), the accumulation of various osmolytes and proteins with a role in repair and protection in synergy with an efficient antioxidant system [2]. The physiological mechanisms that allow plants to overcome the stress condition and to grow during episodes of stress have been extensively studied [3]. They include maintaining cell turgor pressure and reducing water loss by the accumulation of molecules such as betaine, proline, sorbitol, and so on [4]. As for physiological and biochemical mechanisms, many drought-related genes with different roles have also been identified through molecular and genomic analysis of Arabidopsis, rice, tomato and other species [5-7]. The large-scale analysis of the transcriptome has demonstrated the presence of hundreds of genes that are activated or repressed in response to osmotic and water stress [8,9]. To address the complexity of plant responses to drought, it is vital to understand the physiological and genetic basis of this response [10].

Despite significant progress during the past decade in our understanding of pathways affected by drought stress, limited information is available regarding the dynamic of gene networks in tomato under stress conditions. Com-

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pared to other tomato abiotic stresses, less mapping research has been conducted on tomato drought tolerance [11]. In effect, in the past years, breeding efforts to improve drought tolerance have been hindered by its quantitative genetic control and by the poor understanding of the physiological basis of yield in water-limited conditions [12].

Nowadays, thanks to the completed sequencing of tomato genome [13], dozens of genes important for tomato breeding have been mapped and molecular markers are available online (http://sgn.cornell.edu). Knowing the candidate genes for important traits and the exact functional nucleotide polymorphism within these genes, breeders can identify useful alleles in the available germplasm. In addition, breeders can create novel genotypes through the introgression/pyramiding of favourite unused natural alleles and/or by shuffling and re-organizing genomic sequences. In addition, plant breeders can consider manipulating transcription and regulation factors to generate a pool of new trait variations.

Our goal was to study the effects of water deficit on the tomato plant, both at level of plantula and of fruit production. In order to better understand the tomato response when grown under drought conditions, field and greenhouse trials were combined with molecular analyses, and various genetic resources were explored. Among these, some wild species and some local ecotypes were chosen, where a wide genetic variability could be still exploited.

2. Materials and Methods

2.1. Plant Material

Twenty-seven tomato genotypes (**Table 1**), including

Table 1. List of the 27 tomato genotypes analyzed. For each genotype, the species in the genus *Solanum*, the category (wild or cultivated), the country of origin and the source providing seeds are reported.

ACCESSION/NAME	SPECIES	CATEGORY	COUNTRY OF ORIGIN	SOURCE
AD17	*S. lycopersicum*	advanced line	Italy	Univ. of Naples
AL-22/041	*S. lycopersicum*	ecotype	Albany	Univ. of Bari
AL-22/044	*S. lycopersicum*	ecotype	Albany	Univ. of Bari
AL-22/046	*S. lycopersicum*	ecotype	Albany	Univ. of Bari
AL-22/057	*S. lycopersicum*	ecotype	Albany	Univ. of Bari
AL-22/059	*S. lycopersicum*	ecotype	Albany	Univ. of Bari
AL-22/064	*S. lycopersicum*	ecotype	Albany	Univ. of Bari
AL-22/070	*S. lycopersicum*	ecotype	Albany	Univ. of Bari
AL-22/076	*S. lycopersicum*	ecotype	Albany	Univ. of Bari
Casarbore	*S. lycopersicum*	ecotype	Italy	Univ. of Naples
Chile	*S. lycopersicum*	cultivar	Chile	Chilean market
GiaGiù	*S. lycopersicum*	ecotype	Italy	Univ. of Naples
IL9-2-5	*S. pennellii* in *S. lycopersicum*	introgression line	Israel	TGRC
IT-22/005	*S. lycopersicum*	ecotype	Italy	Univ. of Bari
IT-22/025	*S. lycopersicum*	ecotype	Italy	Univ. of Bari
IT-22/030-13	*S. lycopersicum*	ecotype	Italy	Univ. of Bari
LA0462	*S. peruvianum*	wild species	Chile	TGRC
LA0716	*S. pennellii*	wild species	Perù	TGRC
LA1421	*S. lycopersicum* var. *cerasiforme*	wild species	Ecuador	TGRC
LA1579	*S. pimpinellifolium*	wild species	Perù	TGRC
LA1959	*S. chilense*	wild species	Perù	TGRC
LA2711	*S. lycopersicum* cv Edkawi	old cultivar	Egypt	TGRC
M82	*S. lycopersicum*	cultivar	USA	TGRC
Parminatella	*S. lycopersicum*	ecotype	Italy	Univ. of Naples
Sel6	*S. lycopersicum*	ecotype	Italy	Univ. of Naples
Siccagno	*S. lycopersicum*	ecotype	Italy	Univ. of Naples
Vesuvio2001	*S. lycopersicum*	ecotype	Italy	Univ. of Naples

wild species, ecotypes and varieties, were selected for physiological and molecular analyses. Among the wild species, *S. pennellii* (LA0716), *S. pimpinellifolium* (LA1579), *S. chilense* (LA1972), *S. peruvianum* (LA0462) and *S. lycopersicum* var. *cerasiforme* (LA1421) were obtained from the Tomato Genetic Resource Center (TGRC), together with the old cultivar *S. lycopersicum* cv. Edkawi (LA2711). Seventeen local ecotypes come from South Italy and Albany; some were kindly provided by Prof. Ricciardi, University of Bari, Italy. In addition, two cultivars (Chile, M82), one advanced selected line (AD17) and one *S. pennellii* introgression line (IL9-2-5) were added to our collection. Seeds were sown directly in the alveolar plateau containing a mixture of soil and peat, seedlings were then transplanted into larger pots about two weeks after sowing and were grown in greenhouse. Seeds that showed a reduced germination rate were pre-germinated in Petri dishes by a solution of KNO_3 (2 g/l) or GA_3 (3000 ppm).

2.2. Desiccation Test

Tomato plants were subject to a short desiccation test and both Water Loss Rate (*WLR*) and Relative Water Content (*RWC*) were evaluated. Seedlings having similar leaf size and number (at least four true leaves) were selected within each genotype and detached for assaying *WLR* and *RWC*. As for treated plants, fresh weight (*FW*) was immediately recorded (time 0), and then seedlings were placed on dry paper for 24 h at room temperature and the weight (W_{24}) was measured again. After, leaves were soaked in distilled water for 24 h at room temperature in darkness and the turgid weight (*TW*) was recorded. Finally, total dry weight (*DW*) was recorded after drying for 24 h at 80˚C. As for control plants, only the fresh weight (*FW*) at time 0, the turgid weight (*TW*) and the dry weight (*DW*) were recorded. In both cases, *WLR* and *RWC* were evaluated. The water loss rate (*WLR*) was measured as described by [14] using the formula:

$$WLR\left(\mathrm{g}\cdot\mathrm{h}^{-1}\mathrm{g}^{-1}\,DW\right)=\left(FW-W_{24}\right)/\left(DW\times 24\right).$$

The RWC was calculated according to [15]:

$$RWC\left(\%\right)=\left[\left(FW-DW\right)/\left(TW-DW\right)\right]\times 100.$$

2.3. Field Trial

Eight tomato cultivars were utilized for a drought test in field conditions (Acerra, Campania region in the Southern Italy). Control and stressed plants were grown under a plastic tunnel under standard agricultural practices and the same watering volume was applied until 50% of plants appeared to develop fruit set on the first inflorescences. After this time the tested plants were not watered anymore. RWC of control and stressed plants was evaluated weekly. Leaf tissues of three plants per genotype were harvested from control and stressed plants for five consecutive weeks after treatment, frozen in liquid nitrogen, and stored at −80˚C for RNA extraction. Marketable and scrap fruit harvested from all the genotypes were recorded. The analysis of variance (ANOVA) was carried out using the SPSS (Statistical Package for Social Sciences) Package 6 version 15.0. In particular, the General Linear Model (UNIVARIATE ANOVA with a Duncan Post-Hoc test, $P < 0.05$) was used to ascertain the differences among genotypes and between stressed and not stressed plants.

