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Touch-Induced Transcriptional Changes in Flower Buds of a Non-Model Horticultural Plant *Dianthus hybrida*

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Abstract: Touch stimulus responses are common in plants. Some flowering plants sense the arrival of their pollinators and secrete nectar or release pollen sacs, facilitating successful pollination. Molecular mechanisms for mechanical stimulus responses in plants are well characterized in *Arabidopsis* leaves, but not in non-model plants or other organs such as flowers. Here, we performed RNA-seq analysis of touched flower buds of *Dianthus hybrida*, a major ornamental plant. Upon touch treatment, 931 and 132 genes were upregulated and downregulated, respectively. GO enrichment analysis revealed that genes encoding serine/threonine protein kinases were significantly abundant among the upregulated genes, which is consistent with previous studies that demonstrated the pivotal role of protein phosphorylation in the touch stimulus response of *Arabidopsis* leaves. In comparison with the gene expression profile of touched *Arabidopsis* leaves, the same families but different homologs of the representative touch-induced genes encoding protein kinases were upregulated, showing that phosphorelay signaling was the common mechanism for touch stimulus response in flowers and leaves, but the players of the phosphorelay signaling were different. These results will contribute to further studies on the mechanical stimulus responses of ornamental flowers and the utilization of this mechanism for breeding programs.

Keywords: tactile stimulus; ornamental plant; RNA-seq

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1. Introduction

Plants continuously encounter and can sense environmental mechanical stimuli such as wind, rainfall, sounds, and touch to control their growth and development [1,2]. Mechanical stimuli contribute to the successful pollination of some flowers. For example, in the legume *Desmodium setigerum*, flower color and shape are changed by pollination and bee visits [3]. In the case of *Oenothera drummondii*, flowers produce nectar that is sweeter than usual after being treated with the playback sound of a flying bee [4]. The male flower of the dioecious *Catasetum* releases pollen sacs when pollinators touch the center of the flower, helping to effectively pollinate this species [5]. Although flowers are thought to sense mechanical stimuli, their response to them is less documented.

Molecular mechanisms of touch stimulus responses in plants have been studied exclusively in *Arabidopsis thaliana*. Many signaling molecules, including potential secondary messengers and plant hormones, have been implicated in touch-induced responses. First, a very rapid change in cytosolic free Ca^{2+} concentration occurs after the stimulation of plant cells [6–12]. An increase in Ca^{2+} concentrations is sensed by Ca^{2+}-binding proteins that regulate downstream molecular processes [8–12] such as the phosphorylation of proteins, resulting in the modulation of protein conformation [13–15]. In *Arabidopsis*, 24 touch-responsive phosphopeptides, including kinases, phosphatases, cytoskeleton proteins, membrane proteins, and ion transporters, were identified after initiation of touch

treatments [16]. A non-phosphorylated isoform of touch-regulated phosphoprotein 1 (TREPH1) exhibited insensitive phenotypes to touch treatments and loss of the induction of touch-induced gene expression [16].

Touch-induced gene expression occurred 10 to 40 min after the initiation of touch treatments, while the increase in Ca^{2+} concentrations and Ca^{2+}-independent protein phosphorylation occurred shortly after treatment. The representative touch-induced genes, *TCHs*, were originally isolated as responsible genes after stimuli, such as touch, wind, rain, wounding, and darkness [1]. *TCH1* encodes an *Arabidopsis* calmodulin, CAM2, whereas *TCH2* and *TCH3* encode calmodulin-like (CML) proteins, CML24 and CML12, respectively [1,17–19], which is consistent with the above description that Ca^{2+}-dependent pathways are involved in touch stimulation. Touch-induced genes were also identified throughout the genome, and a representative gene, *CML39* was isolated [20]. *TCH4* encodes xyloglucan endotransglucosylase/hydrolase [21].

The phytohormone jasmonate (JA) is also an important molecule involved in touch-induced responses in plants. An *Arabidopsis* mutant defective in allene oxide synthase (*aos*) and JA did not show touch-induced morphological changes [22]. In contrast, the expression of touch-induced genes was detected in the mutant, indicating that this was not sufficient to show touch-induced morphological changes, and that the expression of these genes was independent of the JA signaling pathway or upstream of the pathway [22].

