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Predominant Expression of Diploid Mandarin Leaf Proteome in Two Citrus Mandarin-Derived Somatic Allotetraploid Hybrids

ANNE-LAURE GANCEL,† JÉRÔME GRIMPLET,‡ FRANÇOIS-XAVIER SAUVAGE,‡ PATRICK OLLITRAULT,† AND JEAN-MARC BRILLOUET*,†

Département FLHOR, Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), TA50/16, F-34398 Montpellier Cedex 5, France, and UMR Science pour l'Œnologie, INRA, 2 Place Viala, F-34060 Montpellier Cedex 1, France

Fusion of citrus diploid parental protoplasts generates allotetraploid hybrids which do not retain their parental traits with regard to leaf aroma compound biosynthesis. The aim of this study was thus to examine hybrid leaf proteomes in comparison with their parents. Leaf soluble proteins from two citrus allotetraploid hybrids (mandarin + lime and mandarin + kumquat) and their diploid parents (mandarin, lime, and kumquat) were submitted to 2-D gel electrophoresis. Leaf proteome maps of the tetraploid hybrids were compared with those of their parents on the basis of the presence/absence of spots and of their spot relative volumes. The two allotetraploid hybrid maps were found closer to that of their mandarin parent than to those of their nonmandarin parents in terms of the presence/absence of spots as well as at a quantitative level. This approach has to be related to the already observed dominance of mandarin in allotetraploids with regard to volatile compound biosynthesis in leaves.

KEYWORDS: Tetraploid citrus somatic hybrids; *Citrus deliciosa* Ten.; *Citrus aurantifolia* (Christm.) Swing.; *Fortunella margarita* (Lour.) Swing.; leaf proteome 2-D electrophoresis map

INTRODUCTION

Commonly, allopolyploid plants result from the combination of two or more distinct genomes, thus maintaining diploid sets of chromosomes from each of their parental species. Such hybrids are present in nature and are genetically stable. However, allopolyploidization under both synthetic (colchicine doubling, ...) and natural conditions is often accompanied by rapid epigenetic processes (modifications in DNA cytosine methylation, transposable element activation, ...) and genetic remodeling caused by DNA rearrangements and transposition, triggering gene activation, gene loss, or silencing (*1*−*4*). These rapid genomic changes may lead to phenotypic modifications. They have been observed in synthetic allopolyploids in *Brassica* (*5*), wheat (*6*, *7*), and *Arabidopsis* (*8*−*11*). Recent studies of *Gossypium* allotetraploids showed that, among 40 gene pairs examined from ovules, 27 homeologues (duplicated genes) contributed equally to the transcriptome and 13 of them exhibited biased expression or silencing (*12*). Moreover, the expression levels and silencing of homeologues were dependent on the gene and organ examined (*12*, *13*). Other studies on diploid, tetraploid, and hexaploid wheats (*14*) and more recently on cabbage autopolyploids (*15*) tackled a new approach, the proteome analysis, for a better understanding of duplicated genome interactions in the protein expression.

For two decades, a new category of polyploids has been obtained by fusion of two parental diploid protoplasts, generating among others somatic allo- and autotetraploids. The somatic hybridization has been extensively applied to citrus to create plants with improved character (*16*). The main application of citrus somatic hybridization is the production of rootstocks able to resist pathogens (*Phytophtora*, tristeza, ...) or to adapt to specific environments (alkaline or acid soils, coldness, ...) (*17*). Varietal improvement also relies on the creation of citrus plants producing fruits with original and good sensory qualities (sugar content, acidity, aroma compounds, ...) for both fresh consumption and processing. Two other criteria are of interest: production of easy-peeling and seedless fruits. Tetraploid plants can be, for example, crossed with diploids, resulting in the formation of seedless triploid cultivars (*18*, *19*).

The aromatic quality of citrus somatic hybrids has been studied for less than 10 years (*20*−*24*). In our previous study on leaf volatile compounds of seven citrus somatic allotetraploid hybrids sharing willow leaf mandarin as their common parent (*23*), systematic behaviors were encountered: all hybrids were unable to synthesize monoterpene aldehydes (neral, geranial, citronellal) and monoterpene alcohols (nerol, geraniol, citronellol) like their mandarin parent and unlike their nonmandarin parents. These hybrids did retain the ability, although strongly reduced, of their nonmandarin parents to synthesize sesquiterpene hydrocarbons, sesquiterpene alcohols, and sesquiterpene aldehydes (α- and β-sinensals). These results suggested that complex forms of dominance originating from the mandarin

* To whom correspondence should be addressed. Phone: 33-(0)4-67-61-75-81. Fax: 33-(0)4-67-61-44-33. E-mail: brillouet@cirad.fr.
† CIRAD.
‡ INRA.