2.4. DNA Sequencing

Leaf samples were collected from seedlings, frozen in liquid nitrogen and stored at −80˚C. DNA extraction was performed using the commercial kit DNeasy® Plant Mini Kit provided by QIAGEN following manufacturer's instructions. DNA quality and quantity was checked by agarose electrophoresis and by spectrophotometric quantification utilizing NanoDrop 1000 Spectrophotometer v 3.60. DNA amplification was carried out by PCR using specific primers designed on the gene sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/nuccore) and/or available at the Solgenomics website (http://solgenomics.net). PCR products were purified by the QIAquick PCR Purification Kit of QIAGEN. DNA fragments were then sequenced using the BigDye® Terminator Cycle Sequencing Kit v.3.1 based on the Sanger method. Samples were purified using BigDye® XTerminator TM Purification Kit Applied Biosystems and analyzed on a capillary sequencer ABI PRISM 3130 Genetic Analyzer.

2.5. RNA Analysis

Total RNA was isolated from homogenized powdered tomato leaves stored at −80˚C utilizing the TRI Reagent Solution Ambion with some modifications. Approximately, 0.14 g of ground leaves were added to 1 ml TRI Reagent Solution, tubes were shaken and incubated for 5 min at room temp. 200 µl of chloroform were added and samples were incubated on ice for 10 min, and then centrifuged at 13,000 rpm (15 min, 4˚C). The aqueous phase was transferred to a clean tube, the nucleic acid was precipitated in the aqueous phase with the addition of 500 µl of isopropanol *per* 1 ml of TRI Reagent solution, vortexed for 5 - 10 sec, and incubated on ice for 5 - 10 min. Following centrifugation at 13,000 rpm (15 min, 4˚C) the pellet was washed in 1 ml of 75% ethanol. The samples were then re-dissolved in 300 µl of RNase-free water. RNA samples were quantified finally using a NanoDrop 1000 Spectrophotometer *v* 3.60 and stored at −80˚C. 1 µg of total RNA was reverse transcribed with the Transcrip-

tor High Fidelity cDNA Synthesis Kit, Roche. Amplification of Real-TimePCR products was carried out with a 7900HT Fast Real-Time PCR System (Applied Biosystems) using Power SYBR® Green Master Mix (Applied Biosystems) as detection system in a reaction mixture of 25 µl containing: 0.5 µM of each primer, 12.5 µl of SYBR GreenPCR master mix. Preparation of reactions was automated using the liquid handler Freedom Evo 150 (Tecan). Relative quantification was achieved by the ΔΔCt method [16].

2.6. Bioinformatic Tools

PCR primers were designed using Primer3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Sequences for RT-qPCR primers were retrieved from the Universal Probe Library Assay Design Center (http://www.roche-applied-science.com) if available, or alternatively designed *ad hoc* by using Primer express software version 3.0 (Applied Biosystems). Sequences were processed by ABI PRISM SeqScape software.

3. Results and Discussion

In order to gain a deeper understanding of drought response mechanisms in tomato, a collection of 27 tomato genotypes was studied under different water deficit conditions. The collection included five wild species and 17 local ecotypes. In particular, the wild species were selected by exploring the Tomato Genetic Resource Center (TGRC) database (http://tgrc.ucdavis.edu) using the "abiotic stress tolerance" key-word. These species evolved in extreme habitats of South America, where is the tomato origin center. As for the local ecotypes, they were mainly selected both for fruit yield and quality in southern areas of Italy and Albany.

3.1. Desiccation Test

The effect of drought during plant growth was evaluated by performing a Short-Term (ST) desiccation test on young plantlet, where the Relative Water Content (RWC) and Water Loss Rate (WLR) were recorded. Indeed, the Relative Water Content is considered as a measure of plant water status showing the metabolic activity in the leaf tissue [17], since the plant resistance/tolerance to drought is related to its ability to maintain higher relative water content in the leaves under water stress. In addition, the WLR could allow the selection of more tolerant genotypes, as also reported in wild barley and wheat where a preliminary screening of genotypes with different response to water deficit was realized on the basis of WLR values [14,18].

For all genotypes analyzed, the RWC values (**Figure 1**) showed a significant reduction after 24 hours of desiccation and no differences were observed within genotypes belonging to the two groups considered (the first including wild genotypes coming from South America and the second mainly ecotypes from South Italy and Albania). In particular, in the first group no significant difference ($P = 0.271$) was detected for RWC values among well-irrigated plants. In addition, all genotypes showed a significant ($P = 0.001$) reduction in their RWC 24 hours after the dehydration treatment was applied and also a different response to water stress. Unfortunately, among wild species *S. pennellii* plants did not produce enough leaves for the RWC test because of their low growing rate; therefore, the leaves collected only allowed performing the molecular analysis. Within the second group of genotypes, significant differences in the RWC values were found both before and after 24 hours of dehydration treatment ($P < 0.001$).

The values of WLR were variable among tested genotypes and ranged from 0.09 to 0.18 $g \cdot h^{-1} g^{-1}$ DW in the first group of genotypes. Univariate ANOVA displayed significant differences among genotypes ($P < 0.001$). In particular, *S. pimpinellifolium* and *S. lycopersicum* cv. Edkawi showed a lower value compared to *S. lycopersicum* var. *cerasiforme*, which showed the highest value. The ANOVA also showed significant differences among genotypes of the second group ($P < 0.05$). The values were variable among genotypes and ranged from 0.06 to 0.13 $g \cdot h^{-1} g^{-1}$ DW; AL-22/057 and M82 showed the lowest values whereas Parminatella showed the highest.

