

纽荷尔脐橙疑似耐寒突变体的初步鉴定与评价

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摘要:【目的】鉴定纽荷尔脐橙疑似耐寒突变体是否发生了遗传性状变化,评价其耐寒能力是否较野生型增强。【方法】以纽荷尔脐橙突变体与野生型为试材,观测比较春梢叶片性状和果实外观性状,分析比较果实内在品质,应用SSR分子标记鉴定遗传变异,石蜡切片观测比较叶片组织结构,分析比较-6℃低温处理叶片的电解质渗出率、脯氨酸含量、超氧化物歧化酶活性等抗寒性相关指标。【结果】纽荷尔脐橙突变体春梢叶片、翼叶、叶柄等性状,果实形状、果皮颜色、果实大小、油胞等性状与野生型没有差异;果实含酸量连续2a(年)较野生型低24.7%~38.6%;1对SSR引物扩增显示突变体与野生型在250 bp左右存在2条差异带;突变体叶片细胞结构紧密度、栅栏组织与海绵组织厚度的比值均极显著大于野生型;突变体叶片的电解质渗出率未低温处理以及-6℃处理2~10 h均低于野生型,-6℃处理8 h的电解质渗出率相当于野生型叶片-6℃处理6 h的水平;突变体叶片中脯氨酸含量极显著高于野生型,-6℃低温处理使突变体与野生型叶片脯氨酸含量差异增大;突变体超氧化物歧化酶活性未低温处理(0 h)和低温处理2 h至10 h均显著高于野生型。【结论】该突变体可能是果实含酸量较低,耐寒性增强,DNA水平发生了遗传改变的纽荷尔脐橙芽变。

关键词:脐橙;抗寒;突变体;SSR分子标记;叶片组织结构;电解质渗出率;脯氨酸;超氧化物歧化酶

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Preliminary identification and evaluation of putative cold-resistant mutant in Newhall Navel orange

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Abstract:【Objective】Citrus is a perennial woody plant with a long growth cycle, and once freezing damage occurs, it will cause irreparable loss to the industry. It is difficult to breed cold-resistant cultivar by conventional breeding and marker-assisted breeding in citrus for its long cycle of polyembryonic and sexual generations. Most commercial cultivars of citrus were selected from bud mutants. The mutations obtained after low temperature and freezing injury are very valuable for breeding new cold-resistant citrus varieties. The mutant used in this study was a branch without obvious cold damage under freezing injury of grade 3-4, and it was found by our research group in a Newhall orchard, which was highly likely to be a cold-resistant mutant of Newhall navel orange and was preserved in the Changsha Branch of National Citrus Improvement Center. In this paper, it was an attempt to identify whether the suspected cold-resistant mutant of Newhall navel orange had changes in hereditary characteristics and to evaluate whether the cold-resistant ability is enhanced, compared with the wild type.【Methods】Genetic variation of mutants was mainly identified by a combination of morphological and SSR-molecular-markers. Morphological and structural observation can easily and visually reveal the genetic differences of mutants, while SSR molecular markers can quickly and stably detect the differences between the mutant