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genome determine, to some extent, the biosynthesis pathways of some of the volatile compounds in allotetraploid hybrids. These results raised a question: is this (these) form(s) of dominance of the mandarin genome affecting the biosynthesis pathways of volatiles the consequence of a more global regulation of the leaf proteomes of the considered hybrids? Proteome analysis of polyploid, diploid, tetraploid, and hexaploid wheats has already been published (*14*): the authors showed a differential protein expression in individuals linked to genomic interactions. Thus, we thought that examination of leaf proteomes of parents and their allotetraploid hybrids could be a preliminary approach for a better understanding of inheritance and regulation rules in citrus somatic hybridization.

Thus, instead of studying the hybrid genomic constitution with classical tools (RFLPs, SMTS), we engaged a phenotypical approach by analyzing the hybrid proteomes. We comparatively analyzed the leaf proteome 2-D electrophoresis maps of two somatic allotetraploid hydrids obtained by the CIRAD (mandarin + lime and mandarin + kumquat) sharing mandarin (*Citrus deliciosa* Ten.) as their common parent and those of their other parents, lime [*Citrus aurantifolia* (Christm.) Swing.] and kumquat [*Fortunella margarita* (Lour.) Swing.], and the results are presented herein.

MATERIALS AND METHODS

Plant Materials. The two year old parents, all grafted onto volkameriana rootstock (*Citrus limonia* Osb.) and growing in the same field of the Station de Recherches Agronomiques (INRA-CIRAD) of San Ghjulianu (Corsica, France), were of the following species: mandarin (cv. Willow Leaf, hereafter designated in the tables and figures as WLM), lime (cv. Mexican Lime, ML), and kumquat (cv. Nagami, NK). We also analyzed two year old somatic allotetraploid hybrids, obtained by the fusion of protoplasts from the nucellar callus line of mandarin (the common parent) and, respectively, callus-derived protoplasts of lime (WLM + ML) and leaf-derived protoplasts of kumquat (WLM + NK). These hybrids were shown to be allotetraploid ($4n = 36$) hybrids by flow cytometry and isozyme analysis (*16*, *25*) and were more recently characterized by STSM markers confirming they result from the effective addition of both parental genomes (data not published). The hybrids were all grafted onto volkameriana rootstock and were, as their parents, randomly planted the same week in the same field. Three individual shrubs were sampled for each parent and hybrid. Batches of mature leaves (more than six months old) were randomly hand-picked, revolving around the shrubs on the same day (September 2003), and stored overnight at 4 °C before protein extraction.

Protein Extraction. Leaves from the three batches per genotype were cut with scissors into small pieces (3 × 3 mm) and mixed. Leaf pieces (~200 mg) were crushed and homogenized with a mortar at 4 °C in 50 mM Tris−HCl buffer (pH 7.5) containing 25 mM β-mercaptoethanol and 10 M urea. The homogeneous paste was centrifuged (19000*g*, 5 min, 4 °C); then the supernatant was added with trichloracetic acid (TCA) at a 15% final concentration. After precipitation of proteins (1 h, −20 °C) and centrifugation (19000*g*, 5 min, 4 °C), the protein pellet was rinsed twice with 15% TCA with intermittent sonication and centrifugation. The pellet was then rinsed three times with acetone containing 25 mM β-mercaptoethanol with intermittent centrifugation. The protein pellet was vacuum-dried and then solubilized in 4% (w/v) CHAPS buffer containing 7 M urea, 2 M thiourea, 1% Triton X-100 (w/v), 65 mM DTT, and 2% (v/v) IPG buffer (pH 4−7). The protein content was measured with the Bradford method (*26*).

Electrophoresis and Staining. Each electrophoresis was conducted using 1 mg of proteins from each genotype. The first dimension of the 2-D electrophoresis was performed with an immobilized linear pH gradient from 4 to 7 (Amersham Pharmacia Biotech) using a Multiphor II IEF (LKB, Pharmacia); the strip length was 18 cm. Strip rehydration in the presence of the sample was achieved after 10 h at room temperature. The following running conditions were used: from 0 to 300 V in 1 h, 300 V for 1 h, from 300 to 600 V in 1 h, 600 V for 1 h, from 600 to 3500 V in 3 h, 3500 V for ~33 h until a total of ~120 kVh was reached. The strips were then incubated for 20 min at room temperature in 50 mM Tris−HCl buffer (pH 8.8) containing 6 M urea, 2 M thiourea, 30% (w/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTT. The strips were then incubated for 20 min in the same buffer containing 2.5% (w/v) iodoacetamide instead of DTT.