Based on WLR results, tomato genotypes could be roughly classified in susceptible and tolerant as well as done in wild barley and wheat [14,18]. Accordingly, most of the genotypes belonging to the second group (AL22/041, AL22/044, AL22/046, AL22/059, AL22/076, IL9-2-5, IT-22/005, IT-22/025, Parminatella, Sel6) were classified as more susceptible, since they had a high WLR after 24 hours of desiccation on the laboratory bench. By contrast, AL22/057, GiaGiù, IT-22/30-013, M82, Siccagno and Vesuvio 2001 showed the lowest values of WLR. Indeed, it is known that the ecotype Siccagno performs better under reduced water supply and the genotype M82 is considered semi tolerant [19]. Therefore, the two genotypes M82 and Siccagno confirmed their tolerance to water deficit, whereas this behavior is new for Vesuvio 2001 and for GiaGiù, which also showed a low WLR value. It is noteworthy that both genotypes come from the same geographical area, which is from Vesuvio slopes.

3.2. Field Trial

In order to evaluate the response to drought in terms of yield, a group of local ecotypes was also tested in a field trial, whereas the wild species were excluded since they usually have fruit size very different from the cultivated

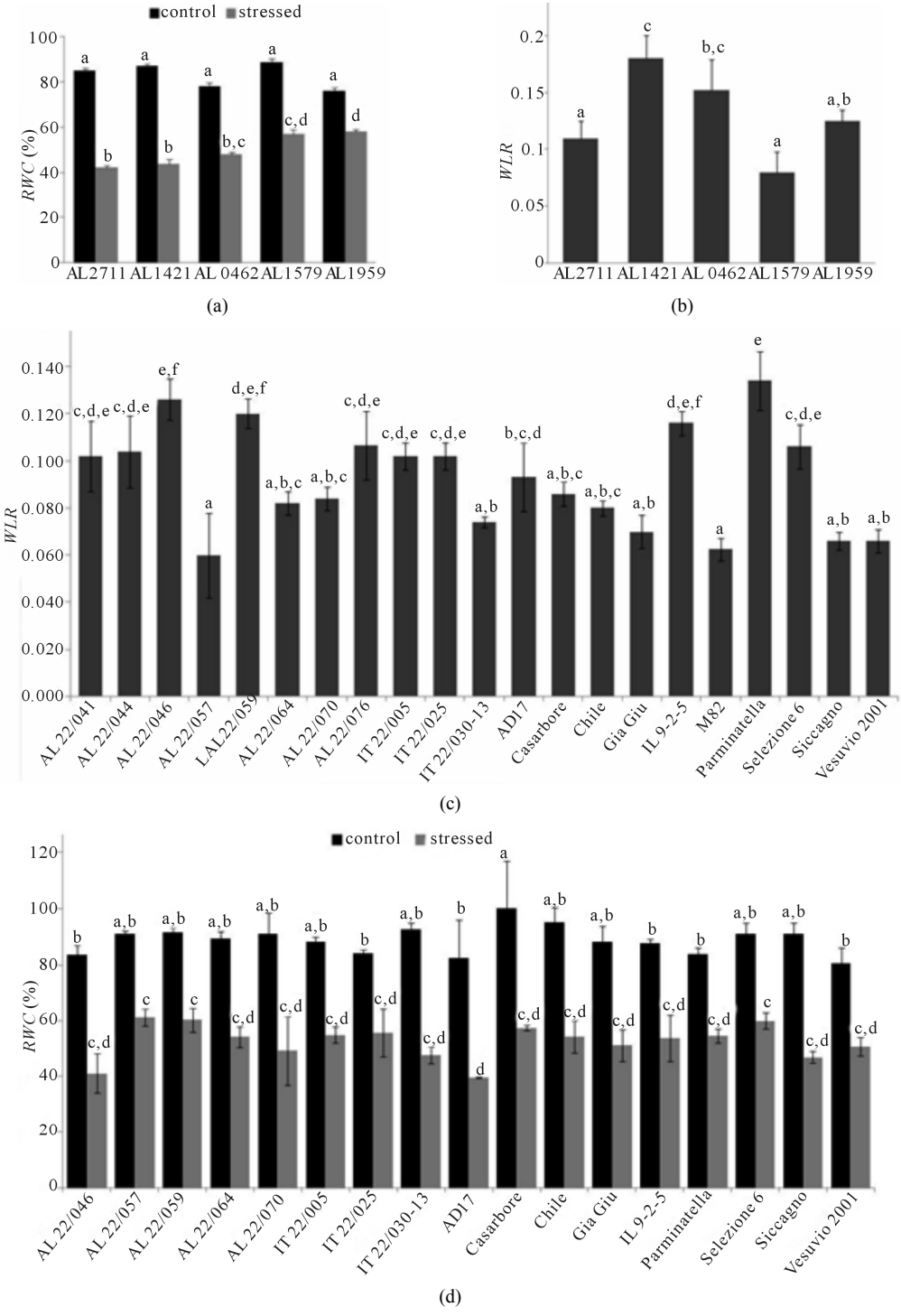

Figure 1. Relative Water Content (*RWC*) and Water Loss Rate (*WLR*) of wild tomato species ((a), (b)), ecotypes and cultivar ((c), (d)) measured after a short term desiccation test. Same letter indicates not significant difference among genotypes (Post Hoc test; Duncan P < 0.05).

genotypes and also reduced yield, not comparable with those of ecotypes and varieties. The experiment was carried out with eight genotypes (Casarbore, GiaGiù, Siccagno, AL-22/057, AL-22/059, IT-22/005, IT-22/030-13, IT-22/025) chosen on the basis of previously field data (data not shown) and of the desiccation test carried out in the present work. Indeed, the rapid screening of a high number of genotypes carried out by the laboratory test allowed to select a restricted number of genotypes to submit to the field trial. In particular, the desiccation test

led to choose four more tolerant genotypes (GiaGiù, Siccagno, AL-22/057, IT-22/030-13), three susceptible (AL-22/059, IT-22/005 and IT-22/025) and one exhibiting an intermediate performance (Casarbore).

The RWC was recorded in consecutive weeks. Overall, most of genotypes did not respond to water deprivation since they did not show any significant decrease in leaf RWC. AL-22/059, IT-22/005 and Siccagno responded early showing a significant reduction in RWC under un-watered conditions limited to the first week whereas IT-22/025 and Casarbore performed a later response.

Red-ripe fruits were harvested from both watered and un-watered plants on August 4th, 19th and on September 1st and yield parameters were recorded on each harvesting (**Figure 2**). Overall, Casarbore, IT-22/005, IT-22/030-13 and Siccagno showed the highest marketable production when grown in watered conditions. Unfortunately, as often reported for tomato grown in stressful conditions [20], they also showed the strongest decrease under water deprivation, except than Siccagno. Indeed, without water supply Siccagno performed better than the other genotypes. As for the level of marketable produc-