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and wild-type at DNA levels. For two consecutive years, the leaf and fruit appearance traits of the high-grafting mutants were observed to analyze and the fruit quality traits were also compared. The DNA level was identified using SSR molecular markers to investigate the genetic variation between the mutant and the wild type. The evaluation of cold resistance adopted the method of laboratory identification that was less restricted than field identification. The tissue structure characteristics of leaves were observed by paraffin section, and the electrolyte leakage rate s, proline content and superoxide dismutase activity of leaves were analyzed and compared at -6 °C. These indexes were used to comprehensively compare the difference in cold-tolerant ability between the mutant and wild type. **【Results】**The mutant of Newell navel orange had no difference with the wild type in the characteristics of spring shoot leaves, winged petioles, petioles, fruit shape, peel color, fruit size, oil cells and so on. In 2020, the acid content of the mutant fruit was 24.7% lower than that of the wild type, and in 2021, the acid content of the mutant fruit was 38.6% lower than that of the wild type, showing a trend of lower acid content than that of the wild type. After amplification with SSR primer P68, a difference band appeared at 100–250 bp, indicating that there were genetic differences between the mutant and wild type. Both mutant and wild-type leaves were composed of upper epidermis, palisade tissue, spongy tissue and lower epidermis. Their spongy tissues had 2–3 layers, and most of them had 2 layers. Palisade tissue thickness was 72.682 mm, leaf thickness was 323.089 mm, and the ratio of palisade tissue to leaf thickness was 0.225. The ratio of palisade tissue to sponge tissue was 0.328, and the thickness of sponge tissue was 223.713 mm, which was significantly higher than that of the wild type. The electrolyte leakage rate of the mutant and wild-type leaves changed in an "S" shape with the increase of treatment time at -6 °C. The electrolyte leakage rate of mutant leaves was always lower than that of wild-type leaves during treatment, and there was no significant difference between them during 0–4 h treatment. The electrolyte leakage rate of mutant leaves after 6 h treatment was significantly lower than that of wild-type leaves. The electrolyte leakage rate with mutant leaves treated at -6 °C for 8 h was equal to that with the wild-type leaves treated at -6 °C for 6 h, and that was more than 50%. The proline content in leaves of the mutant was 1.4–2.3 times more than that of the wild type, which was significantly higher. Under treatment at -6 °C, the difference in proline content between the mutant and wild-type leaves was enlarged. Superoxide dismutase activity of the mutant was significantly higher than that of the wild type under both 0 h and 2–10 h cold treatment. The change trend of SOD activity with the mutants and wild type was basically the same. The SOD activity of mutants and wildtype showed an increasing trend during 0–6 h treatment at -6 °C, and then showed a decreasing trend after 6 h treatment, but the decrease in SOD activity of mutants was slower than that of the wild type. Combined with the characteristics of electrolyte permeability, proline content, superoxide dismutase activity and leaf tissue structure under -6 °C treatment, it was concluded that the mutant had stronger cold resistance than the wild type. **【Conclusion】**The fruit acid content of the mutant was lower than that of the wild type in two consecutive years. The results of genetic identification and cold resistance evaluation showed that the mutant was a cold-resistant bud mutation of Newhall navel orange.

Key words: Navel orange; Cold resistance; Mutant; SSR molecular marker; Leaf tissue structure; Electrolyte leakage rate; Proline; Superoxide dismutase

新中国成立七十多年来,我国柑橘产业因低温冻害造成的损失高达上百亿^[1-2]。培育柑橘抗寒新品种、了解柑橘抗寒机制成为领域内的热点研究方向^[3]。柑橘由于多胚和有性世代周期长,常规育种和分子标记辅助育种培育抗寒品种难度较大^[4]。柑橘的商用栽培品种多数通过芽变选种而来,低温冻害后筛选获得柑橘耐寒芽变材料,对培育柑橘耐寒新品种十分珍贵。2018年12月至2019年1月我国湘北地区遭受了大范围的低温冰冻和辐射霜冻,石门县秀平镇和夹山镇最低温度达到-8.6℃和-9.6℃,导致柑橘大面积遭受3级至4级冻害。冻后恢复救灾时,课题组从石门城郊一个冻害严重的纽荷尔脐橙果园发现1根疑似耐寒突变体的芽变枝条,其上着生的数条一年生春梢叶片仍为绿色,同一株树上其他枝条及同一果园其他树均枝叶枯死,该材料2019年春嫁接繁殖保存。

植物抗寒性研究通常有田间直接鉴定和实验室间接鉴定^[5-6]。实验室间接鉴定主要根据植物叶片结

构差异和低温胁迫下细胞膜受损程度、渗透调节物质、过氧化物酶系统等指标判断其抗寒性差异^[7-9]。笔者对疑似耐寒突变体的纽荷尔脐橙芽变材料进行DNA遗传鉴定以及叶片和果实时性状的鉴定,测定叶片组织结构以及低温胁迫下电解质渗透率、脯氨酸含量、超氧化物歧化酶活性等耐寒相关指标,初步评价该材料是否发生了遗传性状变化,其耐寒能力是否较对照增强,为材料的下一步研究和利用提供必要依据。