2-D SDS−PAGE was performed on 11% (w/v) acrylamide gels. The strips from the first dimension electrophoresis were sealed at the top of the running gels with low-melting-point agarose. Electrophoresis was conducted at 40 V for 3 h, 70 V for 3 h, and 100 V for 10 h using 25 mM Tris−HCl (pH 7.5) buffer containing 192 mM glycine and 0.1% (w/v) SDS.

Nine gels were simultaneously run for each combination [(two parents and their hybrid) × 3 replicates] and stained with Coomassie Brilliant Blue G-250. For each genotype, the gels were run in triplicate for each protein extract to take into account coloration effects. A total of 18 gels were obtained.

Scoring Methods. 2-D electrophoresis gels were scanned with a Bio-Rad GS 710 calibrated imaging densitometer, and comparison of the protein patterns was achieved using Melanie III and IV viewer software (Swiss Institute of Bioinformatics, Switzerland). Spots were detected and quantified by the Gaussian method (*27*). For each matched spot the relative volume was calculated as its volume divided by the total volume of matched spots. The spot volume is defined by its surface and intensity.

Multicolored gels (see **Figures 3** and **4**) were drawn using the PhotoShop 6.0 software (Adobe Systems Inc.).

Statistical Analysis. Hierarchical clustering analyses based on the relative volumes of all spots present in the gels from the parental genotypes (see **Figure 2**) and the three genotypes of both (WLM, ML) and (WLM, NK) combinations (814 and 832 spots, respectively) (see **Figure 5A$_1$,A$_2$**) and on the relative volumes of spots common to the three genotypes of each combination [343 spots for the (WLM, ML) combination and 196 spots for the (WLM, NK) combination] (see **Figure 5B$_1$,B$_2$**) were performed using STATISTICA software (StatSoft Inc.). Both analyses were conducted using the Euclidian distances and UPGMA method.

RESULTS AND DISCUSSION

Mature leaf protein patterns of the somatic allotetraploid hybrids (WLM + ML, WLM + NK) and their parents (mandarin, lime, and kumquat) were obtained by 2-D gel electrophoresis (**Figure 1**).

Parental Leaf Proteomes. The mandarin, lime, and kumquat proteomes are defined, respectively, by 604, 571, and 434 visible spots (**Table 1**). With the aim of comparing hybrids to their respective parents, we chose to group into three classes spots appearing in gels from each parental pair [i.e., (WLM, ML), (WLM, NK)]: those observed in both parental gels (common spots), and those specific to one or the other parent. The sum of these three classes represents 814 different spots for the (WLM, ML) parent pair and 832 for the (WLM, NK) parent pair (see "total in parents" in **Table 1**). In the (WLM, ML) parent pair, 44% of these 814 spots (361 spots) are common to both parents, which represents around 60% of the WLM and ML leaf proteomes. Among these 814 spots, 30% are specific to the WLM parent and 26% to the nonmandarin parent, that is to say, around 40% for each proteome. Thus, the distributions of common spots and parent-specific spots (around 60%/40%, respectively) are similar in the WLM and ML parents. The distribution is different for the (WLM, NK) parent pair. Indeed, only 25% of the 832 spots are common to the WLM and the NK proteomes, which represents 34% and 47% of the WLM and NK leaf proteomes, respectively. Among these 832 spots, 48% are specific to the WLM parent and 27% to the NK parent, which represents 66% and 53% of the WLM and NK total spots, respectively. Thus, with regard to the qualitative data, it seems