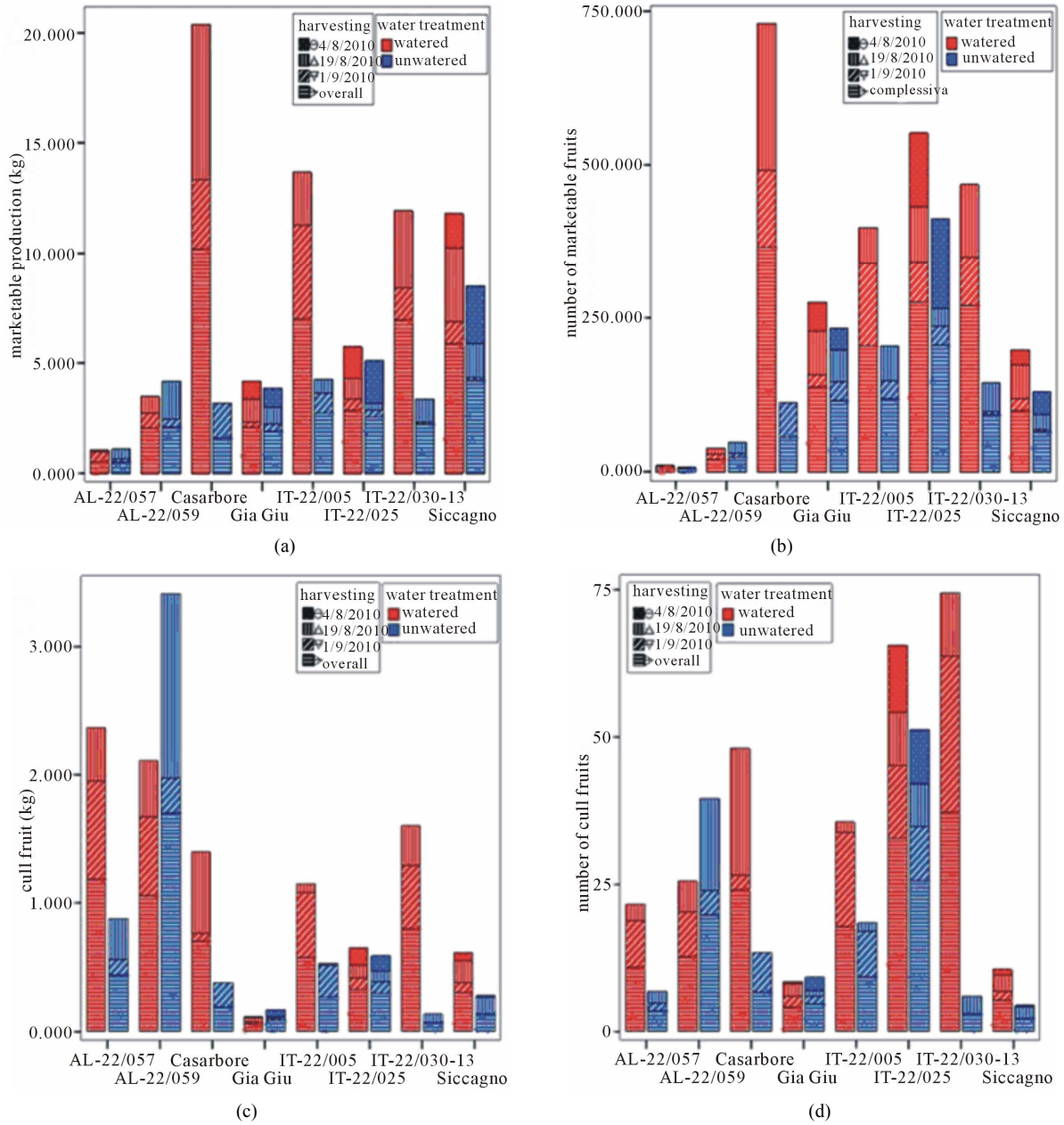

Figure 2. Yield descriptors (marketable production, number of marketable fruits, cull fruit and number of cull fruits) of tested tomato genotypes in the field trial as recorded according to water treatments and harvest period. (a) Marketable production (Kg); (b) Number of marketable fruits; (c) Cull fruit (Kg); (d) Number of cull fruits.

tion *per* plant is concerning, AL-22/057 and AL-22/059 showed an extremely low yield in both watered and un-watered conditions, whereas Casarbore, IT-22/005, IT-22/030-13 confirmed a very deep decrease following un-watering. Finally, GiàGiù, was almost unaffected by water deprivation and Siccagno accounted for a reduced decrease.

Also, the cull fruit *per* plant both in term of weight and number decreased in the un-watered treatment in all genotypes except than for AL-22/059, which showed an opposite trend (**Figure 2**). In addition, the reduced water availability affected the average fruit weight of AL-22/059 and AL-22/057 showing the highest decrease when water deprivation occurred.

Comprehensively, all tested genotypes were graphically discriminated (**Figure 3**), according to the water treatment, by Principle Component Analysis (PCA) performed on yield descriptors (marketable production, number of marketable fruits, cull fruit and number of cull fruits, average fruit weight). Two factors explained 84% of the overall variability. In particular, factor 1 mainly described marketable production and fruits, whereas factor 2 mainly described cull fruit and average fruit weight. A drought-tolerant genotype is expected to gain higher score on factor 1 and lower score on factor 2 regardless of water deprivation. Given this, IT-22/025 and Siccagno ranked best for their ability to grow and produce in low-water input systems. As a whole, the only genotype that showed good performances both in the desiccation test and in the field trial was Siccagno.

3.3. Polymorphisms in Stress Responsive Genes

All the genotypes were analyzed at molecular level with the aim of detecting structural polymorphisms in some selected stress responsive genes (**Table 2**). The sequences of six stress responsive genes, which were previously studied in tomato response to different abiotic stresses, were obtained from the Heinz 1706 complete tomato genome sequence available at the Solgenomics website (www.solgenomics.net). In particular, a BLAST search allowed the identification of the corresponding gene model (identified by mean of a Solyc ID) for *ars*2, *mkp*1 and *cip*1, whereas for the remaining three genes (*tsw*12, *tas*14, *erd*15) only the corresponding scaffold was found. Afterwards, these genes were completely re-sequenced in the selected collection of 27 genotypes and polymorphisms were fully characterized. **Table 3** reports primer sequences designed for the amplification of each gene, the annealing temperature used and the size of the amplified fragment. The resulting sequences were processed by ABI PRISM SeqScape software that allows the analysis of re-sequencing data by comparing consensus

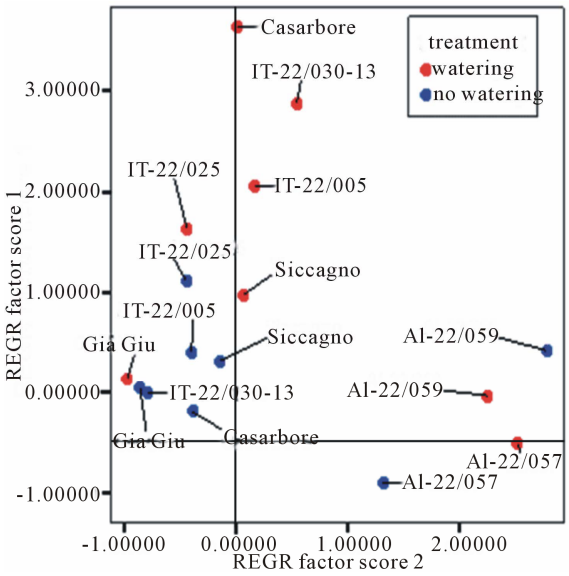

Figure 3. Graphical discrimination of tomato genotypes grown under two water management systems performed by Principle Component Analysis (PCA) based on yield descriptors. Factor 1 and 2 included 84% of the overall variability. In particular, factor 1 mainly described marketable production, number of marketable fruits, number of cull fruits, whereas factor 2 mainly described cull fruit and average fruit weight.