1 材料和方法

1.1 试验材料与处理

2019年春将纽荷尔脐橙疑似耐寒芽变(突变体,图1)的枝条以及对照品种纽荷尔脐橙(野生型)枝条高接于3年生盆栽冰糖橙树上(砧木为枳),2020年春从高接树上取突变体材料及对照枝条嫁接于1年生枳容器苗上扩繁群体。盆栽繁殖材料均放置在露地,常规水肥管理和病虫害防治。高接的



图1 纽荷尔脐橙疑似耐寒突变体

Fig. 1 Newhall navel orange putatively cold resistant mutant

变异和对照材料于2020年和2021年少量开花结果,进行叶片性状与果实时性状观测;枳砧扩繁材料未开花结果,取嫩叶提取DNA进行SSR分子标记,取成熟春梢叶片制作石蜡切片观察叶片组织结构,12月取成熟春梢叶片用去离子水洗净晾干后置于人工气候箱(型号 LEDR-400)-6℃处理0、2、4、6、8、10 h用于电解质渗出率、脯氨酸含量和超氧化物歧化酶活性的测定。

1.2 观测指标与方法

采用改良CTAB^[10]法提取DNA,SSR分子标记参照李益等^[11]的方法;叶片和果实时性状观测参考柑橘DUS测试指南^[12],果实时品质分析参照GB/T 8210—2011^[13];石蜡切片观测叶片解剖结构参照帅焕丽等^[14]的方法,将染色步骤改为0.5%甲苯胺蓝染色液稀释100倍浸泡3 min;电解质渗出率的测定参考徐新娟等^[15]的方法;脯氨酸含量和超氧化物歧化酶活性的测定参照试剂盒方法(脯氨酸试剂盒、超氧化物歧化酶试剂盒为苏州科铭生物技术有限公司产品)。

2 结果与分析

2.1 纽荷尔脐橙突变体遗传变异性鉴定

纽荷尔脐橙突变体树势中等,树姿开张,刺少,春梢叶片披针形,叶缘波状缘,叶端急尖且有缺刻,其翼叶较为窄,呈楔形,与野生型无明显区别(图2)。突变体和野生型的春梢长度、春梢叶片长度、叶片宽度、翼叶长度、翼叶宽度、叶柄长度等均无显著性差异(表1)。

纽荷尔脐橙突变体果实椭圆形,果顶浑圆,基部钝圆,闭脐,果面橙红色,平滑光亮,油胞较稀疏,微凸,与野生型果实性状无异(图3)。连续两年的果实品质比较发现,突变体果实含酸量较野生型偏低(表2)。

表1 纽荷尔脐橙突变体和野生型春梢长度和叶片性状观测

Table 1 Observation of spring shoot length and spring-flush leaves of Newhall navel orange mutant and wild type

基因型 Genotypes	春梢长度 Spring shoot length/mm	翼叶长度 Petiole wing length/mm	翼叶宽度 Petiole wing width /mm	叶片长度 Leaf lamina length /mm	叶片宽度 Leaf lamina width /mm	叶柄长度 Petiole length/ mm
突变体 Mutant	80.3±17.9 a	5.8±1.1 a	2.4±0.6 a	52.2±4.7 a	24.5±3.2 a	11.3±1.9 a
野生型 Wild type	78.2±17.3 a	6.8±1.3 a	2.7±0.3 a	54.5±6.6 a	23.7±3.2 a	12.3±1.6 a

注:表中同一指标不同小写字母表示基因型之间显著性差异为 $p < 0.05$ 差异水平。

Note: For each index, different lowercase letters show significant differences among genotypes at $p < 0.05$.



图2 纽荷尔脐橙突变体和野生型叶片比较

Fig. 2 Comparison of spring-flush leaves between Newhall navel orange mutant and wild type



图3 纽荷尔脐橙突变体和野生型果实比较

Fig. 3 Comparison of fruit orange between Newhall navel orange mutant and wild type

表2 纽荷尔脐橙突变体和野生型果实品质比较

Table 2 Comparison of fruit quality of Newhall navel orange between mutant and wild type