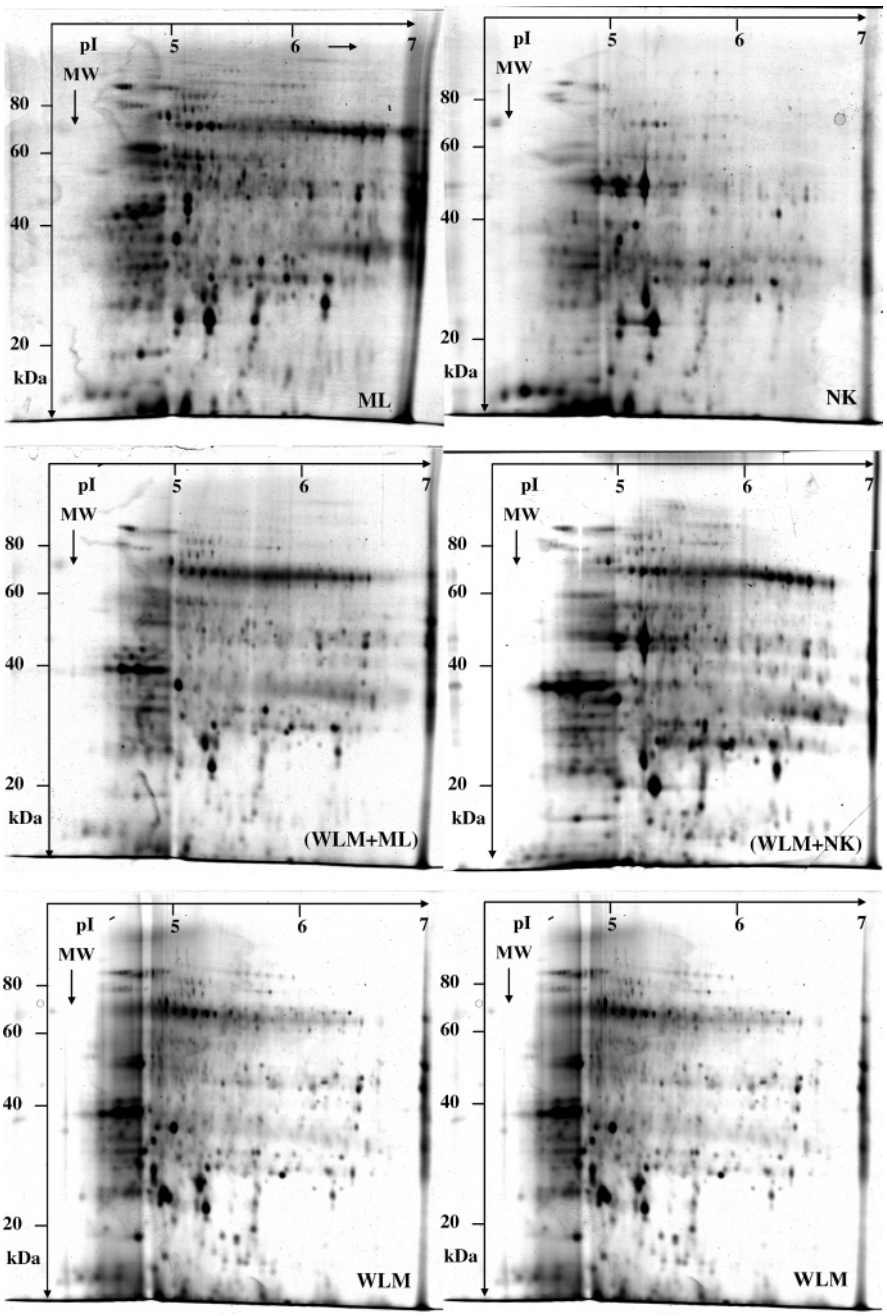

Figure 1. 2-D electrophoresis gels of soluble leaf proteins from the somatic allotetraploid hybrids (WLM + ML, WLM + NK) and their parents (mandarin, lime, and kumquat). WLM = Willow Leaf mandarin, ML = Mexican Lime, and NK = Nagami kumquat.

Table 1. 2-D Electrophoresis Gel Characteristics of the Parents (Mandarin, Lime, and Kumquat)

	(WLM,[a] ML[b]) parent pair			(WLM,[a] NK[c]) parent pair		
category of spots	total in parents	WLM	ML	total in parents	WLM	NK
common to both parents	361 (44%)[e]	60%	63%	206 (25%)	34%	47%
present in only one parent						
mandarin	243 (30%)	40%		398 (48%)	66%	
nonmandarin	210 (26%)		37%	228 (27%)		53%
total	814[d]	604	571	832	604	434

[a] Willow Leaf mandarin. [b] Mexican Lime. [c] Nagami kumquat. [d] Total spots from both genotypes. [e] Percentage of specified spots relative to the number marked with footnote *d*.

that the mandarin and lime parents are closer to each other than the mandarin and kumquat parents, because of the higher percentage of common spots in the first parent pair. This observation could be explained by the closer genetic proximity, on one hand, between the mandarin and lime parents than, on the other hand, between the mandarin and kumquat parents: indeed, mandarin and lime are from the *Citrus* genus, while the kumquat belongs to the *Fortunella* genus (*28*). The result based on quantitative data is not as clear as the previous one observed with qualitative data: a dendrogram built with the relative volumes of all spots detected in the three parental gels using the Euclidian distances and the UPGMA method (**Figure 2**) supports this observation. Indeed, when we consider protein expression by way of spot relative volumes, the lime is almost as distant from the mandarin as the kumquat is. This suggests that the spots common to the three genotypes exhibit different regulation patterns.