Table 2. Characteristics of six sequenced genes potentially involved in stress response. For each gene, the gene or scaffold ID of ITAG2.3 annotation in the Solgenomics databases, the chromosome mapping and the length in bp are also reported.

NAME	ITAG2.3 GENE /SCAFFOLD ID	GENE LENGTH (bp)	DESCRIPTION	FUNCTIONAL CLASS	REFERENCE
*mkp*1	Solyc05g054700 ch5	3605	MAP kinase phosphatase	Signal transduction	[21,22]
*asr*2	Solyc04g071580 ch4	811	abscicic acid stress ripening 2	Transcription factor	[23,24]
*tas*14	SL2.40sc03665 ch2	746	abscisic acid and environmental stress-inducible protein	Protein response and damage repair	[25,26]
*tsw*12	SL2.40sc04199 ch10	891	non-specific lipid-transfer protein 1 precursor	Protein response and damage repair	[27,28]
*erd*15	SL2.40sc04199 ch10	974	dehydratation induced protein	Defense protein	[29,30]
*cip*1	Solyc06g073180 ch6	3344	zinc-finger protein CONSTANS interacting protein 1	DNA synthesis, cell growth and division	[31]

Table 3. Sequence of forward (F) and reverse (R) primers used to amplify and sequence the different exons of six stress-responsive genes. The annealing temperature (Ta) and the amplified fragment size (bp) are also reported.

GENE	PRIMER SEQUENCE (5'-3')	Ta(°C)	FRAGMENT SIZE (bp)	SEQUENCED EXON
*mkp*1	F: GGATGCAGTGGCAAGAGATAAGG R: AACAATCTGGCAAACAGCCCCA	57	620	1
*asr*2	F: TGT GTG AAA TGC CAA ACC AT R: ATC CAA ACG GGC TCA AAG TA A	54	991	1-2
*tas*14	F: AGATGGCACAATACGGCAAT R: TCATTAAACACGGGACACCA	51	888	1-2
*tsw*12	F: CAATATGGAAATGGTTAGCAAAA R: TCGATACAAGACCCCCAAAA	54	800	1-2
*erd*15	F: AATTGGTTTATTAGGCCAGGAAA R: GTATTTGGGTGGACCAATGG	52	880	1-2
*cip*1I	F: GTATTTCAAACCCCCACCAA R: GCTAAAAAGGTAACAAGACACACG	53	1222	1
*cip*1II	F: TTGTGATGTTCCTTTTGATTAGACTC R: CTGTGGAGGCATTTTCAAGC	50	1382	2
*cip*1III	F: AAACCAACTGCAGCCACAAC R: GGCGCTTGGAACATGAAT	50	1245	3

sequences to a known reference sequence. Identified polymorphisms (SNP or IN/DEL) were characterized for type of variation and localization. Moreover, polymorphisms in the Open Reading Frames (ORF) were analyzed to evaluate their possible effect on the amino acid composition of the corresponding protein. **Table 4** reports the number of genotypes exhibiting polymorphisms in the exon regions for all sequenced genes. This varied from a minimum of six genotypes for the gene *tas*14 to a maximum of 22 genotypes for the gene *asr*2. As the whole, mutations observed were mainly SNPs (228 out of 236 mutations, 96.2%) and only nine INDELs were detected, which cause frameshift in the protein synthesis with respect to that of the reference Heinz 1706. Among the SNPs, 88 (38.6%) were synonymous mutations, thus not causing amino acid changes in the protein, whereas among the non-synonymous both missesense and nonsense mutations were observed, the latter with a less extent. **Table 5** lists the non-synonymous SNPs and the INDELs detected in the six sequenced genes with the consequent amino acid changes. These mutations are described below gene by gene.

Gene *mkp*1 The gene *mkp*1 is a member of MAP kinase phosphatases (MKPs), which are potent inactivators of MAP kinases, and are considered important regulators of MAP kinase signaling [21]. In higher plants, MAP kinases are implicated in a multitude of cellular responses to signals such as plant hormones, and both biotic and abiotic stress factors. The mutant *mkp*1 was studied in *A. thaliana* and this gene was isolated and sequenced in maize and tomato to examine the evolutionary conservation of its structure [22]. The tomato gene *mkp*1 is a 3605 bp sequence, including one intron and mapping on the chromosome 5. Since its big size, the sequenced region was restricted to the part of exon 1 carrying the active site motif of the Arabidopsis gene, which is highly conserved in different plant species [22]. Overall, only seven genotypes (**Table 4**) were polymorphic among the 27 analyzed. A total of 31 mutation events were identified: two were INDELs, 11 were synonymous and 18 were not synonymous. The only wild species exhibiting three non-synonymous SNPs was *S. peruvianum* and the most polymorphic cultivated genotype was AL-22/064 with nine events of mutations that deeply might affect the amino acid sequence (**Table 5**).

Gene *ars*2 This gene encodes a putative transcription factor likely involved in one of the signaling pathways of ABA [23] and belongs to the *asr* gene family (named after abscicic acid [ABA], stress, ripening), exclusively present in plant genomes. The members of this family are up-regulated in roots and leaves of water- or salt-stressed plants. The DNA sequence of *asr*2 is of 811 bp with two exons (331-489, 602-787) and located on chromosome 4. It has been previously studied in the two wild species *S. chilense* and *S. arcanum* by an evolutive point of view, since they evolved in habitats with different precipitation regimes [24]. In our collection, almost all genotypes (22 out of 27) revealed polymorphism and a total of 105 mutation events were identified, 26 of these were synonymous, 79 not synonymous (**Table 4**). Unexpected, when compared to the reference genome of Heinz 1706, all genotypes showed the same four consecutive mutations (66 c > T, 67 a > G, 68 t > C, 69 g > A), the first of which was silent whereas the others were missense mutations causing the amino acid change M76A (**Table 5**). This might imply that a mutation occurred in Heinz 1706 in this genomic region during its evolutive history. Thus, excluding these nucleotide changes, AL-22/046 was the cultivar

Table 4. Number of polymorphic events detected in the re-sequenced tomato genes among the 27 selected genotypes.