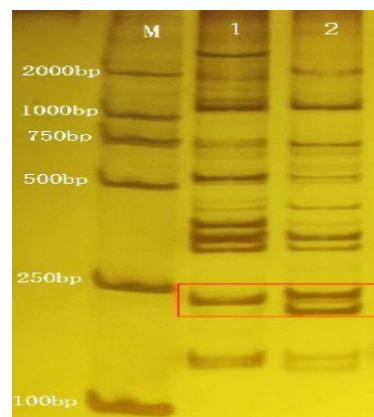
基因型 Genotypes	分析日期 Date	单果质量 Single fruit mass/g	果实横径 Fruit diameter/mm	果实纵径 Fruit length/ mm	w(可溶性固形物) Total soluble solids/%	$\rho(V_c)$ Ascorbic acid/(mg·L ⁻¹)	w(酸) Titratable acidity/%
突变体 Mutant	2020-11-25	280.76	81.50	84.74	11.00	68.00	0.64
野生型 Wild type	2020-11-25	277.54	74.36	81.94	11.70	62.00	0.85
突变体 Mutant	2021-12-14	225.20	77.49	73.81	11.30	71.52	0.35
野生型 Wild type	2021-12-14	275.00	76.09	82.01	12.20	70.73	0.57

采用李益等^[1]筛选的140对区分橙类的SSR引物以纽荷尔脐橙突变体和野生型叶片DNA为模板进行PCR扩增,产物经SDS-PAGE胶分离,1对引物扩增出差异条带(图4),引物P68(F: GCTGTT-TAGGGAAAGCAGTC; R: TATCCACCATCACCG-GCTGT)扩增的差异带位于100~250 bp。

2.2 纽荷尔突变体抗寒性初步评价

石蜡切片观察发现,纽荷尔脐橙突变体和野生型叶片栅栏组织均为2~3层,多数为2层(图5)。在同一显微镜下以相同放大倍数通过电脑软件测量各组织厚度(表3),表明突变体叶片、栅栏组织、海绵组织等厚度、细胞结构紧密度,栅栏组织与海绵组织的厚度的比值均显著或极显著大于野生型。

图6表明,纽荷尔脐橙突变体和野生型叶片电



M. DNA ladder; 1. 野生型; 2. 突变体。

M. DNA ladder; Lane 1. Wild type; Lane 2. Mutant.

图4 纽荷尔突变体和野生型 SSR 分子标记 PAGE 凝胶

Fig. 4 SSR molecular marker band pattern of Newhall navel orange mutant and wild type on PAGE gel

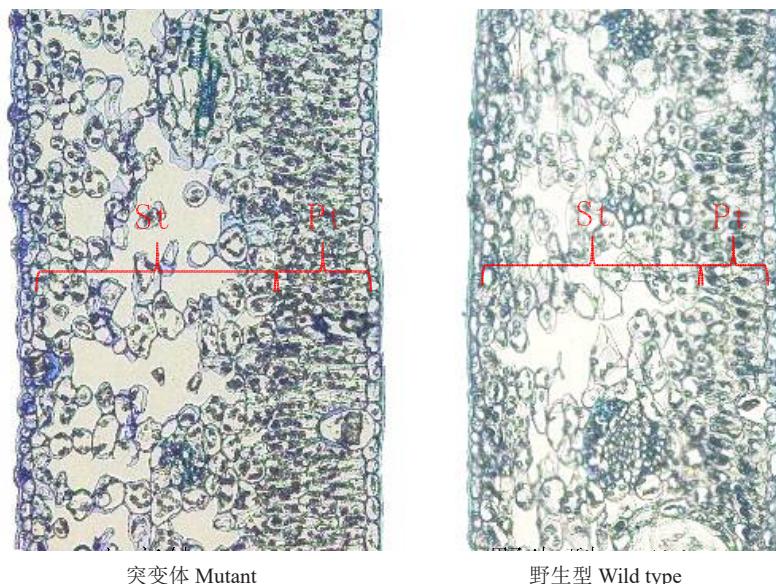


图5 纽荷尔突变体和野生型叶片横切剖面图

Fig. 5 Cross-section of Newhall navel orange mutant and wild type paraffin section

表3 纽荷尔脐橙突变体和野生型叶片结构比较

Table 3 Comparison of leaf structure between Newhall navel orange mutant and wild type