Hybrid Leaf Proteomes Compared to Those of Their Respective Parents. A color code was used to draw multicol-

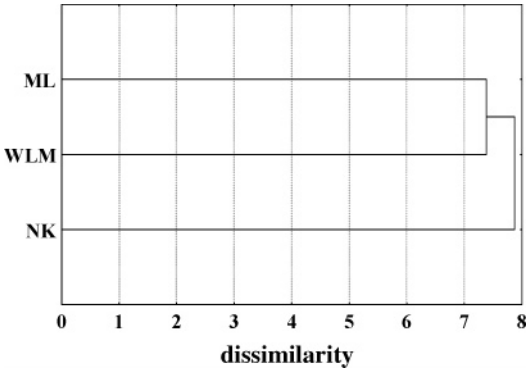

Figure 2. Dendrogram built with the relative volumes of all spots detected in the three parental gels using the Euclidian distances and the UPGMA method.

ored gels from the Coomassie Brilliant Blue G-250 stained gels: the various cases of the absence/presence of spots in hybrids and their respective parents (**Figures 3** and **4**) were thus more easily visualized.

The WLM + ML and WLM + NK hybrid proteomes were, respectively, defined by 568 and 677 visible spots (**Table 2**).

If one considers the presence and/or the absence of spots in the parents and their corresponding tetraploid hybrids, three classes of spots can be established.

Class 1. Spots were common to both parents. In the corresponding hybrids, two possibilities exist: (1) The spots were also present (black spots) (in both cases, 95% of the respective parental common spots, i.e., 343/361 and 196/206, respectively). They constitute 60% and 29% of the total spots from WLM + ML and WLM + NK hybrid proteomes, respectively. While in cabbage *auto*tetraploids green tissue proteomes do not differ significantly from their parental diploid proteomes (*15*), citrus *allo*tetraploids behave differently: in the WLM + ML tetraploid resulting from the merger of two parents belonging to the same *Citrus* genus, parental common spots represented around two-thirds of the total proteins, and in WLM + NK originating from the fusion of a mandarin (*Citrus* genus) and a kumquat (*Fortunella* genus), they constitute only one-third. Thus, the more genetically distant the parents, in the resulting tetraploids, the less representative the common parental proteins. (2) The spots were absent (orange spots) (in both cases, 5% of the respective parental spots, i.e., 18/361 and 10/206, respectively). The absence of these spots in the hybrids is probably the result of a reciprocal silencing of genes coding for these proteins triggered by the merger of both parental genomes (*1*, *3*); gene loss could have also occurred (*7*). Finally, since the gels were run with equal amounts of proteins (1 mg) whatever the individuals, spots can be present but not detectable by densitometry (*29*).

Class 2. Spots were present in only one parent. For spots present in mandarin, in the corresponding hybrids, two possiblities exist: (1) The spots were also present (blue spots) [for the WLM + ML and the WLM + NK hybrids, 42% (102/243) and 55% (220/398) of mandarin-specific spots, respectively]. They constitute 18% and 32% of the total spots from WLM + ML and WLM + NK hybrid proteomes, respectively. (2) The spots were absent (red spots) [for the WLM + ML and the WLM + NK hybrids, 58% (141/243) and 45% (178/398) of mandarin-specific spots, respectively]. For spots present in the nonmandarin parent, in the corresponding hybrids, two possibilities exist: (1) The spots were also present (yellow spots) (for both hybrids, around 25% of nonmandarin-specific spots, 46/210 and 66/228, respectively). They constitute around 10%