GENE	PRIMER SEQUENCE (5'-3')	Ta(˚C)	FRAGMENT SIZE (bp)	SEQUENCED EXON
*mkp*1	F: GGATGCAGTGGCAAGAGATAAGG R: AACAATCTGGCAAACAGCCCCA	57	620	1
*asr*2	F: TGT GTG AAA TGC CAA ACC AT R: ATC CAA ACG GGC TCA AAG TA A	54	991	1-2
*tas*14	F: AGATGGCACAATACGGCAAT R: TCATTAAACACGGGACACCA	51	888	1-2
*tsw*12	F: CAATATGGAAATGGTTAGCAAAA R: TCGATACAAGACCCCCAAAA	54	800	1-2
*erd*15	F: AATTGGTTTATTAGGCCAGGAAA R: GTATTTGGGTGGACCAATGG	52	880	1-2
*cip*1I	F: GTATTTCAAACCCCCACCAA R: GCTAAAAAGGTAACAAGACACACG	53	1222	1
*cip*1II	F: TTGTGATGTTCCTTTTGATTAGACTC R: CTGTGGAGGCATTTTCAAGC	50	1382	2
*cip*1III	F: AAACCAACTGCAGCCACAAC R: GGCGCTTGGAACATGAAT	50	1245	3

with the higher number of mutations even though most of them were heterozygous and thus not stable to be further taken into consideration. AL-22/076 showed a deletion on exon 2 (92 delG), that determines a frameshift in translation. Among wild species, *S. peruvianum* only showed one missense mutation in exon 1.

Gene *tas*14 *Tas*14 is a dehydrin that accumulates in response to mannitol, NaCl or abscisic acid (ABA) treatments [25]. The protein encoded by the *tas*14 gene is present in various phosphorylated forms and it was found to be localized both in the cytosol and, preferentially, in the nucleus by immunocytochemistry [26]. The genomic sequence is estimated of 746 bp with an intron of 218 bp and the gene maps on chromosome 2: the primers designed amplified both exons (**Table 3**). Six genotypes exhibited polymorphisms in exons 1 and 2, accounting a total of 48 mutation events (**Table 4**), but these were non-synonymous SNPs or INDELs only in the two wild species *S. pennellii* and *S. chilense*. In both cases, besides missense mutations, insertions and deletions occurred both in exons 1 and/or 2 (**Table 5**). In particular, the same nucleotide insertion (109-110insGAGCTGGAG, exon 2) occurred in both species and causes three amino acids GAG in-frame insertion at position 113 of the protein. *S. pennellii* showed also one additional in frame deletion (130-132delATG, exon 1) and one insertion (177-178insGGAACTCAAGGCATGGGTACTGGT, exon 1). Also, in common between these genotypes a missense base change (144 g > C, exon 2) was observed.

Gene *tsw*12 The high similarity between the TSW12 deduced amino acid sequence and the reported lipid transfer proteins suggests that *tsw*12 encodes a lipid transfer protein [27]. *tsw*12 mRNA is accumulated during tomato seed germination and its level increases after NaCl treatment or heat shock. In mature plants, *tsw*12 mRNA is only detected upon treatment with NaCl, mannitol or ABA and its expression mainly occurs in stems. The *S. lycopersicum tsw*12 mRNA reported in GenBank (X56040) is 675 bp long. It aligns with a scaffold mapping on chromosome 10, with an estimated genomic sequence length of 893 bp and with the presence of one intron. Nine genotypes (**Table 4**) were polymorphic and a total of 48 mutation events were identified, 17 of these are synonymous, 28 not synonymous SNPs, and three are INDELs. Noteworthy, in all the cultivated genotypes (**Table 5**) the presence of different insertions around the position 104 - 106 bp of exon 1 was revealed. Indeed, five genotypes (AD17, AL-22/070, AL-22/076, IT-22/005 and Sel6) showed a deletion ranging from 1 to 4 bases, which in any case causes a frameshift effect. In addition, three wild species (*S. peruvianum*, *S. pennellii* and *S. chilense*) showed two substitutions (166 A > G; 167 A > G) that cause an amino acid change from asparagine to glycine. This result is supported by Trevino and O'Connell [28], who studied the TSW12 protein in *S. pennellii*.

Gene *erd*15 The ERD15 (Early Responsive to Dehydration 15) protein is a small, acidic protein with an unknown function and is one of the key negative regulators of ABA responses in plants [29]. The gene *erd*15 was originally described as a rapidly drought-responsive gene in Arabidopsis [30]. The relative sequence in tomato, estimated to be 974 bp long, is localized on chromosome 10 and presents one intron. Overall, only seven genotypes (**Table 4**) were polymorphic with a total of 21 mutation events, 13 were synonymous while eight were not synonymous. Most SNPs were silent, and common missense SNPs occurred at positions 3, 4 and 6 bp of exon 2 in genotypes AL-22/064, IT-22/005, and *S. lycopersicum* cv. Edkawi. These SNPs cause an amino acid change from lysine to tyrosine (**Table 5**).

Table 5. Type of non-synonymous SNP and INDEL mutations detected in the polymorphic genotypes for the re-sequenced genes. For each mutation event the position of changed nucleotide (nt) and of the related changed amino acid (aa) are reported. Asterisk refers to stop codons.

GENOTYPE	NT MUTATION	EXON	TYPE OF MUTATION	AA CHANGE
Gene *mkp*1				
AL-22/064	446 t > A	1	Nonsense	L149*
	447 a > G	1	Nonsense	L149*
	448 g > T	1	Missense	D150C
	449 a > G	1	Missense	D150C
	450 t > C	1	Missense	D150C
	451 t > C	1	Missense	S151P
	454 del 4 bp	1	Frameshift deletion	-
	486 g > K	1	Missense	S162[R,L]
AL-22/070	1 a > T	1	Missense	N1Y
IT-22/005	1 a > T	1	Missense	N1Y
	49 delT	1	Frameshift deletion	-
S. peruvianum	134 g > A	1	Missense	R45K
	481 t > G	1	Missense	S161A
	483 a > T	1	Missense	S161A
Gene *asr*2				
AL-22/046	49 a > Y	2	Missense/nonsense	K70[Q,*]
AL-22/076	92 delG	2	Frameshift deletion	-
S. peruvianum	153 g > C	1	Missense	L51F
*Gene tas*14				
S. pennellii	125 g > C	1	Missense	G24A
	130-132 delATG	1	Frameshift deletion	Mdel
	177-178 insGGAACTCAAGCATGGGTACTGGT	1	Frameshift insertion	GTQGMGTG59-60ins
	82 g > A	2	Missense	G105S
	109-110 insGAGCTGGAG	2	Frameshift insertion	GAG-113ins
	144 g > C	2	Missense	K125N
S. chilense	73 c > G	2	Missense	Q102E
	109-110 insGAGCTGGAG	2	Frameshift insertion	GAG-113ins
	144 g > S	2	Missense	K125[K,N]
Gene *tsw*12				
ADVF	104 delC	1	Frameshift deletion	-
AL-22/070	101 t > G	1	Missense	L34W
	103 g > A	1	Missense	A35I
	104 c > T	1	Missense	A35I
	105-106 delTC	1	Frameshift deletion	-
	108 t > K	1	Missense	P36[V,L]
AL-22/076	100 t > G	1	Missense	L34G
	101 t > G	1	Missense	L34G