Although these characteristics have been well documented in *Arabidopsis* leaves, touch-induced gene expression in flowers remains mostly unknown. Here, we investigated the gene expression profiles after exposure to touch stimulus of the flowers of an ornamental plant, *Dianthus hybrida*, which is an interspecific hybrid between *D. chinensis* and *D. barbatus*. The genus *Dianthus*, a member of the family Caryophyllaceae [23], is one of the major ornamental flowers of commercial importance, with production similar to that of *Chrysanthemum* and *Rosa*. The *Dianthus* is commonly known as "carnation," which refers to *Dianthus caryophyllus* and several intra/interspecific hybrids. *Dianthus* flowers vary widely in colors and shapes [24–27]. Molecular understanding of physiological mechanism underlying environmental stimulus effects on flower morphology will facilitate to develop new varieties. The genome project of *D. caryophyllus* is almost complete [28], allowing us to perform transcriptome analyses. In this report, we aimed to list the differentially expressed genes after touch stimulation in *D. hybrida* flowers and indicate directions for future research that will serve as a basis for further development and utilization of this response in breeding programs.

2. Materials and Methods

2.1. Plant Materials and RNA Extraction

D. hybrida cultivar "Telstar Scarlet" plants were grown in plastic pots (10.5 cm diameter and 9.5 cm height) in a wind- and vibration-free PVC box placed in a growth chamber under the following conditions; 14 h light/10 h dark, temperature of 28 °C and relative humidity of 70% (Figure 1A). Photosynthetic photon flux density (PPFD) was 315 µmol m^{-2} s^{-1}. Three untouched flower buds were sampled as controls, and another three buds were touched by hand for 40 s with hands and sampled 40 min later, following a previous study on *Arabidopsis* leaves [20]. All biological replicates were sampled from different plants. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Germantown, MD, USA) following the manufacturer's instructions.

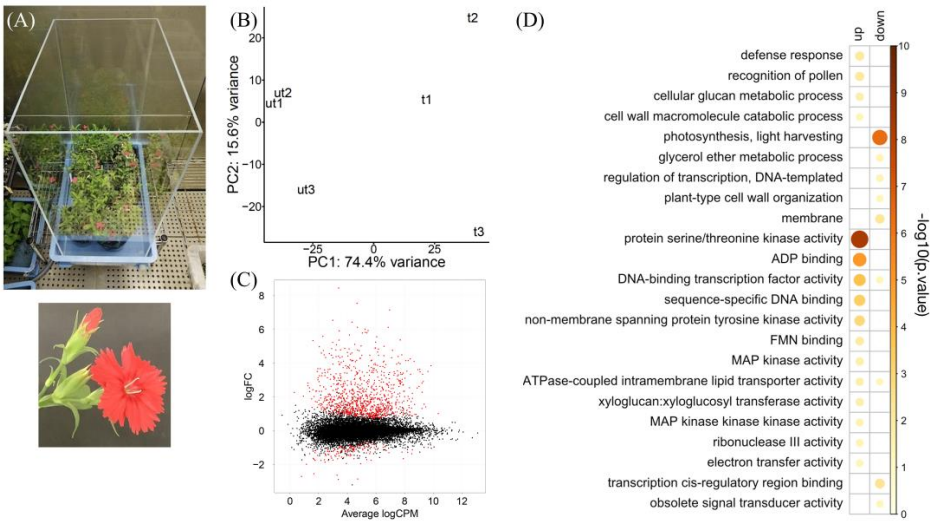

Figure 1. RNA-seq summary of touched *D. hybrida* flower buds. (**A**) *D. hybrida* plants grown in a wind- and vibration-proofed box. The bottom panel shows a flower and a bud. (**B**) PCA plot of three replicates each of touched (t) and untouched (ut) samples using uniquely mapped reads. (**C**) MA plot of expressed genes (TPM > 2.5 at least in all three replicates of either touched or untouched sample). Red dots indicate DEGs (FDR < 0.001). (**D**) GO enrichment analysis of DEGs.