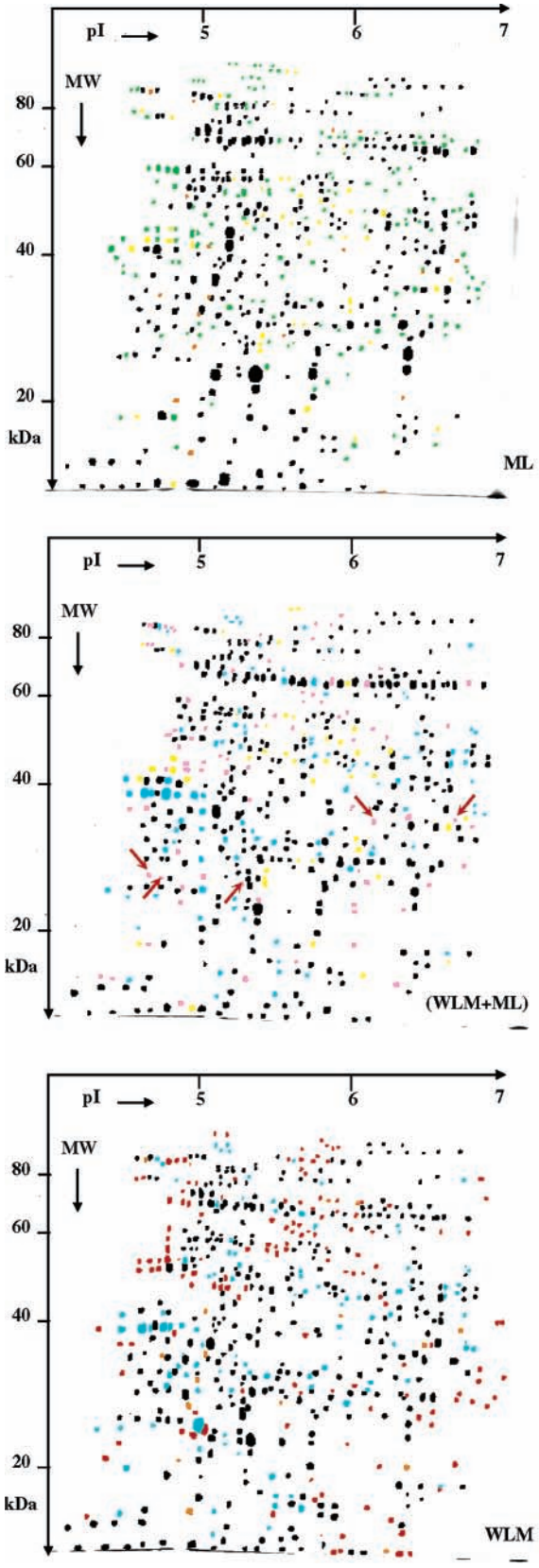

Figure 3. Multicolored 2-D electrophoresis maps of soluble leaf proteins from the WLM + ML somatic allotetraploid hybrid and its parents (mandarin and lime). Color code: black, spots common to the three genotypes; red, spots specific to the mandarin; green, spots specific to the lime; pink, spots specific to the hybrid; blue, spots common to the mandarin and the hybrid only; yellow, spots common to the lime and the hybrid only; orange, spots common to the mandarin and the lime only. WLM = Willow Leaf mandarin, and ML = Mexican Lime.