Continued

	103-106 delGCTC	1	Frameshift deletion	-
	108 t > G	1	Missense	P36R
IT-22/005	101 t > G	1	Missense	L34W
	105-106 delTC	1	Frameshift deletion	-
	107 c > S	1	Missense	P36[E,G]
	108 t > G	1	Missense	P36[E,G]
	109 t > R	1	Missense	C37[P,A]
	111c > S	1	Missense	C37[P,A]
Sel6	104 delC	1	Frameshift deletion	-
S. peruvianum	154 g > A	1	Missense	G25N
	155 g > A	1	Missense	G25N
	166 a > G	1	Missense	N56G
	167 a > G	1	Missense	N56G
S. pennellii	166 a > G	1	Missense	N56G
	167 a > G	1	Missense	N56G
S. chilense	166 a > R	1	Missense	N56[S,G]
	167 a > G	1	Missense	N56[S,G]
Gene *erd*15				
AL-22/064	3 t > A	2	Missense	L114Y
	4 a > C	2	Missense	L114Y
IT-22/005	3 t > A	2	Missense	L114Y
	4 a > C	2	Missense	L114Y
	6 c > A	2	Missense	L114Y
S. lycopersicum cv. Edkawi	3 t > A	2	Missense	L114Y
	4 a > C	2	Missense	L114Y
	6 c > A	2	Missense	L114Y
Gene *cip*1				
S. pennellii	2296 g > R	2	Missense	A65[T,A]
	3129 t > C	3	Missense	S261P

Gene *cip*1 The tomato *cip*1 gene is a DNA binding transcription factor involved in response to salt stress, similar to a CONSTANS-like protein studied in *A. thaliana* [31]. It maps to chromosome 6, has a big size of 3344 bp and presents two introns; therefore three different primer pairs were designed (**Table 3**), each for sequencing one exon. Overall, only seven genotypes (**Table 4**) were polymorphic, showing a total of 15 mutation events, 12 were synonymous while three were not synonymous. Among the various mutations, seven SNPs were observed in exon 1, five occurred in exon 2 and three in exons 3 and 4. Two missense mutations were only observed in *S. pennellii* (**Table 5**).

Comprehensively, *S. pimpinellifolium*, which is the wild species most tolerant at the desiccation test, did not carry any non-synonymous SNPs or INDELs in the sequenced genes. Therefore, no structural variation in the proteins these genes code for could be associated to the tolerance evidenced by this species. In the future, other stress-relative genes might be investigated. On the other side, *S. pennellii* and *S. chilense*, whose higher tolerance to drought stress have been previously reported, and confirmed by results of the desiccation test here described, evidenced a common insertion in exon 2 of the gene *tas*14, which might be related to their tolerance. This polymorphism will be in the future searched for in other

wild species/accessions exhibiting drought tolerance. Among the cultivated genotypes, particularly interesting are 1) the deletion observed in exon 1 of the gene *tsw*12 in five genotypes that could be classified as semi-tolerant at the desiccation test, and 2) the missense mutation observed at the beginning of exon 2 of the gene *erd*15, which was revealed in two tolerant (AL-22/064 and *S. lycopersicum* cv. Edkawi) and one semi-tolerant (IT-22/005) genotypes at the desiccation test. All target sequences will be studied in the future in additional species/accessions exhibiting different response to drought.

3.4. Expression Analysis

Three water stress-related genes (*tas*14, *tsw*12, *erd*15) analyzed for structural polymorphisms were also studied for their expression level in leaf tissue collected from four non-stressed and stressed genotypes (IT-22/025, IT-22/030-13, GiaGiù and Siccagno) grown in the field trial at Acerra. These genotypes were selected because they exhibited higher tolerance to water deficit as resulted from some of the phenotypic evaluations carried out in this work. The leaf mRNA relative abundance of the mRNA of the three genes was assayed by *Real Time RT-PCR* (qRT-PCR). In particular, the relative expression, within the specific genotypes, was reported as fold change of the expression in the un-watered plants relatively to the well-watered counterpart. The assay was performed on two different time-points (July 7th and 21st). As for *tas*14 and *tws*12 (**Figure 4**), results did not show any significant difference between 7th and 21st of July ($P > 0.05$). The same trend was observed for the difference