基因型 Genotypes	栅栏组织厚度 Palisade tissue/ μm	海绵组织厚度 Spongy tissue/ μm	叶片厚度 Leaf thickness/ μm	细胞结构紧密度 Tightness of cell structure	细胞结构松弛度 Cellular structure relaxation	栅海比 Ratio of palisade to spongy
突变体 Mutant	72.68±1.54**	223.71±3.40*	323.09±3.66**	0.23±0.004**	0.69±0.006	0.33±0.008**
野生型 Wild type	58.68±2.09	205.44±6.85	292.44±8.29	0.20±0.005	0.70±0.006	0.29±0.009

注:细胞结构紧密度为栅栏组织与叶片厚度的比值,细胞结构松弛度为海绵组织与叶片厚度的比值;*表示差异显著($p < 0.05$),**表示差异极显著($p < 0.01$)。

Note: Tightness of cell structure is the ratio of palisade tissue to leaf thickness, and Cellular structure relaxation is the ratio of sponge tissue to leaf thickness. * means the significant difference at $p < 0.05$, ** means the extremely significant difference at $p < 0.01$.

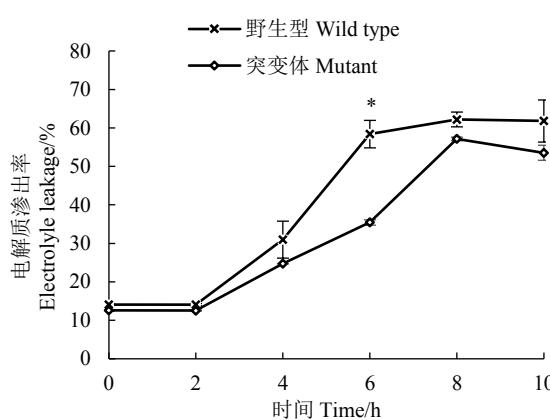


图 6 纽荷尔脐橙突变体和野生型叶片-6 °C不同时间处理下电解质渗出率的变化

Fig. 6 Electrolyte changes of Newhall mutant and wild type leaves treated at -6 °C

解质渗出率随着-6 °C处理时间的增加均呈“S”形变化,突变体叶片的电解质渗出率在处理过程中始终低于野生型,0~4 h两者的差异不大,处理6 h突变体叶片电解质渗出率显著低于野生型,突变体叶片-6 °C处理8 h的电解质渗出率相当于野生型叶片-6 °C处理6 h的水平,即大于50%。

纽荷尔脐橙突变体叶片中脯氨酸含量在-6 °C处理0~10 h时均极显著高于野生型,未低温处理(0 h)时突变体是野生型的1.4倍,低温处理后脯氨酸含量的差异增大,差异最大时前者是后者的2.3倍(图7)。

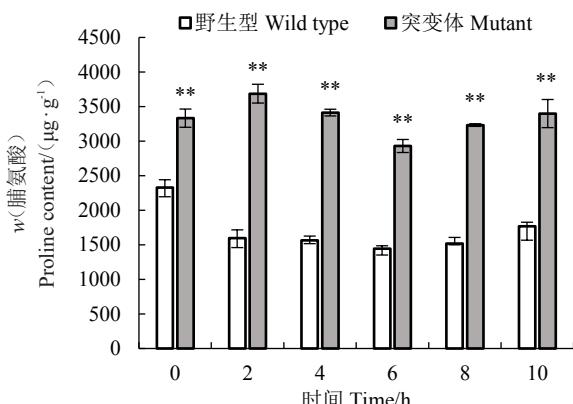


图 7 纽荷尔脐橙突变体和野生型叶片-6 °C不同时间处理下脯氨酸含量的变化

Fig. 7 Proline content in leaves of Newhall mutant and wild type treated at -6 °C