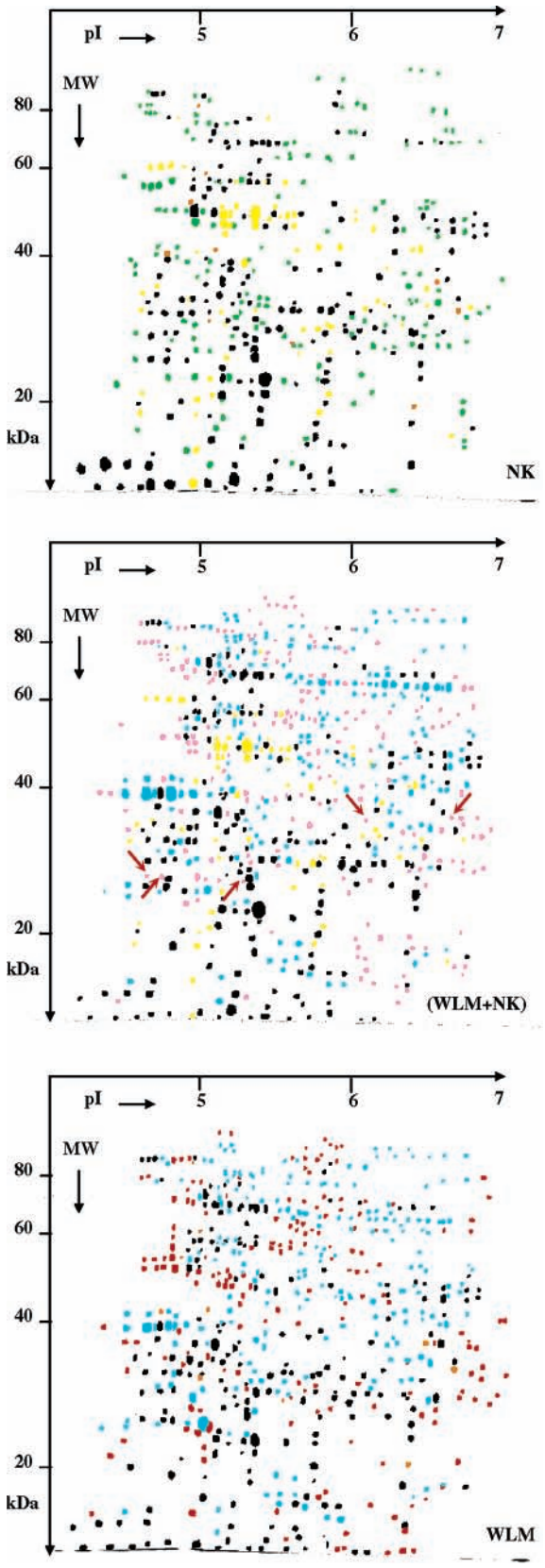

Figure 4. Multicolored 2-D electrophoresis maps of soluble leaf proteins from the WLM + NK somatic allotetraploid hybrid and its parents (mandarin and kumquat). Color code: black, spots common to the three genotypes; red, spots specific to the mandarin; green, spots specific to the kumquat; pink, spots specific to the hybrid; blue, spots common to the mandarin and the hybrid only; yellow, spots common to the kumquat and the hybrid only; orange, spots common to the mandarin and the kumquat only. WLM = Willow Leaf mandarin, and NK = Nagami kumquat.

of the total spots from hybrid proteomes. (2) The spots were absent (green spots) (for both hybrids, around 75% of nonmandarin-specific spots, 164/210 and 162/228, respectively). Therefore, in both combinations, around 50% of spots specific to the mandarin parent are also expressed in the hybrids whereas only 25% of spots specific to the nonmandarin parent are also present in the hybrids. As in class 1, the same hypotheses can be made concerning the nonexpression of spots with regard to one or the other parent.

Class 3. Spots were absent from both parents but present in the hybrid leaf proteome (pink spots). Fourteen percent of the WLM + ML total spots are specific to the hybrid whereas they represent 29% for the WLM + NK total spots. This category of spots (absent in both parents and present in the hybrids) may indicate that numerous genes which were repressed in the diploid parents are newly expressed in the hybrids. Such spots might correspond to proteins encoded by polyploidization-activated genes (*7*, *30*). Indeed, it is now known that polyploidization can induce the expression of sequences in the polyploid plant that were repressed in the diploids (derepression). This phenomenon has been observed in a newly synthesized wheat allotetraploid where 12 out of the 3072 transcripts analyzed were activated (*7*). Among these spots, five are common to both hybrids (**Figures 3** and **4**, arrows). The stronger derepression observed in the WLM + NK hybrid with regard to the WLM + ML hybrid could be compared with the results obtained by Wang et al. (*31*) showing that the combination of two divergent genomes in allotetraploids by interspecific hybridization induces genome-wide nonadditive gene regulation in contrast to the small effects of genome doubling on gene regulation in autotetraploids. They added that nonadditive gene regulation in the allotetraploids largely depends on expression divergence between the parents.

In both cases, when spots were simultaneously encountered in both parents and were absent from the corresponding hybrid and the reciprocal situation, the respective silencing of normally active genes and the activation of genes normally silent in both parental genomes could be explained by different epigenetic mechanisms of the gene regulation such as modifications in DNA cytosine methylation, the interspersion of transposons such as the long terminal repeat (LTR) among genes (*32*), etc.

A first hierarchical classification was performed on the basis of the relative volumes of all spots from the three gels of both (WLM, ML) and (WLM, NK) combinations (respectively, 814 and 832 spots) and using Euclidian distance dissimilarities and the UPGMA method (**Figure 5A$_1$,A$_2$**). It clearly appears that the hybrid leaf proteomes are closer to the mandarin proteome than to those of their other parents.A second hierarchical clustering was performed on the basis of the relative volumes of spots common to the three genotypes of each combination, i.e., those present in the three gels [343 spots for the (WLM, ML) combination and 196 spots for the (WLM, NK) combination]; Euclidian distance dissimilarities and the UPGMA method were used (**Figure 5B$_1$,B$_2$**). Although these spots were present in the gels of the three genotypes (for each combination), we observed a distribution similar to the previous ones (**Figure 5A$_1$,A$_2$**): both hybrid maps are closer to the mandarin map than to their nonmandarin parent maps. Thus, whatever the considered category of spots, either those common to the three genotypes of each combination or total spots, all the statistical analyses show the same distribution of the individuals. The two nonmandarin parents are far from the other individuals; both hybrids are close to their mandarin parent. Thus, it appears that the hybrid leaf proteomes have more similarities with that of

Table 2. 2-D Electrophoresis Gel Characteristics of the Somatic Allotetraploid Hybrids Compared to Their Parents

	distribution of parental spots in the hybrids					
	WLM + ML[a] combination			WLM + NK[b] combination		
category of parental spots	total in parents	present in the hybrid	absent in the hybrid	total in parents	present in the hybrid	absent in the hybrid
common to both parents	361	343 (95%)[c]	18 (5%)	206	196 (95%)	10 (5%)
present in only one parent						
mandarin	243	102 (42%)	141 (58%)	398	220 (55%)	178 (45%)
nonmandarin	210	46 (22%)	164 (78%)	228	66 (29%)	162 (71%)
subtotal	814	491 (60%)	323 (40%)	832	482 (58%)	350 (42%)
absent from both parents		77			195	
total	814	568	323	832	677	350

[a] Willow Leaf mandarin + Mexican Lime. [b] Willow Leaf mandarin + Nagami kumquat. [c] Percentage relative to each category of parental spots.