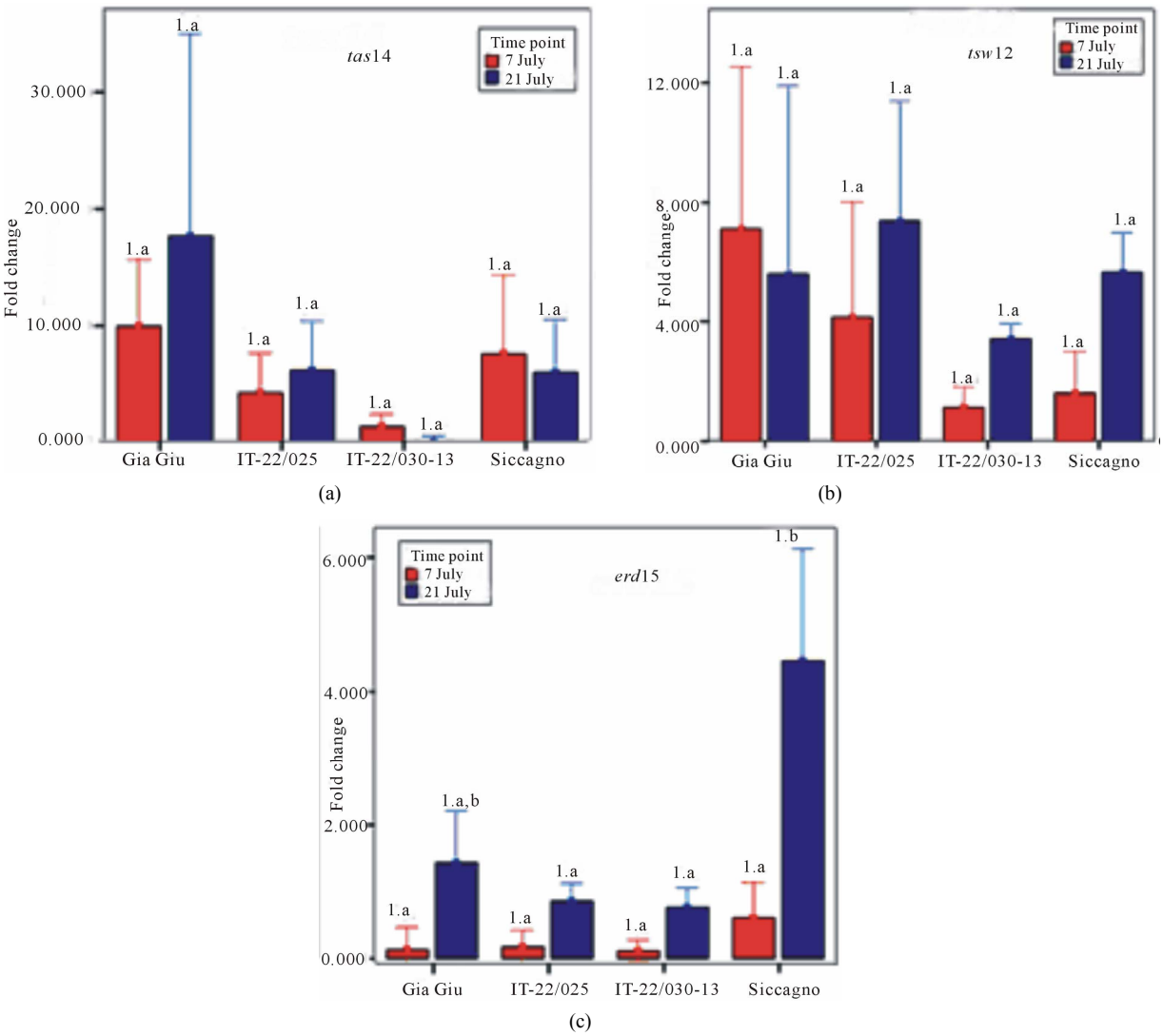

Figure 4. Leaf relative transcription (fold change) of *tas*14, *tsw*12 and *erd*15 genes in un-watered plants of four tomato genotypes compared to the watered counterpart, as resulting by Real Time qPCR. Same letter indicates not significant differences among genotypes within the same time-point (Post Hoc Test LSD. $P < 0.05$). Same number indicates not significant differences between time-point within the same genotypes (Post Hoc Test LSD. $P < 0.05$).

between the two time points within each genotype ($P > 0.05$). No significant differences were also observed between genotypes both on July 7th and 21st. These results did not allowed to associate differences in the level of expression of *tas*14 and *tws*12 to the different response of tested genotypes to water deprivation. By contrast, for *erd*15, an overall significant difference between the two time-points was observed (ANOVA $F_{1,22} = 6.62$; $P < 0.05$). The same result was not observed within each genotypes ($P > 0.05$). Significant differences between Siccagno and IT-22/025 as well as between Siccagno and IT-22/030-13 were observed within the July 21st assay (Post Hoc Test LSD; $P > 0.05$). The latter results allowed arguing a possible implication of higher level of expression of *erd*15 in the higher yield stability performed by Siccagno against water deprivation.

To deeper understand the involvement of the sequenced genes in the drought tolerance response, expression data of *asr*2, *tas*14, *tsw*12, *erd*15 and *cip*1 were also inferred from a microarray analysis performed comparing the Siccagno genotype under stressed and not stressed conditions (data not shown). As result, we found significant differences among mRNA transcription of *tas*14 and *erd*15 genes (**Table 6**). In particular, in Siccagno genotype under stress condition *tas*14 gene was down-regulated while *erd*15 was up-regulated, thus confirming results obtained by RT-qPCR analysis. Other stress-related genes were searched in the Solgenomics database using "water deprivation" as keyword for the Ontology Term. Among all the genes found in the genome database we selected a group of eight genes, whose expression was assayed in the microarray experiment. Comprehensively, all the genes were down-regulated in the Siccagno genotype under stress conditions with respect to the control. Differences in the expression levels of the genes under study found in the stressed genotype allowed us to strengthen their involvement in the drought tomato response as showed by [7]. However, to completely disclose the molecular mechanisms underlying drought tolerance more studies are required.

4. Conclusions

The results reported allowed driving some general conclusions and designing perspectives for future research activities aimed to dissect the complex genetic control of drought tolerance in tomato. Either exploitable genetic resources or molecular information were obtained. Indeed, the ecotype Siccagno ranks among the best for its response to the desiccation test and for its ability to grow and produce in low-water input systems. For other genotypes, as far as the integration of results obtained by dif-

Table 6. List of stress-related genes differentially expressed in un-watered Siccagno genotype respect to the watered control.

GENE	FOLD CHANGE STRESSED *vs.* NOT STRESSED	SL2.3 ITAG ANNOTATION
*asr*2	−1.133	Unknown Protein (AHRD V1); contains Interpro domain(s) IPR003496 ABA/WDS induced protein
*tas*14	−2.499***	Unknown Protein (AHRD V1); contains Interpro domain(s) IPR000167 Dehydrin
*tsw*12	−1.104	Heat shock protein 4 (AHRD V1 ***- B6U237_MAIZE); contains Interpro domain(s) IPR013126 Heat shock protein 70
*erd*15	0.352**	ERD15 EARLY RESPONSIVE TO DEHYDRATION 15 protein binding (AHRD V1 *-*G AT2G41430.2); contains Interpro domain(s) IPR009818 Ataxin-2, C-terminal
*cip*1	−0.194	Zinc finger protein CONSTANS-LIKE 1 (AHRD V1 * COL1_ARATH); contains Interpro domain(s) IPR000315 Zinc finger, B-box
areb	−0.820**	BZIP transcription factor (AHRD V1 **-* Q0PN11_9FABA); contains Interpro domain(s) IPR011616 bZIP transcription factor, bZIP-1
*bhlh*1	−2.422***	BHLH1 transcription factor (AHRD V1 **** D6BP02_HEVBR); contains Interpro domain(s) IPR001092Basic helix-loop-helix dimerisation region bHLH
*dreb*1	−1.400***	Dehydration responsive element binding protein 1
*gras*7	−1.169**	GRAS family transcription factor (AHRD V1 **-* B9IAQ7_POPTR); contains Interpro domain(s) IPR005202 GRAS transcription factor
*wrky*42	−1.103**	WRKY transcription factor (AHRD V1 ***- D3YEX5_SOLLC); contains Interpro domain(s) IPR003657 DNA-binding WRKY
bhlh	−0.554	Transcription factor (AHRD V1 *--* Q9M4A8_MAIZE); contains Interpro domain(s) IPR011598 Helix-loop-helix DNA-binding
ap2-like	−1.125**	AP2-like ethylene-responsive transcription factor
bhlh	−0.743	Transcription factor (AHRD V1 *-*- D6MKM4_9ASPA); contains Interpro domain(s) IPR011598 Helix-loop-helix DNA-binding

*Statistically significant differences at $P < 0.05$; **$0.01 < P < 0.05$; ***$P < 0.01$.

ferent tests, plant response to water deficit in many cases contrasted when plant growth and fruit production were compared. This might confirm that different mechanisms act in plant response to drought during different developmental stages.

Many polymorphisms were detected in six stress-responsive genes, and some of them could imply significant modifications in the consequent protein structure. The potential involvement of these polymorphisms in a differential response to water deficit should be further investigated. Furthermore, the expression analysis by RT-qPCR of three stress-responsive genes allowed arguing a possible implication of a higher level of expression of gene *erd*15 in the higher yield stability performed by Siccagno against water deprivation. Similarly, the lower expression of eight genes in the same genotype analysed through a microarray experiment confirmed the involvement of these stress-related genes in the tomato response to drought.

Nowadays, the availability of highly performing sequencing techniques, such as the various NGS (Next Generation Sequencing) platforms, will allow in the future targeting re-sequencing many stress-responsive gene contemporarily in drought tolerant and sensitive genotypes, in order to associate structural variation to a better response to water deprivation. In addition, these techniques will allow studying the complete transcriptomic response of the genotype Siccagno at different water supplies, in order to identify those genes whose differential expression might be crucial to enhance the tolerance to stress.

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