图8中,-6 °C处理下纽荷尔脐橙突变体叶片中超氧化物歧化酶活性未低温处理(0 h)显著高于野生型,低温处理2~10 h过程中仍显著或极显著高于

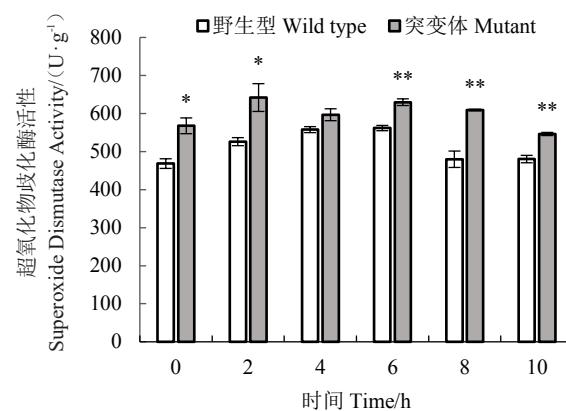


图 8 纽荷尔脐橙突变体和野生型叶片-6 °C不同时间处理超氧化物歧化酶活性的变化

Fig. 8 Changes of superoxide dismutase activity in leaves of Newhall mutant and wild type treated at -6 °C

野生型,两者的超氧化物歧化酶活性变化趋势基本相同,均在-6 °C处理0~6 h酶活性呈上升趋势,之后呈下降趋势。

3 讨 论

纽荷尔脐橙突变体春梢及叶片、果实外观性状等与野生型没有明显差异,果实内在品质由于高接树结果量少,品质数据达不到生物统计分析要求,突变体与野生型的果实内质是否存在明显差异尚不能确定,但2 a重复观测表明纽荷尔突变体果实含酸量有低于野生型的趋势。采用SSR分子标记技术鉴别柑橘芽变材料的遗传变异已有报道,李娜等^[16]和周嘉等^[17]分别用SSR分子标记技术鉴定了普通冰糖橙芽变品种和南丰蜜橘芽变品种的遗传变异,笔者在本研究中也用该技术证明了纽荷尔脐橙突变体与野生型在DNA分子水平存在差异,是发生了遗传改变的突变体。

叶片的组织结构与其抗寒性密切相关,尤其是组织结构紧密度、栅栏组织与海绵组织的比值受环境条件影响较小,是反映抗寒性的常用指标^[18]。吴林等^[19]对18种越橘进行抗寒性比较,发现叶片组织结构紧密度、栅海比与抗寒性强弱呈正相关,田间表现抗寒性强的品种其叶片组织结构紧密度大、栅海比值也大,本研究中纽荷尔脐橙突变体叶片组织结构紧密度和栅海比值极显著高于野生型;马翠兰等^[20]对耐寒性强弱不同的8个柚类品种研究结果也表明,抗寒性强的品种其叶片电解质渗出率低,本

试验中突变体叶片的电解质渗出率未低温处理以及-6 ℃处理2~10 h均低于野生型,-6 ℃处理8 h的电解质渗出率相当于野生型叶片-6 ℃处理6 h的水平;有人对6个石榴品种抗寒性研究发现,叶片中脯氨酸含量高则其抗寒性强^[21],柑橘属植物在遭受低温胁迫时脯氨酸含量增加,抗寒性强的品种,脯氨酸含量高^[22-23],笔者研究发现,纽荷尔脐橙突变体叶片中脯氨酸含量极显著高于野生型,-6 ℃低温处理使突变体与野生型叶片脯氨酸含量差异增大;超氧化物歧化酶活性的变化与植物的胁迫耐受力有关^[24],超氧化物酶活性下降,表示植物细胞酶系统遭受破坏,对细胞内自由基清除能力降低,也有报道抗寒性强的品种超氧化物歧化酶活性高,抗寒性弱的品种超氧化物歧化酶活性始终保持在较低水平^[25-26]。笔者在本研究中发现突变体超氧化物歧化酶活性在未低温处理(0 h)时已显著高于野生型,低温处理2 h至10 h过程中仍显著或极显著高于野生型。因而,综合叶片的组织结构、电解质渗出率、脯氨酸含量、超氧化物歧化酶活性等指标可初步推断,纽荷尔脐橙疑似耐寒突变体的抗寒性较野生型有所增强。

4 结 论

纽荷尔脐橙疑似耐寒突变体春梢叶片性状、果实性状等与野生型没有差异,果实含酸量连续2 a低于野生型,SSR分子标记显示DNA水平发生了遗传变异,叶片的组织结构、电解质渗出率、脯氨酸含量、超氧化物歧化酶活性等抗寒性相关指标显示突变体的抗寒性较野生型强。该突变体可能是果实含酸量较低、耐寒性增强、DNA水平发生了遗传改变的纽荷尔脐橙芽变。

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