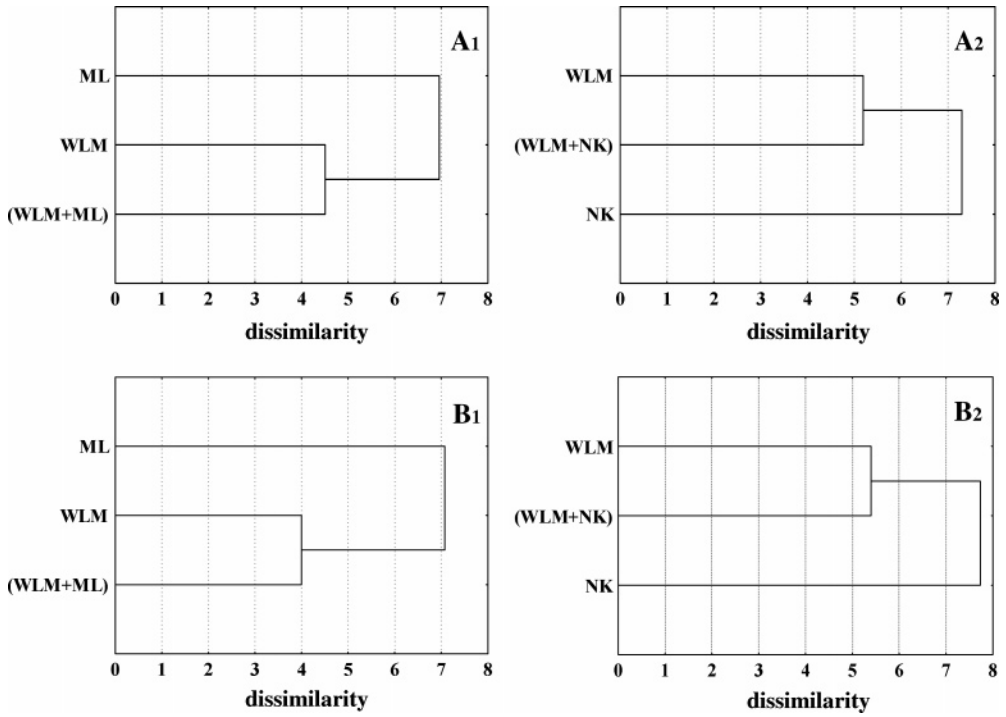

Figure 5. Dendrograms built with the relative volumes of all spots detected in both parents and hybrid gels ($\mathbf{A_1}$ and $\mathbf{A_2}$) and of the spots common to each of both combinations ($\mathbf{B_1}$ and $\mathbf{B_2}$) using the Euclidian distances and the UPGMA method.

their mandarin parent than with those of their other parents, in terms of the presence/absence of spots as well as at a quantitative level.

We can note that when we compare the similarity indexes between the mandarin and the hybrids for each combination (parts $\mathbf{A_1}$/$\mathbf{A_2}$ and $\mathbf{B_1}$/$\mathbf{B_2}$ of **Figure 5**), the WLM + NK hybrid appears to be more distant than the WLM + ML hybrid from the mandarin. This could be explained by the fact that the WLM + NK hybrid is an intergeneric hybrid whereas the WLM + ML hybrid is an interspecific hybrid. The closer to the mandarin the nonmadarin parent is (**Figure 2**), the closer to the mandarin parent the corresponding hybrid is. This result has previously been observed for the amount of spots present in both parents and also in the corresponding hybrid (class 1).

Although the analysis of proteins is less sensitive than that of mRNA would have been, the already observed dominance of mandarin allotetraploids with regard to biosynthesis of volatile compounds is thus reflected in the interspecific and intergeneric hybrid leaf proteomes. The dominance of one parent over the other one has already been observed in *Arabidopsis* allotetraploids with regard to morphological traits (*31*). Indeed, although allotetraploids of *Arabidopsis* were obtained by pollinating *Arabidopsis thaliana* autotetraploid with *Arabidopsis arenosa* tetraploid by opposition to the merger of genomes originated from diploid plants, Wang et al. (*31*) observed that allotetraploids resembled the *A. arenosa* parent.

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