2.2. RNA-seq Analysis

Total RNA sequencing was conducted by Rhelixa (Tokyo, Japan). Strand-specific RNA-seq libraries were prepared using the NEBNext Ultra II Directional mRNA-seq Kit for Illumina (New England Biolabs, Hitchin, Hertfordshire, UK). The libraries were bulked with others and sequenced using the NovaSeq6000 (Illumina, San Diego, CA, USA) with paired-end 150-bp reads.

Due to unavailability of chromosome-level genome assembly of *D. hybrida*, the genome scaffold of the closely related species *Dianthus caryophyllus* (carnation), the highest-quality genome sequence within the genus, was used as a reference. Scaffold, gene annotation, and *Arabidopsis* homolog data for carnation were downloaded from the Carnation DB [28] (http://carnation.kazusa.or.jp, accessed on 7 December 2021). RNA-seq reads were mapped to carnation scaffolds using the STAR aligner ver. 2.7.3a [29] with options "–outFilterMultimapNmax 1 –quantMode GeneCounts". Differentially expressed genes (DEGs) were called with glmLRT of R package edgeR ver. 3.26.8 [30], and transcripts per kilobase million (TPM) values were computed after trimmed mean of M values (TMM) normalization. GO enrichment analysis was performed using the R package clusterProfiler ver 3.12.0 [31] and visualized using the R package corrplot ver 0.92 [32]. To compare the transcriptome profile of *D. hybrida* flower buds with that of *Arabidopsis* leaves, microarray data from a previous study [20] was used.

3. Results and Discussion

To explore the gene expression profiles in the flower buds of *D. hybrida* in response to touch stimulus, we performed transcriptome analysis of the flower buds 40 min after touch treatment. On average, 22.8 million reads were obtained for each sample, and 71.5% of the total reads were uniquely mapped to carnation scaffolds (Table S1). Transcripts from 15,258 genes were detected (TPM $\geq$ 2.5, in all three replicates in either touched or untouched conditions). Principal component analysis based on the uniquely mapped read count showed that touched and untouched samples were clearly separated by the PC1 value with a 74.4% contribution rate (Figure 1B), suggesting touch-induced transcriptional changes in the flower buds. Genes with the highest loading values along the PC2 axis were a *CEP1* homolog and three UDP-glycosyltransferase genes, which are involved in pollen development [33], indicating that the PC2 represented inflorescence developmental

stage. Gene expression analysis showed that 961 and 132 genes were up- or downregulated by touch treatment, respectively (FDR < 0.001, edgeR; Figure 1C; Supplementary Excel File). GO enrichment analysis revealed that genes with GO "protein serine/threonine kinase activity" were significantly upregulated in response to touch stimuli (Figure 1D), which was consistent with previous studies that demonstrated the importance of protein phosphorylation in *Arabidopsis* touch-delayed bolting [16,34]. Other GO terms related to phosphorelay signaling, such as "non-membrane spanning protein tyrosine kinase activity", "MAP kinase activity", and "MAP kinase kinase kinase activity" were also enriched in touch-induced upregulated genes. In addition, GO "xyloglucan:xyloglucosyl transferase activity," a representative mechanosensitive response [21], was detected. Although related GO terms were not enriched significantly, calmodulin genes, such as a *CAM5* homolog, and JA biosynthetic (*LOX1*, *4*, *OPR3*, and *AOC3* homologs) and signaling genes (*JAZ1* and *NINJA* homologs) were upregulated. On the other hand, genes with GO "photosynthesis" were significantly downregulated upon touch stimulus, which resembled responses to other biotic stresses including bacteria, insects, and wounding [35].

To explore whether touch stimulus responses are generally conserved between flowers and leaves, we compared the RNA-seq data of *D. hybrida* flower buds with the microarray data of *Arabidopsis* leaves from a previous study [20]. All DEGs commonly detected in both experiments showed the same expression changes (Figure 2A,B), except for one clock-related gene, *FKF1*, which appeared to reflect the difference in sampling time. *TCH4*, which encodes xyloglucan endotransglucosylase [21], and its two *Dianthus* homologs were commonly upregulated. For calmodulin, *CML49*, but not *CAM5*, was upregulated in touched *Arabidopsis* leaves, while their homologs, *Dca25393* and *Dca20630*, respectively, showed the opposite expression patterns in the flower buds of *D. hybrida*, which implies that different organs utilize different calmodulin family members as touch signaling components. GO enrichment analysis revealed that genes for "protein serine/threonine kinase activity" were enriched in commonly detected and flower bud-specific DEGs, but not in leaf-specific ones (Figure 2C). In fact, 32 out of 44 genes encoding serine/threonine kinases were detected as DEGs only in flower buds, while no kinase gene was differentially expressed specifically in leaves. These kinases might be key players in a flower-specific touch stimulus response. Although it must be considered that these DEGs specific to one condition might reflect the difference between the two species and/or growth condition, these results suggest that an organ-specific molecular mechanism for touch stimulus response might exist, which could be the basis of organ function, as in the case of flowers listening to bees [4].

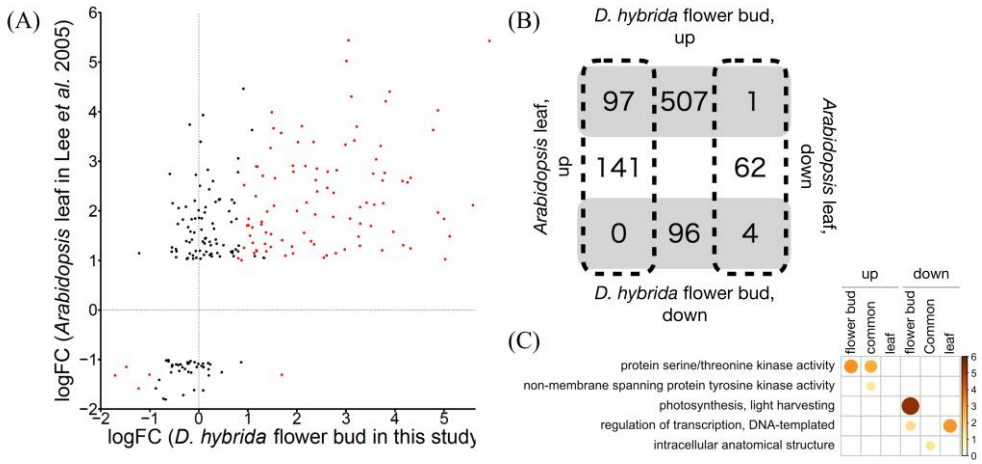

Figure 2. Comparison of transcriptome profile between *D. hybrida* flower buds in this study and *Arabidopsis* leaves [20]. (**A**) Pairwise gene expression comparison, red dots indicating DEGs in this study (FDR < 0.001). (**B**) Venn diagram of up- and downregulated genes. (**C**) GO enrichment analysis of common, *D. hybrida* flower bud-specific, and *Arabidopsis* leaf-specific DEGs.

4. Conclusions

Touch stimuli induced upregulation of genes encoding protein kinase, xyloglucan endotransglucosylase, calmodulin, and JA biosynthetic and signaling components and downregulation of photosynthetic genes in *D. hybrida* flower buds. This response was consistent with that of *Arabidopsis* leaves, in which protein phosphorylation events occur immediately after mechanical stimulation, followed by the increase in Ca^{2+} and JA concentrations [16,20,22], implying that molecular mechanisms for the touch stimulus response are generally conserved among plant organs and among plant species. In contrast, a large proportion of protein kinase genes was induced only in flower buds, suggesting an organ-specific response to tactile stimulation. Our results will contribute to further studies on physiological mechanisms underlying touch stimulus response of ornamental flowers and utilization of this response in future breeding programs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8100918/s1, Table S1: mapping rate of RNA-seq reads to carnation scaffold sequences, File S1: statistical summary of differentially expressed genes.

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